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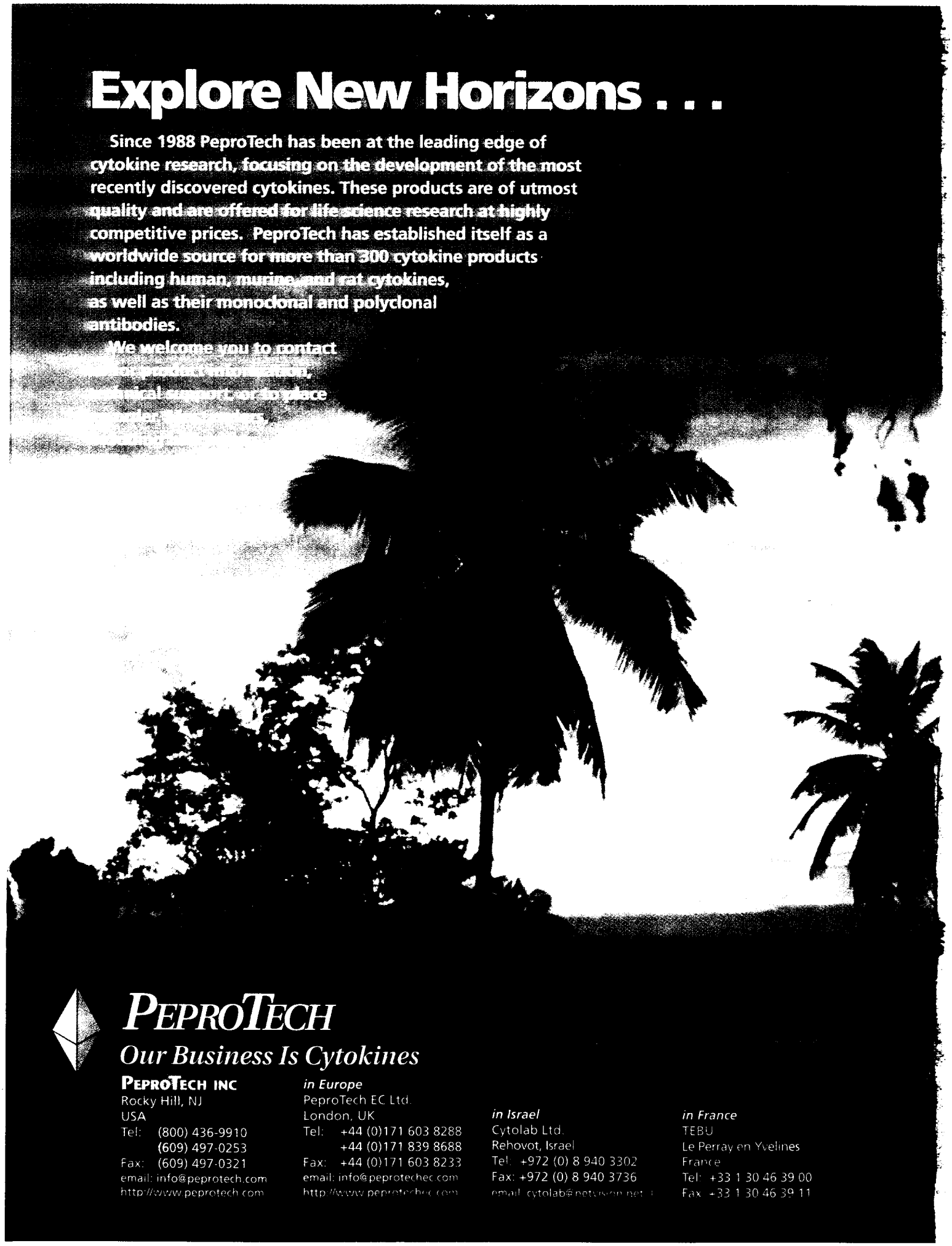
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Summary of Symposia at the 41th International Shock Congress and the Twenty-Second Annual Conference on Shock held in Philadelphia, PA, June 12-16, 1999.

Summary of Symposia at the 7th International Cytokine Conference held in HiltonHead, SC, December 5-9, 1999

Both symposia were very successful. The attached summaries describe the State-of-the-art scientific progress being made in these vital areas. Also attached are abstracts of all the papers delivered at the Symposia.

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The 7th Meeting of the ICS, Hilton Head, December 1999. Meeting Report.

Signal transduction (Udo Junker)

This year's meeting, excellent in scientific merit and brightly organized, nevertheless has no single center of attention: too wide has the field of cytokine research grown to both concentrate on a single topic and cover all cytokines, even considering that interferons, FGF, neurotrophins and others are being analyzed by their respective societies. Signal transduction however might have been the one topic that brought together most of the presentations given at the meeting.

Warren Leonard started out with a talk that might have been called "SCID and the IL2-receptor family". Warren however so much wanted to have it on the very most recent level that he wouldn't even give a title when the presentation was to start. He reported on the effects of various disturbances of the signal cascade triggered from the IL-2-receptor. One of the most elements here is an enhancer site some 3700bp upstream of the TATA motif, containing a dyad GAS site. As he showed, this site is good to bind STAT5-TETRAmers, consisting of two of the commonly known STAT5-dimers. By using oligonucleotide studies he found that for an optimum, more that additive effect of the site, the two binding motifs have to been separated by 6bp, while in the IL2R-alpha promoter they are separated by 11bp.

Lori Glimcher of Harvard took up the theme of regulation of gene-expression in T-cell, reviewing the molecular mechanisms the distinguish a TH1 from a TH2-cell. While the TH2-specific transcription factors c-maf and GATA3 have been detected not so long time ago, Lori found a factor she would call T-bet by screening a TH1 expression library for factors binding to the (relatively TH1-specific) IL2-promoter. This transcription factor while absent in unstimulated cells is expressed in T-cells within 1hr after TcR engagement and in a synergistic manner by IL-12, also found only in NK-cells and B-cells upon triggering CD40. Artificially expressed T-bet is a very powerful promoter of IFN-g, while actually suppressing IL-2, which is only in virtual contradiction with the way it was found:

the initial library screen was only on binding proteins regardless of their effect. T-bet is a powerful factor, able to induce IFNg and shut down IL2, IL4 and IL5 in polarized TH2 and TC2-cells.

Two Minisymposia were specifically designed to deal with signal transduction, another for receptor recycling and signaling. A few of the presentations will be covered here.

The first Minisymposium presentation with major accent in signal transduction was given by Deborah Alpert of N.Y. Medical School, receiving an Outstanding Scholar Award for separating p38-dependent from p38-independent ways sodium salicylate (NaSal) can suppress activation of NF-kB, all of which converge on an inhibition of IkB-alpha phosphorylation. By using MEKK6b(E), a mutated constitutively active upstream activator of p38, and SB203580, an inhibitor of 038, she could show that NaSal always (partially) inhibits IKKa activation in purified proteins, while of the upstream signals it inhibited (again, partially) only TNFa but not IL1, and that this inhibition is mediated by its effect on p38. Interesting was also her observation that NaSal is a rather weak inhibitor of cyclooxygenase II and thus could mediate most if it's effects indirectly via influencing gene expression.

Signaling of IL1 and related cytokines kept coming up during the meeting, after Michael Martin's presentation about the role of Death Domains in IRAK. It turns out IRAK lacking the C-terminal kinase domain as well as a construct consisting of only the IRAK Death Domain are dominant negative inhibitors of IL1 signaling. Also, the kinase domain has autocatalytic activity, so that Michael would envision the sequence of signaling events as follows: binding of IL1 to its receptor, recruitment of MyD88 and of IRAK via the MyD88 Death Domain, auto- (not cross-) phosphorylation of IRAK at the Death Domain and Kinase Domain, release of the MyD88/IRAK complex and association with TRAF6 at a 2IRAK*2TRAF stoichiometry: the TRAF will not enter a complex with the IL1R molecules.

Silvia Bufone of the Free University Berlin received a Young Investigator Award for her research on IL15 receptor signaling. Quite unexpectedly, she could isolate the "spleen tyrosine kinase" Syk as binding partner for the IL15R-alpha chain. Syk, which is usually found in mast cells and basophils, but is most recognized for interacting with the B-cell

antigen receptor, mediates the anti-apoptotic effect of IL15 on ceramide-treated cells; it phosphorylates the IL15Ra and triggers PLC-gamma. It is however not sufficient for IL15R signaling, as reflected by the fact that the IL15R also activates Jak2 in common-gamma-chain negative cells, while in those expressing gamma-common it signals as expected via JAK3.

A tremendous amount of work for everybody working with Ras promised the presentation of John Schrader, University of British Columbia, as he could show that most of the effects commonly regarded as being mediated by H-Ras can in fact be ascribed to a novel member of the Ras family, M-Ras. The confusion stems from the fact that several of the most commonly used H-Ras antibodies do also bind to M-Ras, which in most cells types in fact is more abundant than the "classical" H-Ras. While wild type M-Ras is not tumorigenic, mutated (constantly activated) M-Ras is, and can substitute, e.g., for IL3 in IL3-dependent cell lines. Curiously enough, the combined proliferative effect of IL3 AND mutated M-Ras is less than that of M-Ras alone. M-Ras triggers many pathways that have been described for H-Ras, however it has less Raf-activating capacity. Besides of pro-proliferative effects, M-Ras carries differentiating capacity as it promotes formation of platelets from leukemia cells transfected with activated M-Ras in syngenic animals.

The presentations of Helmut Holtmann from Hannover, Germany and Andy Clark, Kennedy Institute London dealt with regulation of mRNA stability, a topic quite commonly overlooked in signal transduction studies. They find that the AUUUA motifs commonly regarded as the regulators of mRNA stability be themselves are not sufficient to explain all observed effects: Holtmann reported a spatial separation between the AUUUA and the instability-conferring site in the IL8-UTR, while Clark finds that cyclooxygenase mRNA stability is mainly regulated by the first triplet of AUUUA's 3' of the ORF, while the many other repeats further downstream don't seem to play a critical role. He also finds that COX-II mRNA stability can be positively regulated by the MKK6-p38-MAPKAPK2, while there is no such effect in the mRNA of, e.g. c-myc and TNF, which are also destabilized by multiple AUUUA repeats.

A presentation to be held by Roger Rezzonico, but given by J.M. Dayer, reflected the fact that cytokine signaling pathways are being used by other signaling molecules, too.

Integrins (CD11a, CD11b, C11c) do not only share structural elements with cytokine receptors, they also use the same intracellular elements. In short, activation of CD11b, c leads to a quick (within 15min) activation of ERK1 and ERK2 as well as p38, with the consequences of induction of transcription of the IL1beta gene, stabilization of the mRNA and improved translation into protein.

The battle about what protein signals LPS-presence to the cells seems over after Bruce Beutler's (H. Hughes Med. Inst, Dallas) convincing presentation about the tracking down of the gene responsible for the decreased LPS-sensitivity / LPS-nonreactivity of C3H/HeJ and C3H/10ScCr mice, respectively. It turns out that they harbor point mutations and deletions in the TLR4 gene, respectively. TLR2, the other candidate gene was completely ruled out by Bruce; it turns out that obviously binds to and signals proteoglycan and likely Lipid A and/or its derivatives.

A developing field in signaling research is the remodeling process of chromatin structure as an element regulating the accessibility of DNA for transcription factors, and thus a whole symposium was dedicated to these mechanisms. This symposium started with Alan Wolffes (NIH) review of the role of DNA acetylation / deacetylation in transcriptional activity. He stated that a 30% change in acetylation leads to a 20-fold increase in transcription in certain genes. The opposite effect is illustrated by the fact that the MAD/MAX complex, being so to say the antagonist of MYC/MAX, recruits deacetylases (RPD3 in yeast) via SIN3 to the DNA, leading to deacetylation of DNA, shutdown of transcription and thus participating in cell cycle arrest. He further illustrated his review with examples. For instance, in several leukemia cell lines, as a consequence of translocation transcription factors recruit deacetylases to genes that should initiate differentiation and thus prevent the latter. Another important mechanism of DNA shutdown is methylation, which in contrast to acetylation is more or less permanent and thus fixes a cell in a certain state, a mechanism important in, e.g. differentiation of TH1 and TH2 lymphocytes. This was illustrated in the presentation of Anjana Rao, Harvard, who investigated changes in the chromatin structure of the IL4 gene during T-lymphocyte differentiation. By analyzing DNAseI hypersensitivity pattern in T-cells polarized to a various extent she could show that a switch from TH2 to TH1 by high doses of

IFNgamma and anti-CD3 activates the IFNgamma gene without changing the chromatin structure around the IL4, IL5 and IL13 genes, i.e., the changes occur at the level of transcription factors. Vice versa this is not the case: a polarized TH1 cells triggered by high IL4 will (reversibly) shut down the IFNgamma gene but it will not bring back expression of IL4, IL5 and IL13, as these have been closed irreversibly by methylation. She fits her observation into the following time-pattern. In native TH0 cells, the IL4-locus is heavily methylated and thus inactive. Upon first contact at the T-cell receptor, the IL-4 promoter is partially demethylated, and can be accessed. The further fate depends upon exogenous factors: in the presence of IL-4, after 8 weeks the whole gene is demethylated and actively transcribed: in the presence of IFN-gamma, it is completely and irreversibly methylated.

Rik Derynck, UCSF, had to postpone his presentation – he could not make it to the meeting in time. Nevertheless he gave an outstanding overview on TGFb/SMAD signaling. Instead of functioning as autonomous transcription factors, the respective SMAD 2, 3 and 4 cooperate with other transcription factors (AP-1, SP-1 and others) that already may sit on the DNA as in the case p15 induction, the most prominent effect of TGFb. It occurs the p15 promoter has two neighboring sites for SP-1 and SMAD3. The SP-1 site is "preloaded", but weakly active. Upon TGFb signaling, SMAD2, 3 and 4 form a trimer. While SMAD3 binds to its relatively weakly conserved binding motif, SMAD4 interacts with the SP-1 part of the transcription complex and induce a very strong activation.

The last symposium of the meeting was dedicated to apoptosis, not commonly regarded as signal transduction though it is.

Douglas Green, LIAI, gave details on the role and interaction of p53 and cytochrome C in induction of apoptosis. Even though p53 is said to induce bax transcription, activation of p53 triggers bax without necessity of transcription, leading to CytoC release from mitochondria. Interestingly, the amount of time needed for an individual mitochondrium or an individual cell to release all or most of the CytoC in constant, while the time from induction of apoptosis to the actual event of CytoC release may vary between and even within a single cell line.

David Wallach's, Rehovot, Israel presentation called Death & Transcription from the TNF-receptor in fact concentrated on the structural organization of the TNFR signaling complex, especially regarding the NF-kB signalosome, as he would call the complex of NIK, IKK's and NFkB. It turns out, NEMO, a recently described member of this complex binds to the intermediate region of RIP, getting all the signalosome in direct contact with the TNFR-complex. Also, NEMO interacts with A20, a protein known to inhibit NF-kB activation, which binds to TRAF2. Thus, the TNFR and the NF-kB signalosome form a tight, giant intracellular complex, wherein or by the amount of which NF-kB activation is differentially regulated by the amount of available NEMO and A20, which by itself is under positive transcriptional control by NF-kB.

The last review presentation of the Meeting, given by Craig Thompson, University of Chicago, once again centered on the molecular execution mechanisms of apoptosis. He envisions apoptosis as the consequence of both metabolic changes (glucose, energy turnover in the cytoplasm and the mitochondria) and signaling mechanisms. Extending former presentations he sees improper shutdown of ATP/ADP production and transport through the mitochondrial membranes as causing hyperosmolarity, reactive oxygen radicals, lipid oxidation, and pH changes which will ultimately result in rupture of the outer mitochondrial membrane and release of cytochrome C, triggering the Caspase cascade. However, the most important take-home message from his lesson was formulated by Michael Lotze, Pittsburgh, by the short conclusion "I have to re-read my Lehninger", a conclusion that should well be considered by all us cytokinologists: it is all too easy to get detached from external reality of cytokines, such as conventional biochemistry, conventional biology, conventional anatomy of multicellular organisms.

Inflammation (Giamila Fantuzzi)

Martin Nicklin presented data demonstrating that IL-1Ra-deficient mice develop lethal arterial inflammation when crossed into a 129xMF1 genetic background. The effect of IL-1Ra deficiency seems to be highly dependent on the genetic background of the animals, as previous studies had demonstrated either the lack of an evident phenotype or the

development of rheumatoid arthritis (Balb/c strain). Nicklin's data were nicely complemented during the Clinical Symposium by the presentation of Gordon Duff, who demonstrated the association of specific genetic polymoprhism in the IL-1 family with cardiovascular disease. During the same symposium Steven Holland summarized the current evidence for the necessary role of IFN gamma and IL-12 in the protection from mycobacterial infections in humans. Patients with mutations in either the IFN gamma or the IL-12 receptor are extremely susceptible to infections by strains of mycobacteria which are usually harmless to non-immunodeficient individuals. The cloning and characterization of several new interesting molecules has been presented. Among these were TIF (T cell-derived inducible factor) and BLyS (B Lymphocyte Stimulator). As explained by L. Dumoutier, TIF, which is induced by IL-9, shares a 22% aminoacid homology with IL-10, is constitutively expressed in the thymus and brain and activates STAT activity in mesangial, neuronal, and melanoma cell lines. Judith Giri presented results on BlyS, a member of the TNF family which is mainly produced by neutrophils and monocytes and is upregulated by IFN gamma and IL-10. Administration of BlyS to mice induces splenomegaly, alters spleen architecture and increases IgG, IgM and IgA levels. The cloning and characterization of the activity of several isoforms of the human and murine IL-18 binding protein were presented by Soo-Hyun Kim. Some isoforms neutralize the activity of IL-18, whereas others appear to be inactive, raising the possibility that genetic differences in the ratio between active and inactive isoforms might modulate susceptibility to Th1-mediated diseases. S.-H. Kim also presented a tridimensional molecular model for the binding of IL-18 to the IL-18 binding protein. Data on IL-B30, a novel cytokine, were presented by M. Wiekowski. Transgenic mice constitutively expressing IL-B30 show stunted growth and disseminated inflammation, with development of neutrophilia, increase in platelet counts and augmented serum levels of acute-phase proteins. IL-1 and TNF alpha seem to mediate the proinflammatory effects of IL-B30.

Chemokines (Silvano Sozzani)

At difference with past meetings, chemokine communications were not focussed on the

characterization of new chemokines or chemokine receptors, but rather on the mechanisms involved in the regulation of chemokine biology.

Many presentations extended the concept that the signals present at the inflammatory sites can regulate the expression of chemokine receptors. Dr. Gangur showed that IFNg, in addition to induce CXCR3 ligands inhibit the functionality of this receptor in IL-2-activated T lymphocytes, with an apparent new mechanisms that does not involve membrane receptor down regulation. IL-2 can remodel chemokine receptors on NK cells upregulating inflammatory chemokine receptors (e.g. CCR1, CCR2, CCR5, and CCR6) and having an opposite effect on CCR7 (C. Paganin). The local cytokine contest can also induce the expression of "non canonical" receptors in leukocytes. Neutrophils exposed to IFNg in association with TNF become responsive to MIP-3a/LARC, the ligand for CCR6 (T. Yoshimura); and Th2 cytokines (IL-4 or IL-13) induce the expression of functional CXCR1 and CXCR2 in monocytes (S. Sozzani). Finally, A. Sica showed that the alteration of the cell redox states by the use of antioxidants (PDTC or NAC) rapidly inhibits CCR1, CCR5, and CXCR4 expression and function in human monocytes. In contrast, the ROI-generating system Xanthine/Xanthine Oxidase, and H2O2 had the opposite effect. Interesting evidence that one chemokine receptor can be functionally activated by a family secreted proteins, the defensins, were also provided (D. Yang).

Dr. Renauld showed that vMIP-1, in addition to I-309, protect mouse thymic lymphomas against corticoid-induced apoptosis by the activation of CCR8. This effect was inhibited by inhibitors of the MAPK pathway, and by a dominant negative of M-Ras. Since these are signaling pathways very likely activated by all chemokine receptors, the specific mechanism(s) responsible for CCR8 anti apoptotic activity needs still to be elucidated. M-Ras activation is also shown to be activated in MGSA/GRO activated melanocytes, and to responsible for the transforming action of this chemokine. M-Ras activation is also required for the induction of AP-1 and NF-kB transcriptional activity by MGSA/GRO (D. Z. Wang).

Dr. Park presented that targeted disruption of the mig gene resulted in a strong reduction of IgG2a titer in response to Franciscella tularensis, suggesting that MIG plays a role in the activation of the Th1 response against this pathogen.

Finally, Dr. Ruddle presented very interesting evidence that two chemokines SLC and BLC, that are known to play a crucial role for the trafficking of lymphocytes and dendritic cells in lymphoid organs are expressed at sites of chronic inflammatory reactions. This expression may be responsible for the lymphoid neogenesis associated with these diseases and is sustained by the interaction of Lymphotoxin-a with the TNFR1.

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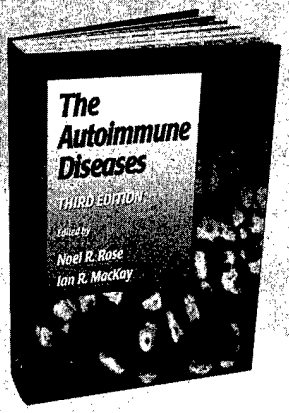

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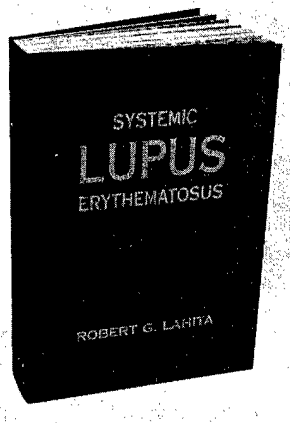

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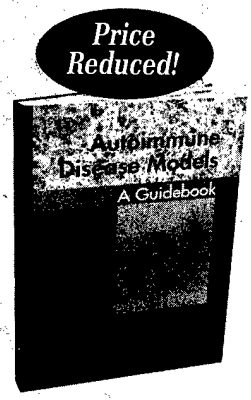

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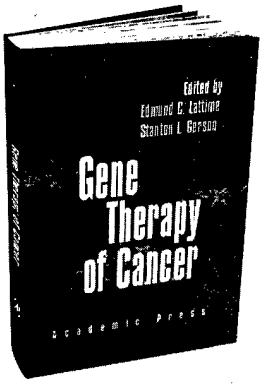

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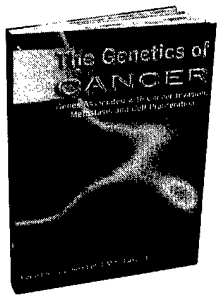

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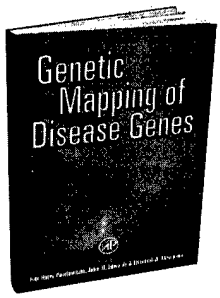

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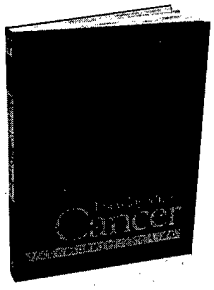

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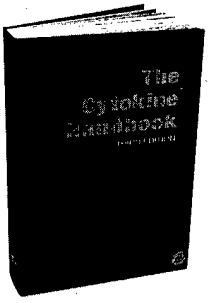

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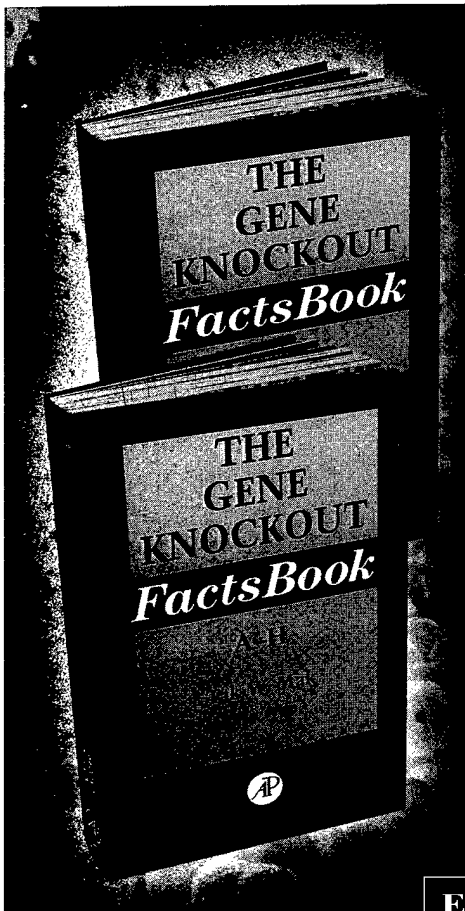

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A recipient of significant national and international awards, Dr. Tak Wah Mak is recognized worldwide for his discovery of the T cell receptor and for the use of genetically engineered animals to understand the immune system. He is a Senior Staff Scientist at the Ontario Cancer Institute, a member of the Departments of Biophysics and Immunology at the University of Toronto, and is Director of the Amgen Institute in Toronto.

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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF FRAG-6, A NOVEL INTERFERON-STIMULATED GENE THAT IS EXPRESSED IN AN IRF-1-INDEPENDENT MANNER

Aristóbolo M. Silva,[1,2] Renata L. A. Bottrel,[1] Luiz F. L. Reis[2]

In order to identify new interferon-stimulated genes that could help in the better understanding of the mechanism of action of interferons (IFNs), we decided to compare, by differential display RT-PCR (DDRT-PCR), the pattern of gene expression between IFN-α treated and untreated mouse embryonic fibroblasts (MEFs). Here we describe the initial characterization of a new cDNA fragment, named FRAG-6, that is expressed only upon IFN stimulation. The IFN-induced expression of this new gene can be observed in both wild-type and IRF-1-deficient MEF. FRAG-6 cDNA hybridizes with an mRNA of 6–9 kb that is induced by IFNs in a time-dependent manner. Analysis of the cloned nucleotide sequence revealed a 174 amino acid (aa) open reading frame (ORF) contained within the 576 bp. No significant homology with known nucleotide or protein sequences was observed. FRAG-6 is induced in vitro upon treatment of wild type or IRF-1-null cells with IFN-α or -γ, but not with TNF or IL-1. Treatment of mice with imiquimod, a potent inducer of IFN, led to induced expression of FRAG-6 mRNA in various organs from wild type or IRF-1-deficient mice, but not from STAT-1 or type I IFN receptor deficient animals. Our results demonstrate that FRAG-6 mRNA induction by interferons is IRF-1-independent and it is likely to be activated by the JAK/STAT pathway. Further characterization of FRAG-6 will help us in the understanding of the mechanism of action of IFNs.

Interferons (IFNs) are a family of pleiotropic proteins that are essential for antiviral defence and also, play a critical role as immune modulators, and in the regulation of cell growth and differentiation.[1] In order to exert their biological functions, IFNs interact with specific cell-surface receptors, initiating a cascade of signalling events that will trigger the expression of several IFN-stimulated genes (ISGs).[2] Type I IFN signalling is mediated primarily via the activation of inactive, cytoplasmic forms of STAT-1 and STAT-2 by JAK-1 and Tyk-2, members of the JAK family of protein kinases. Phosphorylated STAT-1 and STAT-2 will interact with the DNA-binding protein p48 to form the trimeric ISGF-3 complex.[3–7] Type II IFN or IFN-γ signalling is primarily mediated by a homodimer of phosphorylated STAT-1 and utilizes JAK-1 and JAK-2 tyrosine kinases.[8–12]

The crucial role of the JAK/STAT pathway in IFN signalling was substantiated by the findings that mice deficient for STAT-1 lack the ability to respond to both type I and type II IFNs.[13,14] Nonetheless, other signalling molecules have been implicated in the induction of genes by IFNs. For example, CTAII appears to be an essential molecule for the induction of MHC class II by IFN-γ.[15,16] Also, both type I and type II IFNs can induce the expression of the interferon regulatory factor 1 (IRF-1).[17–19]

IRF-1 was originally cloned by screening an expression library with an oligomerized hexanucleotide (AAGTGA) probe derived from a sequence motif present within the promoter region of the IFN-β gene, as well as within the promoter region of several

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ISGs.[17,20] The functional characterization of IRF-1 as a positive regulator of transcription has been very widely documented. For example, cell lines expressing an IRF-1 antisense RNA showed a dramatic reduction in the poly(I).poly(C)- or virus-induced levels of IFN-β mRNA or protein, as well as a reduced expression of some ISGs, suggesting a critical role for IRF-1 in the regulation of the IFN-β and ISG gene expression.[21]

Experiments using mice lacking a functional IRF-1 gene revealed its crucial role in the IFN-γ signalling as demonstrated by the lack of induction of iNOS (inducible nitric oxide synthase)[22,23] and GBP (guanylate-binding protein).[24,25] Moreover, the constitutive expression of LMP-2 and TAP-1 are dramatically reduced in mice deficient for IRF-1.[26] IRF-1-deficient mouse embryonic fibroblasts (MEFs) are also less sensitive to the antiviral action of type I IFN.[24] However, the normal induction of type I IFN genes by both virus or poly(I).poly(C), suggests that, in vivo, IRF-1 is not necessary for the induction of the type I IFN genes,[27] as well as for the induction of type I IFN-stimulated genes such as 2′5′OAS, PKR, etc.[23]

In order to identify new IFN-stimulated genes as well as to further investigate the role of IRF-1 in IFN-α signalling, we compared, by means of differential-display RT-PCR, the mRNA population present in untreated or IFN-α-treated wild-type or IRF-1-deficient mouse embryonic fibroblasts. Here, we describe the characterization of a novel ISG, named FRAG-6. FRAG-6 is induced by type I as well as by type II IFNs in both wild-type and in IRF-1-deficient cells. It is also expressed in vivo in various organs and its IFN-induced expression appears to be mediated by the JAK/STAT pathway and is not affected by the lack of IRF-1.

RESULTS

Isolation and characterization of a cDNA fragment corresponding to a novel IFN-stimulated gene

In the differential-display RT-PCR analysis, we identified a cDNA fragment of 576 bp, that was present in both wild-type and IRF-1-null cells treated with IFN-α for 4 h, but absent in lanes corresponding to untreated wild-type or mutant cells. This cDNA was named FRAG-6 and the corresponding fragment was isolated from the gel, re-amplified and cloned. The cloned fragment was sequenced, revealing a putative open reading frame of 174 amino acids (Fig. 1). Neither the nucleotide sequence nor the putative coded amino acid sequence showed any significant homology with other known sequences deposited in the GenBank.

In order to confirm the presence of this sequence within the mouse genome, we performed Southern blot analysis. Figure 2 shows that the FRAG-6 cDNA probe hybridizes with a single genomic fragment, suggesting that FRAG-6 is a single copy gene.

Induced expression of the FRAG-6 gene in response to cytokine treatment

In order to confirm the inducibility of FRAG-6 by IFN-α, we performed Northern blot analysis with total RNA isolated from wild-type or IRF-$1^{0/0}$ MEFs, untreated or treated with rhuIFN-α2/α1 as indicated, using the labelled FRAG-6 cDNA fragment as probe. Figure 3 demonstrates that a mRNA of about 6–9 kb is detected in both wild-type or IRF-$1^{0/0}$ MEFs treated with IFN-α2/α1, but not in control, untreated cells. We next determined the kinetics of FRAG-6 induction by IFNs. Detectable levels of FRAG-6 mRNA were observed 2 h after induction, and maximal levels of FRAG-6 mRNA were obtained 16 h after induction (data not shown). We also observed a higher accumulation of FRAG-6 mRNA when cells were treated with IFN for 4 or 8 h in the presence of the protein synthesis inhibitor anisomycin (data not shown). This result suggests that induction of the FRAG-6 gene by IFN is not dependent on de novo protein synthesis.

To determine whether the induction of the FRAG-6 gene can also be observed in MEFs stimulated by other pro-inflammatory cytokines, we measured the levels of FRAG-6 mRNA in heterozygous or IRF-$1^{0/0}$ MEFs treated with murine IFN-γ, TNF or IL-1. Figure 4 shows that IFN-γ can increase the levels of FRAG-6 mRNA, albeit less effectively than type I IFN, at least at the length of treatment indicated. We have determined that the primary culture of embryos derived from heterozygous mouse used in this experiments do behave like wild type (not shown). Treatment of MEFs with TNF did not induce the expression of the FRAG-6 gene, at least after 4 or 8 h of treatment, as seen in Figure 5. IL-1 also failed to induce FRAG-6 gene expression under the same circumstances (data not shown).

To investigate whether FRAG-6 mRNA expression could be also observed in tumour cell lines, L-929 cells were treated with recombinant human IFN-α2/α1 or murine TNF. As shown in Figure 6, constitutive levels of FRAG-6 mRNA were observed in control, untreated L-929 cells. Upon IFN-treatment, an increased level of FRAG-6 mRNA was observed already after 4 h of treatment, reaching the maximal induction at 8 h and decreasing thereafter. Once again, as also seen in Figure 5, TNF failed to induce the expression of FRAG-6 mRNA after 4, 8, or 16 h of treatment in these cells.

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                                1   gtgataaccaagtcatggc
20  ctgtgcataacggggagcctgtgtttgacgtggatgagatcttca
                                          M  R  S  S    4
65  aatacctgctcaagtggcccagatgtgaggcagctcttcgaactg
    N  T  C  S  S  G  P  D  V  R  Q  L  F  E  L   19
110 tgtggaaccaatgagaaaatcatagacaacatcacggaggaaggc
    C  G  T  N  E  K  I  I  D  N  I  T  E  E  G   34
155 aggcagctgatggccactgctgagtctgtgttccagaaggtcgca
    R  Q  L  M  A  T  A  E  S  V  F  Q  K  V  A   49
200 ggggaacttgagaatggcaccattgtggttggacagctggagctg
    G  E  L  E  N  G  T  I  V  V  G  Q  L  E  L   64
245 atcctcgagcaccagagtcagtttcttgacatctggaacttaaat
    I  L  E  H  Q  S  Q  F  L  D  I  W  N  L  N   79
290 agaaggcggctgccatcccaagagaaggcctgtgatgtgaggagc
    R  R  R  L  P  S  Q  E  K  A  C  D  V  R  S   94
335 ttgctgaaaaggagaagggatgatctgctgttcctcaagcaagag
    L  L  K  R  R  R  D  D  L  L  F  L  K  Q  E  109
380 aagagatacgtggagagcctcctgaggcagctcggaagagtgaaa
    K  R  Y  V  E  S  L  L  R  Q  L  G  R  V  K  124
425 caccttgtgcaagtggattttggaaacattgaaataatccactct
    H  L  V  Q  V  D  F  G  N  I  E  I  I  H  S  139
470 caagacctcagcaataagaaactaaatgaagccgtgattaagctc
    Q  D  L  S  N  K  K  L  N  E  A  V  I  K  L  154
515 cccaattcctcctcctacaaaagggagacacattactgcctgagc
    P  N  S  S  S  Y  K  R  E  T  H  Y  C  L  S  169
560 cctgacatccgagaaat
    P  D  I  R  E   174
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Figure 1. Nucleotide sequence of the 576 bp of FRAG-6 cDNA fragment (accession no. U76754 in the EMBL, GenBank, and DDBJ nucleotide sequence databases) with the deduced amino acid sequence.

The nucleotide sequence was determined by automated sequencing of both strands (ALF, Pharmacia). The amino acid sequence of the predicted partial coding sequence (numbering on right) is represented with the one-letter code, below the nucleotide sequence.

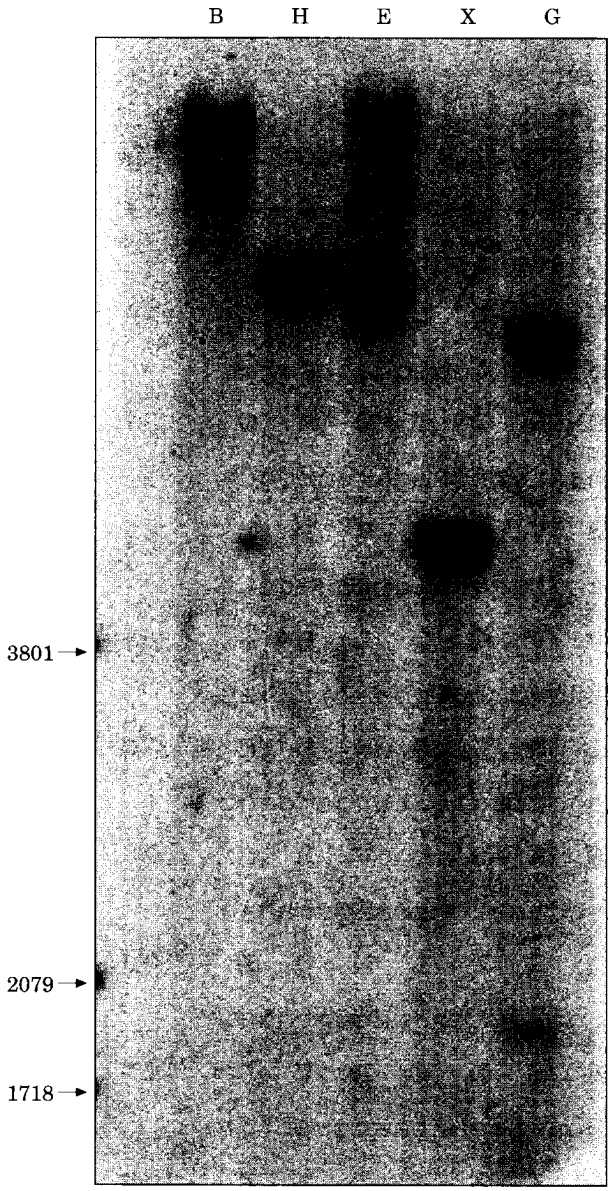

Figure 2. Southern-blot analysis of genomic 129/Sv mouse DNA.

Genomic DNA was isolated from 14 day-old mouse embryo and 20 μg were digested at 37°C, for 18 h, with the restriction enzymes *Bam*HI (B), *Hin*dIII (H), *Eco*RI (E), *Xba*I (X) or *Bgl*II (G). After digestion, DNA was precipitated, fractionated on 0.8% agarose gel, and transferred to a nylon membrane. The filter was hybridized with a ^{32}P-αdCTP-labelled FRAG-6 cDNA. The standard of molecular sizes is indicated at the left in base pairs.

In vivo induction of FRAG-6 mRNA by imiquimod is dependent of STAT-1 and IFN type I receptor

To investigate the in vivo expression of the FRAG-6 gene, we determined the levels of FRAG-6 mRNA in organs of wild type or mutant mice that lack some components of the interferon system after treatment with imiquimod, an immunomodulator that induces high levels of IFN upon oral administration.[28]

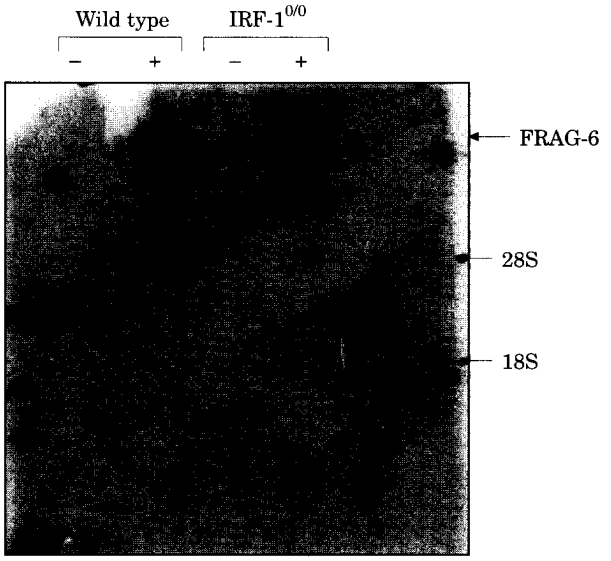

Figure 3. Induction of FRAG-6 mRNA by IFN-α.

Total RNA was extracted from wild type or IRF-1$^{0/0}$ MEFs treated with IFN-α (+) or from untreated controls (–). 15 μg of RNA were fractionated in 1% denaturing-formaldehyde agarose gel, transferred to nylon filter, and hybridized with a ^{32}P-αdCTP-labelled FRAG-6 cDNA.

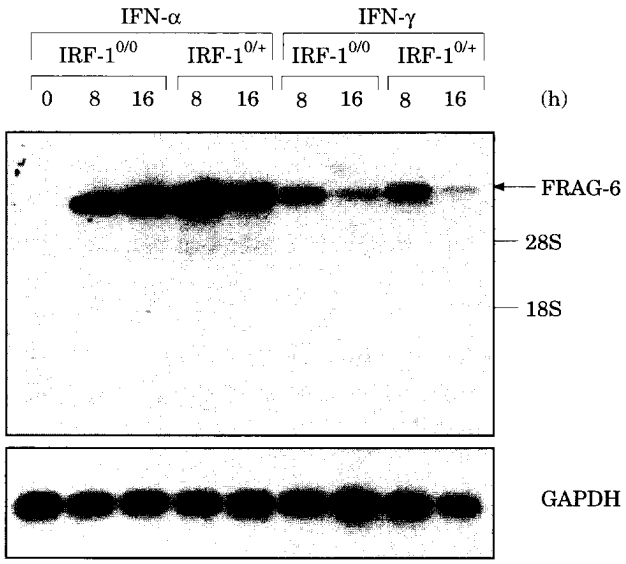

Figure 4. Induction of FRAG-6 by interferons is IRF-1 independent.

Heterozygous or IRF-1$^{0/0}$ MEFs were induced with rhuIFN-α2/α1 (500 IU/ml) or muIFN-γ (100 IU/ml) as indicated and total RNA was isolated. 15 μg total RNA were fractionated on a denaturing 1% agarose gel, transferred to nylon membrane and hybridized with a ^{32}P-αdCTP-labelled FRAG-6 cDNA probe. The filter was then stripped and re-hybridized with the ^{32}P-αdCTP-labelled rat GAPDH cDNA probe to ensure equal loading of RNA.

In wild type mice, there was no detectable level of FRAG-6 mRNA in untreated, control animals. However, either 2 or 8 h after treatment with imiquimod, induced levels of two distinct transcripts of FRAG-6 mRNA were readily detectable in the spleen. When we

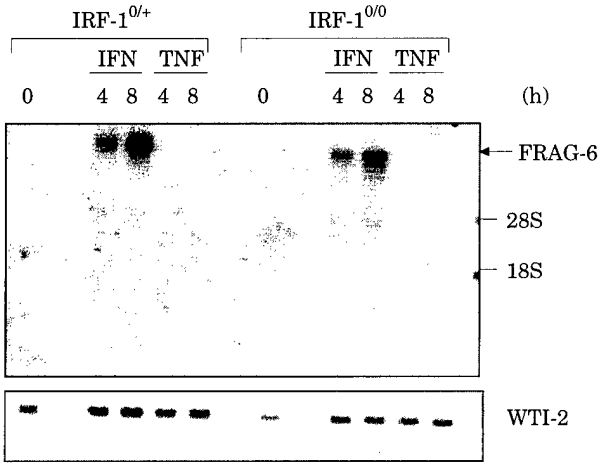

Figure 5. FRAG-6 is induced by IFNs but not by TNF.

Heterozygous or IRF-1$^{0/0}$ cells were treated with rhuIFN-α2/α1 (500 IU/ml) or muTNF (30 ng/ml) as indicated and total RNA was isolated. 15 μg were fractionated on a 1% denaturing agarose gel, transferred to nylon membrane, and hybridized with a ^{32}P-αdCTP-labelled FRAG-6 cDNA probe. In order to ensure equal RNA loading, the filter was stripped and re-hybridized with a ^{32}P-αdCTP-labelled WTI-2 cDNA, a constitutively expressed cDNA fragment that was isolated in our laboratory, whose expression is not modulated by IFNs (accession number gb/U51904).

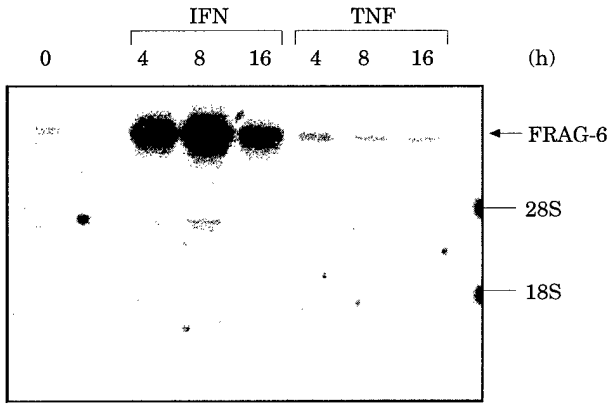

Figure 6. Induced expression of FRAG-6 mRNA in tumour cell line.

L-929 cells were treated with rhuIFN-α2/α1 (500 IU/ml) or muTNF (30 ng/ml) as indicated and total RNA was extracted. 15 μg total RNA were fractionated on a 1% denaturing agarose gel, transferred to nylon membrane, and hybridized with a ^{32}P-αdCTP-labelled FRAG-6 cDNA probe.

compared the in vivo expression of FRAG-6 in wild-type or in IRF-1$^{0/0}$ mice treated with imiquimod, no significant differences in the steady-state levels of FRAG-6 mRNA were observed in the spleen, lung, heart, or thymus (data not shown). However, FRAG-6 induction by imiquimod was impaired in mice deficient for STAT-1 or in mice deficient for the type I IFN receptor with no detectable levels of mRNA in the spleen of these animals as compared with their wild type counterparts (Fig. 7).

DISCUSSION

The mechanism of action of interferons, especially those involved in the inhibition of viral replication is still obscure. Likewise, the physiological role of the transcription factor IRF-1 within the IFN-triggered signalling cascades has not been fully characterized. Taking advantage of cells deficient for IRF-1, we have used the differential display RT-PCR[29,30] to search for new genes that are induced by IFNs and whose expression might be modulated by IRF-1.

Seven cDNA fragments were recovered from the gel, re-amplified, cloned, and sequenced. Homology search was performed using the blast[31] program available at the NCBI Web Site. Five of the sequences show homology to human or mouse ESTs (accession number U47130, U47131, U51904, U46836 and AF074322). One fragment showed high homology (96% similarity) to mouse procollagen type V α-2. Another fragment named FRAG-6, with 576 base pairs showed no significant homology to known sequences and appeared to be differentially expressed in IFN-treated cells. Within this fragment we can identify a putative ORF with two potential initiation codons at positions 53–55 and 164–166 of the nucleotide sequence. Interestingly, since the sequence of anchored oligo $T_{11}VA$ or its complementary sequence was not found within the cloned nucleotide sequence, but rather the random 10-mer 5′-CTTGATTGCC-3′ appears in both the 5′ and the 3′ ends, we cannot speculate that a 3′ untranslated region (UTR) of a cDNA was generated by differential display strategy. This hypothesis is further supported by the absence of a consensus polyadenylation signal within the cloned sequence and also, by the presence of a long internal putative ORF. When analysis of the primary structure of this ORF was performed using the program PSORT/VsII for prediction of protein localization (http://psort.nibb.ac.jp/), a potential nuclear localization signal containing basic residues, Lys-Arg-Arg-Arg, was found at position 97–100 of the predicted coding sequence, according to Chelsky *et al.*[32] Based on Southern blot analysis, the FRAG-6 gene appears to be a single copy gene.

Its differential expression in response to IFN was confirmed by Northern blot analysis where the cDNA fragment hybridized strongly with an mRNA of approximately 6–9 kb in IFN-α-treated wild-type or IRF-1$^{0/0}$ cells, but not in control, untreated cells, suggesting a tightly regulated promoter.

IFNs and other pro-inflammatory cytokines, namely TNF and IL-1, share some of their biological activities, probably because they are capable of inducing a common set of genes.[33–37] In the case of FRAG-6, there was no detectable increase in the steady-state level of mRNA in either wild-type or IRF-1$^{0/0}$ cells treated with TNF or IL-1. The lack of

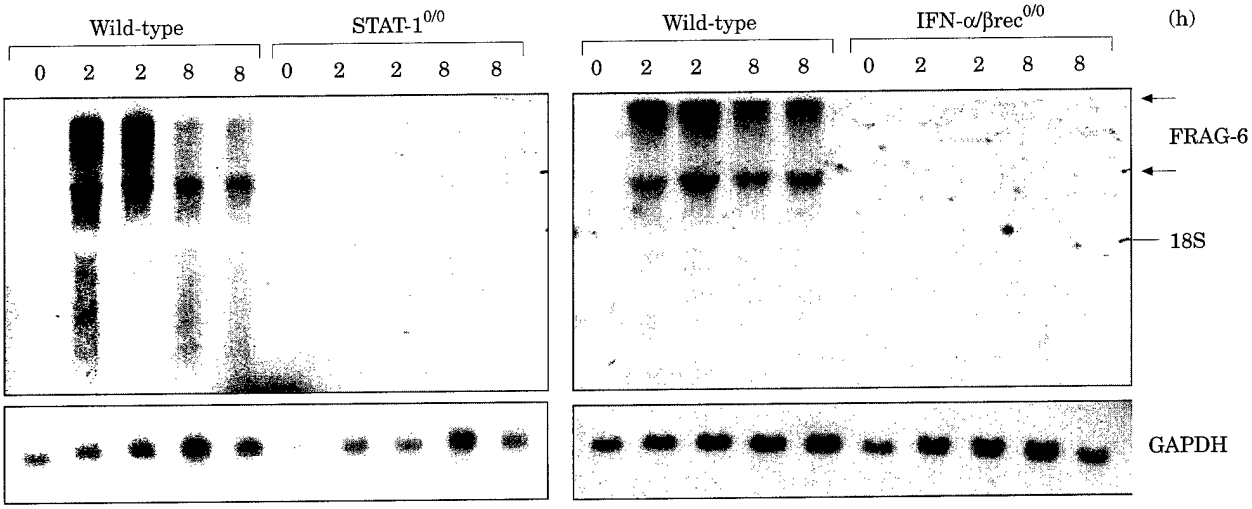

Figure 7. In vivo induction of FRAG-6 by the IFN inducer imiquimod requires STAT-1 and the type I IFN receptor.

Wild type, STAT-1$^{0/0}$ or IFN α/β receptor$^{0/0}$ mice were treated orally with imiquimod (1 mg/kg). Total RNA was prepared from spleen harvested from untreated (0) or from animals treated orally for two or eight hours. 15 μg total RNA were fractionated on a 1% denaturing agarose gel, transferred to nylon membrane, and hybridized with a ^{32}P-αdCTP-labelled FRAG-6 cDNA probe. Arrows indicate the major and minor mRNA transcripts of 6–9 and 4.5 kb, respectively. The filter was then stripped and re-hybridized with a hybridized with a ^{32}P-αdCTP-labelled GAPDH cDNA probe to ensure equal loading of RNA.

induction by TNF or IL-1 suggests that activation of FRAG-6 gene expression is probably not mediated by NF-κB, known as a major component in both TNF and IL-1 signalling cascade,[38–43] although a recent report suggests a second signalling pathway activated by these cytokines.[38]

The induction of FRAG-6 gene expression appears to be a primary cellular response to IFNs. First, the IFN-induced expression of FRAG-6 was not abolished upon induction in the presence of the protein synthesis inhibitor anisomycin, suggesting that protein synthesis is not required for gene activation (data not shown). Moreover, ablation of the IRF-1 gene did not impair the induction of FRAG-6, and both IFN-α and IFN-γ were capable of inducing FRAG-6 gene expression in cells deficient for IRF-1. We have also investigated the induction of FRAG-6 gene expression in vivo. It is well documented that oral treatment of mice with the immune modulator imiquimod leads to higher levels of circulating IFNs as well as the accumulation of mRNA from several ISGs in various organs.[28,43] When we measured the levels of FRAG-6 mRNA in the spleen, lung, heart and thymus of wild-type or IRF-1$^{0/0}$ mice that were treated with imiquimod, there was a comparable and readily detectable induction of FRAG-6 gene expression (data not shown). However, there was no induced expression of FRAG-6 in the spleen of STAT-1$^{0/0}$ or type I IFN receptor$^{0/0}$ mice treated with the drug. Hence, induction of FRAG-6 gene expression by IFN does require an IFN-triggered signal, likely to be the TYK-2/JAK-1 pathway for the activation of STAT-1 and the formation of ISGF-3 complex.[6]

It is noteworthy that, at least in vivo, a shorter mRNA transcript of about 4.5 kb is concomitantly induced with the main transcript of 6–9 kb identified in MEFs. At this point, we are not certain whether this faster migrating mRNA results from a second transcript, generated by an alternative splicing. Further experiments must be done in order to ascertain if the structure of the 4.5-kb transcript is derived from a transcriptional mechanism.

A common feature for several interferon-stimulated genes is the presence of an IFN-stimulated responsive element (ISRE) for type I IFN and a gamma activated sequence (GAS) for type II IFN, present within their promoter region.[45,46] The fact that both type I and type II IFNs induce FRAG-6 gene expression suggests that its promoter region contains these two elements. Thus, the pattern of induction of FRAG-6 follows that of a typical interferon-stimulated gene such as ISG-15, ISG-54, 6–16, etc.[47–50]

Detailed inspections of the putative ORF within FRAG-6 cDNA have not revealed any potentially functional domain that could help us in speculating about possible functions of FRAG-6. Therefore, further studies are now required in order to provide some insight into the physiology of FRAG-6. It will now be necessary to search for the complete coding sequence of FRAG-6 mRNA as well as to determine the structure of the FRAG-6 gene and its promoter region.

MATERIALS AND METHODS

Differential display

Primary cultures of wild type or IRF-1-deficient mouse embryonic fibroblasts (MEFs) were treated or not treated for 4 h with 500 IU/ml of rhuIFN-α2/α1, a recombinant human IFN that is fully active in mouse cells.[51] Total RNA was extracted by standard protocols.[52] 200 ng of DNase-treated total RNA derived from untreated or IFN-α-treated wild type or IRF-$1^{0/0}$ cells were reverse transcribed in a mix containing 20 μM dNTPs, 10 mM DTT, 200 U MMLV-reverse transcriptase and RT-buffer, in the presence of 25 pmoles of anchored oligo T_{11}VA, where V corresponds to A, C or G. One tenth of each cDNA first strand reaction was used as template for the differential display RT-PCR using the same anchored oligo plus a random 10-mer as described.[30] The sequences of the random 10-mer used in the differential-display RT-PCR are P13 (5′-CTGATCCATG-3′), P14 (5′-CTGCTCTCAA-3′), and P15 (5′-CTTGATT GCC-3′). The PCR products were fractionated on a 6% acrylamide sequencing gel or alternatively, some samples were fractionated on 1.2% agarose gel. Bands of interest were recovered from the gel and re-amplified by PCR with the same primers and same conditions used for differential display, except that no radioactive dCTP was added, in a final volume of 40 μl. The re-amplified cDNAs were cloned into pUC18 using the SureClone ligation kit (Amersham Pharmacia Biotech, Sweden).

Sequencing and analysis of cDNA fragments

Cloned cDNA fragments were sequenced using the pUC/M13 forward or reverse sequencing primer using the A.L.F. DNA Sequencer (AmershamPharmacia Biotech, Sweden). Sequences were analysed using the blast[31] program for nucleotides and proteins available at the National Center of Biotechnology Information (NCBI).

Cell culture of primary mouse embryonic fibroblast (MEF) cells and treatment with cytokines

Heterozygous IRF-1 mice[23] were intercrossed and MEFs were prepared from the resulting 12–14 day-old embryos. Individual embryos were transferred to tissue culture dishes containing 10 ml PBS and primary fibroblasts were obtained. Cells were genotyped by RT-PCR from total RNA derived from IFN-induced cells. The heterozygous cells used in some experiments were compared to wild type cells and shown to respond equally well to IFNs. Spontaneously immortalized embryonic fibroblasts derived from wild type, or IRF-$1^{0/0}$ embryos were kindly provided by Drs Yi-Li Yang and Charles Weissmann, University of Zurich, Switzerland. MEFs and L-929 cells were grown in DMEM containing 10% FBS and allowed to grow to near-confluence. For induction, cells were treated with 500 IU/ml of rhuIFN-α2/α1, 100 IU/ml muIFN-γ, 30 ng/ml recombinant muTNF-α or 1 ng/ml recombinant muIL-1α (all cytokines from R&D Systems, Minneapolis, MN, USA) as indicated in each figure.

Mice

All mouse lineages were of a pure 129/Sv background. Mice with disrupted IRF-1 gene were generated by homologous recombination with a targeted deletion in which a gene segment comprising exon 5 and parts of exons 4 and 6 (308 bp of coding sequence) was replaced by an extraneous DNA sequence.[23] Mice deficient in the STAT-1 gene were kindly provided by David Levy, New York University Medical Center, NY, USA.[13] Mice lacking the type I IFN receptor gene were generated by homologous recombination with a targeted disruption on exon 3.[53] All animals were housed in sterile cages and maintained with sterile food and acidified autoclaved water.

In vivo induction of FRAG-6

The compound imiquimod [S-26308 or R-837, 1-(-2-methyl-propyl)-1H-imidazol[4,5-c] quinoline-4-amine] was provided by 3M Pharmaceuticals (St. Paul, MN, USA). Mice were treated orally with 1 mg/kg (20 μl of a 1 mg/ml stock solution). Animals were anaesthetized and killed at different time points and organs were collected and immediately frozen in liquid nitrogen until total RNA extraction.

Northern blot analysis

For RNA isolation, cell cultures were washed twice in PBS (phosphate buffer saline pH 7.4) before RNA extraction. Organs of imiquimod-treated animals were frozen in liquid nitrogen immediately after animals were sacrificed and homogenized in 3 ml of denaturing solution. RNA isolation was performed as described.[52] 15 μg of total RNA were fractionated through a 1% denaturing agarose gel and transferred by capillarity to a Nylon filter (AmershamPharmacia Biotech, Sweden) by standard protocols.[54] Pre-hybridization, hybridization and washes were performed as described previously.[55] ^{32}Pα-dCTP-labelled cDNA probes were prepared using the Rediprime Random primer labeling system (AmershamPharmacia Biotech, Sweden) according to the manufacturer's protocol. The FRAG-6 probe corresponds to the entire 576-bp cDNA fragment, subcloned into pUC18. The rat GAPDH probe[56] corresponds to a 479-bp *Xho*II cDNA fragment subcloned into pSP64 (prepared by G. R. Müller, Institute of Molecular Biology II, Zurich, deceased). The murine WTI-2 cDNA probe (accession number gb/U51904) was cloned in our laboratory and showed constitutive expression not affected by cytokine stimulation. Autoradiography was performed at −70°C, using intensifying screen.

Southern blot analysis of the FRAG-6 gene

Genomic DNA was obtained from fourteen-day old mouse embryos, and digested with restriction enzymes recognizing hexanucleotide sequences (all restriction endonucleases from New England Biolabs). After electrophoresis was done through a 0.8% agarose gel, DNA was transferred by capillarity to a Nylon membrane (AmershamPharmacia Biotech, Sweden) as previously described.[54] The FRAG-6 cDNA probe was the same as described above for Northern blot analysis.

Acknowledgements

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REFERENCES

1. Vilcek J, Sen G (1996) IFNs and others cytokines. In: Fields BN, Knipe DM, Howley PM, Chanok RM, Melnick JL, Monath TP, Roizman B, Straus SE (eds) Virology. Lippincott-Raven Publishers, Philadelphia, pp 375–399.

2. Darnell JE Jr, Kerr IM, Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415–1421.

3. Fu XY, Kessler DS, Veals SA, Levy DE, Darnell JEJ (1990) ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. Proc Natl Acad Sci USA 87:8555–8559.

4. Kessler DS, Levy DE, Darnell JEJ (1988) Two interferon-induced nuclear factors bind a single promoter element in interferon-stimulated genes. Proc Natl Acad Sci USA 85:8521–8525.

5. Kessler DS, Veals SA, Fu XY, Levy DE (1990) Interferon-alpha regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. Genes Dev 4: 1753–1765.

6. Levy DE, Kessler DS, Pine R, Darnell JEJ (1989) Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro. Genes Dev 3: 1362–1371.

7. Levy DE, Raz R, Durbin JE, Bluyssen H, Muzaffar R, Pisharody S (1995) Cytoplasmic transcription factors: mediators of cytokine signaling. Agents Actions Suppl 47:79–85.

8. Darnell JEJ, Kerr IM, Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415–1421.

9. Greenlund AC, Farrar MA, Viviano BL, Schreiber RD (1994) Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). EMBO J 13:1591–1600.

10. Silvennoinen O, Ihle JN, Schlessinger J, Levy DE (1993) Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature 366:583–585.

11. Muller M, Briscoe J, Laxton C, Guschin D, Ziemiecki A, Silvennoinen O, Harpur AG, Barbieri G, Witthuhn BA, Schindler C (1993) The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction [see comments]. Nature 366:129–135.

12. Watling D, Guschin D, Muller M, Silvennoinen O, Witthuhn BA, Quelle FW, Rogers NC, Schindler C, Stark GR, Ihle JN (1993) Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway [see comments]. Nature 366:166–170.

13. Durbin JE, Hackenmiller MC, Simon MC, Levy DE (1996) Targeted disruption of the mouse STAT1 gene results in compromised innate immunity to viral disease. Cell 84:443–450.

14. Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greelund AC, Campbell D, Carver-Moore K, DuBois RN, Clark R, Aguet M, Schreiber RD (1996) Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell 84:431–442.

15. Chang CH, Guerder S, Hong SC, van Ewijk W, Flavell RA (1996) Mice lacking the MHC class II transactivator (CIITA) show tissue-specific impairment of MHC class II expression. Immunity 4:167–178.

16. Steimle V, Siegrist CA, Mottet A, Lisowska-Grospierre B, Mach B (1994) Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. Science 265:106–109.

17. Miyamoto M, Fujita T, Kimura Y, Maruyama M, Harada H, Sudo Y, Miyata T, Taniguchi T (1988) Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell 54:903–913.

18. Pine R, Decker T, Kessler DS, Levy DE, Darnell JEJ (1990) Purification and cloning of interferon-stimulated gene factor 2 (ISGF2): ISGF2 (IRF-1) can bind to the promoters of both beta interferon-and interferon-stimulated genes but is not a primary transcriptional activator of either. Mol Cell Biol 10:2448–2457.

19. Pine R, Canova A, Schindler C (1994) Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN alpha and IFN gamma, and is likely to autoregulate the p91 gene. EMBO J 13:158–167.

20. Fujita T, Sakakibara J, Sudo Y, Miyamoto M, Kimura Y, Taniguchi T (1988) Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN-beta gene regulatory elements. EMBO J 7:3397–3405.

21. Reis LF, Harada H, Wolchok JD, Taniguchi T, Vilcek J (1992) Critical role of a common transcription factor, IRF-1, in the regulation of IFN-beta and IFN-inducible genes. EMBO J 11: 185–193.

22. Kamijo R, Harada H, Matsuyama T, Bosland M, Gerecitano J, Shapiro D, Le J, Koh SI, Kimura T, Green SJ (1994) Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science 263:1612–1615.

23. Reis LF, Ruffner H, Stark G, Aguet M, Weissmann C (1994) Mice devoid of interferon regulatory factor 1 (IRF-1) show normal expression of type I interferon genes. EMBO J 13:4798–4806.

24. Kimura T, Nakayama K, Penninger J, Kitagawa M, Harada H, Matsuyama T, Tanaka N, Kamijo R, Vilcek J, Mak TW (1994) Involvement of the IRF-1 transcription factor in antiviral responses to interferons. Science 264:1921–1924.

25. Briken V, Ruffner H, Schultz U, Schwarz A, Reis LF, Strehlow I, Decker T, Staeheli P (1995) Interferon regulatory factor 1 is required for mouse Gbp gene activation by gamma interferon. Mol Cell Biol 15:975–982.

26. White LC, Wright KL, Felix NJ, Ruffner H, Reis LF, Pine R, Ting JP (1996) Regulation of LMP2 and TAP1 genes by IRF-1 explains the paucity of CD8+ T cells in IRF-1-/- mice. Immunity 5:365–376.

27. Matsuyama T, Kimura T, Kitagawa M, Pfeffer K, Kawakami T, Watanabe N, Kundig TM, Amakawa R, Kishihara K, Wakeham A (1993) Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. Cell 75:83–97.

28. Reiter MJ, Testerman TL, Miller RL, Weeks CE, Tomai MA (1994) Cytokine induction in mice by the immunomodulator imiquimod. J Leukoc Biol 55:234–240.

29. Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967–970.

30. Liang P, Averboukh L, Pardee AB (1993) Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acids Res 21:3269–3275.

31. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410.

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SYNERGISTIC PROLIFERATION AND ACTIVATION OF NATURAL KILLER CELLS BY INTERLEUKIN 12 AND INTERLEUKIN 18

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We investigated the effects of IL-12 and IL-18 on unstimulated murine splenocytes and observed that the two cytokines strongly synergized for their proliferation, whereas IL-12 and IL-18 alone were essentially inactive in this respect. Phenotypical and functional analyses of cells proliferating in response to IL-12 and IL-18 revealed that large granular Ly-49C$^+$ DX5$^+$ CD3$^-$ NK blasts were expanded in these cultures and that they displayed cytotoxic activity against Yac-1 cells, a murine NK cell target. Further analyses indicated three major differences between NK cells appearing in response to IL-12 and IL-18 and those derived in the presence of other NK cell growth factors, such as IL-2 or IL-15. First, a population of T-NK cells, i.e. expressing T cell (TCRαβ, CD3) and NK cell (Ly-49) markers, was detected amongst cells growing in IL-2 or IL-15 but not in cultures supplemented with IL-12 and IL-18. Second, most NK cells derived with IL-2 or IL-15 expressed the NK1.1 antigen, while those derived with IL-12 and IL-18 did not. Finally, striking differences were observed regarding cytokine production. Cells stimulated with IL-12 and IL-18 in combination, but not with IL-2 or IL-15, produced IFN-γ, IL-3, IL-6 and TNF. IFN-γ was not involved in the response of NK cells to IL-12 and IL-18, as indicated by experiments demonstrating that the combination of the two cytokines displayed similar effects on spleen cells from IFN-γR-knock-out mice. Receptor (IL-12Rβ1, IL-12Rβ2 and IL-18R) gene expression studies did not indicate that the mechanism underlying the synergy between IL-12 and IL-18 involved reciprocal induction of their receptors. Taken together, our results demonstrate that IL-12 and IL-18 exert striking synergistic activities for NK cell proliferation and activation, distinct from those induced by IL-2 or IL-15.

Interleukin (IL)12 is a heterodimeric cytokine produced by B cells, macrophages and dendritic cells that plays a well-known immunoregulatory role by promoting cell-mediated immunity, in particular through upregulation of IFN-γ production.[1] Another phagocyte-derived cytokine displaying a potent IFN-γ-inducing activity, provisionally called IGIF (IFN-γ-inducing factor), was recently cloned[2] and re-named IL-18.[3]. Interestingly, IL-12 and IL-18 were found to synergize for IFN-γ production by human and murine T cells.[4–6] We and others unmasked a similar synergy between the two cytokines for IFN-γ production by B cells. Thus, murine naive B cells stimulated with anti-CD40 mAb and IL-4 produce huge amounts of IFN-γ in response to the combination of IL-12 and IL-18.[7] Similarly, activated B cells isolated from mice with chronic graft-vs-host disease produce IFN-γ after stimulation with both cytokines.[8] In these two experimental settings, the synergy between IL-12 and IL-18 for IFN-γ induction accounted for the strong inhibition of immunoglobulin (Ig) production exerted by the two cytokines.[7,8]

These results, by indicating that the synergy between IL-12 and IL-18 was operating on at least two distinct lymphocyte subsets, prompted us to investigate further the effects of IL-12 and IL-18 on normal murine splenocytes. This approach led to the intriguing observation that the two cytokines strongly synergized for the proliferation of unstimulated murine spleen cells, whereas IL-12 and IL-18 alone were inactive in this respect.

Characterization of splenocytes proliferating in response to IL-12 and IL-18 indicated that they belonged to the natural killer (NK) cell lineage and

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that these NK cells were phenotypically and functionally different from those derived in the presence of IL-2 or IL-15.

RESULTS

Synergistic proliferation and activation of NK cells induced by IL-12 and IL-18

Nylon wool-filtered splenocytes were cultured in the presence or absence of IL-12 and/or IL-18. As indicated in Figure 1A, addition of IL-12 and IL-18 alone had no effect on cell proliferation. By contrast, the combination of the two cytokines induced as strong a proliferation as that observed with IL-2 (50 U/ml), with half-maximal stimulation being obtained with 10 pg/ml of IL-12 and 50 ng/ml of IL-18. To characterize the cells responding to IL-12 and IL-18, we performed FACS analyses with T cell (CD3), B cell (CD19), monocyte (Mac-1α) and NK cell (Ly-49C, DX-5) markers. These experiments revealed that a population of large (high FSC), granular (high SSC), Ly-49C$^+$ DX5$^+$ CD3$^-$ NK blasts was present in cultures stimulated with IL-12 and IL-18 (Fig. 1B and 1C). Similar results were obtained with nylon wool-filtered splenocytes prepared from C57BL/6 and 129 mice.

In order to confirm that cells proliferating in response to the combination of IL-12 and IL-18 belonged to the NK cell lineage, we performed cytotoxity assays using NK-sensitive Yac-1 cells as targets and nylon wool-filtered splenocytes as effectors. Spleen cells cultured with IL-12 and IL-18 in combination, exerted a strong cytotoxic activity, comparable to that observed with cells supplemented with IL-2. Again, a strong synergy between IL-12 and IL-18 was unmasked (Table 1). Cells proliferating in response to IL-12 and IL-18 were sorted by flow cytometry on the basis of their FSC/SSC pattern (upper-right region in Figure 1B) and they also displayed a potent cytotoxic activity on Yac-1 targets (Table 1).

Surface markers analysis of cells proliferating in response to IL-12 and IL-18, IL-2 or IL-15

The phenotype of cells proliferating in response to the combination of IL-12 and IL-18 was further studied and compared to that of cells cultured in the presence of IL-2 or IL-15, two other well-known NK cell growth factors. Double-labelling experiments revealed that Ly-49C$^+$ cells proliferating in response to IL-12 and IL-18 consisted of a homogeneous population of Ly-49C$^+$ NK1.1$^-$ CD3$^-$ NK cells (Figs 2A and 2C). While a population of Ly-49C$^+$ blasts was also detected in cultures supplemented with IL-2, these cells differed from those appearing in cultures supplemented with IL-12 and IL-18 regarding CD3 and

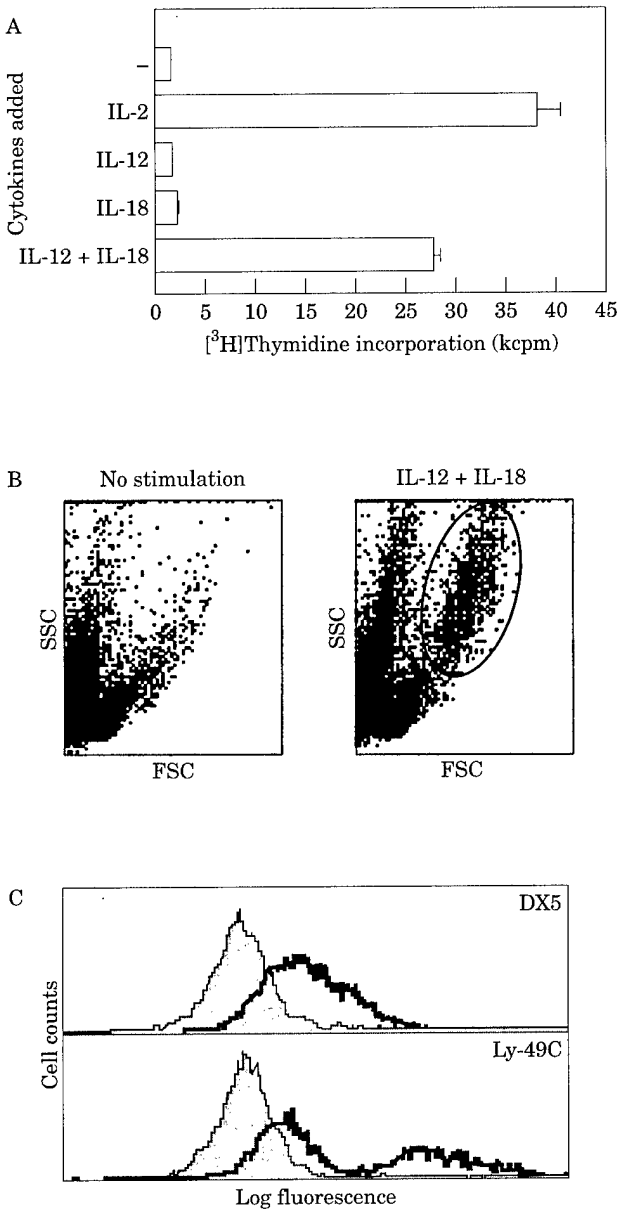

Figure 1. Proliferation of NK cells.

Nylon wool-filtered Balb/c splenocytes were cultured in the presence of the indicated cytokine(s), as described in Materials and Methods. Proliferations (mean kcpm ± SEM) were measured by [^{3}H]-thymidine incorporation (A). The plots shown in (B) indicate the distribution of cells (cultured without or with IL-12 and IL-18) along the forward scatter channel (FSC) and the side scatter channel (SSC) on flow cytometric analysis. FACS analyses (DX5, Ly-49C) shown in (C) were gated on large granular cells appearing in response to IL-12 and IL-18, as defined by the area depicted in (B). Shaded areas correspond to labelling obtained with an isotype-matched control mAb. The data presented in A, B and C are representative of 12, 10 and 8 experiments, respectively.

NK1.1 expression. Thus, most (80%) Ly-49C$^+$ cells present in IL-2 cultures were NK1.1$^+$ (Fig. 2B) and could be categorized according to CD3 expression into a Ly-49C$^+$ CD3$^+$ subset of T-NK cells and a Ly-49C$^+$ CD3$^-$ subset of NK cells (Fig. 2D). These experiments

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TABLE 1. Lytic activity of NK cell populations

Cells	E/T ratio	Cytokines added				
		None	IL-12	IL-18	IL-12 IL-18	IL-2
Total N^+ cells	100	0	19	0	58	62
	30	0	4	0	41	36
	10	0	0	0	18	33
	3	0	0	0	3	16
	1	0	0	0	2	6
Sorted cells*	30	ND	ND	ND	69	61
	10	ND	ND	ND	27	52
	3	ND	ND	ND	17	27
	1	ND	ND	ND	6	13

Figures represent percentage of specific lysis (SD<10%) of ^{51}Cr-labelled Yac-1 cells (1×10^3 cells) measured in quadruplicate (for total N^+ cells) or triplicate (for sorted cells) experiments; effector cells used in the cytotoxity assays have been previously stimulated for three days with the indicated cytokines, as described in Materials and Methods.

*Cells proliferating in response to IL-12 and IL-18 were sorted by flow cytometry on the basis of their FSC/SSC pattern (upper-right region in Fig. 1B).

E/T: effector/target. N^+; nylon wool-filtered.

These data are representative of four and two distinct experiments performed on total N^+ and sorted cells, respectively.

also indicated that cultures supplemented with IL-2 contained a population of $CD3^+$ T cell blasts (Fig. 2D), that were absent in cultures stimulated with IL-12 and IL-18.

We also investigated the effects of IL-15 in our experimental setting. IL-15 strongly stimulated the proliferation of nylon-wool filtered splenocytes (stimulation index: 101 and 142 in two experiments) and FACS analyses indicated that cells proliferating in response to IL-15 were phenotypically similar to those responding to IL-2, with three distinct populations being identified, namely NK cells ($Ly\text{-}49C^+$ $CD3^-$), T cells ($Ly\text{-}49C^-$ $CD3^+$) and T-NK cells ($Ly\text{-}49C^+$ $CD3^+$). Again, most (89%) $Ly\text{-}49C^+$ cells induced by IL-15 were $NK1.1^+$ (data not shown).

Cytokine production in cultures stimulated with IL-12 and IL-18, IL-2 or IL-15

We measured the concentrations of various cytokines in the supernatants from cells stimulated with IL-12 and/or IL-18 and compared them to those determined in cultures supplemented with IL-2 or IL-15. As indicated in Figure 3, IL-12 and IL-18 were essentially inactive on their own but strongly synergized, not only for IFN-γ production but also for IL-3, IL-6 and TNF induction. By contrast, IL-2 and IL-9 were not detected in cultures stimulated with IL-12 and IL-18. Interestingly, addition of IL-2 or IL-15 had no significant effect on the production of IL-3, IL-6, IL-9, IFN-γ and TNF.

In order to determine whether IFN-γ detected in cultures supplemented with IL-12 and IL-18 was produced by cells proliferating in response to these cytokines, we performed cytoplasmic IFN-γ staining experiments by FACS analyses. These experiments confirmed that large granular cells appearing in response to IL-12 and IL-18, and not those proliferating in response to IL-2 or IL-15, produced IFN-γ (Fig. 4). More specifically, double-labelling experiments indicated that $Ly\text{-}49C^+$ blasts produced IFN-γ in response to IL-12 and IL-18 (data not shown).

IFN-γ does not mediate the proliferation and activation of NK cells induced by IL-12 and IL-18

Next, we evaluated whether IFN-γ was involved in the proliferation of NK cells in response to IL-12 and IL-18. Although large amounts of IFN-γ were produced by cells stimulated with IL-12 and IL-18, two lines of evidence argued against the possibility that IFN-γ was involved in the proliferation of NK cells induced by the two cytokines. First, addition of IFN-γ to spleen cells did not induce NK-cell proliferation. Second, splenocytes isolated from IFN-γR knock-out 129 mice responded similarly to IL-12 and IL-18 compared to wild type 129 mice, both in terms of proliferation and cytotoxicity (Fig. 5).

IL-12R and IL-18R gene expression studies

We wondered whether the synergy between IL-12 and IL-18 for the proliferation and activation of NK cells could be explained by reciprocal induction of their receptor. We addressed this issue by RT-PCR analyses performed on cDNA synthesized after RNA extraction from cells cultured in the absence or presence of IL-12 and/or IL-18. Specific murine IL-12Rβ1, IL-12Rβ2 and IL-18R oligonucleotides were used as primers. For the latter, we assumed that the murine IL-18R gene was identical to the previously cloned murine IL-1R related protein (IL-1Rrp) gene, as recently described for human cells.[9] As indicated in Figure 6, IL-12Rβ1, IL-12Rβ2 and IL-18R gene expression was detected in unstimulated cells. Addition of IL-12 and IL-18 increased IL-12Rβ, IL-12Rβ1 and IL-12Rβ2 gene expression but did not influence that of IL-18R. These results did not support the hypothesis that the mechanism underlying the synergy between IL-12 and IL-18 involved reciprocal induction of their receptors.

DISCUSSION

NK cells play a critical role in innate immunity against pathogens, virus-infected cells and tumors through MHC-unrestricted cytotoxicity and their capacity to produce cytokines. Their function is a tightly regulated process, involving activating (CD16, CD2, CD28 and NKR-P1) and inhibitory (Ly-49,

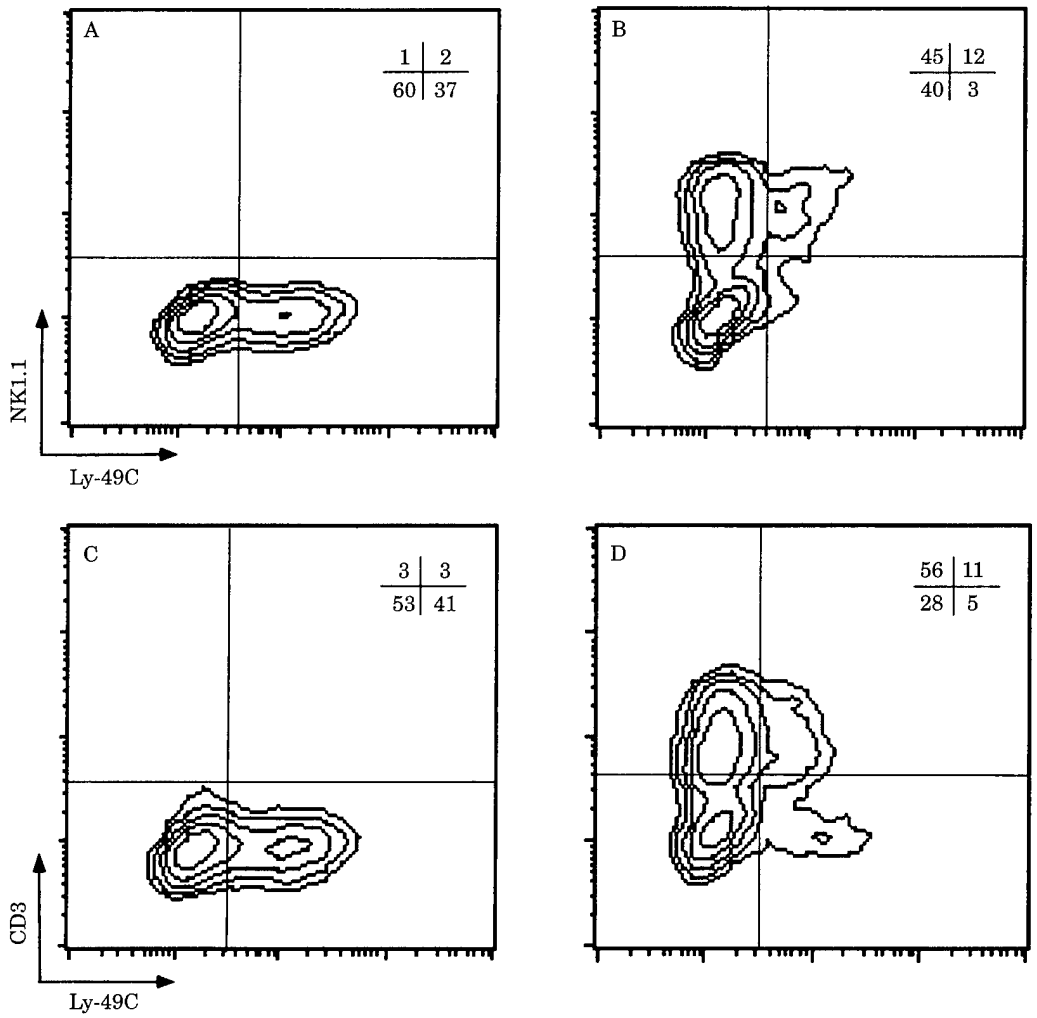

Figure 2. Surface markers analysis.

Nylon wool-filtered C57BL/6 splenocytes were cultured with IL-12 and IL-18 (A and C) or with IL-2 (B and D), as described in Materials and Methods. After 3 days of culture, cells were double-labelled with fluorochrome-conjugated mAb (anti-NK1.1 and anti-Ly-49C in Panels A and B; anti-CD3 and anti-Ly-49C in Panels C and D). FACS analyses were gated on large granular cells appearing in response to IL-12 and IL-18 or to IL-2. Isotype-matched control mAb, using the same cut-off fluorescence values, stained no more than 0.5% of the cells (data not shown). The data are representative of two distinct experiments.

KIR) membrane receptors, the latter recognizing polymorphic MHC class I molecules.[10,11] Several cytokines stimulate NK cell proliferation and activation, in particular IL-2,[12] IL-12,[13,14] IL-15,[15,16] and IL-18.[3,17–19] The experiments presented here demonstrate the existence of a striking synergy between IL-12 and IL-18, not only for the proliferation but also for the activation of NK cells, in particular for cytotoxicity and cytokine production.

Functional comparison of cells appearing in response to the combination of IL-12 and IL-18 on the one hand, and in response to IL-2 or IL-15 on the other hand, revealed several differences. First, Ly-49$^+$ NK cells appearing in response to IL-12 and IL-18 were NK1.1$^-$, while most Ly-49$^+$ NK cells detected in IL-2- or IL-15-supplemented cultures were NK1.1$^+$. NK1.1, the prototypical mouse NK cell antigen,[20,21] is a member of the family of NKR-P1 NK cell receptors.[22] Although its function is currently unknown, some data indicate that NK1.1 plays a role in target recognition[23] and functions as an activating signal. Regarding NK1.1 expression, our results contrast with those published recently by Tomura *et al.*, suggesting that IL-18 plays an obligatory role in inducing NK1.1$^+$ NK cells from murine CD4$^-$ CD8$^-$ sIg$^-$ Ia$^-$ splenocytes.[19] In our experimental setting, IL-2 and IL-15 were capable, on their own, without addition of IL-18, to stimulate the proliferation of NK1.1$^+$ NK cells. The reasons for this discrepancy are unclear but might be related to differences in the splenic cell preparations used to derive NK cells.

Another phenotypical difference between cultures derived in the presence of IL-12 and IL-18 and those derived with IL-2 or IL-15 was the presence, in the

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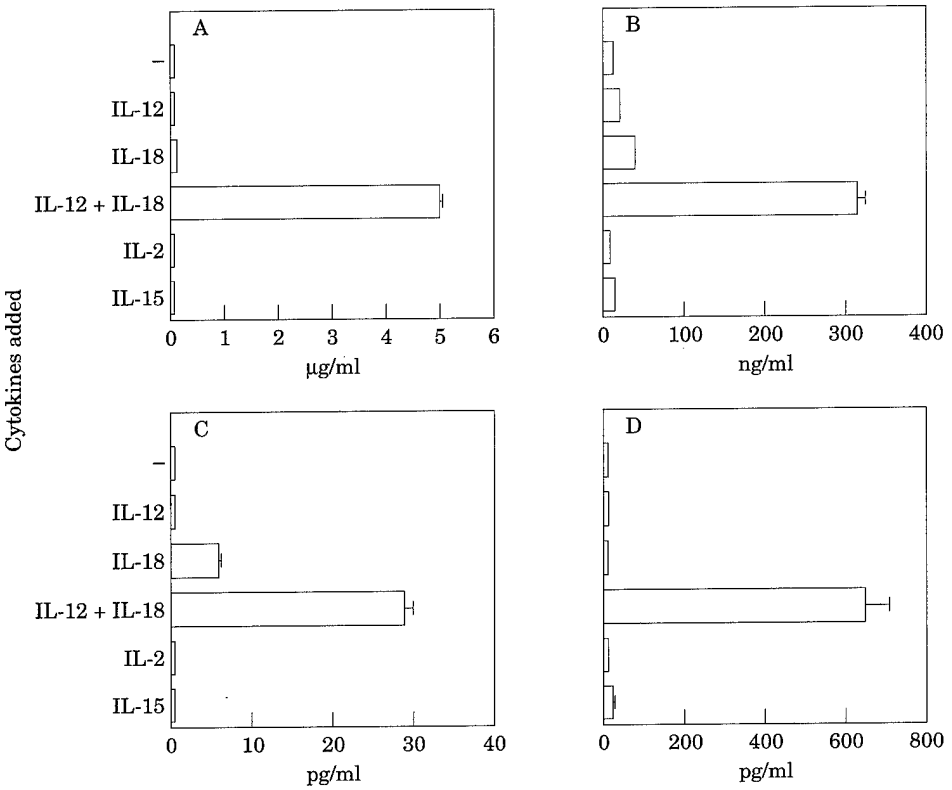

Figure 3. Cytokine production.

Nylon wool-filtered Balb/c splenocytes were cultured with the indicated cytokine(s), as described in Materials and Methods. (A) IFN-γ; (B) IL-3; (C) IL-6; (D) TNF concentrations (mean ± SEM) were measured in supernatants from duplicate or triplicate cultures by specific bioassays performed in quadruplicate cultures. The data are representative of 3 distinct experiments for IFN-γ and IL-6 and 2 experiments for IL-3 and TNF.

latter, of a population of T-NK cells (Ly-49$^+$ CD3$^+$) that was not detected amongst cells responding to IL-12 and IL-18. T-NK cells represent a small percentage of T cells expressing both T cell (TCRαβ, CD3) and NK cell (Ly-49) antigens.[24] Their tissue distribution, i.e. mainly in the bone marrow and the liver, where T-NK cells represent roughly one third of mature T cells, is puzzling and raises some questions regarding their function. T-NK cells have a restricted TCR repertoire, are cytotoxic and secrete large amounts of cytokines, in particular IL-4,[25] which may contribute to skew the immune response towards a Th2 phenotype.

A third difference between cells derived with IL-12 and IL-18 and those grown in IL-2 or IL-15 relates to their pattern of cytokine production, as only the former produced IFN-γ, IL-3, IL-6 and TNF. The role of IL-12 and IL-18 as inducers of cytokine production by NK cells is likely to be of critical importance in innate immune responses against tumour cells, pathogens and virus-infected cells, a process in which cytokine production is thought to be a pivotal physiological event.

We examined several possible mechanisms that could mediate the effects of IL-12 and IL-18 on NK cells. The experiments performed in IFN-γR-knockout mice clearly demonstrated that IFN-γ is not involved in the synergy between the two cytokines. We also tested the possibility that IL-2 or IL-6 mediates NK activation induced by IL-12 and IL-18. Addition of blocking anti-IL2Rα (CD25) or anti-IL6 mAb to cultures stimulated by IL-12 and IL-18 failed to block the proliferative responses induced by the two cytokines (data not shown), thereby indicating that IL-2 and IL-6 were not likely to be involved in the process.

We next investigated whether the synergy between IL-12 and IL-18 was related to the induction by IL-12 of IL-18R gene expression, as recently demonstrated by Ahn *et al.* for IFN-γ production by an IL-12-responsive murine T cell line (2D6) and by naive murine T cells stimulated with anti-CD3 and anti-CD28 mAb, that were shown to express IL-18R after exposure to IL-12.[26] by RT-PCR analyses, we observed that IL-12Rβ1, IL-12Rβ2 and IL-18R were constitutively expressed on unstimulated nylon wool-filtered splenocytes, and not reciprocally induced by IL-12 and IL-18. It should be stressed, however, that this issue is difficult to address in our experimental setting, as NK cell precursors represent only a small

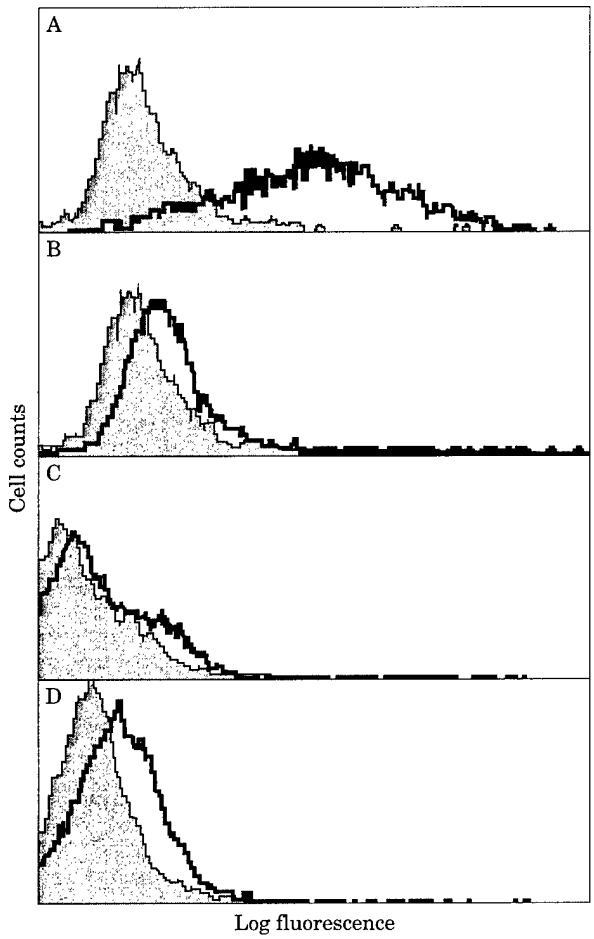

Figure 4. Intracytoplasmic IFN-γ staining.

Nylon wool-filtered Balb/c splenocytes were cultured with IL-12 and IL-18 (A and B), IL-2 (C) or IL-15 (D), as described in Materials and Methods, before being fixed, permeabilized with saponine and labelled with an FITC-conjugated anti-IFN-γ mAb. FACS analyses were gated on large granular cells appearing in response to the corresponding cytokine(s). (B) illustrates the reversion of intracytoplasmic IFN-γ staining in cells cultured with IL-12 and IL-18, by addition of exogenous IFN-γ during labelling procedure. Shaded areas correspond to autofluorescence. The data are representative of 5 distinct experiments.

percentage of splenocyte populations, thereby preventing correct interpretation of the results at the single cell level.

An alternative explanation for the synergy between the two cytokines might be related to "post-receptor" events. In this respect, IL-12 and IL-18 were recently found to differentially regulate the transcriptional activity of the human IFN-γ promoter in primary $CD4^+$ lymphocytes, with IL-18 causing direct activation of AP-1 whereas both AP-1 and STAT4 are required for IL-12-dependent IFN-γ promotor activation.[27]

Taken together, the results reported here demonstrate that the combination of IL-12 and IL-18 displays unique NK-cell activating properties, distinct from those exerted by other NK-cell growth and differentiation factors, in particular regarding cytokine production. These observations might be relevant not only for anti-tumour responses but also in autoimmune diseases. In the latter, an immunoregulatory role for NK cells has been recently postulated, e.g. through up-regulation of TGF-β production and, thereby, downregulation of inappropriate B-cell immune responses.[28]

MATERIALS AND METHODS

Cytokines

Murine rIL-12 and rIL-18 were purchased from R&D Systems Europe Ltd. (Abingdon, UK), as was human rIL-15 (which is active on murine cells). In some experiments, we used rIL-18 obtained from Peprotech EC Ltd. (London, UK). Murine rIFN-γ was a kind gift from Dr W. Fiers (University of Ghent, Belgium) and human rIL-2 was purchased from Eurocetus (Chiron Corporation, Amsterdam, The Netherlands). The following cytokine concentrations were used: 1 ng/ml for IL-12, 400 ng/ml for IL-18 obtained from R&D Systems Europe Ltd., 100 ng/ml for IL-18 obtained from Peprotech EC Ltd., 50 ng/ml for IL-15, 50 U/ml for IL-2, and 100 ng/ml for IFN-γ.

Purification and stimulation of splenocytes

Balb/c, C57BL/6, 129 and IFN-γR-knock-out 129 mice (G129) were bred in our animal facility. The latter, initially derived by Dr S. Huang and Dr M. Aguet[29] were obtained through the courtesy of Dr F. Brombacher (Max Planck Institute for Immunobiology, Freiburg, Germany). Splenocytes from 12-week-old female mice were prepared by Lymphoprep (Nycomed AS, Oslo, Norway) density gradient centrifugation. Adherent cells were removed by nylon wool filtration and the remaining cells were seeded at a density of 3×10^6 cells/well in 24-well plates in DMEM supplemented with 10% FCS, 50 μM 2-ME, 0.55 mM L-arginine, 0.24 mM L-asparagine and 1.25 mM L-glutamine. Cells were stimulated with various additives and harvested three days later for FACS analyses, cytotoxicity assays, and/or RNA extraction. Cytokine concentrations were measured in their supernatants. For proliferation assays, nylon wool-filtered splenocytes were stimulated in triplicate cultures for 48 h at a density of 2×10^5 cells/well in microtitre plates and pulsed overnight with [^{3}H]-thymidine (0.5 μCi/well).

FACS analyses

Cells were washed and incubated first with an anti-CD16/32 (FcγRIII) mAb (Pharmingen, San Diego, CA; 10 μγ/ml) (to prevent aspecific binding of mAb to FcγR) in a sodium phosphate (1 mM, pH=7.4) buffer containing 137 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM $MgCl_2$, 5 mM glucose, 4 mM $NaHCO_3$, 1 mM EDTA, 3% FCS and 20 mM sodium azide. Cells were further stained with a PE- and/or a FITC-conjugated mAb directed against surface antigens (or with a biotinylated mAb followed by streptavidine-PE; Pharmigen, San Diego, CA). Cells were

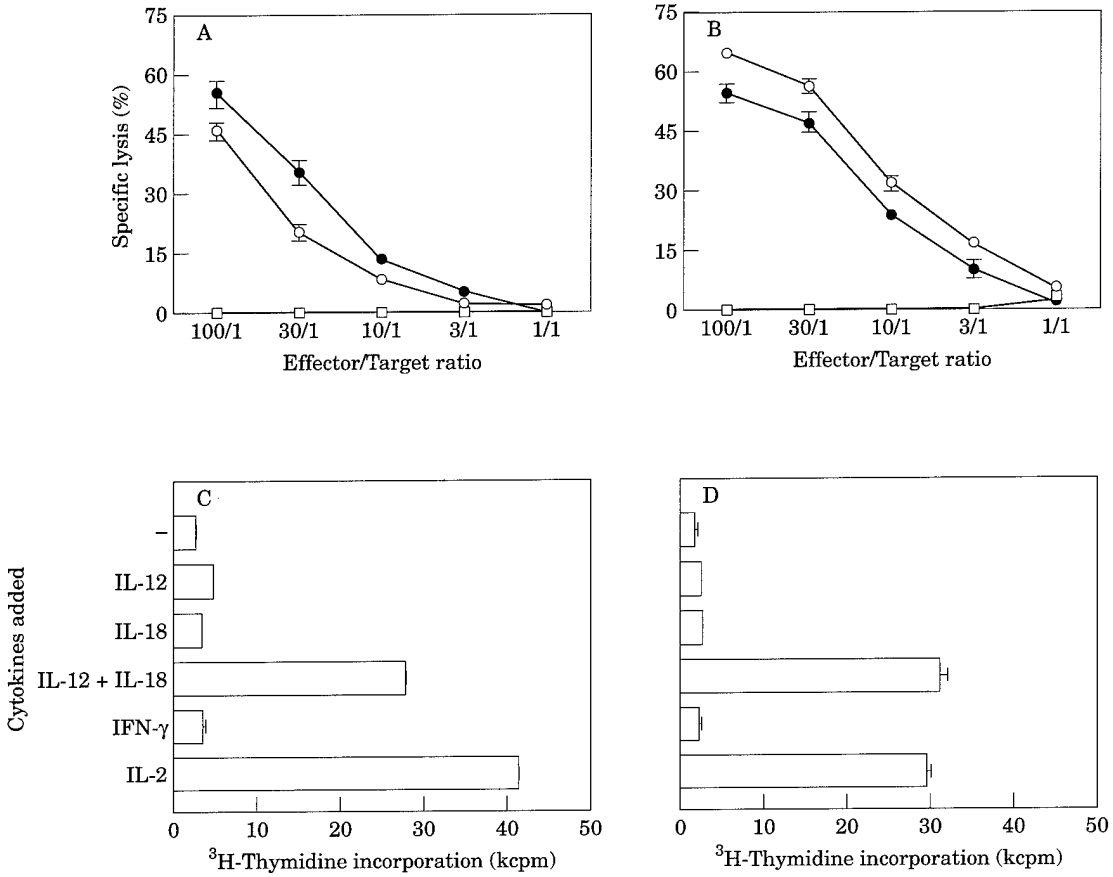

Figure 5. Effects of IL-12 and IL-18 on IFN-γR-knock-out splenocytes.

Nylon wool-filtered 129 (A and C) or IFN-γR-knock-out 129 (B and D) splenocytes were cultured with the indicated cytokines(s), as described in Materials and Methods. Cytotoxic activity against Yac-1 cells was determined at different effector/target (E/T) ratios by ^{51}Cr-release assays. (●), IL-12+IL-18; (○), IL-2; (□), control (A and B). Proliferations (mean ± SEM) were measured by [^{3}H]-thymidine incorporations (C and D).

fixed in paraformaldehyde (0.6%) before being analysed by flow cytometry (Becton Dickinson, Mountain View, CA).

The following fluorochrome-conjugated mAb used for FACS analyses were purchased from Pharmingen (San Diego, CA): anti-DX5, anti-Ly-49C, anti-NK1.1, anti-CD3, anti-CD19, and anti-IFN-γ. Anti-Mac-1α was purified from culture supernatants of ATCC clone M1/70 and biotinylated in our laboratory. Fluorochrome-conjugated unrelevant isotype-matched mAbs (Pharmingen, San Diego, CA) were used as controls.

NK1.1 labelling experiments were performed on C57BL/6 splenocytes, as Balb/c cells do not transcribe any of the known NKR-P1 genes, including NK1.1.[22]

For intracellular IFN-γ staining experiments, cells were fixed in a 4% paraformaldehyde solution for 20 min, before being permeabilized in ice-cold PBS containing 0.1% saponine and 1% FCS. A PE-conjugated anti-IFN-γ mAb (1.25 μg/ml) was added for 30 min. To confirm the specificity of the intracellular labelling, similar experiments were performed in the presence of exogenous IFN-γ (1.5 μg/ml).

Cytotoxicity assays

Yac-1 cells (1×10^3 cells/well), a murine NK cell target, were ^{51}Cr-labelled and incubated for 4 h in U-shaped microtitre wells with effector populations at various effector/target ratios in quadruplicate cultures. ^{51}Cr release was determined in supernatants and specific lysis was calculated as the ratio: [measured ^{51}Cr release minus minimal ^{51}Cr release (targets alone)]/[maximal ^{51}Cr release (targets in 1% triton) minus minimal ^{51}Cr release].

Cytokine assays

IFN-γ titres were measured by PACIA (particle counting immunoassay)[30] using latex particles coated with two rat anti-mouse IFN-γ mAbs (R46A2 and XMG1.2), both purchased from Endogen (Woburn, USA). IL-2, IL-3, IL-6, IL-9 and TNF titres were measured on CTLL-2, Ba/F3, 7TD1,[31] TS1[32] and WEHI 164 cells, respectively.

RNA extraction and reverse-transcriptase PCR analyses

Cells were lysed in TriPur (Bœhringer Mannheim GmbH, Mannheim, Germany) and total RNA was purified by chloroform extraction. cDNA was synthesized by using oligo(dT) primers (Bœhringer Mannheim GmbH) and murine Moloney leukaemia virus reverse-transcriptase (Life Technologie Inc., Grand Island, NY). Diluted cDNA was

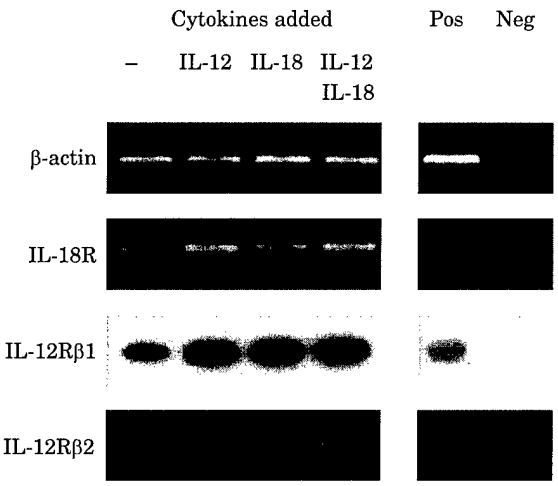

Figure 6. IL-12R and IL-18R gene expression.

Nylon wool-filtered Balb/c splenocytes were cultured with the indicated cytokines(s). RT-PCR analyses were performed with primers specific for the indicated receptors, as described in Materials and Methods. β-actin, IL-12Rβ2 and IL-18R RT-PCR products were analysed by agarose gel electrophoresis while IL-12Rβ1 RT-PCR products were blotted onto nylon filters before hybridization with an ^{32}P-labelled IL-12Rβ1 internal oligonucleotide. cDNA synthesized from con A-stimulated DBA/2 splenocytes was used as a positive control (Pos), while the negative control PCR (Neg) was performed without addition of cDNA. The data are representative of 4, 2 and 3 experiments for IL-18R, IL-12Rβ1 and IL-12Rβ, respectively.

amplified by PCR using recombinant Taq DNA polymerase (Takara, Shiga, Japan) and specific primers for mβ-actin (5′-ATGGATGACATATCGCTGC-3′; 3′-GAGTGACAG GTGGAAGGTCG-5′), mIL-12Rβ1 (5′-CTGCGAGGCTG AAGACGG-3′; 3′-GTCTCCGTCTCCGTCCAC-5′), mIL-12Rβ2 (5′-CATCACGAAGTTTCCCCCAC-3′; 3′-CGTAG CGATAGTAGTGCCAC-5′), and mIL-18R (5′-AGAAGC CATAGACACCAAGA-3′; 3′-TATGAAAACGACACCT CTGC-5′). PCR was performed as follows: 1 min at 94°C, followed by 1 min at 60°C for β-actin, 55°C for IL-12Rβ1, 55°C for IL-12Rβ2, 50°C for IL-18R and by 2 min at 72°C for 25 (β actin) or 35 (IL-12Rβ1, IL-12Rβ2 and IL-18R) cycles. The β-actin, IL-12Rβ2 and IL-18R PCR products were analysed by agarose gel electrophoresis. The IL-12Rβ1 PCR products were blotted on nylon filters and hybridized with a ^{32}P-labelled IL-12Rβ1 internal oligonucleotide (5′-ATTTCCCGTTTATCCATCATT-3′).

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REFERENCES

1. Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U, Presky DH (1998) The interleukin-12/interleukin-12-receptor system; role in normal and pathologic immunes responses. Annu Rev Immunol 16:495–521.

2. Okamura H, Tsutsui H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, Torigoe K, Okura T, Nukada Y, Hattori K, Akita K, Namba M, Tanabe F, Konishi K, Fukuda S, Kurimoto M (1995) Cloning of a new cytokine that induces IFN-γ production by T cells. Nature 378:88–91.

3. Ushio S, Namba M, Okura T, Hattori K, Nukada Y, Akita K, Tanabe F, Konishi K, Micallef M, Fujii M, Torigoe K, Tanimoto T, Fukuda S, Ikeda M, Okamura H, Kurimoto M (1996) Cloning of the cDNA for human IFN-gamma-inducing factor, expression in Escherichia coli, and studies on the biologic activities of the protein. J Immunol 156:4274–4279.

4. Micallef MJ, Ohtuski T, Kohno K, Tanabe F, Ushio S, Namba M, Tanimoto T, Torigoe K, Fujii M, Ikeda M, Fukuda S, Kurimoto M (1996) Interferon-γ-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-γ production. Eur J Immunol 26: 1647–1651.

5. Robinson D, Shibuya K, Mui A, Zonin F, Murphy E, Sana T, Hartley SB, Menon S, Kastelein R, Bazan F, O'Garra A (1997) IGIF does not drive Th1 development but synergizes with IL-12 for interferon-γ production and activates IRAK and NF-κB. Immunity 7:571–581.

6. Okamura H, Kashiwamura S-I, Tsutsui H, Yoshimoto T, Nakanishi K (1998) Regulation of interferon-γ production by IL-12 and IL-18. Curr Opin Immunol 10:259–264.

7. Yoshimoto T, Okamura H, Tagawa Y-I, Nakanishi K (1997) Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-γ production from activated B cells. Proc Natl Acad Sci USA 94:3948–3953.

8. Lauwerys BR, Renauld J-C, Houssiau FA (1998) Inhibition of *in vitro* immunoglobulin production by IL-12 in murine chronic graft-*vs*.-host disease; synergism with IL-18. Eur J Immunol 28:2017–2024.

9. Torigoe K, Ushio S, Okura T, Kobayashi S, Taniai M, Kunikata T, Murakami T, Sanou O, Kojima H, Fujii M, Ohta T, Ikeda M, Ikegami H, Kurimoto M (1997) Purification and characterization of human interleukin-18 receptor. J Biol Chem 272: 25737–25742.

10. Moretta A, Bottino C, Vitale M, Bende D, Biassoni R, Mingari MC, Moretta L (1996) Receptors for HLA class-I molecules in human natural killer cells. Annu Rev Immunol 14:619–648.

11. Lanier LL (1998) NK cell receptors. Annu Rev Immunol 16:359–393.

12. Siegel JP, Sharon M, Smith PL, Leonard WJ (1987) The IL-2 receptor β chain (p70): role in mediating signals for LAK, NK, and proliferative activities. Science 238:75–78.

13. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biological effects on human lymphocytes. J Exp Med 170:827–845.

14. Schoenhaut DS, Chua AO, Wolitzky AG Quinn PM, Dwyer CM, McComas W, Familletti PC, Gately MK, Gubler U (1992) Cloning and expression of murine IL-12. J Immunol 148:3433–3440.

15. Mingari MC, Vitale C, Cantoni C, Bellomo R, Ponte M, Schiavetti F, Bertone S, Moretta A, Moretta L (1997) Interleukin-15-induced maturation of human natural killer cells from early thymic precursors: selective expression of CD94/NKG2-A as the only HLA class I-specific inhibitory receptor. Eur J Immunol 27:1374–1380.

16. Carson WE, Fehniger TA, Haldar S, Eckhert K, Lindemann MJ, Lai CF, Croce CM, Baumann H, Caligiuri MA (1997) A potential role for interleukin-15 in the regulation of human natural killer cell survival. J Clin Invest 99:937–943.

17. Tsutsui H, Nakanishi K, Matsui K, Higashino K, Okamura H, Miyazawa Y, Kaneda K (1996) IFN-γ-inducing factor

up-regulates fas ligand-mediated cytotoxic activity or murine natural killer cell clones. J. Immunol 157:3967–3973.

18. Hunter CA, Timans J, Pisacane P, Menon S, Cai G, Walker W, Aste-Amezaga M, Chizzonite R, Bazan JF, Kastelein RA (1997) Comparison of the effects of interleukin-1α, interleukin-1β and interferon-γ-inducing factor on the production of interferon-γ by natural killer cells. Eur J Immunol 27:2787–2792.

19. Tomura M, Zhou XY, Maruo S, Ahn H-J, Hamaoka T, Okamura H, Nakanishi K, Tanimoto T, Kurimoto M, Fujiwara H (1998) A critical role for IL-18 in the proliferation and activation of $NK1.1^+CD3^-$ cells. J Immunol 160:4738–4746.

20. Glimcher L, Shen FW, Cantor H (1977) Identification of a cell-surface antigen selectively expressed on the natural killer cell. J Exp Med 145:1–9.

21. Ballas ZK, Rasmussen W (1990) $NK1.1^+$ thymocytes. Adult murine $CD4^-$, $CD8^-$ thymocytes contain an $NK1.1^+$, $CD3^+$, CD5hi, CD44hi, TCR-V beta 8^+ subset. J Immunol 145:1039–1045.

22. Giorda R, Weisberg EP, Ip TK, Trucco M (1992) Genomic structure and strain-specific expression of the natural killer cell receptor NKR-P1. J Immunol 149:1957–1963.

23. Kung SKP, Miller RG (1995) The NK1.1 antigen in NK-mediated F1 antiparent killing in vitro. J Immunol 154: 1624–1633.

24. Vicari AP, Zlotnik A (1996) Mouse $NK1.1^+$ T cells: a new family of T cells. Immunol Today 17:71–76.

25. Yoshimoto T, Paul WE (1994) $CD4^+$, $NK1.1^+$ T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. J Exp Med 179:1285–1295.

26. Ahn H-J, Maruo S, Tomura M, Mu J, Hamaoka T, Nakanishi K, Clark S, Kurimoto M, Okamura H, Fujiwara H (1997) A mechanism underlying synergy between IL-12 and IFN-γ inducing factor in enhanced production of IFN-γ. J Immunol 159:2125–2131.

27. Barbulescu K, Becker C, Schlaak JF, Schmitt E, Meyer zum Büschenfelde K-H, Neurath MF (1998) Cutting edge: IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-γ promoter in primary $CD4^+$ T lymphocytes. J Immunol 160:3642–3647.

28. Horwitz DA, Gray JD, Ohtsuka K, Hirokawa M, Takahashi T (1997) The immunoregulatory effects of NK cells: the role of TGF-β and implications for autoimmunity. Immunol Today 18:538–542.

29. Huang S, Hendriks W, Althage A, Hemmi S, Bluethmann H, Kamijo R, Vilcek J, Zinkernagel RM, Aguet M (1993) Immune response in mice that lack the interferon-γ receptor. Science 259:1742–1745.

30. Masson PL, Cambiaso CL, Collet-Cassart D, Magnusson CG, Richards CB, Sindic CJ (1981) Particle counting immunoassay (PACIA). Methods Enzymol 74:106–139.

31. Van Snick J, Cayphas S, Vink A, Uyttenhove C, Coulie PG, Rubira MR, Simpson RJ (1986) Purification and NH2-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. Proc Natl Acad Sci USA 83:9679–9683.

32. Uyttenhove C, Simpson RJ, Van Snick J (1988) Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity. Proc Natl Acad Sci USA 85:6934–6938.

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DIFFERENTIAL REGULATION BY IL-4 AND IL-10 OF RADIATION-INDUCED IL-6 AND IL-8 PRODUCTION AND ICAM-1 EXPRESSION BY HUMAN ENDOTHELIAL CELLS

Anne Van der Meeren, Claire Squiban, Patrick Gourmelon, Hélène Lafont, Marie-Hélène Gaugler

Radiation exposure results in an inflammatory reaction with acute as well as subacute consequences. Leukocyte infiltration is one of the predominant early histological changes and involves both cytokines and adhesion molecules. Endothelial cells play a key role in this reaction. We have previously shown the increased production of interleukin 6 (IL-6) and IL-8 and the upregulation in intercellular adhesion molecule 1 (ICAM-1) expression by HUVEC following γ ray exposure. In the present study, we used the cytokines IL-4 and IL-10 to regulate these radiation-induced manifestations. Human umbilical vascular endothelial cells (HUVEC) were treated with IL-4 and IL-10 (50 pg/ml) either before or after 10- Gy irradiation. Three and seven days after irradiation, IL-6 and IL-8 production by HUVEC (either treated or non-treated) was assessed by enzyme-linked immunosorbent assay (ELISA). Our results show that IL-4, when added after irradiation, reversed the radiation-induced increase in IL-8 production, although slightly increased IL-6 production. IL-10 decreased both IL-8 and IL-6 production when added after irradiation. ICAM-1 expression was evaluated 3 days after irradiation by flow cytometry. The radiation-induced upregulation in ICAM-1 expression remained unaffected by the use of IL-4. Altogether, our results show that radiation-induced endothelial cell activation may be ameliorated by IL-4 and/or IL-10, which is of significance in designing strategies for cytokine-mediated intervention and/or therapy of radiation damage.

Inflammation is a complex response of tissues to injury involving a network of signals leading to endothelial cell activation and leukocyte recruitment. Cytokines and adhesion molecules play a major role in inflammatory response. Inflammatory reaction is also a classical pathophysiological response to radiation exposure and could contribute to tissue damage by exacerbation of the direct damage of ionizing radiation. Histological changes are observed as early as a few hours after irradiation and are characterized by neutrophil infiltration of irradiated tissues.[1–3] Long term consequences of inflammatory reaction, such as fibrosis, are also frequently described. The mechanism leading to the radio-inflammatory process is poorly understood. Endothelial cells (ECs), key effectors of inflammatory reactions, seem to be involved partly through the upregulation of adhesion molecule expression and the release of cytokines. We have recently shown that γ exposure of ECs leads to an enhanced production of the pro-inflammatory cytokines IL-6 and IL-8[4] and to the upregulation of ICAM-1 expression correlated with augmented adhesion of neutrophils to irradiated ECs.[5] These manifestations were dose dependent and maintained up to 10 days after γ exposure, suggesting that these molecules could be implicated in the regulation of the traffic of leukocytes following local irradiation and to the development and maintenance of an inflammatory process.

The inflammatory state of many cell types can be controlled by regulatory cytokines such as IL-4 and IL-10. IL-4 and IL-10 are mainly produced by activated T cells of the TH2 subset (for review, see Refs [6–8]). These agents are potent immuno-regulatory

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TABLE 1. Effect of IL-4 and/or IL-10 on the number of cells either irradiated or not

		IL-4	IL-10	IL-4+IL-10
Non-irradiated cells	Day 3	1.24 ± 0.05**	1.1 ± 0.04	1.17 ± 0.06*
	Day 7	1.37 ± 0.7**	1.15 ± 0.06*	1.64 ± 0.14**
10-Gy irradiated cells	Day 3	1.54 ± 0.13**	1.68 ± 0.01**	1.68 ± 0.15**
	Day 7	1.51 ± 0.14**	1.56 ± 0.12**	2.54 ± 0.24**

*$P<0.05$, **$P<0.01$

IL-4 and/or IL-10 were added 3 h after irradiation. The number of viable cells was determined 3 and 7 days after irradiation. Results are expressed as fold increase in the number of cells in the presence of IL-4 and/or Il-10 as compared to control cells. *P* values represent the result of statistical analysis between the non treated group and the IL-4 and/or IL-10 treated group as determined by the non-parametric Mann–Whitney test.

cytokines and have been shown to attenuate activation of a variety of immunocompetent cells, such as pro-inflammatory cytokine production or expression of adhesion molecules both in vitro and in vivo.[9–12] The role of IL-4 seems to depend on both the nature of target cells and the nature of the activating stimulus on immunocompetent cells and ECs. A large number of authors have studied the effects of IL-4 on ECs showing both activating and anti-inflammatory properties. IL-4 induces the adhesion of T cells on ECs due to the induction of VCAM-1 expression,[13–16] and increases the production of IL-6 and MCP-1.[17–19] However, the IFN-γ induced production of RANTES is inhibited after IL-4 treatment.[20] IL-8 production seems to be unchanged by IL-4 on unstimuled HUVEC but upregulated after LPS activation.[21] The role of IL-4 on ICAM-1 expression is not clear: ICAM-1 is either downregulated[22] or unchanged[15] after addition of IL-4 depending on activation factors and the source of ECs. Only few studies have been carried out on the consequences of IL-10 treatment on ECs. The induction of E-selectin expression is observed after treatment of ECs with IL-10[23] although ICAM-1 and VCAM-1 are downregulated.[24] Contradictory results are obtained concerning IL-8 and IL-6 production depending on the agent used to activate ECs and on the type of ECs.[21,25] In general, IL-10 may be useful in some inflammatory conditions in which overexpression of cytokines plays an important role in pathogenesis. Several potential clinical applications have been proposed.[26] The effects of IL-10 on irradiated microvascular cells resulted in either a decrease in radiation-induced ICAM-1 expression in the presence of LPS or no effect in its absence.[27,28]

In order to limit the development of the radiation-induced inflammatory reaction, we used two cytokines, IL-4 and IL-10, and assessed their effect on IL-6 and IL-8 production and expression of ICAM-1 by in vitro irradiated endothelial cells. Different times of treatment with anti-inflammatory cytokine relative to the time of irradiation were tested. By modulating the response of ECs to ionizing radiation, IL-4 and IL-10 may regulate pro-inflammatory cytokine production and adhesion molecule expression and hence influence the nature and the progression of inflammatory response. Such mechanisms could then be explored to develop intervention and therapeutic approaches following radiation exposure.

RESULTS

Assessment of IL-4 and IL-10 production by HUVEC

IL-4 has been shown to be released after irradiation by alveolar macrophages[29] and lymphocytes.[30] Although IL-4 has never been shown to be produced by ECs, we assessed the presence of IL-4 in the cell supernatant. IL-4 was not detectable by ELISA (detection limit of the assay: 31.3 pg/ml) neither in the control nor in the irradiated cells.

IL-10 has also been shown to be induced by UV exposure both in vivo and in vitro in serum or supernatants of cultures of keratinocytes.[31–33] Therefore, we studied the release of IL-10 in the supernatant of HUVEC either irradiated or not. IL-10 remained undetectable in both conditions (detection of the assay: 15.4 pg/ml).

Effect of IL-4 and IL-10 on cell number

IL-4 causes a slight proliferation of non-irradiated cells (Table 1) as previously described by others[34] although IL-10 had no effect. When used together, IL-4 and IL-10 promoted the growth of HUVEC.

Irradiation exposure to a dose of 10 Gy induces a loss of cells, which was 2-fold three days after γ exposure and 4.1-fold 7 days after (data not shown). We determined if a treatment with regulatory cytokine could have any effect on this loss of cells.

The use of IL-4 or IL-10 3 h after irradiation limited the decrease in the cell number caused by irradiation exposure both 3 and 7 days after irradiation. Furthermore, IL-4 and IL-10 had an additive effect on irradiated cells since there were 2.5-fold more cells in treated than in non-treated cultures 7 days after irradiation ($P<0.001$ when compared to

each group treated with either cytokine alone). However, cytokine stimulation did not reverse fully the damaging effect of irradiation since after treatment, irradiated cells remained in a lower number than the non-irradiated cultures.

IL-4 amplifies the radiation-induced production of IL-6 while inhibiting the radiation-induced overproduction of IL 8 by HUVEC three days after exposure

Effect on IL-6 production

IL-6 secretion was increased by adding of IL-4 to non-irradiated cells, as previously shown by other investigators.[18,19,35] In our culture conditions, constitutive production of IL-6 was 16 pg/10^3 cells. Enhancement following IL-4 treatment was 1.8-fold.

After irradiation alone IL-6 production was 42 pg/10^3 which corresponds to a 2.6 fold increase as compared to non-irradiated cells (Fig. 1A).

To test the consequence of IL-4 treatment on this radiation-induced increase in IL-6 production, IL-4 was added either before or after 10-Gy irradiation. In all experimental conditions, IL-4 increased the radiation-induced production of IL-6. However, IL-6 production was higher when IL-4 was added 1 h before or concomitantly (respectively 3-fold and 2.8-fold increase as compared to irradiated non-treated cells) than 3 h or 24 h after irradiation (respectively 1.7-fold and 1.3-fold increase) (Fig. 1B).

Effect on IL-8 production

The addition of IL-4 on sham irradiated HUVEC was followed by a decrease of 35% in IL-8 production (from 83 to 54 pg/10^3 cells). Irradiation of 10-Gy lead to increased production IL-8 (1.8-fold) (Fig. 1A).

IL-4 was able to reduce the radiation-induced increase in IL-8 production 3 days after radiation exposure whatever the time of treatment (Fig. 1B). However, IL-4 was more efficient when added 3 h after irradiation than 1 h before (respectively 64% and 13% inhibition, $P<0.01$ between both groups). Furthermore, when IL-4 was added 3 h after irradiation, IL-8 production by irradiated cells returned to a level of production lower but not significantly different from basal production by non-irradiated control cells (respectively 57 and 83 pg/10^3 cells).

IL-10 decreases both the radiation-induced IL-6 and IL-8 production 3 days after irradiation

Effect on IL-6 production

IL-10 did not significantly change the constitutive IL-6 production by HUVEC (Fig. 2A). In all experimental conditions, IL-10 was able to reduce the radiation-induced IL-6 production and was still effective when added 24 h after radiation exposure with a

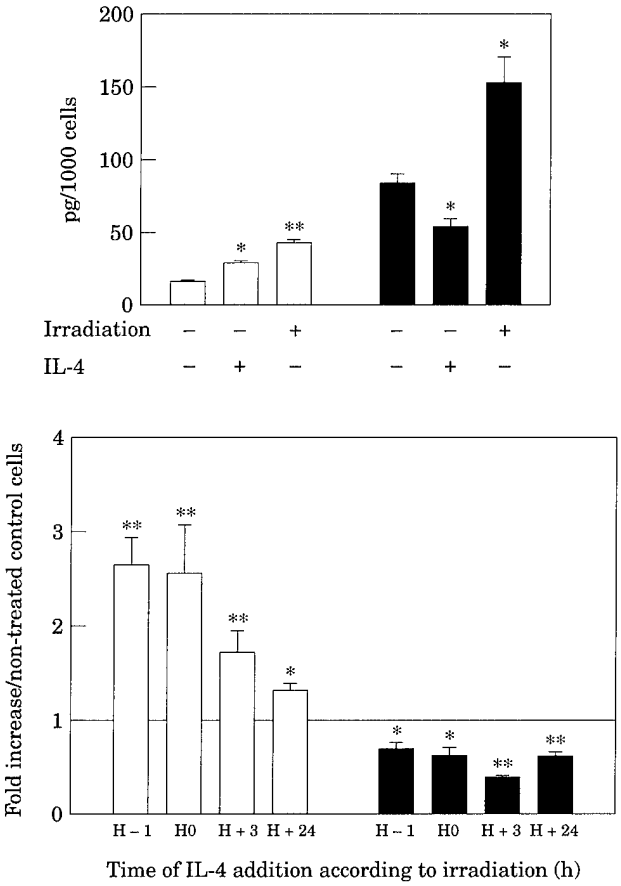

Figure 1. IL-6 (□) and IL-8 (■) production by HUVEC 3 days after IL-4 treatment.

Effect of IL-4 and 10-Gy irradiation on constitutive production of IL-6 and IL-8 assessed by ELISA (A). Values represent the mean ± SEM of three to five independent experiments performed in triplicate. Statistical differences are related to non irradiated non-treated cells. Effect of IL-4 on 10 Gy-irradiated cells (B). Fifty pg/ml of IL-4 were added 1 h before (H-1), concomitantly (H0), 3 h (H+3) or 24 h (H+24) after irradiation. Results are expressed as fold increase in the number of cells in the presence of IL-4 as compared to control cells (mean ± SEM of three to five independent experiments performed in triplicate). Statistical differences are calculated comparing the IL-4 treated groups to the non-treated groups.

17% decrease as compared to non-treated cells (Fig. 2B). When IL-10 was added 3 h after irradiation the efficiency seemed slightly better (34% decrease).

Effect on IL-8 production

The constitutive production of IL-8 was not significantly modified by the use of IL-10, confirming previous data.[21] Similar results were obtained with 10-Gy irradiated cells when cells were treated with IL-10 1 h before irradiation. When IL-10 was added 3 or 24 h after irradiation, a decrease of approximately 40% in the radiation-induced production of IL-8 was observed (Fig. 2B), giving a level of production not significantly different from non-irradiated non treated cells.

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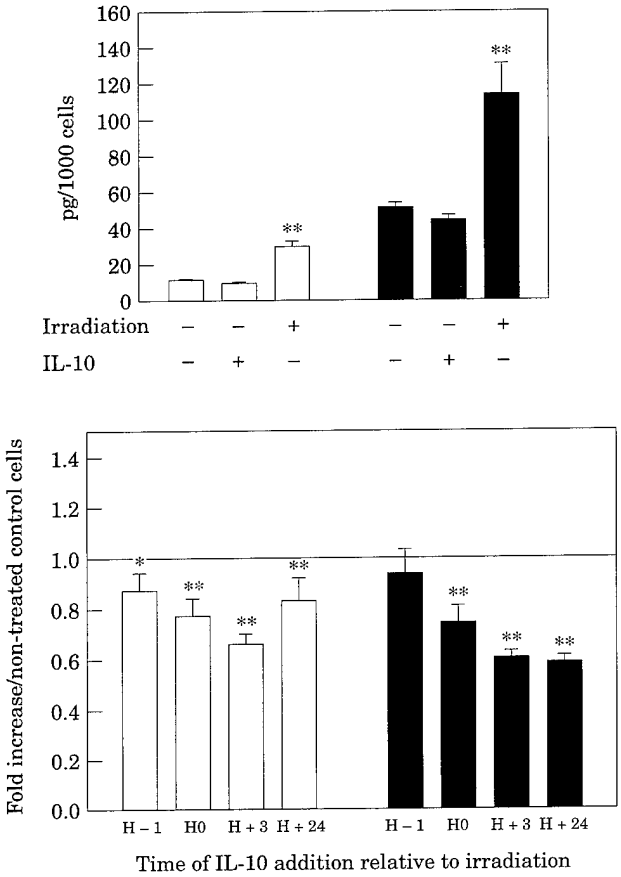

Figure 2. IL-6 (□) and IL-8 (■) production by HUVEC 3 days after IL-10 treatment.

Effect of IL-10 and 10-Gy irradiation on constitutive production of IL-6 and IL-8 assessed by ELISA (A). Values represent the mean ± SEM of three to five independent experiments realized in triplicate. Statistical differences are related to non irradiated non treated cells. Effect of IL-10 on 10-Gy irradiated cells (B). Fifty pg/ml of IL-10 were added 1 h before (H-1), concomitantly (H0), 3 h (H+3) or 24 h (H+24) after irradiation. Results are expressed as fold increase in the number of cells in the presence of IL-10 as compared to control cells (mean ± SEM of three to five independent experiments realized in triplicate). Statistical differences are calculated comparing the IL-10 treated groups to the non-treated groups.

Long term effect of IL-4 and IL-10 on IL-6 and IL-8 production by irradiated HUVEC

To evaluate the sustained regulatory potential of IL-4 and IL-10, we measured the production of IL-6 and IL-8 by HUVEC 7 days after radiation exposure. Three days after exposure, the increase in IL-6 and IL-8 content in irradiated cells as compared to non-irradiated cells was 2.6- and 2-fold, respectively. Seven days after irradiation, the increases were 8- and 7.2-fold respectively (data not shown).

Irradiated cells were first treated with IL-4 or IL-10 3 h after radiation exposure, and not retreated at the medium change 4 days after irradiation.

Seven days after irradiation, both IL-4 and IL-10 decreased the radiation-induced production of IL-6 (Table 2) in proportion similar to what is observed 3 days after irradiation.

TABLE 2. Effect of IL-4 or IL-10 on IL-6 production by HUVEC 7 days after irradiation

IL-4		IL-10	
IL-6	IL-8	IL-6	IL-8
0.52 ± 0.05 $P<0.0001$	0.45 ± 0.07 $P<0.0001$	0.75 ± 0.07 $P<0.05$	0.47 ± 0.06 $P<0.0001$

IL-4 or IL-10 were added 3 h after irradiation and not re-added to the culture at the medium change. Results are expressed as fold increase in IL-6 or IL-8 production by irradiated HUVEC in the presence of IL-4 or IL-10 as compared to irradiated cells in the absence of IL-4 or IL-10 and are the mean ± SEM of 6 to 12 values. *P* values represent the result of statistical analysis between IL-4 or IL-10 treated group and non treated group, as determined by the non-parametric Mann–Whitney test.

The effects of IL-4 and IL-10 on IL-8 production were persistent, although the radiation-induced enhancement of IL-8 production was more pronounced (Table 2).

Effect of IL-4 on ICAM-1 expression

We studied the basal expression of ICAM-1 following IL-4 addition on HUVEC and the activity of IL-4 on the radiation-induced upregulation of ICAM-1 in our experimental conditions. IL-4 was added on non-irradiated cells or on 10-Gy irradiated cells, 3 h after irradiation. Three days after γ exposure, ICAM-1 expression by irradiated cells was 2.3 times higher than of control cells, thus confirming our previously published results obtained on resting cells. However, a change in ICAM-1 level was not observed after adding IL-4 to either non-irradiated cells or to γ-exposed cells.

DISCUSSION

It is generally recognized that counteraction of the inflammatory response to radiation is important to attenuate acute radiation effects and prevent chronic consequences.[36] The most widely available anti-inflammatory drugs are glucocorticosteroids, which, however, do not always give beneficial results. In the last 10 years new insights into inflammation mechanisms have emerged with the identification of pro-inflammatory cytokines and their antagonists. It is of interest to explore these new agents and their mechanisms in overcoming the radiation-induced inflammatory reaction.

ECs have been the target of various treatments for radiation damage.[37–39] Only few studies describe the use of the more recently available cytokines in the treatment of inflammation after radiation exposure. A recent report has shown the inhibiting role of IL-11 on pro-inflammatory cytokine production by alveolar

macrophages following thoracic irradiation, accompanied by an improvement of survival.[40] Some authors have demonstrated the inhibition by IL-10, of LPS-induced apoptosis and ICAM-1 upregulation. However, no effect on these parameters were seen on irradiated ECs.[27,28,41,42] In the present work, we attempted to limit both the inflammatory response of ECs and the direct radiation damage. Irradiation has deleterious effects on cells resulting either from mitotic death or apoptosis as it has been widely shown. This is illustrated in the present study by a progressive diminution in the number of cells in the culture. IL-4 is a mitogenic factor for endothelial cells. More interestingly, it was able to limit the decrease in cell number resulting from the 10-Gy irradiation, even when added up to 24 h after radiation exposure. The protection of ECs towards deleterious action of radiation is of importance from a therapeutic perspective since disruption of endothelium can result in promoting the development of late lesions such as vascular damage and fibrosis.[43] IL-10 did not modify the growth of non-irradiated HUVEC but, similar to IL-4, was able to limit the loss of cells induced by γ exposure. Moreover, when added together, the two cytokines were more efficient than each cytokine used alone.

It has been shown that cytokines as well as adhesion molecules are involved in the recruitment and extravasation of leukocytes following radiation exposure.[2,44] We have previously demonstrated the enhancement of IL-6 and IL-8 production by HUVEC following irradiation. This increase was dose dependent and persistent (up to 10 days after radiation exposure). In the present study we asked whether the use of immunoregulatory cytokines such as IL-4 and IL-10 could limit the radiation-induced inflammatory response of HUVEC by limiting the radiation-induced production of IL-6 and IL-8 and the upregulation of ICAM-1 expression.

To our knowledge, there are no data available concerning the regulation of cytokine production by irradiated ECs.

The anti-inflammatory role of IL-4 on ECs is controversial. Briefly, IL-4 seems to have no effect on non-stimulated cells but mainly promotes inflammatory reactions when used on ECs activated by tumour necrosis factor α (TNF-α), IL-1 or LPS. Because radiation-induced increase in IL-6, IL-8 and ICAM-1 occur independently of TNF-α and IL-1 and because TNF-α and ionizing radiation seemed to have distinct mechanism of action,[4,5] we studied the effect of IL-4 and/or IL-10 on the radiation-induced inflammatory response of HUVEC.

We have shown that IL-10 decreased both IL-6 and IL-8 production although IL-4 diminished only IL-8 production, suggesting differential regulatory mechanisms. When added after irradiation IL-4 increased IL-6 production but to a lesser extent that when added before. Under some conditions, the two regulatory cytokines fully reversed the radiation-induced increase in inflammatory cytokine production. An effect of IL-4 was not seen on the radiation-induced ICAM-1 increase. These results suggest differences in the mechanism of induction of IL-6 and IL-8 production and ICAM-1 expression by γ exposure. Our previously published studies lead to similar primary conclusions, since IL-6 and IL-8 overproduction did not result from transcriptional activation, although upregulation of ICAM-1 did.[4,5]

IL-6 and IL-8 have been shown to be involved in inflammatory reaction. Substantial data are available on the inflammatory effect of IL-6 such as induction of acute phase proteins by the liver and fever.[45] However, IL-6 has also been considered as an anti-inflammatory cytokine because of its ability to induce IL-1 and TNF-α antagonists.[46] IL-8 is a chemoattractant for neutrophils. ICAM-1 mediate firm adhesion of rolling leukocytes to the endothelium and is required for transendothelial migration into tissues.[47,48] A complex network of signals involving sequentially chemokines and adhesion molecules occur during inflammatory process so allowing the migration of leukocytes to damaged tissues. Both IL-8 and ICAM-1 may contribute to the recruitment of leukocytes following irradiation. We have shown the down regulation of IL-6 and IL-8 by IL-10, and IL-8 by IL-4. We and others[27] could not see any effect of IL-4 and IL-10 on radiation-induced ICAM-1 over-expression. However, limiting one step of the inflammatory process cascade, might limit the afflux of leukocytes, and thus control the nature and the progression of the inflammatory reaction. This can be of benefit since it has been widely shown that inflammatory reaction is intrinsically destructive for surrounding tissues. In addition, an early release of cytokines could be responsible for late damage leading to the hypothesis of a perpetual cascade of cytokines initiating radiation-induced late effects.[49]

We also determined if observations made on irradiated HUVEC were still observed 7 days after exposure. In previous studies we showed that ECs inflammatory response to irradiation was more pronounced when time increased at least up to 2 weeks after irradiation (data not shown). The effect of IL-4 and IL-10 was maintained 7 days after irradiation when IL-6 and IL-8 production were increased by 7- and 8-fold, respectively, as compared to non-irradiated cells and even if IL-4 and IL-10 were not readded to the culture at the medium change. This demonstrates that a single addition of regulatory cytokines to irradiated HUVEC is able to maintain a low level of IL-6 and IL-8 production up to 7 days after irradiation.

Our results have underlined the influence of the treatment scheduling according to the time of

irradiation, with a better anti-inflammatory effect when treatment was made after irradiation. This is of great importance when designing new strategies using pharmacological doses of cytokines. Most of the studies use regulatory cytokines in pretreatment or added together with the activating factor. When tested, the time of treatment according to the stimulus did not seem to interfere with the results.[16,50]

We have shown that γ exposure of HUVEC leads to an enhanced production of IL-6 and IL-8, which can be regulated by IL-4 and IL-10. These effects are maintained at least up to 7 days after irradiation and are still observed even when the treatment was made up to 24 h after irradiation exposure. These two observations suggest that the cytokines IL-4 and IL-10 might be good candidates for a prospective study on the therapeutic potential in radiation-induced inflammatory reactions.

MATERIALS AND METHODS

Cell culture

HUVEC were obtained from ATCC and routinely cultured as described previously.[4] Briefly, cells were grown in gelatin-coated dishes in F12K medium (Sigma, France) supplemented with 20% fetal calf serum (Gibco-BRL, France), glutamine, heparin (Sigma, France), endothelial cell growth supplement 60 μg/ml (Sigma, France) and antibiotics referred thereafter as complete medium. They are nearly diploid, non-transformed cells and were free of mycoplasm. Three different batches of cells from passages 4 to 9 after ATCC freezing (passage 12) were used in this study.

Irradiation procedure and cytokine treatment

For irradiation experiments, $1.5–2 \times 10^4$ cells/cm^2 were plated in flasks or 24-well dishes in complete culture medium. Three days thereafter, medium was replaced by fresh complete medium and semi confluent cells were irradiated with a dose of 10 Gy at 1 Gy/min with a ^{60}Co source (ICO 4000). The sham irradiated controls were treated in the same conditions.

To test the effect of anti-inflammatory cytokines IL-4 and IL-10 on IL-6 and IL-8 production by HUVEC, cells were treated either 1 h before irradiation, right before irradiation, 3 h or 24 h after irradiation. Sham irradiated cells were treated immediately after handling. IL-4 ($0.5–2 \times 10^7$ U/mg) and IL-10 ($1–2 \times 10^6$ U/mg) were obtained from R&D Systems (Abingdon, UK) and were both used at a final concentration of 50 pg/ml.

Three and 7 days after irradiation, supernatants were collected, cells trypsinized and viable adherent cells counted in the presence of nigrosine. Supernatants were centrifuged to remove debris and frozen at − 80°C until use. IL-4 and IL-10 were re-added to the culture at the medium change (3 days after irradiation) unless mentioned. The combined effect of IL-4 and IL-10 was also tested on HUVEC. Both cytokines (50 pg/ml) were added 3 h after radiation exposure.

Cytokine immunoassays

A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of the cytokines IL-6, IL-8, IL-4 and IL-10 in culture media was performed according to the manufacturer's recommendations (Amersham, France). Results were expressed in pg/10^3 viable cells because both irradiation and cytokine treatment induced a change in cell number as compared to control cells. The results of the three individual HUVEC batches were standardized by giving results as ratios (fold increase or decrease in cytokine levels).

Flow cytometry analysis

For cell fluorescent staining, HUVEC were incubated with anti-ICAM-1 (Immunotech, France). Cells were then washed twice and labelled with the fluorescein-5-isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibody. Acquisitions were performed on a FACSort® flow cytometer (Becton Dickinson Immunocytometry Systems, France). Cells ($0.5–1 \times 10^4$) were acquired and analysed with CellQuest software (Becton Dickinson). 7-Aminoactinomycin D (1 μg/ml) (Sigma, France) staining was used to gate out the non-viable cells. Marker M1 was set according to negative isotype-matched control thus determining the percentage of fluorescent cells with anti-ICAM-1 antibody. The corrected percentage of fluorescent cells was calculated as follows: for each treatment the percentage of fluorescent cells for the isotype-matched control antibody was substracted from the percentage of fluorescent cells for anti-ICAM-1.

Statistical analysis

Statistical significance was calculated using the Mann–Whitney rank sum test. P values <0.05 were considered significant.

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REFERENCES

1. Buell MG, Harding RK (1989) Proinflammatory effects of local abdominal irradiation on rat gastrointestinal tract. Digest Dis Sci 345:390–399.

2. Panes J, Anderson DC, Miyasaka M, Granger DN (1995) Role of leukocyte-endothelial cell adhesion in radiation-induced microvascular dysfunction in rats. Gastroenterology 108:1761–1769.

3. Fliss H, Menard M (1994) Rapid neutrophil accumulation and protein oxidation in irradiated rat lungs. J Appl Physiol 77:2727–2733.

4. Van der Meeren A, Bertho JM, Vandamme M, Gaugler MH (1997) Ionizing radiation enhances IL-6 and IL-8 production by human endothelial cells. Mediators Inflamm 6:185–193.

5. Gaugler MH, Squiban C, Van der Meeren A, Bertho JM, Vandamme M, Mouthon MA (1997) Late and persistent up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by ionizing radiation in human endothelial cells in vitro. Int J Radiat Biol 72:201–209.

6. Paul WE (1991) Interleukin-4: a prototypic immunoregulatory lymphokine. Blood 77:1859–1870.

7. De Vries JE, De Waal Malefyt R (1995) Interleukin-10, a potent immunosuppressant. Drug News and Perspectives 8:84–88.

8. Mosmann TR (1994) Properties and functions of interleukin-10. Adv Immunol 56:1–26.

9. Standiford TJ, Kunkel SL, Liebler JM, Burdick MD, Gilbert AR, Strieter RM (1993) Gene expression of macrophage inflammatory protein-1α from human blood monocytes and alveolar macrophages is inhibited by interleukin-4. Am J Respir Cell Mol Biol 9:192–198.

10. Standiford TJ, Strieter RM, Chensue SW, Westwick J, Kasahara K, Kunkel SL (1990) IL-4 inhibits the expession of IL-8 from stimulated human monocytes. J Immunol 145:1435–1439.

11. Te Velde AA, Huijbens RJF, Heije K, De Vries JE, Figdor CG (1990) Interleukin-4 (IL-4) inhibits secretion of IL-1β, tumor necrosis factor α, and IL-6 by human monocytes. Blood 76: 1392–1397.

12. Wang P, Wu P, Siegel ML, Egan RW, Billah MM (1995) Interleukin (IL)-10 inhibits nuclear factor κB (NF κB) activation in human monocytes. J Biol Chem 270:9558–9563.

13. Paleolog EM, Aluri GR, Feldman M (1992) Contrasting effects of interferon γ and interleukin 4 on responses of human vascular endothelial cells to tumour necrosis factor α. Cytokine 4:470–478.

14. Masinovsky B, Urdal D, Gallatin WM (1990) IL-4 acts synergistically with IL-1β to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell adhesion molecule-1. J Immunol 145:2886–2895.

15. Iademarco MF, Barks JL, Dean DC (1995) Regulation of vascular cell adhesion molecule-1 expression by IL-4 and TNF-alpha in cultured endothelial cells. J Clin Invest 95:264–271.

16. Thornhill MH, Haskard DO (1990) IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN-γ. J Immunol 145:865–872.

17. Rollins BJ, Pober JS (1991) Interleukin-4 induces the synthesis and secretion of MCP-1/JE by human endothelial cells. Am J Pathol 138:1315–1319.

18. Howells G, Pham P, Taylor D, Foxwell B, Feldmann M (1991) Interleukin 4 induces interleukin 6 production by endothelial cells: synergy with interferon-γ. Eur J Immunol 21:97–101.

19. Colotta F, Sironi M, Borr A, Luini W, Maddalena F, Mantovani A (1992) Interleukin 4 amplifies monocyte chemotactic protein and interleukin 6 production by endothelial cells. Cytokine 4:24–28.

20. Marfaing-Koka A, Devergne O, Gorgone G, Portier A, Schall TJ, Galanaud P, Emilie D (1995) Regulation of the production of the RANTES chemokine by endothelial cells. J Immunol 154:1870–1878.

21. De Beaux AC, Maingay JP, Ross JA, Fearon KCH, Carter DC (1995) Interleukin-4 and interleukin-10 increase endotoxin-stimulated human umbilical vein endothelial cell interleukin-8 release. J Interferon Cytokine Res 15:441–445.

22. Renkonen R, Mattila P, Majuri ML, Paavonen T, Silvennoinen O (1992) IL-4 decreases IFN-γ induced endothelial ICAM-1 expression by a transcriptional mechanism. Scand J Immunol 35:525–530.

23. Vora M, Romero LI, Karasek MA (1996) Interleukin-10 induces E-selectin on small and large blood vessel endothelial cells. J Exp Med 184:821–829.

24. Krakauer T (1995) IL-10 inhibits the adhesion of leukocytic cells to IL-1-activated human endothelial cells. Immunol 45:61–65.

25. Sironi M, Munoz C, Pollicino T, Siboni A, Luisa Sciacca F, Bernasconi S, Vecchi A, Colotta F, Mantovani A (1993) Divergent effects of interleukin-10 on cytokine production by mononuclear phagocytes and endothelial cells. Eur J Immunol 23:2692–2695.

26. Geissler K (1996) Current status of clinical development of interleukin-10. Curr Opin Hematol 3:203–208.

27. Lindner H, Holler E, Gerbitz A, Johnson JP, Bornkamm GW, Eissner G (1997) Influence of bacterial endotoxin on radiation-induced activation of human endothelial cells in vitro and in vivo. Transplantation 64:1370–1373.

28. Eissner G, Lindner H, Behrends U, Kölch W, Hieke A, Klauke I, Bornkamm GW, Holler E (1996) Influence of bacterial endotoxin on radiation-induced activation of human endothelial cells in vitro and in vivo. Transplantation 62:819–827.

29. Buttner C, Skupin A, Reimann T, Rieber EP, Unteregger G, Geyer P, Franck KH (1997) Local production of interleukin-4 during radiation-induced pneumonitis and pulmonary fibrosis in rats: macrophages as a prominent source of interleukin-4. Am J Respir Cell Mol Biol 17:315–325.

30. Galdiero M, Cipollaro G, Folgore A, Cappello M, Giobbe A, Sasso FS (1994) Effects of irradiation doses on alterations in cytokine release by monocytes and lymphocytes. J Med 25:23–40.

31. Enk CD, Sredni D, Blauvelt A, Katz SI (1995) Induction of IL-10 gene expression in human keratinocytes by UV B exposure in vivo and in vitro. J Immunol 154:4851–4856.

32. Rivas JM, Ullrich SE (1992) Systemic suppression of delayed-type hypersensivity by supernatants from UV-irradiated keratinocytes. J Immunol 149:3865–3871.

33. Rivas JM, Ullrich SE (1994) The role of IL-4, IL-10, and TNF-α in the immune suppression induced by ultraviolet radiation. J Leukocyte Biol 56:769–775.

34. Klein NJ, Rigley KP, Callard RE (1993) IL-4 regulates the morphology, cytoskeleton, and proliferation of human umbilical vein endothelial cells: relationship between vimentin and CD23. Int Immunol 5:293–301.

35. Chen CC, Manning AM (1996) TGF-β1, IL-10 and IL-4 differentially modulate the cytokine-induced expression of IL-6 and IL-8 in human endothelial cells. Cytokine 8:58–65.

36. Michalowski AS (1994) On radiation damage to normal tissues and its treatment. II. Anti-inflammatory drugs. Acta Oncologica 33:139–157.

37. Evans ML, Graham MM, Mahler PA, Rasey JS (1987) Use of steroids to suppress vascular response to radiation. Int J Radiat Oncol Biol Phys 13:563–567.

38. Kimura H, Wu NZ, Dodge R, Spencer DP, Klitzman BM, McIntyre TM, Dewhirst MW (1995) Inhibition of radiation-induced up-regulation of leukocyte adhesion to endothelial cells with the platelet-activating factor inhibitor, BN52021. Int J Radiat Oncol Biol Phys 33:627–633.

39. Dion MW, Hussey DH, Osborne JW (1989) The effect of pentoxifylline on early and late radiation injury following fractionated irradiation in C3H mice. Int J Radiat Oncol Biol Phys 17: 101–107.

40. Redlich CA, Gao X, Rockwell S, Kelley M, Elias JA (1996) IL-11 enhances survival and decreases TNF production after radiation-induced thoracic injury. J Immunol 157:1705–1710.

41. Lindner H, Holler E, Ertl B, Multhoff G, Schreglmann M, Klauke I, Schultz-Hector S, Eissner G (1997) Peripheral blood mononuclear cells induced programmed cell death in human endothelial cells and may prevent repair: role of cytokines. Blood 89:1931–1938.

42. Eissner G, Kohlhuber F, Grell M, Ueffing M, Scheurich P, Hieke A, Multhoff G, Bornkamm GW, Holler E (1995) Critical involvement of transmembrane tumor necrosis factor-α in endothelial programmed cell death mediated by ionizing radiation and bacterial endotoxin. Blood 86:4184–4193.

43. Reinhold HS, Fajardo LF, Hopewell JW (1990) The vascular system. Adv Radiat Biol 14:177–226.

44. Langberg CW, Hauer-Jensen M, Sung CC, Kane CJM (1994) Expression of fibrogenic cytokines in rat small intestine after fractionated irradiation. Radiother Oncol 32:29–36.

45. Kishimoto T (1989) The biology of interleukin-6. Blood 74:1–10.

46. Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW (1994) Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. Blood 83:113–118.

47. Adams DH, Shaw S (1994) Leucocyte-endothelial interactions and regulation of leucocyte migration. Lancet 343:831–836.

48. Springer TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76:301–314.

49. Rubin P, Johnston CJ, J.P. W, McDonald S, Finkelstein JN (1995) A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis. Int J Radiat Oncol Biol Phys 33:99–109.

50. Vannier E, Miller LC, Dinarello CA (1992) Coordinated anti inflammatory effects of Interleukin-4: Interleukin-4 suppresses Interleukin-1 production but up-regulates gene expression and synthesis of Interleukin-1 receptor antagonist. Proc Natl Acad Sci USA 89:4076–4080.

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PROTEIN KINASE C-DEPENDENT PATHWAY IS CRITICAL FOR THE PRODUCTION OF PRO-INFLAMMATORY CYTOKINES (TNF-α, IL-1β, IL-6)

Ewa Kontny, Maria Ziółkowska, Alicja Ryżewska, Włodzimierz Maśliński

The authors hypothesized that certain PKC isoforms play an important role in the induction of pro-inflammatory cytokine (TNF-α, IL-1β, IL-6) synthesis. To test this hypothesis, the cytosol-to-membrane translocation of select PKC isoforms with tested cytokine production in human monocytes cultured in vitro was correlated. It is reported that in monocytes treated with phorbol ester (PMA), translocation of PKC isoforms α, βII, δ and ε precede cytokine synthesis. Moreover, specific inhibition of PKC translocation that occurs in the presence of Calphostin C is reflected in downstream events: lack of MAP kinases phosphorylation, loss of DNA binding ability by AP-1 transcription factor, and the reduction of pro-inflammatory cytokine synthesis. Thus, the cytosol-to-membrane translocation of PKC isoforms α, βII, δ and ε with the subsequent activation of: (1) MAP kinases; and (2) AP-1 transcription factor, may represent critical steps in the induction of signalling cascade leading to TNF-α, IL-1β, IL-6 synthesis in human monocytes.

Monocytes play an important role in the initiation of immune response through their ability to synthesize a variety of mediators including pro-inflammatory cytokines: TNF-α, IL-1β and IL-6. These cytokines exert pleiotropic biological effects on a wide range of target cells and, due to their pro-inflammatory and immunoregulatory properties, play crucial role in the inflammation, immune response and haematopoiesis.[1–3] However, in some pathological conditions, e.g. in rheumatoid arthritis, the continuous production of pro-inflammatory cytokines mediates chronic inflammatory responses that may result in the connective tissue damage.[4] Interestingly, TNF-α, IL-1β and IL-6 share many of their pro-inflammatory activities.[1–3] Despite its pro-inflammatory activities, IL-6 exerts also anti-inflammatory properties. For example, IL-6 promotes the acute phase reaction, up-regulates metalloproteinase inhibitors, suppresses TNF-α and IL-1β synthesis in vitro, and induces the synthesis of TNF-α and IL-1β endogenous antagonists (i.e. TNF-α soluble receptor and IL-1 receptor antagonist) in vivo.[5–7]

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria is a potent activator of monocytes/macrophages that induces production of several cytokines, including TNF-α, IL-1β and IL-6. Although some aspects of the intracellular mechanisms triggered by LPS are known, the exact signalling pathways leading to the synthesis of these pro-inflammatory cytokines are not fully understood. Several molecules were suggested to play a role as LPS receptors. Two of them, the CD14 molecule,[8] and the low-affinity LPS-binding protein, represented probably by CD11c/CD18 molecule of the β_2 integrin family,[9] mediate LPS-induced production of numerous cytokines. LPS triggers the activation of several protein tyrosine kinases including p53/56lyn,[10] as well as serine/threonine kinases: Raf-1,[11] mitogen-activated protein kinases (MAPK),[11] and protein kinase C (PKC).[12] The downstream events leading to the production of TNF-α, IL-1β and IL-6 are, at least in part, regulated by the same transcription factors (AP-1, NFκB, NF-IL-6).[13–15] Interestingly, in our preliminary studies we have observed a strong correlation between serum levels of TNF-α and IL-1β in healthy blood donors. These data suggested that activation of monocytes may trigger a common signalling pathway

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that ultimately results in the simultaneous production of pro-inflammatory cytokines.

Based on these data we hypothesized that the induction of these cytokine synthesis in human monocytes utilizes a common signalling pathway that involves certain PKC isoforms. In order to test this hypothesis we have carried out a parallel study in which the amounts of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) produced in human monocytes upon stimulation with PKC activator (phorbol ester, PMA) were correlated with the cytosol-to-membrane translocation of select PKC isoforms. In addition, further downstream events such as the phosphorylation of MAPK and DNA binding activity of AP-1 transcription factor were also investigated.

We report that production of TNF-α, IL-1β and IL-6 by human monocytes cultured in vitro, as well as the translocation of PKC isoforms α, βII, δ and ε in these cells are blocked in the presence of specific PKC inhibitor, Calphostin C. Thus, we propose that PKC isoforms α, βII, δ and ε may play important role in the induction of pro-inflammatory cytokine synthesis.

RESULTS

Activation of PKC induces production of pro-inflammatory cytokines

In order to determine whether the activation of PKC alone is sufficient to trigger the production of pro-inflammatory cytokines, monocytes were stimulated with an activator of PKC (PMA; 1 nM) for 21 h, and the synthesis of secreted, as well as cell-associated forms of TNF-α, IL-1β and IL-6 were assayed by ELISA. Monocytes treated with a non-active phorbol ester analogue (4αPMA; 1 nM) or stimulated with LPS (5 μg/ml) were used as a negative or positive control, respectively. As shown in Figure 1, LPS induces strong production of both secreted and cell-associated forms of all tested cytokines. The optimal concentration of PMA (1 nM) partially mimics this effect with the production of TNF-α and IL-1β reaching ~40% and ~22% of LPS-triggered levels, respectively. Interestingly, PMA induced the production of secreted form of IL-1β only, while both secreted and cell-associated forms of TNF-α were detected. The influence of PMA on IL-6 synthesis was even more complex. Although average IL-6 production remained unchanged upon PMA stimulation (Fig. 1), there were two distinct groups of donors that differ in their response to stimuli (data not shown). In seven out of 15 volunteers, PMA treatment resulted in a significant increase of IL-6 production (both secreted and cell-associated form of protein) to the level ~17% of LPS-triggered response. In contrast, monocytes from the remaining eight donors responded to PMA

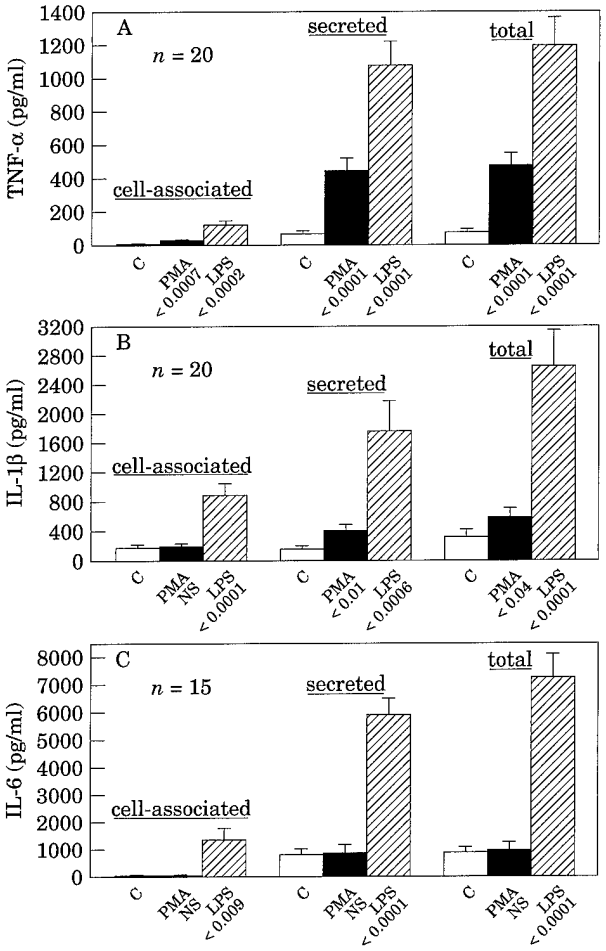

Figure 1. PMA partially mimics LPS-triggered cytokine production.

Monocytes were culture for 21 h in the absence (control-C) or presence of 1 nM PMA or 5 μg/ml LPS. Concentration of cytokines was determined in cell lysates (cell-associated) and culture supernatants (secreted), by ELISA. Results are the mean ± SEM of 20 (TNF-α and IL-1β) or 15 (IL-6) experiments. Statistical comparison between samples with and without stimuli is shown as a *P* value. PMA raised production of TNF-α (A) and IL-1β (B) to the levels of 40% and 22% of the LPS-triggered responses, respectively. Upon PMA treatment production of IL-6 (C) was significantly raised (to 17% of LPS-triggered response) only in seven out of 15 volunteers.

with decrease rather than increase of baseline production of IL-6. Interestingly, the latter group of volunteers generally exerts lower production of cytokine triggered not only by PMA but also by LPS treatment. The non-active PMA analogue (4αPMA) had no effects on the production of tested cytokines (data not shown), confirming the specificity of PMA as a PKC activator.

These results show that activation of PKC only partially mimics LPS-induced response leading to the sub-optimal synthesis of pro-inflammatory cytokines. We also observed that among tested cytokines TNF-α is the most, while IL-6 is the least dependent on the sole activation of PKC upon PMA stimulation.

Selective PKC inhibitor, Calphostin C, blocks the production of pro-inflammatory cytokines

In order to further explore the role of PKC-dependent pathway in the cytokine production, a highly selective inhibitor of PKC, Calphostin C, was used. Calphostin C binds to the unique phorbol ester/DAG-binding site and inhibits various PKC isoforms to the same extent (IC_{50} for purified enzymes equals 50 nM). At the concentration range used in our studies (10–50 nM), Calphostin C is a specific inhibitor of PKC; much higher concentration of Calphostin C (>50 μM) is required to affect the activity of other protein kinases.[16]

In our study, the pre-treatment of monocytes with 50 nM Calphostin C strongly suppressed production of TNF-α, IL-1β and IL-6 in both LPS- and PMA-treated cells (Fig. 2). Similar results were obtained when Western blotting technique was used to assess cytokine production (data not shown).

The lower concentration of Calphostin C (10 nM) exerts more discrete influence on the LPS induced production of TNF-α, IL-1β and IL-6. Pretreatment of monocytes with 10 nM Calphostin C resulted in a strong reduction of LPS-triggered TNF-α synthesis (~80%), while weaker inhibition of IL-1β and IL-6 production (~60%) were observed (Fig. 2). These results support an earlier observation (Fig. 1) that the production of IL-1β and IL-6 is less dependent on the activation of PKC than TNF-α. Interestingly, both secreted and cell-associated forms of all tested cytokines were inhibited by Calphostin C to the same extent (data not shown).

It is well known that inhibition of PKC by Calphostin C requires photoactivation.[16] In our control cell cultures shielded from the light, Calphostin C did not affect cytokine production (data not shown), indicating its selectivity in blocking PKC.

Calphostin C reduces transcription of TNF-α and IL-1β

The synthesis of cytokines is regulated at the level of transcription and modified by post-transcriptional events. Therefore, we tested the influence of Calphostin C on the transcription of genes encoding TNF-α and IL-1β. This was done employing a semi-quantitative RT-PCR technique. Consistently with the results shown in Figure 1, the expression of mRNA encoding TNF-α and IL-1β was lower in PMA- than in LPS-treated monocytes (Fig. 3). In the presence of Calphostin C, both PMA- and LPS-induced expression of mRNA coding for tested cytokines was significantly diminished (Fig. 3).

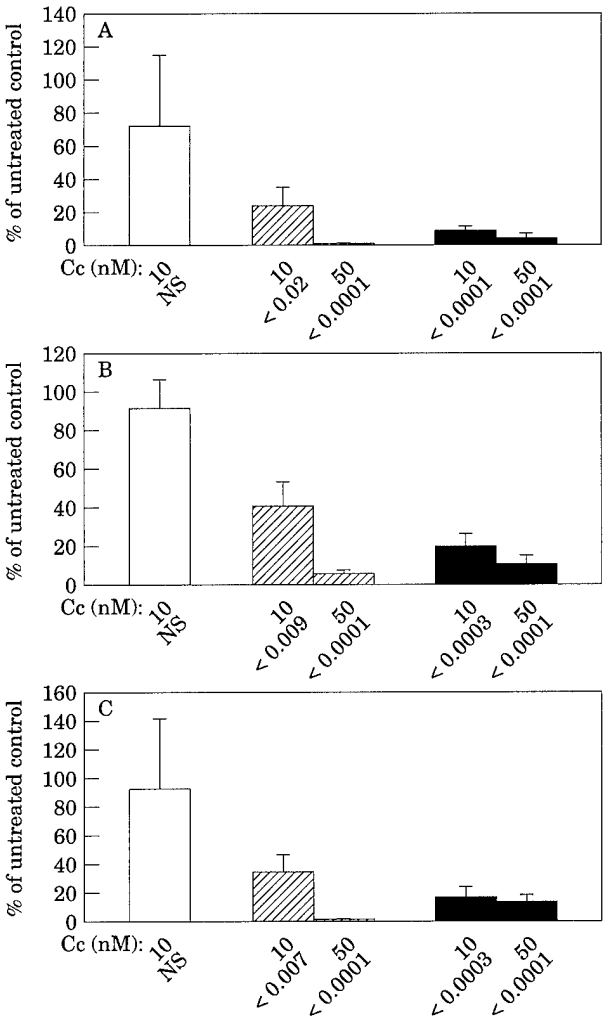

Figure 2. Calphostin C blocks production of cytokines.

Monocytes pre-treated for 2 h with 10 or 50 nM Calphostin C (Cc), were stimulated for 21 h with 5 μg/ml LPS or 1 nM PMA. Total production of cytokines (A: TNF-α; B: IL-1β; C: IL-6) was determined by ELISA. Results are expressed as the percentage of cytokine levels in cell cultures not exposed to Calphostin C and represent the mean ± SEM of five (PMA treatment, ▨) or 10 (LPS treatment, ■) experiments. Statistical comparison between cells pre-treated with Calphostin C and not treated, is shown as a *P* value. (□), unstimulated cells.

Expression of PKC isoenzymes in PMA- and Calphostin C-treated monocytes

Depending on the dose and time of the treatment, PMA exerts different effects on cellular PKC proteins, leading to either activation or degradation of the enzyme.[17] Upon activation, PKC isoenzymes translocate from the cytosol to the particular cellular fractions (e.g. plasma membrane, nuclear envelope).[17,18] In order to evaluate the activation of several PKC isoforms, we compared their distribution among the cytosolic, membrane and SDS/glycerol extractable particulate protein fractions isolated from monocytes stimulated with PMA in the presence or absence of

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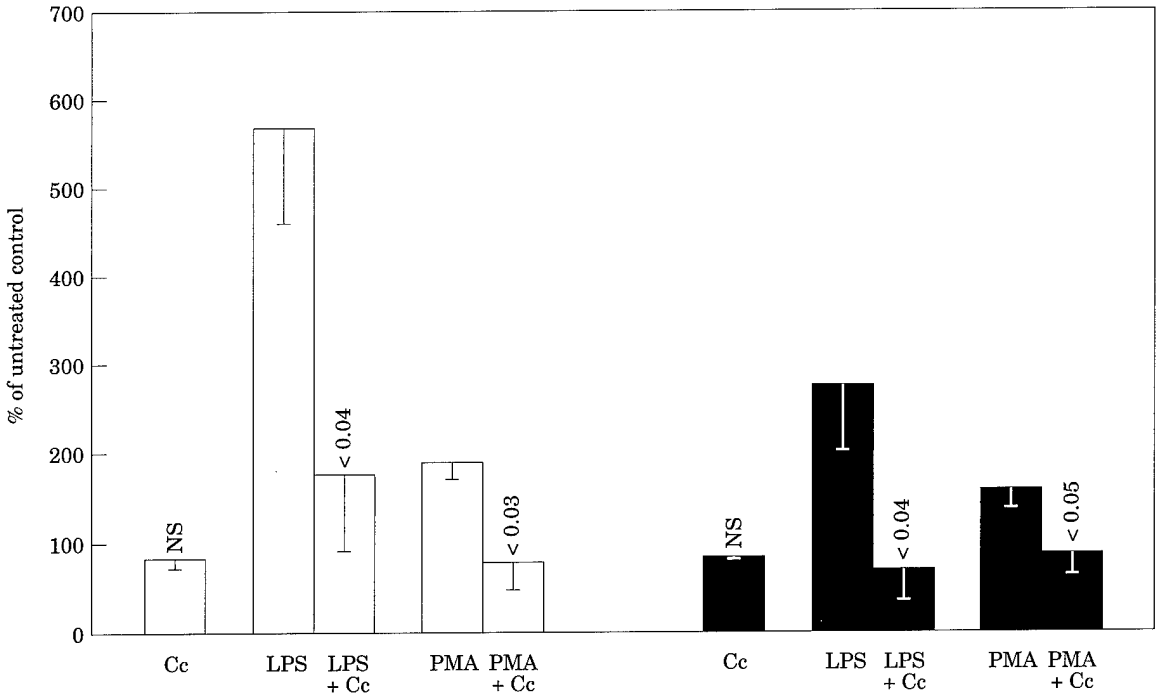

Figure 3. Calphostin C diminishes expression of mRNAs encoding TNF-α (□) and IL-1β (■).

Monocytes pre-treated for 2 h with 10 nM Calphostin C (Cc) were stimulated for another 2 h with 5 μg/ml LPS or 1 nM PMA. RNA was isolated and the amount of tested cytokine mRNA was measured using semiquantitative RT-PCR technique. Transcript levels were measured by densitometry and the ratio of monocyte cDNA to standard cDNA was calculated. Results (the mean ± SEM of four experiments) are expressed as a percentage of the response stated in untreated control (C) cells. *P* values, showing differences between the cells treated versus non-treated with Calphostin C are shown above the bars.

Calphostin C. After 21 h of stimulation with 1 nM PMA (optimal time to measure cytokine production), the translocation of PKC α, δ and ε isoenzymes from the cytosolic to membrane and particulate fractions were clearly observed (Fig. 4). In contrast, PKCβII, although transiently translocated (Fig. 5), was almost completely degraded upon prolonged PMA treatment (Fig. 4). Pretreatment of monocytes with Calphostin C (2 h; 50 nM) (Fig. 5) blocks PMA-triggered translocation of all tested PKC isoenzymes. Moreover, in these conditions PKC isoenzymes were completely (ε and βII) or partially (δ and α) degraded. Therefore, Calphostin C blocked translocation of PKC α, βII, δ and ε precedes the inhibition of cytokine production.

Calphostin C inhibits phosphorylation of MAP kinases

LPS is known to activate the MAP kinase cascade.[11,19,20] Although this event is distal from PKC activation, in some systems it may be PKC dependent.[21] Phosphorylation of MAP kinases in tyrosine/threonine residues is prerequisite for their activation.[21] To determine whether the activation of MAP kinases depends on the PKC activation, we tested the effect of Calphostin C on the phosphorylation status of MAP kinases isolated from PMA- and LPS-treated monocytes. While the PMA- or LPS-treatment results in the phoshorylation of MAP kinases (Erk1/Erk2) on tyrosine and threonine residues, Calphostin C (50 nM) completely blocked LPS- and PMA-triggered phosphorylation of these enzymes (Fig. 6).

These results show that phosphorylation of MAP kinases, an important event in LPS triggered signal transduction, is also dependent on the activation of PKC pathway.

Calphostin C blocks DNA-binding activity of AP-1 transcription factor

The AP-1 transcription factor regulates the transcription of cytokine genes in either a positive (TNF-α and IL-1β) or negative (IL-6) manner.[13,14,22,23] In some systems AP-1 represents also one of the downstream targets on the PKC-dependent pathway.[24] Therefore, we tested whether the effects of Calphostin C on PKC translocation would reflect on AP-1 activity in stimulated monocytes. The DNA-binding activity of AP-1 was probed in cells pre-treated with 50 nM Calphostin C followed by 2-h stimulation with PMA or LPS. In resting cells the AP-1 binding activity was weak or undetectable but it was significantly induced upon stimulation (Fig. 7). Calphostin C reduced DNA binding activity of AP-1 in both PMA- and LPS-treated

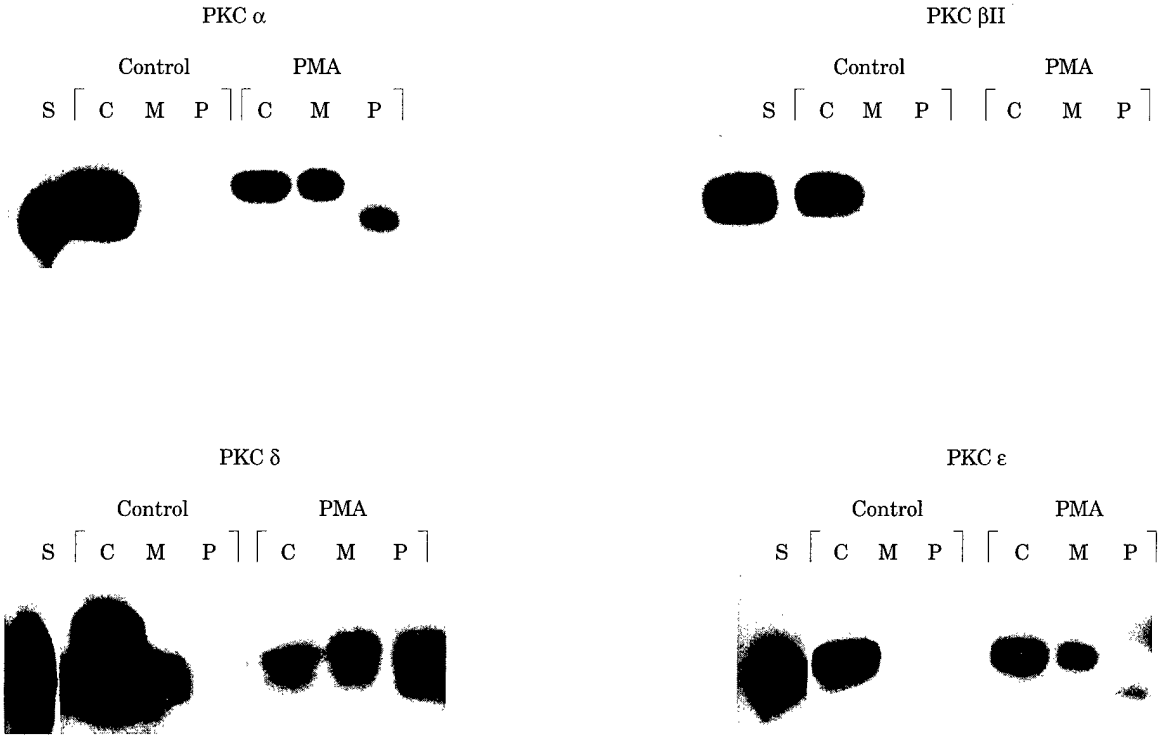

Figure 4. PMA induces long-lasting translocation of PKC α, δ and ε isoenzymes.

Monocytes were stimulated for 21 h with 1 nM PMA. The cytosolic (C), membrane (M) and particulate (P) protein fractions were isolated from the cells and proteins (40 μg/lane) were tested for the presence of PKC isoform by Western blotting. Control, non-stimulated cells; S, standard of the respective human recombinant PKC isoenzyme.

cells. In contrast, the DNA-binding activity of housekeeping Oct-1 transcription factor was not affected by any of these treatments.

These results support the notion that AP-1 DNA-binding activity also depends on the PKC pathway. Thus, Calphostin C reduced cytokine production may, at least in part, be mediated by impaired activation of AP-1.

DISCUSSION

In the present study we first confirmed that in our experimental conditions the PKC-dependent pathway plays an important role in the synthesis of pro-inflammatory cytokines. The results of experiments where selective PKC inhibitor, Calphostin C, significantly blocked PMA- as well as LPS-induced cytokine production (Fig. 2), support this hypothesis. Interestingly, PMA, a potent activator of PKC, only partially mimics LPS-induced synthesis of these cytokines exerting the following dependency on the sole activation of PKC: TNF-α>IL-1β>IL-6 (Fig. 1). These results suggest that PKC activation is necessary, but not sufficient for the optimal production of pro-inflammatory cytokines. Therefore, we propose that the co-operation of at least two different signalling pathways—one that requires PKC activation and another, exerting a dominant role for IL-6, and to a lesser extent participating in IL-1β and TNF-α—is required for the optimal (e.g. triggered by LPS) induction of pro-inflammatory cytokines synthesis. The synergism between PKC- and cAMP-dependent pathways has been proposed for the induction of IL-1β production in human myeloid cell lines.[25] However, in human monocytes the elevation of cAMP was shown to inhibit rather than increase the LPS-triggered production of TNFα and IL-1β.[26,27] Therefore, it is likely that previously reported co-operation between PKC- and another, PTK-dependent pathways is more relevant in monocytes.[12,20]

Interestingly, we also observed that upon PMA stimulation most, if not all, of de novo synthesized IL-1β protein is released into the media. This result corroborates with the report of others that not only the synthesis but also the secretion of IL-1β is regulated by PKC.[28]

The IL-6 production is dependent on PKC in a variety of non-haemopoietic cells.[29,30] In contrast, in human monocytes the synthesis of this cytokine was reported to be PKC independent.[31] However, the latter finding was based on the: (1) failure of PMA to induce IL-6 production and (2) elevation of IL-6 transcription by serine/threonine kinase inhibitor, staurosporin. Our observation that only some individuals produce IL-6

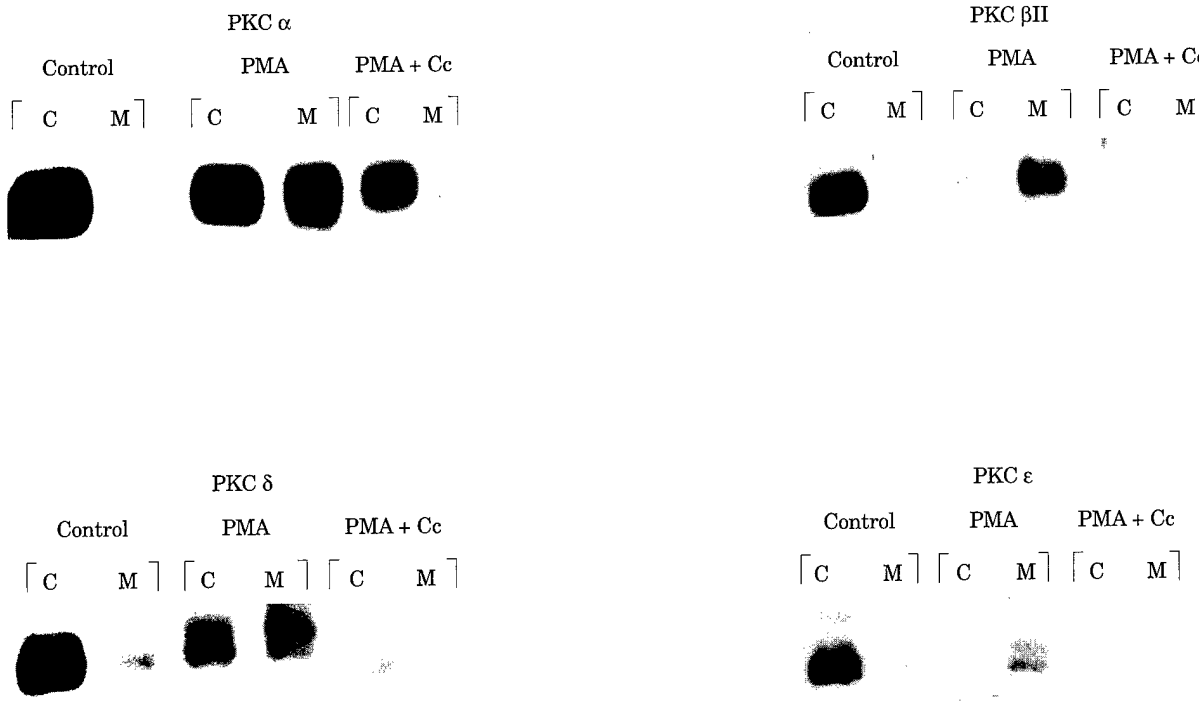

Figure 5. Calphostin C inhibits PMA-triggered translocation and induces degradation of PKC isoenzymes.

Monocytes pre-treated for 2 h with 50 nM Calphostin C (Cc), were stimulated for 15 min with 1 nM PMA. Proteins (10 μg/lane) of cytosolic (C) and membrane (M) fractions were tested for the presence of PKC isoforms using Western blotting technique. Calphostin C inhibited PMA-triggered translocation of all tested PKC isoforms (disappearance from M fraction). Upon Calphostin C treatment PKC isoenzymes were completely or partially (e>βII>δ>α) lost also from C fraction (degradation of the isoenzymes).

upon PMA treatment, as well as the evidence that staurosporin may exert partial PKC agonistic activity in some systems,[17] give support to the notion that PKC activation may play an important role in the induction of IL-6 synthesis. Taken together these data provide the evidence that activation of PKC is necessary for LPS-stimulated production of all tested pro-inflammatory cytokines.

Next, we explored the activation of different PKC isoforms during the stimulation leading to select cytokine production. The PKC family is heterogeneous and consists of isozymes that differ in their structure, cofactor requirement, tissue distribution and subcellular localization.[32] Although there are reports suggesting that biological functions of PKC are also isozyme selective,[32–35] the role of individual PKC isoenzymes in cellular processes, especially in the induction of cytokine synthesis, only recently received attention. For example, there is evidence indicating that PKCβ activation plays an important role in LPS-induced production of TNF-α in mouse peritoneal macrophages.[36] Although the LPS-triggered activation of PKCζ in human monocytes was reported, the role of this PKC isoform in the induction of pro-inflammatory cytokine synthesis has not been tested.[37] In the present study we have focused on the role of classical (α and βII) and novel (δ and ε) PKC isoenzymes in this process. We report, that at the time of maximal cytokine production (21 h), the PMA triggered translocation of PKC α, δ and ε to membrane fraction was still evident, while most of PKCβII protein was already degraded (Fig. 4). Interestingly, although more than 80% of monocytes cultured in the presence of Calphostin C for 21 h were alive (see Materials and Methods), all tested PKC isoforms were either partially or completely degraded (Fig. 5). These results show that the induction of pro-inflammatory cytokines is associated with PKC α, δ, ε and βII activation and suggest that the importance of particular PKC isoenzyme is not equal. Whether the activation of all or only some of these PKC isoenzymes is important for cytokine synthesis, is currently tested in our laboratory using isoenzyme-selective PKC inhibitors.

Activation of MAP kinase cascade represents an important signalling event triggered by LPS.[11,19,20] MAP kinases are activated by various up-stream signalling events, e.g. by Ras/Raf kinase pathway,[21] and/or PKC.[21,38] In addition, MAP kinases were demonstrated to participate in the activation of $cPLA_2$ leading to the release of arachidonic acid.[19] The activation of MAPK results, in turn, in the induction of several transcription factors crucial for transcription of genes encoding pro-inflammatory cytokines, e.g. c-*fos* component of AP-1 transcription factor and

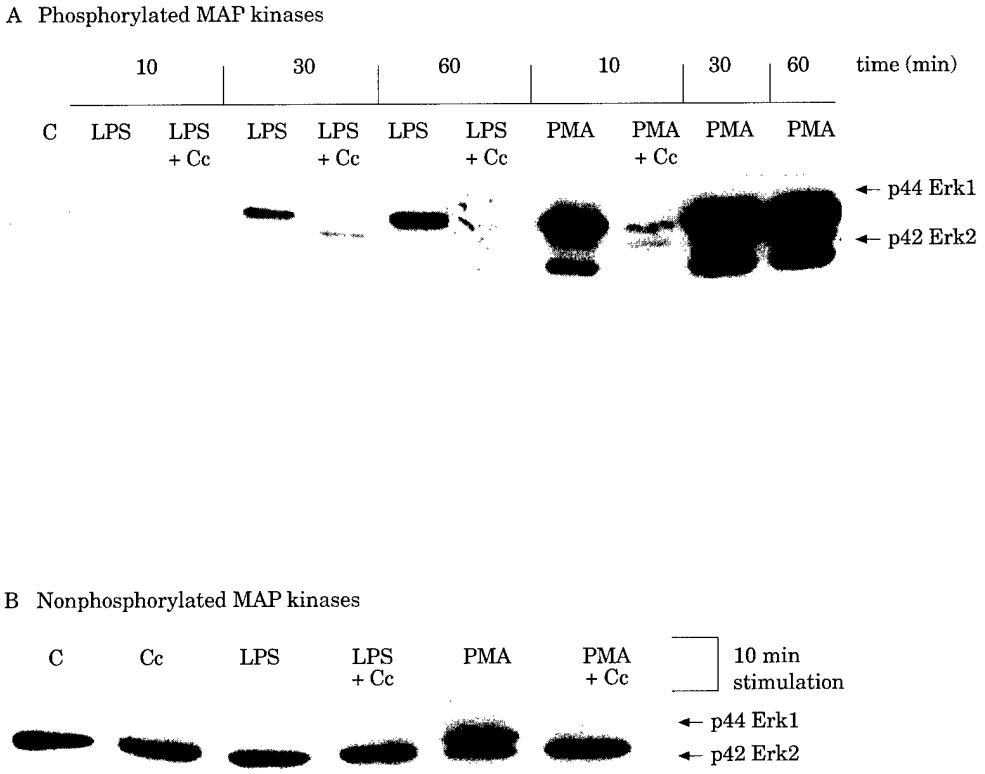

Figure 6. Calphostin C inhibits phosphorylation of MAP kinases.

Monocytes pre-treated with 50 nM Calphostin C (Cc), were stimulated with 5 μg/ml LPS or 1 nM PMA for the indicated time. Total protein fraction was isolated and the expression of phosphorylated (A) and nonphosphorylated (B) MAP kinases was determined in the same samples using Western blotting. Position of Erk1 and Erk2 isoenzymes is indicated. C-non-stimulated control cells. This is a representative experiment selected from three performed.

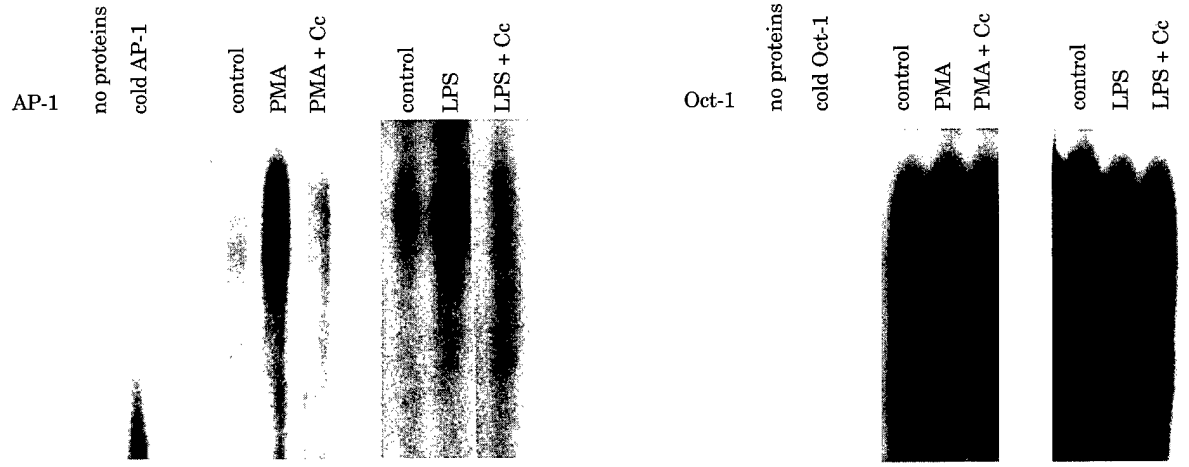

Figure 7. Calphostin C reduces DNA-binding activity of AP-1 transcription factor.

Monocytes pre-treated with 50 nM Calphostin C (Cc) were stimulated for 2 h with 5 μg/ml LPS or 1 nM PMA. Nuclear protein fraction was isolated and assayed by EMSA, using AP-1 and Oct-1 consensus oligonucleotides. Specificity of the binding was controlled by excluding protein samples and by adding 100-fold excess of unlabelled probes. Control, non-stimulated cells. This is a representative experiment selected from three performed.

NF-IL6.[21,24] Our data show that inhibition of PKC α, δ, ε and βII translocation result in the blockade of MAPK phosphorylation that precede impaired production of pro-inflammatory cytokines (Fig. 6). Interestingly, recent reports show that PKC α and δ isoenzymes act as the activators of MAPK in various

types of cells.[39–41] The possibility that these PKC isoforms may activate MAPK in human monocytes is presently tested in our laboratory.

Transcription of human genes encoding TNF-α, IL-1β and IL-6 expression is a complex process that requires participation of several transcription factors including AP-1. Although AP-1 regulates transcription of TNF-α and IL-1β genes in a positive manner, it is also considered as a negative regulator of IL-6 gene transcription.[23] AP-1 exists as a homo- or heterodimer formed by proteins of the Fos and the Jun families and is a target molecule for PKC and the enzymes of MAP kinase family.[24]

Our results show that Calphostin C-blocked PKC translocation is reflected downstream in diminished AP-1 binding activity in monocytes stimulated with LPS or PMA (Fig. 7). Thus, it is likely that inhibitory effects of Calphostin C on the induction of TNF-α and IL-1β synthesis is, at least in part, mediated by depressed activity of AP-1. In contrast, PMA triggered induction of AP-1 may account for a negligible production of IL-6 observed in monocytes of some individuals. However, similar to TNF-α and IL-1β, the production of IL-6 in LPS-treated cells was also blocked by Calphostin C. This result suggests that in LPS-stimulated response, PKC pathway may also modulate the activity of other transcription factor(s) crucial for IL-6 gene transcription.

Although we have not tested the activity of other key transcription factors, our results imply that PKC dependent pathway participates in TNF-α and IL-1β synthesis acting via AP-1. Whether all or some of PKC isoforms associated with the induction of these cytokine synthesis are important for AP-1 transcriptional activity, remains to be elucidated. Interestingly, it has recently been reported that PKC α regulates the composition of AP-1 complexes in mouse keratinocytes,[42] while PKC-theta selectively stimulates AP-1 in murine T cell lines.[43] Thus, it is tempting to speculate that transcriptional activity of AP-1 during the process leading to cytokine synthesis may be controlled by PKC in an isoenzyme-specific manner.

In summary, our data: (1) demonstrate the key role of PKC in the induction of pro-inflammatory cytokine synthesis in human monocytes; (2) suggest a possible role of PKC α, δ, ε and βII isoenzymes in this process; and (3) support the notion that the activation of MAPK and AP-1 transcription factor participate in the PKC-dependent pathway controlling several aspects of inflammatory response.

MATERIALS AND METHODS

Cell culture and treatment

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood of healthy adult volunteers on density gradient using Gradisol L (Polfa, Poland), then incubated on plastic Petri dishes for 1 h at 37°C in RPMI 1640 medium supplemented with 10% FCS (Gibco, Paisley, UK), 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 1 mM HEPES (Gibco). The non-adherent cells were removed by vigorous washing with RPMI. The remaining adherent cells (monocytes) contained less than 10% of $CD2^+$ T lymphocytes and $CD19^+$ B lymphocytes, as estimated by immunofluorescent staining and flow cytometric analysis. Monocytes isolated from 2×10^6 (for ELISA) or from 10×10^6 (for Western blotting, EMSA and for RNA isolation) of PBMCs were cultured in RPMI medium containing 1% FCS and 2.5×10^{-5} M of β-mercaptoethanol. Cells were stimulated with 5 μg/ml of lipopolisaccharide (LPS; *E. coli* 055:B5; Difco, Detroit, MI), 1 nM of phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO) or 1 nM of non-active PMA analog (4αPMA; Sigma). Selective PKC inhibitor, Calphostin C (Sigma), was added 2 h before stimuli and cell cultures were exposed to light to activate the compound.[16] Control cultures were kept in the darkness.

Cell viability, assayed by trypan blue exclusion, exceeded 95% after adherence, and 80% after 21-h incubation in the presence or absence of stimuli and inhibitor.

Cytokine detection using enzyme-linked immunosorbent assay (ELISA)

Cytokine production was assayed after 21 h of culture. Culture supernatants were used to measure production of secreted forms of cytokines. The production of cell-associated cytokines was determined in cell lysates, obtained by three cycles of freezing-thawing of cells resuspended in 1 ml of freshly added culture medium. The concentration of cytokines was determined using specific ELISA as previously described.[44] Goat polyclonal, neutralizing antibodies specific to human TNF-α, IL-1β or IL-6 (R&D Systems, Minneapolis, MN) were used as capture, while cytokine specific rabbit polyclonal antibodies (anti-TNF-α from Genzyme, Cambridge, MA; anti-IL-1β and anti-IL-6 from Sigma) were used as detection antibodies, followed by horseradish peroxidase conjugated goat anti-rabbit immunoglobulins and *O*-phenylenediamine dihydrochloride (both from Sigma) as a substrate. Human recombinant cytokine standards were from R&D Systems. Optical density was measured at 492 nm using an automatic ELISA reader (LP 400, Diagnostics Pasteur, France). The detection limit was 4 pg/ml for TNF-α and 15 pg/ml for both IL-1β and IL-6.

Protein analysis by Western blotting

Expression of PKC isoenzymes were analysed in the cytosolic, membrane and particulate protein fractions according to the method previously described.[35] The cytosolic fraction was prepared from cells treated with the lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml digitonin, 10 μg/ml leupeptine, 10 μg/ml pepstatin A). The remaining protein pellets were treated with the lysis buffer supplemented with 1% of Triton X-100 to obtain the membrane fraction. The particulate fraction was extracted from the pellets remaining after collection of

cytosolic and membrane fractions by boiling for 5 min in a 125 mM Tris-HCl (pH 6.8) buffer supplemented with 2.5% SDS and 10% glycerol. Protein contents was measured using Bio-Rad Protein Assay kit (Bio-Rad, Germany) and adjusted to the same level in tested samples. Proteins were than acetone precipitated, boiled in Laemli buffer and samples containing 10 or 40 mg of protein were separated on 8% SDS-PAGE. After blotting, the PVDF membranes (BioRad, Germany) were treated overnight with the rabbit polyclonal antibodies specific to PKC α, βII, δ or ε isoenzymes (Calbiochem, La Jolla, CA), respectively. Cytokine-bound antibody was detected using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins and the ECL system (Amersham, UK). The specificity of primary antibody binding was assured by pre-incubation of the antibody with 0.5 mg/ml of isoform-specific peptide (Calbiochem) for 10 min at room temperature, prior to use. Human recombinant PKC α, βII, δ and ε isoenzymes (Calbiochem) were used as the positive controls.

Phosphorylation of mitogen-activated protein kinases (MAP kinases; Erk1/Erk2) was detected using the PhosphoPlus p44/42 MAP kinase antibody kit, containing antibodies specific to either non-phosphorylated or phosphorylated (recognizing Thr 202/Tyr 204) forms of MAP kinases (New England BioLabs, USA), according to the manufacturer protocol.

Analysis of cytokine mRNA by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from monocytes using TRIZOL reagent (Gibco BRL, UK), according to the manufacturer protocol. All reagents used for reverse transcription and PCR amplification were from Perkin-Elmer, USA. Total RNA (25 ng or 50 ng for IL-1β or TNF-α mRNA, respectively) served as a template for cDNA preparation, using GeneAmp RNA PCR kit. Copies of pAW109 RNA 1×10^5, used as an internal standard, were transcribed in the same reaction. The reverse transcription reactions were performed at 42°C for 40 min. Ten microlitres of these cDNA solutions were amplified by PCR using 2.5 U of Taq DNA polymerase and 0.15 μM of each primer (AW112/AW113 primers for TNF-α and DM155/DM156 primers for IL-1β) in 1 × PCR buffer II containing 2 mM $MgCl_2$, in a total volume of 40 μl. The thermoamplification program consisted of an initial denaturation (1 min at 95°C), followed by 35 cycles of 1 min denaturation (95°C), 2 min annealing (55°C) and 3 min elongation (72°C), with a final extension period of 5 min at 72°C. Products were separated on 2% agarose gel containing 3% (v/v) ethidium bromide and detected by trans-illumination with UV light. Gels were photographed, bands representing amplified products were scanned and the ratio of tested to standard RNA was calculated.

Estimation of DNA-binding activity of AP-1 transcription factor by the electromobility gel-shift assay (EMSA)

The nuclear protein extracts were prepared by micropreparation technique.[45] The protein concentration was measured, samples normalized for protein contents and stored at −70°C. The AP-1 oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA) were ^{32}P end-labelled, using T4 polynucleotide kinase and [γ-^{32}P]ATP (both reagents from Amersham, UK). Gel-shift assay was performed as previously described.[46] 2.5 μg of protein extract was incubated in ice-water bath for 1 h with AP-1 labelled probes (0.1 ng; 50 000 cpm). The specificity of probe binding was assured by adding 100-fold excess of unlabeled probe. Binding of proteins to Oct-1 ("house-keeping" sequence) probe (Santa Cruz Biotechnology) served as a control. The protein-DNA complexes were resolved for 2 h at 250 V on pre-electrophoresed 5% non-denaturating polyacrylamide gel in Tris-borate-EDTA (TBE) buffer, containing 0.02% NP-40. The gels were autoradiographed on X-ray films (Amersham, UK).

Statistical analysis

The paired *t*-test was applied to evaluate the effect of stimuli and Calphostin C. Probability values less than 0.05 were considered as statistically significant.

Acknowledgements

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REFERENCES

1. Rink L, Kirchner H (1996) Recent progress in the tumour necrosis factor-α field. Int Arch Allergy Immunol 111:199–209.
2. Dinarello CA (1996) Biological basis for interleukin-1 in disease. Blood 87:2095–2147.
3. Lotz M (1995) Interleukin-6: a comprehensive review. Cancer Treat Res 80:209–233.
4. Müller-Ladner U (1996) Molecular and cellular interactions in rheumatoid synovium Curr Opin Rheumatol 8:210–220.
5. Koch AE, Kunkel SL, Strieter RM (1995) Cytokines in rheumatoid arthritis. J Invest Med 43:28–38.
6. Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW (1994) Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumour necrosis factor receptor p55. Blood 83:134–118.
7. Tilg H, Dinarello CA, Mier JW (1997) IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. Immunol Today 18:428–432.
8. Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, Busse LA, Zukowski MM, Wright SD (1994) Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med 179:269–277.
9. Ingalls RR, Golenbock DT (1995) CD11c/CD18, a transmembrane signaling receptor for lipopolysaccharide. J Exp Med 181:1473–1479.
10. Stefanova I, Corcoran ML, Horak EM, Wahl LM, Bolen JB, Horak ID (1993) Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase $p53/56^{lyn}$. J Biol Chem 268:20725–20728.
11. Reimann T, Bücher D, Hipskind RA, Krautwald S, Lohmann-Matthes ML, Baccarini M (1994) Lipopolysaccharide induces activation of the Raf-1/MAP kinase pathway. J Immunol 153:5740–5749.

12. Shapira L, Takashiba S, Champagne C, Amar S, Van Dyke TE (1994) Involvement of protein kinase C and protein tyrosine kinase in lipopolysaccharide-induced TNFα and IL-1β production by human monocytes. J Immunol 153:1818–1834.

13. Aggarwal BB, Natarajan K (1996) Tumor necrosis factors: development during the last decade Eur Cytokine Netw 7:93–124.

14. Auron PE, Webb AC (1994) Interleukin-1: a gene expression system regulated at multiple levels Eur Cytokine Netw 5: 573–592.

15. Narazaki M, Kishimoto T (1994) Interleukin-6 (IL-6). In Nicola NA (ed) Guidebook to cytokines and their receptors. A Sambrook and Tooze publication, Oxford University Press, pp. 56–58.

16. Brund RF, Miller FD, Merriman RL, Howbert JJ, Heath WF, Kobayashi E, Takahashi I, Tamaoki T, Nakano H (1991) Inhibition of protein kinase C by calphostin C is light-dependent. Biochem Biophys Res Commun 176:288–293.

17. Basu A (1993) The potential of protein kinase C as a target for anticancer treatment. Pharmac Ther 59:257–280.

18. Newton A (1995) Protein kinase C: structure, function, and regulation. J Biol Chem 270:28495–28498.

19. Chow CW, Grinstein S, Rotstein OD (1995) Signaling events in monocytes and macrophages. New Horiz 3:342–351.

20. DeFranco AL, Hambleton J, McMahon M, Weinstein SL (1995) Examination of the role of MAP kinase in the response of macrophages to lipopolysaccharide. Prog Clin Biol Res 392:407–420.

21. Seger R, Krebs EG (1995) The MAPK signaling cascade FASEB J 9:726–735.

22. Janaswami PM, Kalvakolanu DVR, Zhang Y, Sen GC (1992) Transcriptional repression of interleukin-6 gene by adenoviral E1A proteins. J Biol Chem 267:24886–24891.

23. Hsu W, Kerppola TK, Chen PL, Curran T, Chen-Kiang S (1994) Fos and Jun repress transcriptional activation by NF-IL6 through association at the basic zipper region. Mol Cell Biol 14:268–276.

24. Foletta VC (1996) Transcriptional factor AP-1, and the role of Fra-2. Immunol Cell Biol 74:121–133.

25. Serkkola E, Hurme M (1993) Synergism between protein-kinase C and cAMP-dependent pathways in the expression of the interleukin-1β gene is mediated via the activator-protein-1 (AP-1) enhancer activity Eur J Biochem 213:243–249.

26. Molnar-Kimber K, Yonno L, Heaslip R, Weichman B (1993) Modulation of TNFα and IL-1β from endotoxin-stimulated monocytes by selective PDE isozyme inhibitors. Agents Actions 39:C77–C79.

27. Sinha B, Semmler J, Eisenhut T, Eigler A, Endres S (1995) Enhanced tumor necrosis factor suppression and cyclic adenosine monophosphate accumulation by combination of phosphodiesterase inhibitors and prostanoids Eur J Immunol 25:147–153.

28. Bakouche O, Moreau JL, Lachman LB (1992) Secretion of IL-1: role of protein kinase C. J Immunol 148:84–91.

29. Kim GS, Kim CH, Choi CS, Park JY, Lee KU (1997) Involvement of different second messengers in parathyroid hormone- and interleukin-1-induced interleukin-6 and interleukin-11 production in human bone marrow stromal cells. J Bone Miner Res 12:896–902.

30. Kozawa O, Suzuki A, Tokuda H, Uematsu T (1997) Prostaglandin F2alpha stimulates interleukin-6 synthesis via activation of PKC in osteoblast-like cells. Am J Physiol 272 (2Pt 1): E208–E211.

31. Gross V, Zhang B, Geng Y, Villiger PM, Lotz M (1993) Regulation of interleukin-6 (IL-6) expression: evidence for a tissue-specific role of protein kinase C. J Clin Immunol 13:310–320.

32. Jaken S (1996) Protein kinase C isozymes and substrates Curr Opin Cell Biol 8:168–173.

33. Kiley SC, Parker PJ (1995) Differential localization of protein kinase C isozymes in U937 cells: evidence for distinct isozyme functions during monocyte differentiation. J Cell Sci 108:1003–1016.

34. Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses FASEB J 9:484–496.

35. Assender JW, Kontny E, Fredholm BB (1994) Expression of protein kinase C isoforms in smooth muscle cells in various states of differentiation FEBS Lett 342:76–80.

36. Shinji H, Akagawa KS, Tsuji M, Maeda M, Yamada R, Matsuura K, Yamamoto S, Yoshida T (1997) Lipopolysaccharide-induced biphasic inositol 1,4,5-trisphosphate response and tyrosine phosphorylation of 140-kilodalton protein in mouse peritoneal macrophages. J Immunol 158:1370–1376.

37. Herrera-Velit P, Knutson KL, Reiner NE (1997) Phosphatidylinositol 3-kinase-dependent activation of protein kinase C-zeta in bacterial lipopolysaccharide-treated human monocytes. J Biol Chem 272:16445–16452.

38. Büscher D, Hipskind RA, Krautwald S, Reimann T, Baccarini M (1995) Ras-dependent and -independent pathways target the mitogen-activated protein kinase network in macrophages. Mol Cell Biol 15:466–475.

39. Young SW, Dickens M, Tavare JM (1996) Activation of mitogen-activated protein kinase by protein kinase C isotypes alpha, beta I and gamma, but not epsilon FEBS Lett 384:181–184.

40. Yamaguchi K, Ogita K, Nakamura S, Nishizuka Y (1995) The protein kinase C isoforms leading to Map-kinase activation in CHO cells. Biochem Biophys Res Commun 210:639–647.

41. Ueda Y, Hirai Si, Osada Si, Suzuki A, Mizuno K, Ohno S (1996) Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J Biol Chem 271:23512–23519.

42. Rutberg SE, Saez E, Glick A, Dlugosz AA, Spiegelman BM, Yuspa SH (1996) Differentiation of mouse keratinocytes is accompanied by PKC-dependent changes in AP-1 proteins. Oncogene 13:167–176.

43. Baier-Bitterlich G, Uberall F, Bauer B, Fresser F, Wachter H, Grunicke H, Utermann G, Altman A, Baier G (1996) Protein kinase C-theta isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. Mol Cell Biol 16:1842–1850.

44. Ziolkowska M, Kontny E, Dudzinska E, Filipowicz-Sosnowska A, Ryzewska A (1996) Effect of adrenaline on IL-1β and TNFα production by peripheral blood mononuclear cells from patients with rheumatoid arthritis. Int J Thymol 4:1–6.

45. Andrews NC, Faller DV (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499.

46. Kontny E, Ciruela F, Svenningsson P, Ibàñez CF, Fredholm BB (1997) On the role of the low-affinity neurotrophin receptor $p75^{LNTR}$ in nerve growth factor induction of differentiation and AP-1 binding activity in PC12 cells. J Mol Neurosci 8:29–44.

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GM-CSF RESCUES TF-1 CELLS FROM GROWTH FACTOR WITHDRAWAL-INDUCED, BUT NOT DIFFERENTIATION-INDUCED APOPTOSIS: THE ROLE OF BCL-2 AND MCL-1

Lidija Klampfer, Jin Zhang, Stephen D. Nimer

Cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-3 promote the survival and stimulate the proliferation of haematopoietic cells. Using the GM-CSF-dependent TF-1 myeloid leukaemia cell line, the authors show that the endogenous levels of BCL-2 and MCL-1 are downregulated upon GM-CSF withdrawal, whereas the levels of BCL-x_L and Bax are unchanged. Re-exposure of growth factor deprived cells to GM-CSF resulted in an early and transient increase in MCL-1 expression, and prolonged induction of BCL-2, which prevented apoptosis. In contrast, the expression of BCL-2 and MCL-1 were not modulated during TPA-induced differentiation of TF-1 cells, which was followed by apoptosis despite the presence of GM-CSF. TF-1 cells overexpressing BCL-2 or MCL-1 underwent delayed apoptosis upon growth factor withdrawal, but displayed no impaired apoptosis in response to TPA. Erythropoietin (Epo) induced the expression of BCL-2 and MCL-1 protein in TF-1 cells, however it did not support their long term proliferation, further demonstrating that upregulation of these anti-apoptotic genes is insufficient for the long term proliferation of TF-1 cells.

Programmed cell death (apoptosis) is a genetically determined response of cells to certain stimuli, such as growth factor withdrawal, DNA-damaging agents and differentiation inducing agents.[1] Programmed cell death is regulated by the expression of many genes; some of them, such as BCL-2, BCL-X_L, BCL-w and MCL-1, can prevent or delay the onset of apoptosis, while others (e.g. Bax, Bik, Bak, Bad and BCL-X_S) antagonize the function of antiapoptotic genes and can induce or accelerate cell death.[2,3] There is extensive crosstalk between BCL-2 family members by virtue of their protein–protein interactions and the ratio of proapoptotic to antiapoptotic proteins has been shown to be a major determinant of the cells' propensity to undergo apoptosis.

The MCL-1 gene was initially cloned as a gene differentially expressed in TPA treated myeloid cells.[4] MCL-1 shows extensive homology to BCL-2; it can heterodimerize with Bax[5] and thus plays an important role in preventing apoptosis.[6–8] Recent studies have shown that myeloblasts from patients with acute myeloid leukaemia (AML) express higher levels of MCL-1 at relapse than at first presentation of the disease,[9] suggesting that, like BCL-2, MCL-1 may play a role in resistance to chemotherapy. In contrast to MCL-1, BCL-2, and BCL-X_L, Bax promotes apoptosis, and its ability to dimerize with several members of the BCL-2 family is essential for its proapoptotic activity.[5,10]

Failure of cells to undergo apoptosis has been linked to developmental abnormalities, autoimmune diseases and cancer, while enhanced apoptosis has been linked to the pathogenesis of certain viral infections and neurodegenerative diseases.[11] Recently, several cytokines, in addition to controlling cell proliferation, have been shown to regulate the process of apoptosis by modulating the expression of BCL-2 family members and/or by inducing postranscriptional modifications of these proteins.[12–15] Hence, the survival and proliferation of myeloid leukaemia cells commonly depends on the presence of exogenous growth factors, such as GM-CSF, IL-3 or G-CSF.

GM-CSF is a potent growth factor for a variety of haematopoietic cells. Upon binding to its heterodimeric

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receptor it activates several signaling pathways, including the Ras-raf-MAPK and the JAK-STAT pathways, resulting in activation of several genes, such as c-myc and A1,[16,17] and generating both mitogenic and anti-apoptotic signal. We studied the role of GM-CSF in supporting the viability of TF-1 cells by examining its effect on the expression of several genes involved in apoptosis. We found that GM-CSF withdrawal results in the downregulation of both BCL-2 and MCL-1 which coincides with induction of apoptosis. We also demonstrate that TPA-induced differentiation of TF-1 cells is coupled to induction of apoptosis, as shown by the activation of DEVD specific caspases and cleavage of PARP in TPA-treated cells. Whereas re-exposure of growth factor deprived TF-1 cells to GM-CSF increased BCL-2 and MCL-1 levels and prevented apoptosis, GM-CSF did not rescue cells from TPA-induced cell death. TF-1 cells transfected with a BCL-2 or MCL-1 expression vector underwent delayed apoptosis upon GM-CSF withdrawal, but did not proliferate in the absence of cytokine. Likewise, although Epo upregulates BCL-2 and MCL-1 expression as efficiently as GM-CSF or IL-3, it cannot support the continuing proliferation of TF-1 cells. Overexpression of BCL-2 or MCL-1 did not interfere with TPA-induced cell death, suggesting that distinct signaling pathways mediate growth factor withdrawal induced and TPA-induced apoptosis.

RESULTS

GM-CSF withdrawal or TPA treatment induces apoptosis in TF-1 cells

TF-1 cells maintained in GM-CSF depleted media for 24 h show classical morphological features of apoptosis, including cytoplasmic boiling and nuclear fragmentation (Refs 20, 21, and data not shown). We demonstrated a characteristic pattern of DNA fragmentation in TF-1 cells maintained in growth factor depleted media for 24 h (Fig. 1, lane 1), but not in cells grown in media containing GM-CSF (Fig. 1, lane 2). TPA induces the rapid differentiation of TF-1 cells into macrophages (Ref. 22, and data not shown) and generates a similar pattern of DNA fragmentation as growth factor withdrawal (Fig. 1, lane 3). GM-CSF did not completely block TPA-induced DNA fragmentation (Fig. 1, lane 4), suggesting that TPA activates an irreversible cell death program in these cells.

We also demonstrated activation of DEVD-specific caspase (caspase-3 like) in cells grown in GM-CSF depleted media and in cells treated with TPA for 48 h (Fig. 1B). Accordingly, PARP, a substrate for caspase-3, is cleaved in both GM-CSF starved cells (Fig. 1C, lane 2) and in cells undergoing TPA-induced differentiation (Fig. 1C, lane 3). Although we measured higher DEVD caspase specific activity in

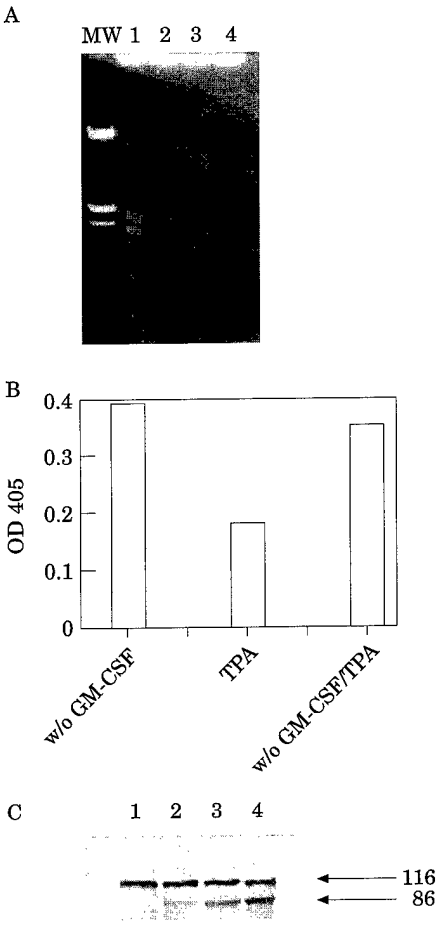

Figure 1. GM-CSF withdrawal or TPA stimulation induces apoptosis of TF-1 cells.

A: DNA fragmentation. Cells were cultured for 24 h in the absence (lane 1) or presence (lane 2) of GM-CSF (10 ng/ml). Other cells were stimulated with TPA (0.2 ng/ml) for 24 h in the presence (lane 3) or absence (lane 4) of GM-CSF. DNA fragmentation was monitored by electrophoresis in 2% agarose gel. B: Caspase-3 activation in growth factor deprived and/or TPA treated cells. Caspase-3 activity was determined in cells deprived of GM-CSF (w/o GM-CSF) or treated with TPA (0.2 ng/ml) for 60 h in the presence (middle bar) or absence (far right bar) of GM-CSF. Background caspase activity in cells grown in the presence of GM-CSF was assigned the value 0. C: Cleavage of PARP in cells grown in the presence (lane 1), or absence (lane 2) of GM-CSF for 48 h, or in cells treated with TPA (0.1 ng/ml) in the absence (lane 3) or presence of GM-CSF (lane 4). The 118 kDa (intact form) and 86 kDa (cleavage form) of PARP were detected using a polyclonal anti-PARP antibody (Pharmingen) in a Western blot assay.

GM-CSF-deprived cells than in TPA-treated cells, PARP cleavage products were more abundant in TPA-stimulated cells, suggesting that caspases outside of the DEVD-specific family contribute to the cleavage of PARP during TPA-induced cell death.

GM-CSF, but not TPA, regulates the expression of BCL-2 and MCL-1

Various members of the BCL-2 family have been shown to play important roles in the GM-CSF dependent survival of myeloid leukaemia cell lines.[18,19,21] We

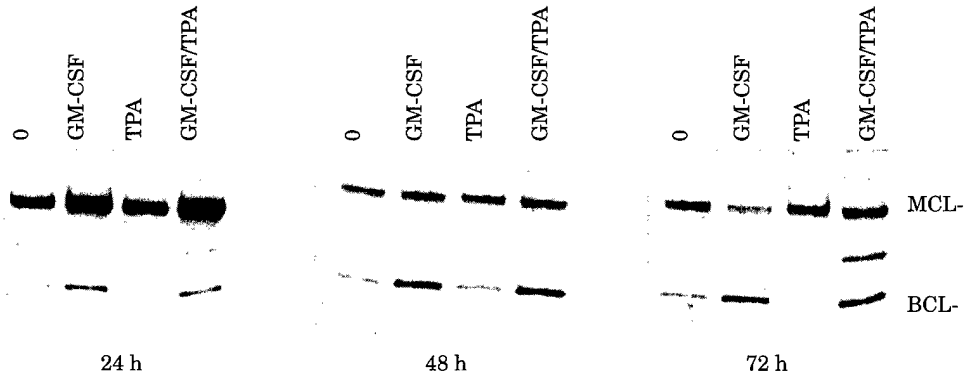

Figure 2. GM-CSF upregulates the expression of MCL-1 and BCL-2 proteins.

Cells were grown in the absence of GM-CSF for 24 h and then GM-CSF (10 ng/ml), TPA (0.1 ng/ml) or both GM-CSF and TPA were added. Cells lysates were prepared 24, 48 or 72 h after stimulation, and the levels of MCL-1 were determined by Western blot analysis. Membranes were then reprobed with an antibody specific for BCL-2. The SDS-PAGE gel was stained with Coomassie blue to confirm the equal loading and transfer of proteins in all lanes.

used TF-1 cells in order to examine the effects of GM-CSF (and several other cytokines) on the expression of numerous BCL-2 family members. TF-1 cells were deprived of GM-CSF for 24 h and were either left untreated or were stimulated with GM-CSF, TPA or the combination of GM-CSF and TPA, for 24, 48 or 72 h and analysed for the expression of BCL-2, MCL-1, BCL-x, and Bax. As previously reported, GM-CSF potently induces BCL-2 expression, which in TF-1 cells reaches its peak 48–72 h after cytokine reexposure (Fig. 2). Western blot analysis demonstrated that the level of MCL-1 protein declined significantly in cells deprived of GM-CSF, most notably between 24 and 48 h after growth-factor deprivation (Fig. 2). Re-exposure of growth-factor deprived cells to GM-CSF induced a rapid but transient expression of MCL-1 protein, which reached maximal levels 24 h after GM-CSF stimulation and, in contrast to BCL-2, returned to its basal level 48 h after GM-CSF treatment.

Cytokines such as GM-CSF play an important role in maintaining the survival of mature differentiated cells, thus we examined whether GM-CSF can prevent differentiation-induced apoptosis in TF-1 cells. TPA induces the terminal differentiation of TF-1 cells in the presence of GM-CSF, ultimately leading to apoptosis. TF-1 cells treated with GM-CSF alone, or the combination of GM-CSF and TPA, have comparable level of BCL-2 and MCL-1 (Fig. 2), nevertheless, cells treated with TPA and GM-CSF undergo apoptosis (Fig. 1). This suggests that upregulation of BCL-2 or MCL-1 by GM-CSF is insufficient to rescue cells from differentiation induced apoptosis. This finding also suggest that TPA triggers apoptosis independent of changes in the levels of BCL-2 or MCL-1.

Both GM-CSF and IL-3 support the long-term proliferation of TF-1 cells, whereas TF-1 cells proliferate for only a limited time in the presence of Epo.[22] We examined whether IL-3 and Epo modulate the expression of BCL-2 and MCL-1 in a similar fashion as GM-CSF. Cells were deprived of growth factor for 12 h and were either left untreated (Fig. 3A, lane 1) or stimulated with cytokines as indicated (lanes 2–6). GM-CSF (lane 2), IL-3 (lane 3) and Epo (lane 4) all induce the expression of MCL-1 and BCL-2, whereas TNF (lane 5) and IL-1 (lane 6), which cannot support the growth of TF-1 cells, did not. We also examined

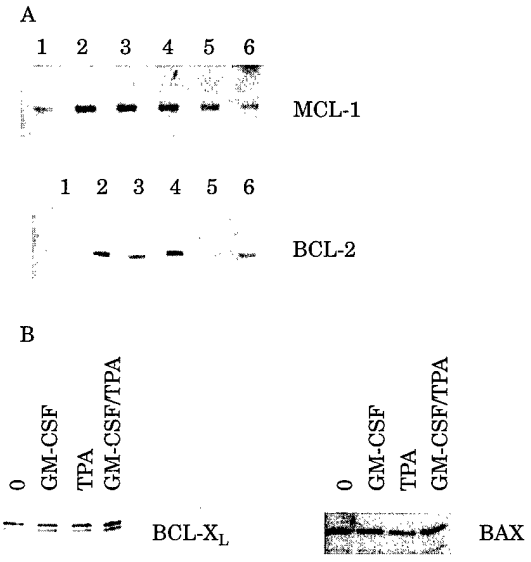

Figure 3. GM-CSF, IL-3 and EPO upregulate the expression of MCL-1 and BCL-2 protein.

A: Cells deprived of growth factors for 24 h were left untreated (lane 1) or were stimulated with GM-CSF (10 ng/ml) (lane 2), IL-3 (5 ng/ml) (lane 3), EPO (10 U/ml) (lane 4), TNF (20 ng/ml) (lane 5) or IL-1 (2 ng/ml) (lane 6). The level of MCL-1 protein was determined 4 h and the level of BCL-2 protein 24 h after cytokine stimulation. B: The levels of Bax and BCL-x protein were determined by Western blot analysis in cells stimulated for 24 h with GM-CSF, TPA or the combination of both.

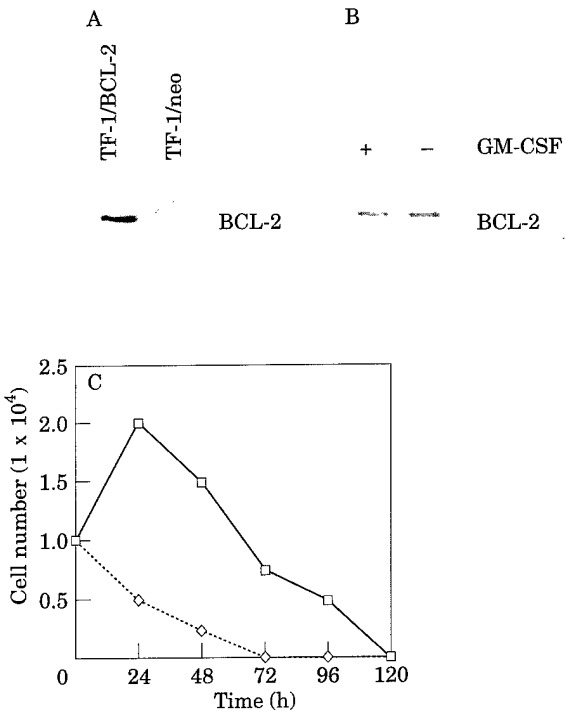

Figure 4. Overexpression of BCL-2 delays growth-factor withdrawal-induced apoptosis.

A: Expression of BCL-2 was determined in TF-1 cells transfected with an empty vector (pBKCMV) and in TF-1 cells transfected with the pBKCMV-BCL-2 plasmid. B: TF-1/BCL-2 cells maintain their level of BCL-2 in the absence of GM-CSF (24 h) as determined by Western blot. C: TF-1/BCL-2 (□) and TF-1/neo (◇) cells were grown in GM-CSF depleted media; the number of viable cells was determined over a period of 5 days. Experiments were repeated at least three times and similar results were obtained.

the expression of BCL-x_L and Bax, and as shown in Figure 3B, GM-CSF or TPA did not modulate the expression of BCL-x and Bax in TF-1 cells.

Overexpression of BCL-2 and MCL-1 delays growth factor withdrawal induced apoptosis of TF-1 cells

To determine whether BCL-2 overexpression can substitute for the presence of GM-CSF, we stably transfected TF-1 cells with an expression plasmid containing the human BCL-2 c-DNA (pBKCMV-BCL-2) or with an empty vector (pBKCMV). Western blot analysis of whole cell lysates demonstrated that cells transfected with the pBKCMV-BCL-2 plasmid (TF-1/BCL-2) have higher levels of BCL-2 than cells transfected with the empty vector (TF-1/neo) (Fig. 4A). The levels of Bax protein were not significantly different in these cell lines (data not shown). TF-1/BCL-2 cells deprived of GM-CSF for 24 h maintained their level of BCL-2 protein, demonstrating that the transfected gene is not under normal regulatory control (Fig. 4B). While the growth rate of TF-1/BCL-2 and TF-1/neo cells was comparable in the presence of GM-CSF (data not shown), TF-1/BCL-2 cells underwent delayed

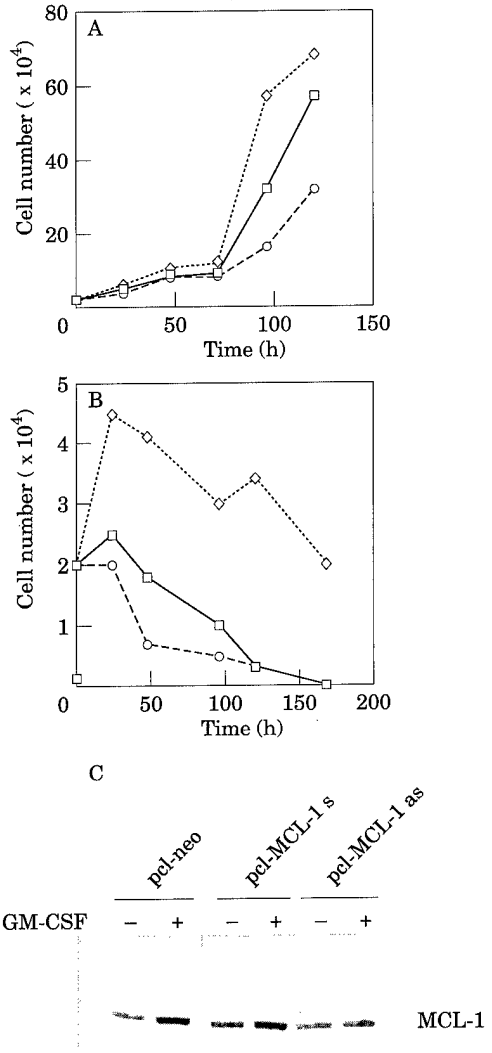

Figure 5. Overexpression of MCL-1 delays growth-factor withdrawal-induced apoptosis.

Cells, stably transfected with MCL-1 in the sense (MCL-1s) (◇) or antisense (MCL-1as) (○) orientation were grown in the presence (A) or absence (B) of GM-CSF. Viability was determined by the trypan blue exclusion assay. (□), neo. C: The expression level of MCL-1 protein in TF-1 cells transfected with pcI-neo, p-cI-MCL-1s and pcI-MCL-1as in the absence (–) or the presence (+) of GM-CSF was determined by Western blotting. Results shown are representative of at least three independent experiments.

apoptosis when deprived of GM-CSF (Fig. 4C). TF-1/BCL-2 cells did not proliferate in the absence of GM-CSF, consistent with the notion that BCL-2 provides a survival rather than a proliferative signal.

We also studied the survival potential of TF-1 cells transfected with the MCL-1 cDNA in the sense (MCL-1s) or antisense orientation (MCL-1as). In the presence of GM-CSF, TF-1/MCL-1s cells grow more rapidly compared to the parental cell line, whereas TF-1/MCL-1as cells grow more slowly (Fig. 5A). Following growth factor withdrawal, TF-1/MCL-1s cells survive longer, whereas TF-1/MCL-1as cells undergo

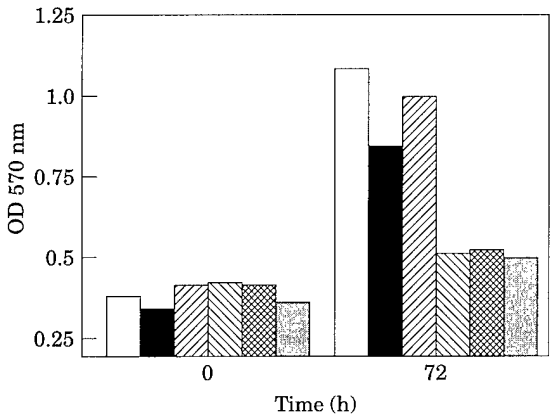

Figure 6. Overexpression of BCL-2 or MCL-1 does not rescue cells from TPA-induced cell death.

TF-1 cells, TF-1/BCL-2 and TF-1/MCL-1 cells were left untreated or were treated with TPA (1 ng/ml) for 72 h, then growth rates were measured using the MTT assay (Boehringer Mannheim). (□), TF-1; (■), TF-1/BCL-2; (▨), TF-1/MCL-1; (▧), TF-1/TPA; (▩), TF-1/BCL-2/TPA; (▢), TF-1/MCL-1/TPA.

more rapid cell death (Fig. 5B). The TF-1/MCL-s cells show only a modest increase in MCL-1 protein levels (most likely due to high endogenous levels of MCL-1 in TF-1 cells), whereas TF-1/MCL-1as cells show impaired induction of MCL-1 in response to GM-CSF (Fig. 5C).

In addition, we have tested the ability of BCL-2 and MCL-1 to prevent TPA-induced cell death. Wild-type TF-1 cells, and TF-1 cells overexpressing BCL-2 or MCL-1 were treated with TPA (1 ng/ml) and cell growth was monitored by the MTT assay. As shown in Figure 6, neither BCL-2 nor MCL-1 protected cells from the TPA-induced apoptosis, further confirming that the pathways of growth factor withdrawal and TPA-induced apoptosis are distinct.

DISCUSSION

Although many human acute leukaemia cell lines require exogenous growth factors for their continued proliferation, cell lines that retain the ability to differentiate are rarely cytokine dependent. TF-1 cells are dependent on GM-CSF or IL-3 for their survival, yet they can differentiate into megakaryocytic,[22] erythroid[20] and monocyte/macrophage[20] lineages. TF-1 cells therefore represent a relatively unique reagent to compare the pathways of growth factor withdrawal-induced apoptosis with differentiation-induced apoptosis. As expected, some components of these apoptotic pathways are common, whereas others are stimulus-specific.

Both growth factor withdrawal and TPA-treatment resulted in the activation of DEVD specific caspases and cleavage of PARP. However, apoptosis of TF-1 cells induced by growth factor withdrawal, but not TPA, coincided with the rapid downmodulation of MCL-1 and BCL-2 proteins. Cytokines, such as IL-3 and Epo, have been shown to rescue haematopoietic cells from γ-irradiation induced cell death,[23] however we have demonstrated that GM-CSF does not prevent TPA-induced cell death in TF-1 cells.

MCl-1 is an anti-apoptotic member of the BCL-2 family. In addition to its role in apoptosis, MCL-1 most likely plays a role in the differentiation of myeloid cells, as it was originally cloned as a TPA-inducible gene; the expression of MCl-1 has been shown to correlate with the survival of haematopoietic cells, including peripheral blood B lymphocytes.[7] Furthermore, MCL-1 overexpression in CHO cells prolongs their viability upon c-myc induction.[6] We have shown that cytokines which support the viability of TF-1 cells (GM-CSF, IL-3 and Epo) induce MCL-1 (and BCL-2) protein expression, whereas cytokines that do not support the growth of TF-1 cells (e.g. TNF or IL-1) fail to modulate the level of MCL-1. Hence, our results demonstrate that MCL-1 expression is modulated by survival/proliferative signals, supporting the notion that MCL-1 functions as an inhibitor of cell death. The increase in MCL-1 expression after GM-CSF stimulation is rapid and transient, providing further support for the idea that MCL-1 acts as a "short-term" regulator of cell viability, which can influence the early phase of apoptosis.[26] Overexpression of MCL-1 in TF-1 cells clearly delayed apoptosis upon growth factor withdrawal, whereas expression of the MCL-1 cDNA in the antisense orientation accelerated apoptosis upon growth factor withdrawal. Another group has similarly found that MCL-1 expression is an important mediator of the response to GM-CSF.[24] Likewise, activation of MCL-1 by IL-13 has been shown to inhibit apoptosis of B cells.[25] MCL-1 levels increase in ML-1 cells after treatment with TPA, however we could not detect a substantial increase in MCL-1 in response to TPA treatment in TF-1 cells. Treatment of ML-1 cells with TPA results in differentiation, without substantial apoptosis, whereas TF-1 cells undergo rapid apoptosis following TPA-induced differentiation, despite continued cytokine exposure (our data and Ref. 19).

We have preliminary data that GM-CSF stimulates BCL-2 promoter activity in TF-1 cells (data not shown). Although we have not yet localized the region of the BCL-2 promoter that confers responsiveness to GM-CSF, our preliminary results demonstrate that GM-CSF regulates BCL-2 expression at least in part at the level of transcription. GM-CSF can also activate MCL-1 promoter activity.[24] Although BCL-2 overexpression cannot substitute for the proliferative signals provided by GM-CSF, cells overexpressing BCL-2 do survive longer in the absence of growth factor. The

ectopic expression of BCL-2 has been previously shown to prolong the viability of lymphoid cells,[27] and the HCD-57 erythroid progenitor cell line,[28] without affecting cell proliferation.

The long-term proliferation of haematopoietic cells requires that cells receives both proliferative and survival signals. Several cytokines can provide both stimuli, however experiments in 32D and Baf/3 cells have demonstrated that the signaling pathways involved in these processes are distinct; genestein inhibited IL-3 induced DNA synthesis, but did not compromise the anti-apoptotic function of IL-3.[17] Similarly, a mutant GM-CSF receptor, which cannot activate the Ras signaling pathway, failed to support the survival of 32D cells but still promoted DNA synthesis in response to GM-CSF.[17] The ability of GM-CSF to activate MAPK has been linked to its anti-apoptotic activity, whereas activation of STAT-5 may be more important in the GM-CSF mediated proliferation of TF-1 cells.[16]

TF-1 cells overexpress a truncated Epo receptor which cannot efficiently activate the STAT5 pathway despite efficient JAK2 activation;[29] accordingly Epo fails to support the long-term proliferation of TF-1 cells.[20] Interestingly, we demonstrated that Epo can upregulate BCL-2 and MCL-1 in TF-1 cells as efficiently as GM-CSF or IL-3. This demonstrates that the region of the Epo receptor required to upregulate cell survival genes is intact in TF-1 cells and that the regions of the Epo receptor that signal for proliferation and survival are distinct.

Altogether, our data demonstrate that pathways of cytokine withdrawal-induced apoptosis and differentiation-induced apoptosis are distinct and underscore the importance of both anti-apoptotic stimuli and proliferative stimuli in the long-term proliferation of haematopoietic cells.

MATERIALS AND METHODS

Cell culture and transfection studies

TF-1 cells (kindly provided by Toshio Kitamura) were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 1% glutamine, penicillin, streptomycin and rhGM-CSF (10 ng/ml) (kindly provided by Sandoz Pharmaceuticals). Growth factor withdrawal was accomplished by extensively washing the cells with media lacking GM-CSF. Subsequently, these cells were re-stimulated with growth factors, either rhGM-CSF (10 ng/ml), rhIL-3 (10 ng/ml), rhEpo (100 U/ml), rhTNF (10 ng/ml) or rhIL-1 (1 ng/ml) for the time indicated.

To prepare a TF-1 cell line that stably overexpresses BCL-2, cells were electroporated with the empty pBKCMV vector or the pBKCMV-BCL2 expression plasmid (kindly provided by Andrew Zelenetz) and then selected in G418 (800 μg/ml). The MCL-1 c-DNA (kindly provided by Ruth Craig) was cloned into the pcI expression vector (*Promega*), in the sense and antisense orientation, and electroporated into TF-1 cells. Mixed, polyclonal populations of G418 resistant cells were established, and analysed for BCL-2, or MCL-1, expression by Western blotting.

DNA fragmentation assay

Apoptosis was induced by culturing TF-1 cells in media lacking GM-CSF for 24 h, or by stimulating cells for 24 h with 12-phorbol-myristate-13-acetate (TPA) (0.2 ng/ml). Then, 10^6 cells per lane were directly loaded onto an agarose gel, containing 2% SDS and 60 μg/ml of proteinase K as described by McGahon *et al.*[30] Samples were electrophoretically separated for 6–10 h at 60 V and the gel was stained with ethidium bromide.

Analysis of caspase-3 activity

TF-1 cells were either deprived of GM-CSF for 48 h, or were treated with TPA (0.1 ng/ml) for 48 h, and then the enzymatic activity of caspase-3 was determined using an ApoAlert™ cpp32/caspase-3 assay kit (Clontech, Palo Alto, CA) as suggested by the manufacturer. Briefly, 2×10^6 cells were resuspended in 50 μl of lysis buffer and then reaction buffer and a chromogenic cpp32 substrate (DEVD-pNA) were added. Reactions were incubated at 37°C for 1 h and the chromogenicity of the samples was measured at 405 nm.

Western blot analysis

TF-1 cells were either grown in the presence of growth factors (GM-CSF, IL-3, Epo, TNF or IL-1), or were washed twice in media without GM-CSF and cultured in the absence of exogenous growth factors. TF-1 cells were also treated with TPA in the presence or absence of GM-CSF for the times indicated. The TF-1 cells were then resuspended in SDS-lysis buffer and the samples were electrophoretically separated on a 12% SDS-PAGE gel. After transfer to a nitrocellulose membrane, the gel was stained with Coomassie blue to demonstrate equal loading of proteins in all lanes. The presence and amount of BCL-2 protein were detected using a monoclonal antibody that specifically recognizes BCL-2 (Oncogene Science, Cambridge, MA). The membrane was reprobed with a polyclonal anti-MCL-1 antibody (Pharmingen, San Diego, CA), an anti-Bax antibody (kindly provided by John Reed) or an anti-BCL-X antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Western blots were developed using the Amersham ECL kit and exposed to Kodak XAR film for 30 s (for BCL-2, Bax and BCL-x detection) or for 1 min (for MCL-1 detection).

Acknowledgements

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REFERENCES

1. Wyllie AH, Kerr JF, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306.

2. Yang E, Korsmeyer SJ (1996) Molecular thanatopsis: a discourse on the BCL2 family and cell death. Blood 88:386–401.

3. White E (1996) Life, death, and the pursuit of apoptosis. Genes Dev 10:1–15.

4. Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW (1993) MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc Natl Acad Sci USA 90:3516–3520.

5. Sedlak TW, Oltvai N, Yang E, Wang K, Boise LH, Thompson CB, Korsmeyer SJ (1995) Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proc Natl Acad Sci USA 92:7834–7838.

6. Reynolds JE, Yang T, Qian L, Jenkinson JD, Zhou P, Eastman A, Craig RW (1994) Mcl-1, a member of the Bcl-2 family, delays apoptosis induced by c-Myc overexpression in Chinese hamster ovary cells. Cancer Res 54:6348–6352.

7. Lomo J, Smeland EB, Krajewski S, Reed JC, Blomhoff HK (1996) Expression of the Bcl-2 homologue Mcl-1 correlates with survival of peripheral blood B lymphocytes. Cancer Res 56:40–43.

8. Zhou P, Qian L, Kozopas KM, Craig RW (1997) Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood 89:630–643.

9. Kaufmann SH, Karp JE, Svingen PA, Krajewski S, Burke PJ, Gore SD, Reed JC (1998) Elevated expression of the apoptotic regulator Mcl-1 at the time of leukemic relapse. Blood 91:991–1000.

10. Oltvai ZN, Milliman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609–619.

11. Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. Science 267:1456–1462.

12. Broome HE, Dargan CM, Krajewski S, Reed JC (1995) Expression of Bcl-2, Bcl-x, and Bax after T cell activation and IL-2 withdrawal. J Immunol 155:2311–2317.

13. Nass SJ, Li M, Amundadottir LT, Furth PA, Dickson RB (1996) Role for Bcl-xL in the regulation of apoptosis by EGF and TGF beta 1 in c-myc overexpressing mammary epithelial cells. Biochem Biophys Res Commun 227:248–256.

14. Blume-Jensen P, Janknecht R, Hunter T (1998) The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. Curr Biol 8: 779–782.

15. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 87:619–628.

16. Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, Hennighausen L, Finbloom DS (1997) STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. Blood 90: 1768–1776.

17. Kinoshita T, Yokota T, Arai K, Miyajima A (1995) Suppression of apoptotic death in hematopoietic cells by signalling through the IL-3/GM-CSF receptors. EMBO J 14:266–275.

18. Han JH, Gileadi C, Rajapaksa R, Kosek J, Greenberg PL (1995) Modulation of apoptosis in human myeloid leukemic cells by GM-CSF. Exp Hematol 23:265–272.

19. Rinaudo MS, Su K, Falk LA, Halder S, Mufson RA (1995) Human interleukin-3 receptor modulates bcl-2 mRNA and protein levels through protein kinase C in TF-1 cells. Blood 86:80–88.

20. Kitamura T, Tange T, Terasawa T, Chiba S, Kuwaki T, Miyagawa K, Piao YF, Miyazono K, Urabe A, Takaku F (1989) Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. J Cell Physiol 140:323–334.

21. Bradbury D, Zhu Y-M, Russel N (1994) Regulation of BCL-2 expression and apoptosis in acute myeloblastic leukemia cells by granulocyte-macrophage colony-stimulating factor. Leukemia 8:786–791.

22. Testa U, Grignani F, Hassan HJ, Rogaia D, Masciulli R, Gelmetti V, Guerriero R, Macioce G, Liberatore C, Barberi T, Mariani G, Pelicci PG, Peschle C (1998) Terminal megakaryocytic differentiation of TF-1 cells is induced by phorbol esters and thrombopoietin and is blocked by expression of PML/RARa fusion protein. Leukemia 12:563–570.

23. Quelle FW, Wang J, Feng J, Wang D, Cleveland JL, Ihle JN, Zambetti GP (1998) Cytokine rescue of p53-dependent apoptosis and cell cycle arrest is mediated by distinct Jak kinase signaling pathways. Genes Dev 12:1099–1107.

24. Chao JR, Wang JM, Lee SF, Peng HW, Lin YH, Chou CH, Li JC, Huang HM, Chou CK, Kuo ML, Yen JJ, Yang-Yen HF (1998) mcl-1 is an immediate-early gene activated by the granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling pathway and is one component of the GM-CSF viability response. Mol Cell Biol 18:4883–4898.

25. Lomo J, Blomhoff HK, Jacobsen SE, Krajewski S, Reed JC, Smeland EB (1997) Interleukin-13 in combination with CD40 ligand potently inhibits apoptosis in human B lymphocytes: upregulation of Bcl-xL and Mcl-1. Blood 89:4415–4424.

26. Zhan Q, Bieszczad CK, Bae I, Fornace AJ Jr, Craig RW (1997) Induction of BCL2 family member MCL1 as an early response to DNA damage. Oncogene 14:1031–1039.

27. Nunez G, London L, Hockenbery D, Alexander M, McKearn JP, Korsmeyer SJ (1990) Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immunol 144:3602–3610.

28. Silva M, Grillot D, Benito A, Richard C, Nunez G, Fernandez-Luna JL (1996) Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-XL and Bcl-2. Blood 88:1576–1582.

29. Chretien S, Varlet P, Verdier F, Gobert S, Cartron JP, Gisselbrecht S, Mayeux P, Lacombe C (1996) Erythropoietin-induced erythroid differentiation of the human erythroleukemia cell line TF-1 correlates with impaired STAT5 activation. EMBO J 15:4174–4181.

30. McGahon AJ, Martin SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ, Nishioka WK, Green DR (1995) The end of the (cell) line: methods for the study of apoptosis in vitro. Methods Cell Biol 46:153–185.

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IL-1β INDUCES SERINE PROTEASE INHIBITOR 3 (SPI-3) GENE EXPRESSION IN RAT PANCREATIC β-CELLS. DETECTION BY DIFFERENTIAL DISPLAY OF MESSENGER RNA

Meng-Chi Chen, Frans Schuit, Daniel G. Pipeleers, Décio L. Eizirik*

Immune-mediated β-cell damage induces diverse intracellular signals, leading to transcription of different genes which may either contribute to β-cell repair and/or defence or lead to cell death. The cytokine interleukin-1β (IL-1) is a potential mediator of β-cell dysfunction and damage in type 1 diabetes mellitus. To understand the molecular actions of this cytokine upon β-cells, this study aimed at the cloning of genes induced in FACS-purified rat pancreatic β-cells by a 6- or 24-h exposure to IL-1 by using differential display of mRNA with reverse transcription-polymerase chain reaction (DDRT-PCR). Among these cytokine-induced genes, a gene encoding for rat serine protease inhibitor (SPI-3) was isolated. SPI-3 may be involved in cellular defence responses against inflammatory stress. RT-PCR analysis confirmed that SPI-3 mRNA expression in rat β-cells is increased by IL-1 at an early stage (2 h), with maximal accumulation during 6–12 h and decline after 24 h. Similar observations were made in mouse pancreatic islets and in the rat insulinoma cell line RINm5F. IFN-γ neither increased SPI-3 gene expression nor potentiated its induction by IL-1 in rat β-cells. The stimulatory effects of IL-1 on SPI-3 mRNA expression were decreased by co-incubation with an inhibitor of gene transcription (actinomycin D), an inhibitor of protein synthesis (cycloheximide) or an inhibitor of NF-κB activation (PDTC). On the other hand, a blocker of inducible nitric oxide synthase (iNOS) activity (N^G-methyl-L-arginine) did not prevent IL-1-induced SPI-3 expression. Thus, SPI-3 mRNA expression following IL-1 exposure depends on gene transcription, protein synthesis and activation of the nuclear transcription factor NF-κB, but it is independent of NO formation.

Dysfunction and damage of β-cells in early insulin dependent diabetes mellitus (IDDM) may result from a direct contact with islet infiltrating mononuclear cells (reviewed in Ref. 1) and/or exposure to inflammatory mediators produced by these cells, such as cytokines and toxic free radicals (reviewed in (Refs 2, 3, 4). In vitro studies have shown that the cytokine interleukin 1β (IL-1) inhibits glucose-stimulated insulin secretion and biosynthesis in a concentration and time dependent manner and, together with interferon γ (IFN-γ) and/or tumour necrosis factor α (TNF-α), leads to β-cell destruction.[2] Exposure of β-cells to IL-1 induces diverse intracellular signals, leading to transcription of different genes which may either contribute to cell death or support processes leading to cell defence and repair.[2,5]

In order to further clarify the mechanisms involved in IL-1-induced β-cell dysfunction, we searched for new cytokine-related genes using the differential display technique (DDRT-PCR).[6,7] This method is based on comparison of mRNAs expressed in purified rat β-cells exposed to control condition or cytokines, by running their reverse transcribed PCR products in adjacent lanes in sequencing gels. Bands showing differential expression are retrieved, amplified and used for cloning and sequencing. Following this approach, we identified a cDNA with high homology to the rat gene encoding for serine protease inhibitor SPI-3.[8] SPI-3 was previously described as an acute phase reactant secreted by hepatocytes in response to acute inflammation,[8] and expression of this gene is induced in the hippocampus after transient forebrain ischaemia.[9] In both cases, it was suggested that this

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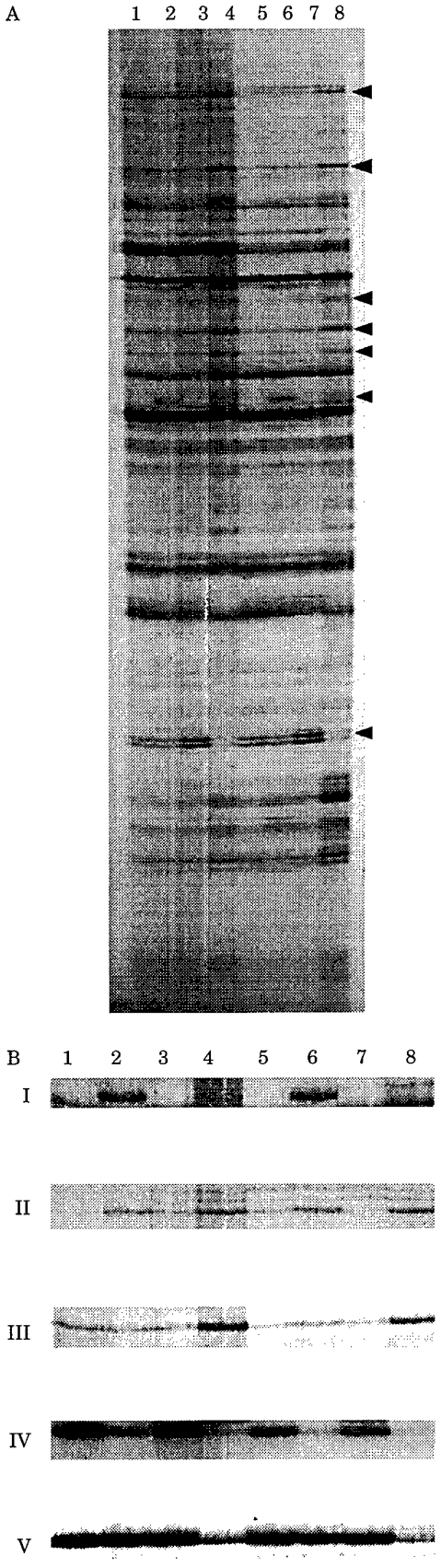

serine protease inhibitor may play a role in cellular protection against injury. This, and the observation that synthetic serine protease inhibitors protect rat pancreatic islets against IL-1β-induced inhibition of β-cell function,[10,11] prompted us to presently characterize the molecular regulation of the natural serine protease inhibitor SPI-3 in FACS-purified rat β-cells.

RESULTS

DDRT-PCR

As described in the original paper by Liang and Pardee,[6] about 100 discreet bands were detected in each lane with different primer combinations (Fig. 1A). The sensitivity of the method in our study, using a cDNA amount equivalent to 1200 and 5000 cells, is comparable to that described in previous studies.[6,12] The PCR signals were reproducible and abundance of the product correlated well with the starting cDNA concentration (Fig. 1A and B). IL-1β affected gene expression in a complex way, leading either to stimulation or inhibition of mRNA expression. Moreover, there was a clear temporal pattern on the effects of the cytokine on mRNA levels (Fig. 1B). We have already observed more than 30 fragments differentially displayed following IL-1β exposure, and we are in the process of cloning and DNA sequencing to identify their nature.

SPI-3 expression

One of the clones detected in the experiment outlined above (DD10) showed high homology (>98%) to the rat serine protease inhibitor-3 gene at the position 1190-1620 (GenBank X16359), the region encompassing the antiprotease-reactive center and 3′ untranslated sequence, which is specific for this gene.[8] Using gene specific primers and RT-PCR we confirmed that SPI-3 gene is inducible by IL-1 in rat β-cells (Fig. 2). DNA sequencing of the SPI-3 PCR product confirmed the identity of the gene (data not shown). Control β-cells (i.e. not exposed to cytokines)

Figure 1. Detection of IL-1β-induced changes in gene expression in rat pancreatic β-cells by DDRT-PCR.

Rat β-cells (10^6 cells) were exposed to IL-1β (30 U/ml) or control condition for 6 or 24 h. Poly $(A)^+$ mRNA was extracted and DDRT-PCR was performed as described in Materials and Methods. (A) Characteristic pattern of polyacrylamide gel electrophoresis of amplified cDNA. Arrowheads indicate bands differentially displayed following IL-1β exposure; (B) IL-1β may affect gene expression by: (I) early and transient induction; (II) early and prolonged induction; (III) late induction; (IV) early suppression; (V) late suppression. Lanes 1 and 5, 6 h control; lanes 2 and 6, 6 h IL-1β; lanes 3 and 7, 24 h control; lanes 4 and 8, 24 h with IL-1β. Samples loaded on lanes 5, 6, 7 and 8 were amplified from one fourth of the amount of cDNA used in lanes 1, 2, 3 and 4.

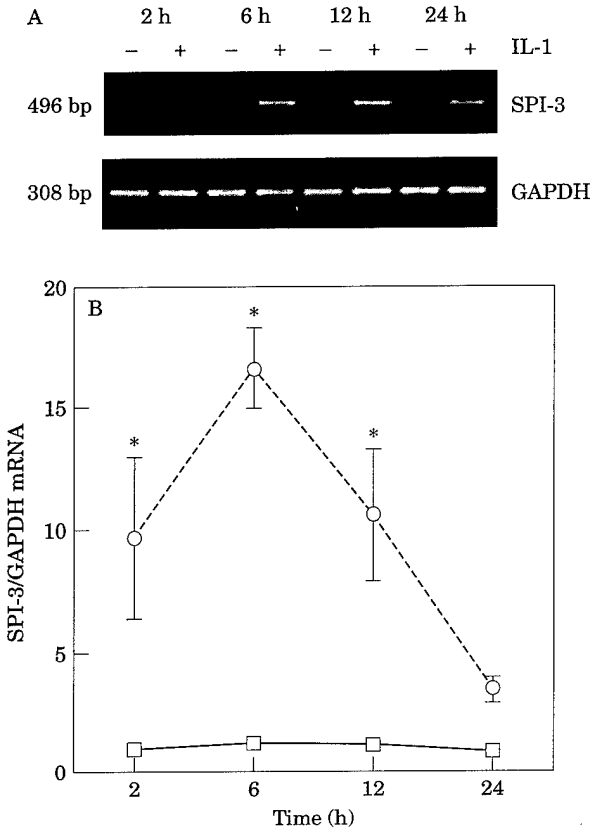

Figure 2. Time course of IL-1-induced SPI-3 gene expression in rat β-cells.

(A) Rat β-cells (5×10^4 cells/well) were exposed to IL-1 (30 U/ml) or control condition for the indicated time periods. SPI-3 and GAPDH mRNA levels were analysed by RT-PCR using cDNA aliquot equivalent to 3×10^3 cells as described in Materials and Methods. The pictures shown are representative of three independent experiments with similar results. (B) Semi-quantitative analysis of RT-PCR where band intensities for SPI-3 were corrected for intensities of GAPDH and expressed as arbitrary units of optical intensity (O.D.). The results are means ± SEM of three independent experiments. *$P<0.05$ vs control. (□), control; (○), IL-1.

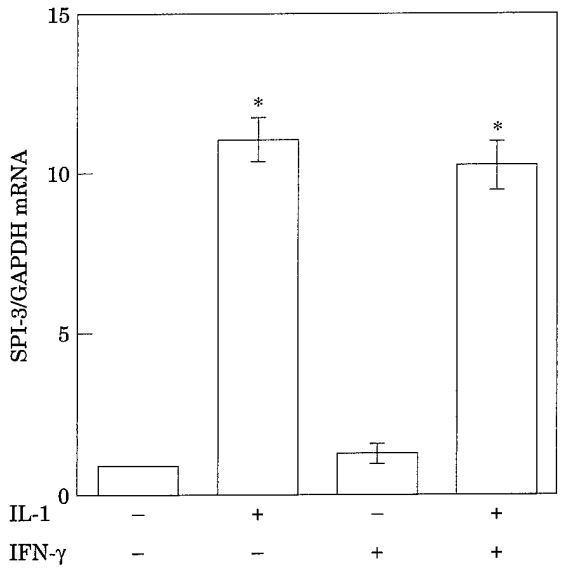

Figure 3. Effect of IL-1β and IFN-γ on SPI-3 mRNA expression in rat β-cells.

Rat β-cells were exposed to IL-1 (30 U/ml) and/or IFN-γ (1000 U/ml) for 6 h. SPI-3 and GAPDH mRNA levels were analysed by RT-PCR using gene-specific primers as described in Figure 2. Data are expressed as O.D. of SPI-3 corrected for GAPDH expression. The results are means ± SEM of four independent experiments. *$P<0.01$ vs control cells.

expressed very low or undetectable levels of SPI-3 mRNA (Fig. 2). Treatment with IL-1β for 2 h increased gene expression by about 5-fold. There was further increase in SPI-3 expression after 6 h (12-fold compared to basal levels), followed by a progressive decrease in mRNA levels after 12- and 24-h continuous exposure to IL-1β. On the other hand, preparations of rat islet non-β-cells (containing >80% α-cells) presented higher basal expression of SPI-3 but did not increase the expression of this mRNA following 6 h exposure to IL-1 (n=3; data not shown). The rat insulinoma cell line RINm5F also showed an early increase in SPI-3 expression in response to IL-1. After 2- and 6-h exposure to IL-1 there was a 4- and 6-fold increase, respectively in SPI-3 mRNA content (values corrected per GAPDH, as described in Fig. 2; results as means of two similar experiments). Mouse pancreatic islets exposed to IL-1 for 2 or 24 h showed a 2–3-fold and 10-fold increase, respectively in SPI-3 mRNA expression (values corrected per GAPDH, as described in Fig. 2; $n=4$). Based on the findings described in Fig. 2, we selected 6 h for further studies on the regulation of SPI-3 expression in rat β-cells (see below).

Culture of rat β-cells for 6 h in the presence of IFN-γ did not cause detectable changes in SPI-3 mRNA content (Fig. 3). Moreover, IFN-γ did not potentiate the stimulatory effect of IL-1 on SPI-3 expression. Similar results were obtained when we prolonged the exposure time to IFN-γ and/or IL-1 to 24 h, i.e. IFN-γ again failed to increase SPI-3 expression (data not shown).

In a subsequent series of experiments we investigated whether gene transcription and protein synthesis are necessary steps for SPI-3 induction. Both actinomycin D (an inhibitor of gene transcription) and cycloheximide (an inhibitor of protein biosynthesis) decreased IL-1-induced SPI-3 mRNA levels by 70–90% (Fig. 4). To test whether the nuclear transcription factor NF-κB is required for SPI-3 mRNA expression, rat β-cells were exposed to IL-1β in the presence of the NF-κB inhibitor PDTC. This agent induced a partial decrease (50%) in SPI-3 expression (Fig. 4; $P<0.01$ vs controls and $P<0.01$ vs IL-1; ANOVA). It has been previously shown that IL-1β, under conditions similar to the ones used in the present experiments, induces expression of iNOS mRNA and synthesis of NO by

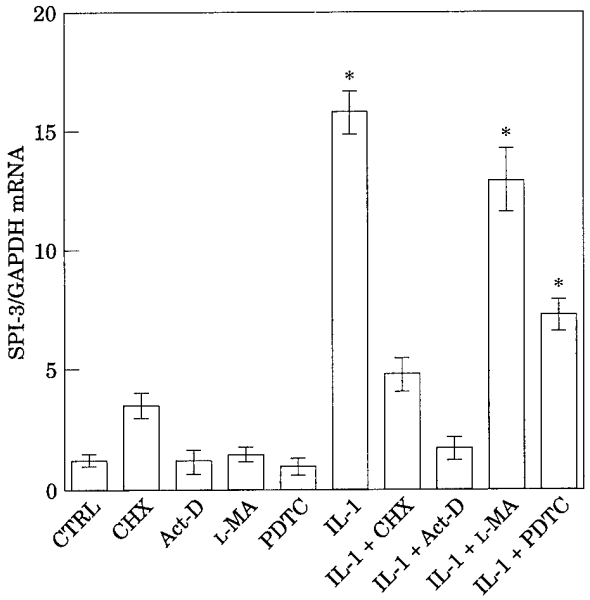

Figure 4. Effects of cycloheximide (CHX), actinomycin D (Act-D), L-MA and PDTC on IL-1-induced SPI-3 mRNA expression in rat β-cells.

Rat β-cells were pretreated with cycloheximide (20 mg/ml) for 30 min, actinomycin D (5 μg/ml) for 30 min or PDTC (100 μM) for 1 h before addition of IL-1 (30 U/ml) and subsequent culture for 6 h. L-MA (1 mM) was added together with IL-1β. RT-PCR for SPI-3 and GAPDH mRNAs were performed as described in Figure 2. Data are expressed as O.D. of SPI-3 corrected for GAPDH expression. The results are means ± SEM of 3–8 independent experiments. *$P<0.01$ vs control cells, ANOVA and paired t-test.

FACS-purified rat β-cells,[13,14] a finding reproduced in the present study (data not shown). To evaluate whether NO mediates the stimulatory effect of IL-1β on SPI-3 expression, rat β-cells were exposed to the cytokine in the presence of the iNOS inhibitor N^G-methyl-L-arginine (L-MA). This agent, at a concentration (1 mM) which fully inhibits IL-1β-induced nitrite production (data not shown) failed to significantly decrease SPI-3 expression (Fig. 4).

DISCUSSION

The DDRT-PCR method has been widely used to detect altered gene expression in eukaryotic cells.[6,12,15] This method allows the simultaneous identification of up- and down regulated mRNAs in multiple samples. It is more sensitive and requires less RNA material than conventional subtractive hybridization.[16,17] These advantages allow us to study various early and late responsive genes regulated by IL-1β in rat β-cells, aiming to find new candidate genes which might be involved in β-cell defence or damage. The use of FACS-purified β-cells provides an homogeneous and well-defined cell population, and obviates the problem of detecting non-β-cell genes, inherent to DDRT-PCR or similar methods performed in whole islet preparations. Previous studies on purified β-cells showed that exposure to IL-1β for >20 h strongly suppress their main glucose-inducible functions, namely insulin synthesis and release,[18] while it stimulates the expression of other proteins, such as iNOS, heat shock protein 70, Mn superoxide dismutase and haem oxygenase.[13] In this study we described the detection and identification of a novel IL-1-inducible gene in β-cells, the serine protease inhibitor SPI-3. Other IL-1 regulated genes identified by the DDRT-PCR will be reported elsewhere.

The gene product of SPI-3 has been reported as an acute phase protein, and its expression is induced by both IL-6 and glucocorticoids in hepatic cells.[8] SPI-3 is also induced in gerbil astrocytes following transient forebrain ischaemia.[9] In both cases, it was suggested that this protein blocks protease activity leaking from degenerative cells, and may thus decrease the local inflammatory response. Moreover, it has been previously observed that chemical serine protease inhibitors prevent IL-1β-induced β-cell dysfunction.[10,11] The present observation that SPI-3 is also induced by IL-1 in pancreatic β-cells suggests that this gene may be part of a general cellular response to inflammatory-mediated cellular dysfunction. Unfortunately, the lack of specific antibodies against SPI-3 has up to now hampered studies to characterize the expression of this protein under different in vivo conditions of autoimmune assault.

As previously described for iNOS and argininosuccinate synthetase (two genes involved in NO production by β-cells),[19,20] the IL-1-induced increase in SPI-3 mRNA abundance requires gene transcription, protein synthesis and activation of the transcription factor NF-κB (present data). Similarly to iNOS,[5] the promoter region of SPI-3 contains oligonucleotide elements homologous to sequences for the binding of several transcription factors, including NF-κB,[21] thus reinforcing the putative relevance of this transcription factor for the expression of both genes (Ref. 14, present data). It is noteworthy that IFN-γ, which has been reported to induce SPI-3 mRNA expression in rat hepatocytes via STAT activation,[22,23] did not have a similar effect on rat β-cells (present data). We have previously shown that IFN-γ activates STAT and IRF-1 in rodent insulin-producing cells,[5,14] but it is conceivable that the SPI-3 promoter in β-cells respond to different transcription factors than those described for hepatocytes. Further studies are required to clarify this issue.

The observation that SPI-3 expression is affected by agents which also inhibit iNOS mRNA expression[19,24] raises the question on whether IL-1 increases SPI-3 mRNA levels via NO formation. The present

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findings that SPI-3 is already induced by IL-1 in β-cells after 2 h, while medium nitrite (a stable by product of NO formation) is only detected after 4–6 h of exposure to the cytokine (Ref. 19; Pavlovic *et al.*, unpublished data), and that L-MA, an iNOS blocker, fail to significantly decrease SPI-3 expression, argue against this possibility.

In conclusion, we presently describe a rapid stimulatory effect of IL-1β on the expression of SPI-3 mRNA in pancreatic β-cells. The protein product of this gene may have protective effects against β-cell damage in the course of insulitis, and further studies will be devoted to the characterization of β-cell SPI-3 expression under in vivo conditions and on the potential protective role for up-regulation of this gene against cytokine-induced β-cell damage.

MATERIALS AND METHODS

Cell isolation and culture

Pancreatic islets were isolated from 10-week-old male Wistar rats by collagenase digestion followed by dissociation into single cells as was previously described in detail.[25] Briefly, islet β-cells were isolated on the basis of high light scatter and high flavin adenine dinucleotide (FAD)-fluorescence at 2.8 mM glucose using autofluorescence-activated cell sorting (FACStar, Beckton-Dickinson, Sunnyvale, USA).[25] Purified rat β-cells (>95% purity) were precultured overnight at 37°C in Ham's F-10 medium (GIBCO BRL-Life Technologies, UK) supplemented with 5 mg/ml charcoal-treated bovine serum albumin (BSA; fraction V, RIA grade, Sigma, St Louis, MO), 0.1 mg/ml streptomycin, 12.5 U/ml penicillin, 0.3 mg/ml L-glutamine and 10 mM glucose. This was followed by culture at 37°C in Ham's F-10 medium (GIBCO) supplemented with 10 mM glucose, 5 mg/ml BSA, 0.1 mg/ml streptomycin, 12.5 U/ml penicillin, 0.3 mg/ml L-glutamine and 50 μM 3-isobutyl-1-methylxanthine (IBMX; Jansen Chimica, Beerse, Belgium).[26]

For DDRT-PCR, 10^6 cells were treated with 30 U/ml recombinant human IL-1β (kindly provided by Dr C. W. Reynolds, National Cancer Institute, USA) for 6 or 24 h before mRNA isolation (see below). These time points were selected to provide information on both early and direct induction of genes by IL-1β (6 h) and subsequent cellular responses to IL-1β-induced stress (24 h).[5] Control cells were cultured for the same time period in the absence of cytokines.

To characterize SPI-3 mRNA expression, rat β-cells (10^5 cells) were exposed for either 2, 6, 12 or 24 h to IL-1β alone (30 U/ml), or for 6 h to the cytokine in the additional presence of the protein synthesis inhibitor cycloheximide (20 μg/ml; Sigma Chemicals, St Louis, MO), the gene transcription inhibitor actinomycin D (5 μg/ml; Sigma Chemicals), the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC, 100 μM; Sigma Chemicals) or the iNOS inhibitor N^G-methyl-L-arginine (L-MA, 1 mM; Sigma Chemicals). In some experiments the cells were also exposed to 1000 U/ml recombinant murine interferon γ (Life Technologies, Gent, Belgium). The choice of cytokine and inhibitor agent concentrations was based on our previous data on insulin-producing cells and pancreatic islets, having as biological end point stimulation (cytokines) or inhibition (all other agents) of cytokine-induced iNOS expression and/or activity.[19,20,24,27]

The insulinoma cell line RINm5F (provided by Å. Lernmark, then at the Hagerdon Institute, Copenhagen, Denmark) was cultured at 5×10^4 cells/well in medium RPMI-1640 supplemented with 10% (V/V) fetal calf serum (FCS), 0.1 mg/ml streptomycin, and 12.5 U/ml penicillin.[28] Islets from C57 BL/6 mice were isolated by collagenase digestion, and the islets cultured for 24 h in Ham's F10 as described above.

mRNA isolation

Poly(A)+RNA was isolated from cell aggregates using oligo$(dT)_{25}$-coated polystyrene Dynabeads (Dynal, Oslo, Norway). The yield of mRNA in each sample was evaluated by RT-PCR amplification of the "house keeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We have previously shown that IL-1 does not affect GAPDH expression.[29]

Differential display of mRNA (DDRT-PCR)

The DDRT-PCR procedure was modified from the original protocol described by Liang and Pardee.[6] The reverse transcription reaction was performed at 42°C for 1 h and contained (per 10 μl) mRNA equivalent to 8×10^4 cells, 1 × reverse transcription buffer, 5 mM $MgCl_2$, 250 mM of each deoxyribonucleoside triphosphate, 2 μM of either $T_{12}MG$, $T_{12}MA$, $T_{12}MT$ or $T_{12}MC$ (where M is a mixture of dA, dC and dG) and 100 U of Moloney murine leukaemia virus reverse transcriptase (Perkin Elmer, Norwalk, CT, USA). The cDNAs then obtained were diluted into two concentrations equivalent to 2500 and 600 cells per μl. PCR reactions were performed in a DNA thermal cycler 9700 (Perkin Elmer) in 20 μl reaction solution containing 2 μl cDNA, 2.5 μM of the original $T_{12}MN$ primers, 0.5 μM arbitrary upstream primers,[12] 25 μM of each deoxyribonucleoside triphosphate, 1 × PCR buffer, 1.5 mM $MgCl_2$, 10 μCi [α-^{33}P]dATP (1000–3000 Ci/mmol, Amersham, Little Chalfont, Bucks, UK), and 1 U AmpliTaq DNA polymerase (Perkin Elmer). The thermal cycling included 40 cycles of denaturation for 30 s at 94°C, annealing for 2 min at 42°C, extension for 30 s at 72°C, followed by 5-min post extension at 72°C. Each sample was run in parallel at two starting cDNA concentrations (see above). This use of duplicate samples, with a 4-fold difference in concentration, decreases the risk of false-positive findings during the DDRT-PCR (data not shown). The amplified cDNAs were separated on a 6% polyacrylamide gel and then blotted onto a piece of Whatman 3-mm filter paper and dried without fixation. The gels were exposed to X-ray film (Kodak, Rochester, NY, USA) for 2 days. Bands showing differential expression in both duplicates and having a fragment length of 300–600 bp were excised, eluted in boiling water and reamplified using the same primer combination as in the condition described above, except that no radioisotope was added. Reamplified cDNAs were first visualized on a 1.5% agarose gel to verify size and then cloned into the pCR 2.1 vector using the TA cloning system (Invitrogen, San Diego, CA). Bacterial cells

picked from positive colonies were mixed with PCR mixture and the plasmid DNA were amplified using M13 and T7 primers. The sizes of amplified fragments were examined to verify the presence of the cDNA inserts. Clones of interest were further analysed by automated DNA sequencing (ABI PRISM 310 Genetic Analyzer) using M13 primer and the Dye Terminator Cycle Sequencing kit (Perkin Elmer). The cDNA sequences were compared to those contained in the GenBank and EMBL database using GCG sequencing analysis software (Genetics Computer Group, Madison, USA)

RT-PCR

Due to limited availability of purified rat β-cells, we performed RT-PCR instead of Northern blot to confirm the differential expression of the SPI-3 gene detected during DDRT-PCR. mRNA was prepared as described above from 10^5 cells. The reverse transcription reaction was performed at 42°C for 1 h, and contained (per 10 µl) mRNA equivalent to 6×10^3 cells, 1 × reverse transcription buffer, 5 mM $MgCl_2$, 1 mM of each deoxyribonucleoside triphosphate, 2.5 µM random hexamer primers and 100 units of Moloney murine leukaemia virus reverse transcriptase (Perkin Elmer). The subsequent PCR reaction contained (in 25 µl reaction solution) 5 µl cDNA, 0.4 µM of forward and reverse primers, 200 µM of each deoxyribonucleoside triphosphate, 1 × PCR buffer, 2 mM $MgCl_2$, and 0.625 U AmpliTaq Gold DNA polymerase (Perkin Elmer). PCR specificity and efficiency was improved by using hot start PCR with 12 min predenaturation at 95°C and then 28 (GAPDH) or 31 (SPI-3) cycles of: 94°C for 45 s, 58°C for 45 s and 72°C for 80 s. For semi-quantitative PCR, the housekeeping gene GAPDH was included as control. To enable comparisons between the relative amounts of SPI-3 mRNA among different samples, external standards for SPI-3 and GAPDH cDNA were used in the PCR reactions. PCR external standards were prepared from a dilution series of PCR products (4 points), each containing increasing amounts of target cDNA. These serially diluted standards were amplified simultaneously with cDNA samples. The primer sequences were SPI3-F: 5′-CAA ATT TGT TCC AAT GTC TGC-3′; SPI3-R: 5′-AAT CGT GCA CAG TCC CAT CAA-3′; GAPDH-F 5′-TCC CTC AAG ATT GTC AGC AA-3′; GAPDH-R 5′-AGA TCC ACA ACG GAT ACA TT-3′. The identity of PCR fragments of SPI-3 gene were confirmed by DNA sequencing. The ethidium bromide-stained agarose gel was photographed under UV-transillumination using Kodak Digital Science DC40 camera (Kodak, Rochester, NY, USA). Abundance of the PCR product of interest (SPI-3) was assessed by Biomax 1D Image analysis software (Kodak) and expressed in pixel intensities (O.D.), normalized for the abundance of the GAPDH signal amplified from the same cDNA sample.

Statistical analysis

Values are expressed as means ± SEM. Group comparison were performed by Student's paired or unpaired *t*-test, and multiple comparisons were performed by analysis of variance (ANOVA) followed by paired or unpaired t-tests with the Bonferroni correction for multiple comparisons.[30]

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REFERENCES

1. Bach JF (1995) Insulin-dependent diabetes mellitus as a beta-cell targeted disease of immunoregulation. J Autoimmun 8:439–463.

2. Mandrup-Poulsen T (1996) The role of interleukin-1 in the pathogenesis of IDDM. Diabetologia 39:1005–1029.

3. Eizirik DL, Pavlovic D (1997) Is there a role for nitric oxide in β-cell dysfunction and damage in IDDM? Diabetes Metab Rev 13:293–308.

4. Rabinovitch A, Suarez-Pinzon WL (1998) Cytokines and their roles in pancreatic islet β-cell destruction and insulin-dependent diabetes mellitus. Biochem Pharmacol 55:1139–1149.

5. Eizirik DL, Flodström M, Karlsen AE, Welsh N (1996) The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. Diabetologia 39:875–890.

6. Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 274:787–789.

7. Liang P, Averboukh L, Pardee AB (1993) Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acids Res 21:3269–3275.

8. Pages G, Rouayrenc JF, Le Cam G, Mariller M, Le Cam A (1990) Molecular characterization of three rat liver serine-protease inhibitors affected by inflammation and hypophysectomy. Eur J Biochem 190:385–391.

9. Tsuda M, Kitagawa K, Imaizumi K, Wanaka A, Tohyama M, Takagi T, (1996) Induction of SPI-3 mRNA, encoding a serine protease inhibitor, in gerbil hippocampus after transient forebrain ischemia. Mol Brain Res 35:314–318.

10. Welsh N, Bendtzen K, Sandler S (1991) Influence of protease on inhibitory and stimulatory effects of interleukin 1β on β-cell function. Diabetes 40:290–294.

11. Eizirik DL, Bendtzen K, Sandler S (1991) Short exposure of rat pancreatic islets to interleukin-1β induces a sustained but reversible impairment in β-cell function: influence of protease activation, gene transcription, and protein synthesis. Endocrinology 128:1611–1616.

12. Bauer D, Müller K, Reich J, Riedel H, Ahrenkiel V, Warthoe P, Strauss M (1993) Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). Nucleic Acids Res 21:4272–4280.

13. Ling Z, Chen MC, Smismans A, Pavlovic D, Schuit F, Eizirik DL, Pipeleers DG (1998) Intercellular differences in interleukin-1β-induced suppression of insulin synthesis and stimulation of noninsulin protein synthesis by rat pancreatic β-cells. Endocrinology 139:1540–1545.

14. Darville MI, Eizirik DL (1998) Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. Diabetologia 41:1101–1108.

15. Rafaellof R, Qin XF, Barlow SW, Rosenberg L, Vinik AL (1996) Identification of differentially displayed genes induced in pancreatic islet neogenesis. FEBS Lett 378:219–223.

16. Yaswen P, Smoll A, Peehl DM, Trask DK, Sager R, Stampfer M (1990) Down-regulation of a calmodulin-related gene

during transformation of human mammary epithelial cells. Proc Natl Acad Sci USA 87:2319–2323.

17. Lee SW, Tomasetto C, Sager R (1991) Positive selection of candidate tumor-suppressor genes by subtractive hybridization. Proc Natl Acad Sci USA 88:2825–2829.

18. Ling Z, In't Veld PA, Pipeleers DG (1993) Interaction of interleukin-1 with islet β-cells. Distinction between indirect, aspecific cytotoxicity and direct, specific functional suppression. Diabetes 42:56–65.

19. Eizirik DL, Björklund A, Welsh N (1993) Interleukin-1-induced expression of nitric oxide synthase in insulin-producing cells is preceded by c-fos induction and depends on gene transcription and protein synthesis. FEBS Lett 317:62–66.

20. Flodström M, Niemann A, Bedoya FJ, Morris Jr SM, Eizirik DL (1995) Expression of the citrulline-nitric oxide cycle in rodent and human pancreatic β-cells: induction of argininosuccinate synthetase by cytokines. Endocrinology 136:3200–3206.

21. Rossi V, Rouayrenc JF, Paquereau L, Vilarem MJ, Le Cam A (1992) Analysis of proteins binding to the proximal promoter region of two rat serine protease inhibitor genes. Nucleic Acids Res 20:1061–1068.

22. Kordula T, Travis J (1995) Activation of the rat serine proteinase inhibitor 3 gene by interferon γ via the interleukin 6-responsive element. Biochem J 309:63–67.

23. Kordula T, Travis J (1996) The role of Stat and C/EBP transcription factors in the synergistic activation of rat serine protease inhibitor-3 gene by interleukin-6 and dexamethasone. Biochem J 313:1019–1027.

24. Bedoya FJ, Flodström M, Eizirik DL (1995) Pyrrolidine dithiocarbamate prevents IL-1-induced nitric oxide mRNA, but not superoxide dismutase mRNA, in insulin producing cells. Biochem Biophys Res Commun 210:816–822.

25. Pipeleers DG, In't Veld PA, Van De Winkel M, Maes E, Schuit FC, Gepts W (1985) A new in vitro model for the study of pancreatic A and B cells. Endocrinology 117:806–816.

26. Ling Z, Hannaert JC, Pipeleers DG (1994) Effect of nutrients, hormones and serum on survival of rat islet B cell. Endocrinology 117:834–840.

27. Eizirik DL, Sandler S, Welsh N, Cetkovic-Cvrlje M, Niemann A, Geller DA, Pipelleers D, Bendtzen K, Hellerström C (1994) Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. J Clin Invest 93:1968–1974.

28. Niemann A, Björklund A, Eizirik DL (1994) Studies on the molecular regulation of the inducible form of nitric oxide synthase (iNOS) in insulin-producing cells. Mol Cell Endocrinol 106:151–155.

29. Eizirik DL, Björklund A, Cagliero E (1993) Genotoxic agents increase expression of growth arrest and DNA damage-inducible genes gadd 153 and gadd 45 in rat pancreatic islets. Diabetes 42:738–745.

30. Wallestein S, Zucker CL, Fleiss JL (1980) Some statistical methods useful in circulation research. Circ Res 47:1–9.

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BIOLOGICAL FUNCTIONS OF RECOMBINANT BOVINE INTERLEUKIN 6 EXPRESSED IN A BACULOVIRUS SYSTEM

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The cDNA encoding bovine interleukin 6 (IL-6) was obtained from messenger RNA extracted from lipopolysaccharide-stimulated bovine Kupffer cells by the reverse transcription polymerase chain reaction (RT/PCR), and cloned into the baculovirus vector pVL 1392. Insect cells (Sf21AE derived from *Spodoptera frugiperda*) infected with the recombinant baculovirus secreted a large amount of 23.7 kD protein into the culture medium. This protein was capable of causing increased haptoglobin production and decreased albumin production in primary cultured bovine hepatocytes. The swine and human IL-6s were also able to decrease albumin production in bovine hepatocytes. This recombinant IL-6 did not stimulate the proliferation of 7TD1 cells (a murine IL-6-dependent cell line), whereas the recombinant swine IL-6 which was expressed in the same baculovirus system, and recombinant human IL-6 derived from *Escherichia coli* were each capable of stimulating proliferation of 7TD1 cells, respectively. This suggests a species restriction between bovine IL-6 and murine IL-6 dependent cell lines.

Interleukin 6 (IL-6) is a multifunctional cytokine with roles in the regulation of the immune response and the host defence reaction, and it is produced by a variety of cell types.[1] The biological activities of IL-6 include differentiation of B cells, activation of T cells, growth promotion of hybridomas/plasmacytomas, proliferation of haematopoietic stem cells and stimulation of the acute phase response by hepatocytes.[2–4] Over-expression of IL-6 is known to be an important feature of the pathogenesis of a number of inflammatory diseases, and may also be a good marker for the diagnosis of systemic inflammatory response syndrome (SIRS) and hypercytokinaemia.

Human IL-6 consists of a single polypeptide chain of 184 amino acids. It is known that the IL-6 produced by mammalian cells is N- and O-glycosylated.[5] The structural sequences of the IL-6 gene are less well conserved among mammalian species. At the protein level, bovine IL-6 shows 65, 53 and 42% homology to the porcine, human and murine IL-6s, respectively.[6]

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Human IL-6 is capable of stimulating the proliferation of murine IL-6-dependent hybridoma cell lines,[7] although IL-6 genes are also less conserved between human and mouse (42% at the amino acid level). However, a bioassay using a murine IL-6-dependent cell line is not adequate for measurement of the bovine IL-6 level.[8] A sensitive and accurate method for detecting the level of IL-6 is needed for the diagnosis of many inflammatory diseases in cattle, as well as basic studies of IL-6 functions. We have cloned and expressed the amino acids-coding region of bovine IL-6 in a baculovirus system, as an initial step to establish a sensitive assay for IL-6 in cattle.

RESULTS

Expression of bovine IL-6 in the baculovirus system

The single-stranded cDNA encoding bovine IL-6 was amplified using PCR. The PCR products were cloned into pVL 1392 and sequenced. The derived sequence contained an open reading frame of 624 bp, encoding 208 amino acids with a predicted molecular weight of 23 758 Da. The nucleotide sequence was entirely identical to the bovine IL-6 reported by Droogmans *et al.*[6] The resultant transfer vector pVLBoIL-6 was co-transfected with a baculovirus

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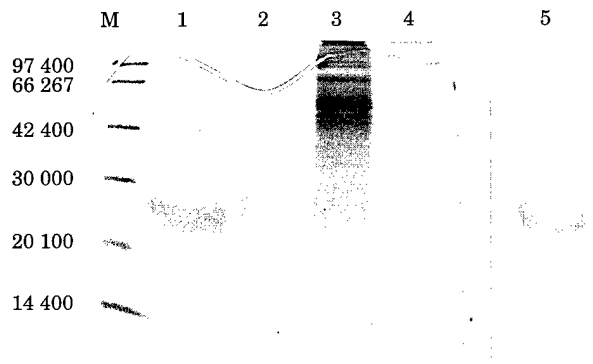

Figure 1. Expression of rBoIL-6 protein by recombinant baculovirus.

Proteins were analysed by electrophoresis on SDS-12.5% PAGE, followed by staining with Coomassie brilliant blue R-250. Lane M: marker proteins. Lane 1: supernatant of pVLBoIL-6-infected cells 3 days after infection. Lane 2: supernatant of pVLBoIL-6-infected cells 4 days after infection. Lane 3: 10-fold concentrated supernatant of non-infected cells 4 days after infection. Lane 4: supernatant of wild-type AcNPV-infected cells 4 days after infection. Lane 5: Western blot of the same samples as lane 1 with a rabbit anti ovine IL-6 antiserum.

(AcNPV) on Sf21AE cells. After cloning, the recombinant baculovirus containing bovine IL-6 (AcBoIL-6) was obtained. The secreted proteins reactive with anti-ovine IL-6 antiserum in the concentrated supernatant were detected by a Western blot analysis (Fig. 1). In addition, the secreted protein cross-reacted with anti-human IL-6 rabbit serum (data not shown). The molecular size of the secreted protein was about 23.7 kDa.

Biological activity of rBoIL-6

In order to examine the activity of recombinant bovine IL-6 (rBoIL-6) on bovine cells, the induction of acute phase proteins by IL-6 was measured in primary cultured bovine hepatocytes. As shown in Figure 2, the supernatant of the cell culture that had been infected with AcBoIL-6 showed reduced albumin production (measured in an ELISA assay) in a dose-dependent manner. The same bovine hepatocytes were much less responsive to control supernatant which was derived from AcNPV wild-type virus-infected cells. Recombinant human IL-6 (rHuIL-6) expressed in *E. coli*, dose-dependently reduced albumin production by bovine hepatocytes, but the degree of reduction at rHuIL-6 concentrations of 1000 ng/ml was less than that obtained with supernatant containing rBoIL-6. Recombinant swine IL-6 (rSwIL-6) expressed in the baculovirus system, produced in the same baculovirus system, also reduced albumin production by bovine hepatocytes.

In contrast to the decrease in albumin production, rBoIL-6 induced haptoglobin production by bovine hepatocytes (Fig. 3). Although primary cultured bovine hepatocytes secreted a small amount of haptoglobin in the non-stimulated state, rBoIL-6 increased

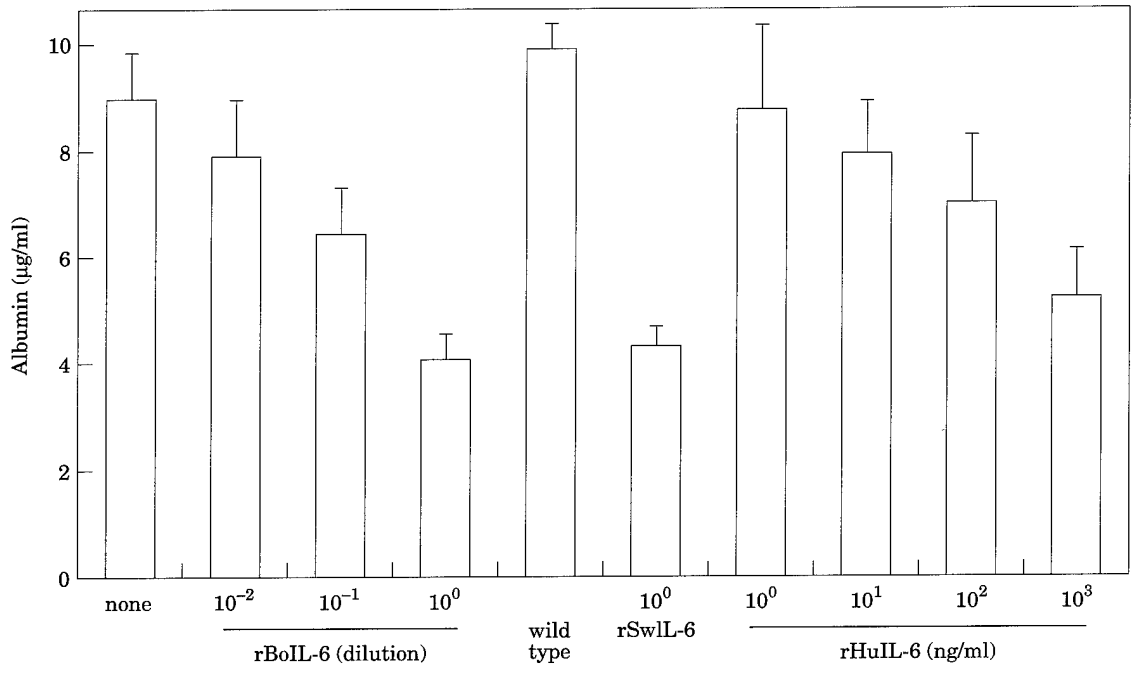

Figure 2. Albumin production by bovine hepatocytes in response to IL-6.

Albumin concentration of the culture supernatant of hepatocytes stimulated with IL-6 samples was determined with an ELISA. Samples were (i) serially diluted culture supernatant of AcBoIL-6 (rBoIL-6), (ii) AcSwIL-6 (rSwIL-6), (iii) wild type AcNPV infected cells (wild type), and (iv) rHuIL-6 expressed in *E. coli* (1000, 100, 10, 1, 0 ng/ml). Means and standard deviations are shown.

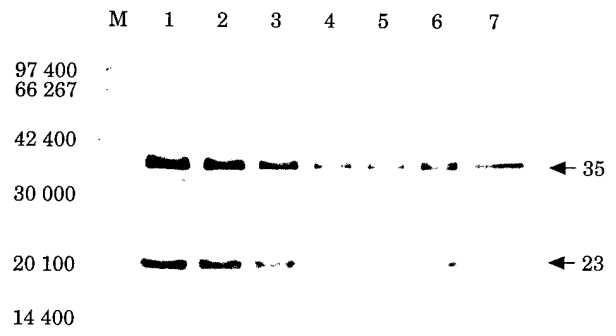

Figure 3. Immunoblot analysis of haptoglobin secretion by bovine hepatocytes in response to rBoIL-6.

The haptoglobin in the culture supernatant from hepatocytes stimulated with IL-6 was detected by immunoblotting. 10 µl culture medium concentrated to a quarter of the original volume was separated by SDS-PAGE, blotted and reacted with anti-bovine haptoglobin. Lane M: marker proteins. Lane 1: rBoIL-6. Lane 2: 10-fold dilution of rBoIL-6. Lane 3: 100-dilution of rBoIL-6. Lane 4: Supernatant of wild-type AcNPV-infected cells. Lane 5: Supernatant of non-infected cells. Lane 6: medium control. Lane 7: positive control (500-fold dilution acute-phase calf serum).

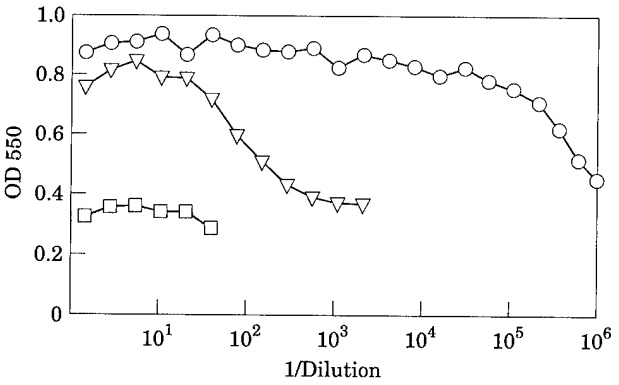

Figure 4. Proliferative response of 7TD1 cells to recombinant IL-6s.

The culture supernatant of Sf21AE cells infected with AcBoIL-6 was used to stimulate the proliferation of 7TD1 cells for 72 h. The colorimetric assay was done by the MTT method. Recombinant HuIL-6 expressed in *E. coli*, and rSwIL-6 expressed in baculovirus system, were simultaneously assayed as positive controls. (□), rBoIL-6; (○), rSwIL-6; (▽), rHuIL-6 (10 ng/ml); shaded area indicates negative control range.

haptoglobin secretion dose dependently. The supernatants derived from either non-infected cells or AcNPV wild type virus-infected cells, as a negative control, failed to increase haptoglobin production.

Proliferation assays for the baculovirus-expressed rBoIL-6 were done using 7TD1 cells, which offer a highly sensitive proliferation assay for human, porcine and murine IL-6. As shown in Figure 4, the supernatant of Sf21AE cell culture that had been infected with AcBoIL-6, poorly stimulated proliferation of 7TD1 cells. Recombinant HuIL-6 and rSwIL-6 were assayed as positive controls. In the assay shown in Figure 4, activity of rHuIL-6 at 100 pg/ml was converted into 1 standard unit. The supernatant containing rSwIL-6 strongly stimulated proliferation of 7TD1 cells, and its biological activity was calculated at over 1×10^5 U/ml when compared with human IL-6 as a standard. This proliferation induced by rSwIL-6 was inhibited by anti-human IL-6 antiserum (data not shown). A combination of the supernatant containing rBoIL-6 and rHuIL-6 did not affect proliferation activity of rHuIL-6 (data not shown), suggesting that the supernatant containing rBoIL-6 was not cytotoxic to 7TD1 cells. The supernatant of Sf21AE cells infected with AcNPV wild type virus did not proliferate 7TD1 cells.

DISCUSSION

In the present study, we succeeded in cloning and expressing bovine recombinant IL-6 in a baculovirus system. The sequence is identical to that reported by Droogmans *et al.*[6] Recombinant BoIL-6 was unable to stimulate the proliferation of 7TD1 cells. However, this protein retained other functional activities of IL-6, in the induction of acute phase proteins (increase of haptoglobin, decrease of albumin) by bovine primary cultured hepatocytes.

We tried to assess the bioactivity of bovine IL-6 by proliferation assay using 7TD1 cells, but failed to detect IL-6 in the serum or milk of cows. There are other reports in which this assay was not effective in measuring ruminant IL-6.[8] On the other hand, the 7TD1 cell line has been used to test for bovine IL-6 activity.[9,10] We have been able to detect chicken or swine IL-6 in serum using this cell line.[11] Recently, it has been reported that a recombinant caprine IL-6 expressed in a baculovirus system stimulates the growth of 7TD1 cells.[12] However, that report did not confirm that the antibodies to human IL-6 inhibited the proliferation of 7TD1 cells induced by the caprine IL-6. Rabbit antibodies to human IL-6 (Genzyme, Cambridge, MA, USA) inhibited the proliferation of 7TD1 cells induced by human IL-6, but had no effect on bovine serum-induced proliferation of 7TD1 cells (unpublished data).

Amino acid sequences of mammalian IL-6s are not extensively conserved. This is especially true for the bovine and murine IL-6 sequences, which show only 42% homology. However, there is a high level of homology between the bovine IL-6 and the ovine IL-6 (95%).[6] There may be a partial species barrier between ovine IL-6 and murine cells which is not present between human IL-6 and murine cells.[8] In that report, the recombinant ovine IL-6 expressed by a yeast system did not cause extensive proliferation of B9 murine myeloma cells, and human IL-6 did not stimulate immunoglobulin synthesis by ovine peripheral blood

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mononuclear cells. In the present study, rBoIL-6, in addition to human and swine IL-6, was shown to induce acute phase protein synthesis. Human IL-6 could induce acute phase protein in cattle by continuous infusion,[4] and stimulate the secretion of serum amyloid-A and haptoglobin of acute phase protein in bovine hepatocytes in vitro.[13] A similar species barrier has been noted between human and murine IL-6. Human IL-6 binds to the murine IL-6 receptor to stimulate murine IL-6-dependent cell lines, while murine IL-6 is unable to induce acute phase protein in human hepatoma (Hep3B) cells.[14] In this study, we also observed that recombinant murine IL-6 expressed in *E. coli* failed to induce acute phase protein in bovine hepatocytes (data not shown).

It has been proposed that IL-6 folds into a bundle of four α-helices (A, B, C and D).[15] Four cysteine residues forming disulfide bridges at positions 72–78 and 101–111 are conserved in the IL-6 of cow as well as in other species.[16] IL-6 requires an α-helical structure and a positive charge at the C-terminus for its bioactivity.[17,18] Two sets of leucine residues spaced at 7-residue intervals (Leu 168, 175, 182 and Leu 152, 159, 166 in the human sequence) play an important role in the receptor binding and bioactivity of IL-6.[19] Compared with human IL-6, bovine IL-6 has some deleted amino acids in the mature sequence.[20] Only Leu 168 and Leu 175 in the human sequence have equivalents in the bovine sequence, but Leu 182 is not present in most other IL-6 sequences. However, the substitution of Leu 168 with several other residues has no effect on bioactivity. A set of leucine residues in the human sequence (Leu 152, 159, 166) has equivalents in the rodent sequences. These absences of leucines may be important in the observed species barrier.

The IL-6 receptor is composed of two polypeptides, the α chain (IL-6r/gp80), an 80 kDa transmembrane glycoprotein that binds IL-6 with low affinity, and the β chain (gp130), a 130 kDa transmembrane glycoprotein that binds to the IL-6-IL-6r heterodimer to form a high affinity signal transducing complex.[21] Critical residues in IL-6 for specific binding to IL-6r or gp130 have been identified by site-directed mutagenesis.[22] Human IL-6 binds to both human and rodent IL-6r, whereas rodent IL-6 interacts only with rodent cells.[23] The common signal-transducing subunit gp130 does not discriminate between different IL-6 or IL-6r species.[23,24] One region of human IL-6 (residues Gly77–Glu95) is important for the interaction of IL-6 with the IL-6r, and another region (residues Lys41–Ala56) is essential for the activation of gp130.[25,26] The midregion of IL-6 that recognizes IL-6r may be involved in the species barrier between bovine and murine IL-6s.

MATERIALS AND METHODS

Construction of the bovine IL-6 cDNA recombinant transfer vector

Bovine Kupffer cells were prepared by the centrifugal elutriation method as previously described,[27] and were stimulated with 1 μg/ml of lipopolysaccharide (LPS; O111:B4, Sigma, St Louis, MO, USA) for 3 h in RPMI1640) medium supplemented with 2% fetal calf serum. The messenger RNA was extracted from the stimulated Kupffer cells with an mRNA purification kit (Pharmacia, Uppsala, Sweden). A first strand cDNA was synthesized from 50 ng of the mRNA with an RNA PCR kit (Takara Shuzo, Kyoto, Japan). The entire coding region of bovine IL-6 cDNA was amplified by PCR with primers that were designed from the cDNA sequence reported by Droogmans *et al.*[6] Then, *Eco*RI and *Bam*HI restriction enzyme sites were added to 5′ and 3′ ends of the PCR products. The sequences of the forward and reverse primers were 5′-TCCG*GAATTC*GAACAGCTAT GAACTCCCGCTT-3′, and 5′-TGTA*CCTAGG*ATGCCCA GGAACTACCACCATC-3′, respectively. The PCR product was purified with a PCR Purification kit (Qiagen, Hilden, Germany), and ligated into the *Eco*RI- *Bam*HI site of the baculovirus transfer vector pVL1392 (Pharmingen, San Diego, CA, USA), according to the protocol of the ligation kit (Pharmacia; Ready-To-Go T4 DNA Ligase). The recombinant transfer vector (pVLBoIL-6) containing bovine IL-6 cDNA was digested with *Eco*RI and *Bam*HI enzymes and analysed by gel electrophoresis to certify the size and integrity of the inserted genes. Furthermore, the DNA sequence of the inserted portion was analysed by using an Autocyle Sequencing kit (Pharmacia).

Expression of Bovine IL-6

Purified DNA of the transfer vector pVLBoIL-6 and Baculogold Linearized Baculovirus DNA (Pharmingen) were mixed with Lipofectin (Gibco-BRL, Gaithersburg, MD, USA), then added to the *Spodoptera frugiperda* cell line Sf21AE.[28] After 4 days incubation at 27°C, the supernatant fluid containing recombinant baculoviruses was subjected to plaque purification. The recombinant baculovirus AcBoIL-6 that was obtained from pVLBoIL-6 and that contained bovine IL-6 cDNA was used for production of recombinant protein. Sf21AE cells were infected with AcBoIL-6 and cultured with serum-free medium Sf900II (Gibco-BRL) at 27°C.

Biochemical characterization of recombinant bovine IL-6

The culture medium of Sf21AE cells infected with recombinant virus AcBoIL-6 was harvested after 3 days incubation, and centrifuged at 600 × ***g*** at 4°C for 10 min. The culture supernatant was further centrifuged at 40 000 × ***g*** for 1 h, and then purified by a concentrator (300 kDa cut-off; Gottingen, Germany) to remove the virus and concentrated by ultrafiltration (10 kDa cut-off; Sartorius). The resultant culture supernatant was mixed with sample buffer (50 mM Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 20% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue and 10% glyerol), boiled for 3 min, and subjected to an SDS-PAGE

analysis followed by staining with Coomassie brilliant blue R-250. Proteins separated by SDS-PAGE were also electro-transferred to nitrocellulose membranes by the standard method. Transferred membranes were blocked with a blocking reagent (Block Ace; Dainippon Seiyaku, Osaka, Japan) and were incubated with rabbit anti-recombinant ovine IL-6 antiserum (Serotech, Kidlington, UK). An alkaline phosphatase-conjugated goat antibody to rabbit IgG (Sigma) was used as a secondary antibody and then alkaline phosphatase activity was visualized by developing with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride (BCIP/NBT) kit (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA).

Cell proliferation assay

Biological activity of recombinant bovine IL-6 was assayed by using the IL-6 dependent murine hybridoma 7TD1 cells as described by Van Snick *et al.*[7] with modifications. In brief, 7TD1 cells were incubated at a density of 5×10^4 cells per well in 96-well microplates with the test sample in RPMI1640 with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, in a total volume of 200 μl for 3 days at 37°C. The proliferation of 7TD1 cells was evaluated by using the MTT assay. Recombinant HuIL-6 developed in *E. coli* and SwIL-6 expressed by the baculovirus system were included in this assay as positive controls.

Primary culture of bovine hepatocytes

Five male Holstein calves (up to 1 week old) were studied, and details of culturing have been described.[29] Briefly, 2 ml of cell suspension (5×10^5 cells/ml) in William's E medium containing 5% calf serum, 10 M insulin, 5 IU/ml aprotinin, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) were seeded on 35-mm plastic dishes (Sumitomo Bakelite, Tokyo, Japan), and the cells were incubated at 37°C for 4 h in a humidified atmosphere of 5% CO_2 in air. The medium was replaced, and monolayer cultures were further maintained for 24 h. The cells were washed 3 times with fresh medium and cultured for an additional 24 h in serum-free medium. Then the medium was exchanged with fresh serum-free medium and the test samples as stated in the figure legends were added. The supernatants were harvested after 24 h incubation, stored at − 20°C after centrifugation at 700 $g \times 10$ min for bioassays.

ELISA assay of albumin

The bovine albumin content of the culture medium was determined using an ELISA assay by the method of Yamanaka *et al.*[30] Bovine serum albumin (Seikagaku Co., Tokyo, Japan) or diluted culture medium were used to coat a flat-bottom 96-well microplate, and were reacted with a purified rabbit anti-bovine albumin IgG antibody as the first antibody, and then alkaline phosphatase-conjugated anti-rabbit IgG goat serum (Sigma) as the second antibody for colour development.

Immunoblot analysis of haptoglobin

The haptoglobin in the culture medium was identified by immunoblot analysis. Proteins separated by SDS-PAGE (5% stacking gel and 12.5% separation gel) were transferred to a nitrocellulose membrane at 0.8 mA per 1 cm^2 for 1 h. The transferred haptoglobin was reacted with purified anti-bovine haptoglobin rabbit IgG, for 30 min at room temperature. After washing with 10 mM sodium phosphate buffer (pH 7.4) containing 0.14 M NaCl and 0.05% tween 80, the membrane was treated with alkaline phosphatase substrate system.

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REFERENCES

1. Van Snick J (1990) Interleukin-6: An overview. Annu Rev Immunol 8:253–278.

2. Geiger T, Andus T, Klapproth J, Hirano T, Kishimoto T, Heinrich PC (1988) Induction of rat acute-phase proteins by interleukin-6 in vivo. Eur J Immunol 18:717–721.

3. Heinrich PC, Castell JV, Andus T (1990) Interleukin-6 and acute phase response. Biochem J 265:621–636.

4. Nakajima Y, Momotani E, Murakami T, Ishikawa Y, Morimatsu M, Saito M, Suzuki H, Yasukawa K (1993) Induction of acute phase protein by recombinant human interleukin-6 (IL-6) in calves. Vet Immunol Immunopathol 35:385–391.

5. Lotz M (1993) Interleukin-6. Cancer Invest 11:732–742.

6. Droogmans L, Cludts I, Cleuter Y, Kettmann R, Burny A (1992) Nucleotide sequence of bovine interleukin-6 cDNA. DNA Sequence 2:411–414.

7. Van Snick J, Cayphas S, Vink A, Uyttenhove C, Coulie PG, Rubria MR, Simpson RJ (1986) Purification and NH_2-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. Proc Natl Acad Sci USA 83: 9679–9683.

8. Ebrahimi B, Roy DJ, Bird P, Sargan DR (1995) Cloning, sequencing and expression of the ovine interleukin 6 gene. Cytokine 7:232–236.

9. Andrews AE, Barcham GY, Ashman K, Meeusen ENT, Brandon MR, Nash AD (1993) Molecular cloning and characterization of ruminant interleukin-6 cDNA. Immunol Cell Biol 71: 341–348.

10. Jian Z, Yang Z, Miller MS, Carter CD, Slauson DO, Bochsler PN (1995) Interleukin-6 secretion by bacterial lipopolysaccharide-stimulated bovine alveolar macrophages in vitro. Vet Immunol Immunopathol 49:51–60.

11. Nakamura K, Mitarai Y, Yoshioka M, Koizumi N, Shibahara T, Nakajima Y (1998) Serum levels of interleukin-6, alpha 1-acid protein, and corticosterone in the two-week-old chickens inoculated with *Escherichia coli* lipopolysaccharide. Poultry Science 77:908–911.

12. Takakura H, Mori Y, Tatsumi M (1997) Molecular cloning of caprine IL-6 cDNA and its expression in insect cells. Int Arch Allergy Immunol 113:409–416.

13. Alsemgeest SP, Van T Klooster GA, Van Miert AS, Hulskamp Koch CK, Gruys E (1996) Primary bovine hepatocytes in the study of cytokine induced acute-phase protein secretion in vitro. Vet Immunol Immunopathol 53:179–184.

14. Fiorillo MT, Cabibbo A, Iacopetti P, Fattori E, Ciliberto G (1992) Analysis of human/mouse interleukin-6 hybrid proteins: both amino and carboxy termini of human interleukin-6 are required for in vitro receptor binding. Eur J Immunol 22:2609–2615.

15. Bazan JF (1990) Hemopoietic receptors and helical cytokines. Immunol Today 11:350–354.

16. Simpson RJ, Moritz RJ, Van Roost E, Van Snick J (1988) Characterization of a recombinant murine interleukin-6: Assignment of disulfide bonds. Biochem Biophys Res Commun 88:364–372.

17. Lutticken C, Kruttgen A, Moller C, Heinrich PC, Rose-John S (1992) Evidence for the importance of a positive charge and a α-helical structure of the C-terminus for biological activity of human IL-6. FEBS Lett 282:265–267.

18. Leebeek FW, Kariya K, Schwabe M, Fowlkes DM (1992) Identification of a receptor binding site in the carboxyl terminus human interleukin-6. J Biol Chem 267:14 832–14 838.

19. Nishimura C, Ekida T, Nomura K, Sakamoto K, Suzuki H, Yasukawa K, Kishimoto T, Arata Y (1992) Role of leucine residues in the C-terminal region of human interleukin-6 in the biological activity. FEBS Lett 311:271–275.

20. Savino R, Lahm M, Giorgio M, Cabibbo A, Tramontano A, Ciliberto G (1993) Saturation mutagenesis of the human interleukin 6 receptor-binding site: Implication for its three-dimensional structure. Proc Natl Acad Sci USA 90:4067–4071.

21. Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, Kishimoto T (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp 130. Cell 58:573–581.

22. Xu GY, Yu HA, Hong J, Stahl M, McDonagh T, Kay LE, Cumming DA (1997) Solution structure of recombinant human interleukin-6. J Mol Biol 268:468–481.

23. Sugita T, Totsuka T, Saito M, Yamasaki K, Taga T, Hirano T, Kishimoto T (1990) Functional murine interleukin 6 receptor with the intracisternal A particle gene product at its cytoplasmic domain: Its possible role in plasmacytomagenesis. J Exp Med 171:2001–2009.

24. Hibi M, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T (1990) Molecular cloning and expression of an IL-6 Signal Transducer, gp 130. Cell 63:1149–1157.

25. Van Dam M, Mullberg J, Schwabe M, Fowlkes DM (1993) Structure function analysis of interleukin-6 utilizing human/murine chimeric molecules. J Biol Chem 267:15 285–15 290.

26. Ehlers M, Grotzinger J, DeHon FD, Mullberg J, Brakenhoff JP, Liu J, Wollmer A, Rose-John S (1994) Identification of two novel regions of human IL-6 responsible for receptor binding and signal transduction. J Immunol 153:1744–1753.

27. Yoshioka M, Nakajima Y, Ito T, Mikami O, Tanaka S, Miyazaki S, Motoi Y (1997) Primary culture and expression of cytokine mRNAs by lipopolysaccharide in bovine Kupffer cells. Vet Immunol Immunopathol 58:155–163.

28. Felgner PL, Gadek TR, Holm M, Roma R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M (1987 Lipofection: A highly efficient. lipid-mediated DNA-transfection procedure) Proc Natl Acad Sci USA 84:7413–7417.

29. Higuchi H, Katoh N, Miyamoto T, Yuasa A, Takahashi K (1994) Dexamethasone-induced haptoglobin release by calf liver parenchymal cells. Am J Vet Res 55:1080–1085.

30. Yamanaka N, Kitani H, Mikami O, Nakajima Y, Miura K (1997) Serum-free culture of adult chicken hepatocytes; morphological and biochemical characterization. Res Vet Sci 62:233–237.

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FLOW CYTOMETRICAL DETERMINATION OF INTERLEUKIN 1β, INTERLEUKIN 6 AND TUMOUR NECROSIS FACTOR α IN MONOCYTES OF RHEUMATOID ARTHRITIS PATIENTS; RELATION WITH PARAMETERS OF OSTEOPOROSIS

Ann Verbruggen, Luc S. De Clerck, Chris H. Bridts, Jan F. Van Offel, Wim J. Stevens

Experimental data suggest that pro-inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α) are important in the pathogenesis of osteoporosis in rheumatoid arthritis. Therefore we compared the production of these cytokines by monocytes in 10 rheumatoid arthritis patients and 10 controls. Cytokine levels in rheumatoid arthritis patients were related to disease activity parameters, bone mineral density (BMD) corrected for age and sex (Z scores) and osteocalcin as a laboratory parameter of bone remodelling. Cytokines were determined by a flow cytometrical technique. There was a tendency for higher IL-1β levels in patients compared with controls. A positive correlation between erythrocyte sedimentation rate and spontaneous production of monocytic cytokines was found. Z scores of the lumbar spine showed a negative correlation with spontaneous production of IL-1β and IL-6. Plasma osteocalcin levels were positively correlated with spontaneous production of IL-1β, IL-6 and TNF-α. In conclusion, the correlation of the levels of these cytokines with parameters of bone metabolism and osteoporosis suggest that especially IL-1β and IL-6 are associated with more pronounced osteoporosis in active rheumatoid arthritis.

Cytokines are major triggers of inflammation and joint destruction in rheumatoid arthritis.[1,2,3] Monocytic cytokines such as IL-1β, IL-6 and TNF-α are especially potent pro-inflammatory mediators, and they are prevalent both in serum and synovial fluid and tissue.[2,4] Experimental data suggest that these cytokines might be important in the pathogenesis of osteoporosis in rheumatoid arthritis.[1,5,6] More pronounced osteoporosis is often seen in active rheumatoid arthritis, as stated by Emery *et al.*[7] To our knowledge there are little quantitative data on intracellular or cell-associated levels of cytokines in monocytes in rheumatoid arthritis. Cytokine levels in supernatants are not necessarily an adequate parameter of total cytokine production, as they may immediately be metabolized or bound by mononuclear cells. Therefore we studied cytokines at a cellular level. Furthermore, we correlated monocytic cytokine levels of rheumatoid arthritis patients with articular disease activity parameters, laboratory parameters of osteoporosis and bone remodelling and bone mineral content in lumbar spine and hip.

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RESULTS

Determination of cell-associated cytokines

Total cell-associated cytokines were defined as the combination of intracellular and cell surface bound molecules. Cytokines were determined flow cytometrically. Saponin permitted the determination of intracellular cytokines in addition to cell membrane-bound cytokines. Without saponin, only the membrane bound cytokines were measured.

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IL-1β

Although we could not detect IL-1β expression on the cell surface in basal conditions (data not shown), LPS stimulation induced two populations of cells with surface bound IL-1β (Fig. 1A). 40% of the monocytes showed a bright and 60% a dim fluorescence intensity: mean total IL-1β fluorescence intensity was tenfold higher than mean cell surface-associated IL-1β fluorescence intensity. Total cell-associated IL-1β production increased markedly after LPS-stimulation. The mean fluorescence intensity (MFI) of IL-1β augmented from 41 MFI without LPS to 227 MFI after LPS stimulation.

IL-6

We could not detect spontaneous IL-6 expression on the cell surface in basal conditions (data not shown). After LPS-stimulation, histograms of cell surface-bound IL-6 revealed two populations: 12% of the cells had a bright and 88% of the cells had a dim pattern of fluorescence intensity (Fig. 1B). Mean total IL-6 fluorescence intensity was fourfold higher than mean cell surface-associated IL-6 fluorescence intensity. Total cell-associated IL-6 showed an increase after LPS-stimulation: from 21 MFI to 553 MFI.

TNF-α

We could not detect cell surface-bound TNF-α, neither in basal nor in stimulated conditions (Fig. 1C). Total cell-associated TNF-α production of all monocytes was low but consistently present in both basal conditions and after stimulation, respectively 10 and 15 MFI.

Comparison between rheumatoid arthritis patients and controls

Figure 2 shows the mean fluorescence intensity in controls and patients in basal conditions (Fig. 2A) and after an 8 h culture without (Fig. 2B) and with LPS-stimulation (Fig. 2C). IL-1β production after 8 h showed a tendency for higher levels in rheumatoid arthritis patients as compared to controls ($P=0.09$). For IL-6 and TNF-α values, patients and controls were not different.

Relation with disease activity in rheumatoid arthritis patients

A positive correlation between erythrocyte sedimentation rate (ESR) and basal cytokine production was found (Table 1). Other disease activity parameters [C-reactive protein (CRP), and clinical parameters] were not significantly correlated with monocytic cytokine levels. Plasma osteocalcin levels were positively correlated with spontaneous production (basal and 8 h culture) of IL-1β and IL-6, and with basal

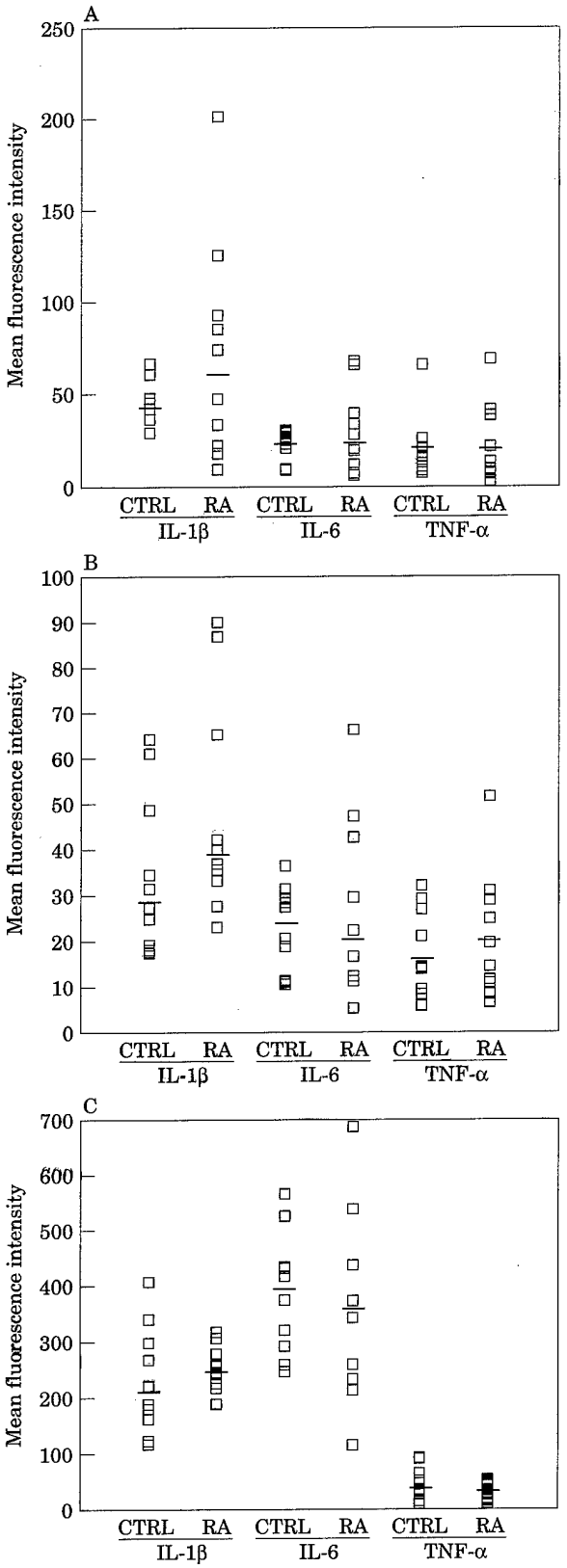

Figure 1. Monocytic cytokine levels.

(A) Basal conditions; (B) 8 h cultured without LPS; (C) 8 h cultured with LPS. MFI, mean fluorescence intensity; RA, rheumatoid arthritis patients; CTRL, control persons; LPS, Lipopolysaccharide; (—), median.

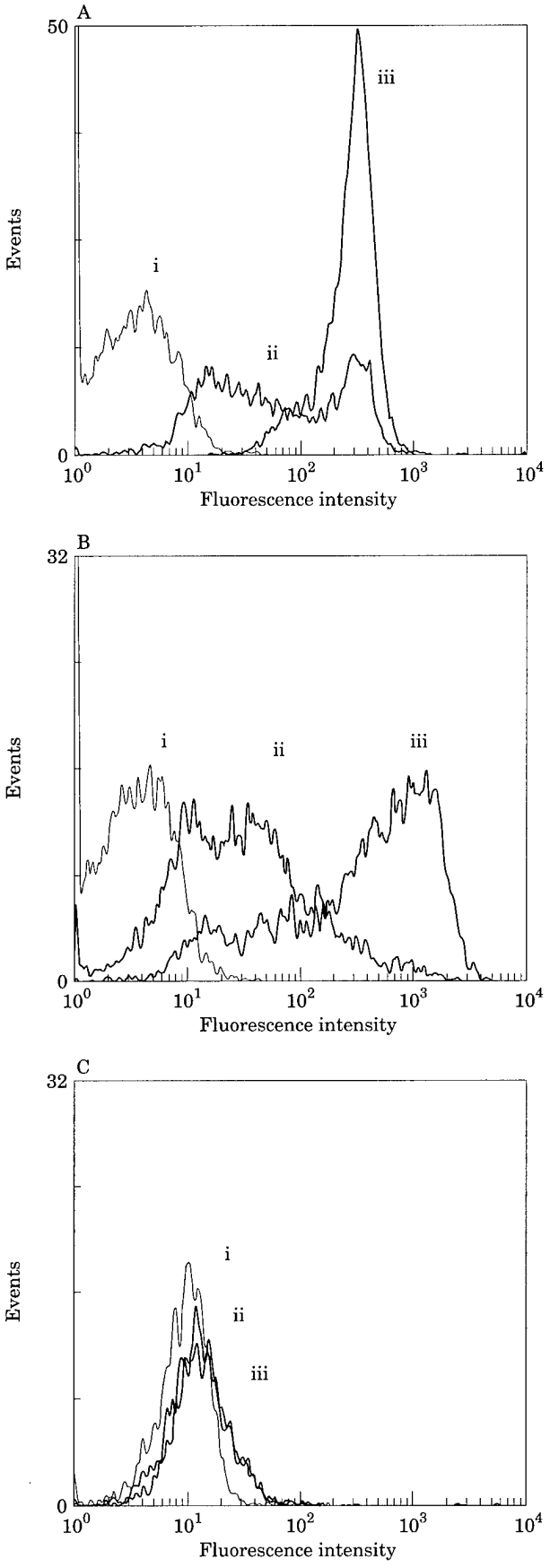

TABLE 1. Spearman rank correlation coefficient between monocyte cytokine levels and erythrocyte sedimentation rate

	Basal (before culture)	Cultured without LPS* (8 h)	Cultured with LPS* (8 h)
IL-1β**	0.466	0.576	0.357
IL-6**	0.745	0.721	−0.563
TNF-α**	0.406	0.127	−0.115

*LPS, Lipopolysaccharide.
**IL-1β, interleukin 1β; IL-6, interleukin 6; TNF-α, tumour necrosis factor α.

TABLE 2. Spearman rank correlation coefficient between monocyte cytokine levels and osteocalcin

	Basal (before culture)	Cultured without LPS* (8 h)
IL-1β**	0.136	0.475
IL-6**	0.433	0.621
TNF-α**	0.481	0.178

*LPS, Lipopolysaccharide.
**IL-1β, interleukin 1β; IL-6, interleukin 6; TNF-α, tumour necrosis factor α.

TABLE 3. Spearman rank correlation coefficient between monocyte cytokine levels and BMD (L2–L4)

	Basal (before culture)	Cultured without LPS* (8 h)
IL-1β**	0.006	−0.600
IL-6**	−0.430	−0.672
TNF-α**	0.187	−0.200

*LPS, Lipopolysaccharide.
**IL-1β, interleukin 1β; IL-6, interleukin 6; TNF-α tumour necrosis factor α.

TNF-α production (Table 2). Bone mineral density of the lumbar spine correlated negatively with basal and 8 h cultured cytokine levels, especially for IL-6 (Table 3). In LPS-stimulated monocytes, no correlation was found between cytokine levels and Z scores (data not shown).

DISCUSSION

In this study, a flow cytometrical method was used to measure IL-1β, IL-6 and TNF-α in peripheral blood monocytes of patients with rheumatoid arthritis under treatment with non-steroidal anti-inflammatory drugs. Experimental data suggest that these cytokines

Figure 2. Histogram showing log scale of fluorescence intensity.

(A) IL-1β; (B) IL-6; and (C) TNF-α after 8 h LPS stimulation. (i) Isotype matched irrelevant antibody; (ii) cell surface cytokines; (iii) cytokines in permeablized cells. LPS; Lipopolysaccharide; IL-1β, interleukin 1β; IL-6, interleukin 6; TNF-α, tumour necrosis factor α.

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might be important in the pathogenesis of osteoporosis.[1] As monocytes are the main producers of these cytokines, we examined the hypothesis that increased production of these cytokines could be related to clinical and laboratory parameters of inflammation (e.g. ESR and CRP). In our study, a consistent but rather low production of TNF-α was found in controls and rheumatoid arthritis patients. Others using different techniques have also found low or undetectable TNF-α levels in serum: Saarinen *et al.*[11] who measured TNF-α in serum in haematological malignancies, using an ELISA with a detection level of 10 ng/l, could not detect TNF-α in healthy children and those patients in complete remission. Markham *et al.*, who described a more sensitive ELISA for TNF-α, with a detection limit of 1 ng/l, found very low TNF-α levels in women with gynaecological tumours, the mean TNF-α concentration was 10 ng/l.

We found higher spontaneous production of cell-associated IL-1β in rheumatoid arthritis patients, suggesting that monocytes in rheumatoid arthritis are activated, and also involved in systemic inflammation, not only in local inflammation of the joint space. Findings of Lioté *et al.*[13] who reported higher monocyte adhesiveness, integrin expression and cytokine release in blood monocytes of rheumatoid arthritis patients, point in the same direction. Since we found no differences in IL-1β production in LPS-stimulated monocytes between patients and controls, we presume that monocytes of rheumatoid arthritis patients have the same maximal production capacity as monocytes of controls, although the spontaneous IL-1β production is somewhat higher in rheumatoid arthritis.

Our data show that, after stimulation, IL-1β and IL-6 were also associated with the cell surface. Jung *et al.*[9] reported IL-2 and IFN-γ associated with the cell surface of non-permeabilized T cells. In our study, membrane associated IL-6 and IL-1β are probably bound to their receptor, since such membrane receptors have been described on human monocytes. Wognum *et al.*[14] reported a significant expression of IL-6 receptors on human peripheral blood monocytes with a high affinity for IL-6. IL-1 receptors and IL-1 production in controls and haemodialysis patients were extensively studied by Donati *et al.*[15] They found higher expression of IL-1 receptors on monocytes of patients compared with controls. As far as the relationship between cytokines and inflammatory parameters is concerned, data in blood of rheumatoid arthritis patients are controversial. Dasgupta *et al.*[16] reported a correlation between IL-6 in serum and erythrocyte sedimentation rate; in contrast, Holt *et al.*[17] found no correlations between plasma IL-6 and clinical disease activity. In our study we measured cytokines more directly on the cells; this enabled us to avoid discrepancies between serum and plasma. This also gave us the advantage of studying monocytic cytokine levels after LPS stimulation. Cell associated IL-6, as well as cell associated IL-1β, levels were strongly positively correlated with the erythrocyte sedimentation rate, which strengthens the hypothesis that peripheral blood monocytes are activated and involved in systemic inflammation in rheumatoid arthritis. Another important feature of rheumatoid arthritis is generalized osteoporosis.[18] Interestingly, in our study we were able to link spontaneous IL-1β and IL-6 production by monocytes to diffuse osteoporosis, as measured by bone mineral density of the lumbar spine. In addition, spontaneous IL-1β and IL-6 production was positively correlated to plasma osteocalcin levels, which is a marker of bone remodelling.[19,20]

In summary, we demonstrated that peripheral blood monocytes in rheumatoid arthritis are activated and produce pro-inflammatory cytokines, which are linked to generalized inflammation and osteoporosis.

MATERIALS AND METHODS

Reagents and antibodies

AIM-V medium (Life Technologies, Paisley, Scotland), Mouse anti-human CD14 fluorescein isothiocyanate (FITC) (Becton Dickinson, San Jose, CA) Rat anti-human IL-1β phycoerythrin (RPE), mouse anti-human IL-6 phycoerythrin and mouse anti-human TNF-α phycoerythrin (Biosource International, Camarillo, CA, USA). Rat IgG2a negative control phycoerythrin and mouse IgG1 negative control phycoerythrin (RPE) (Serotec Ltd, Oxford, England). LinearFlow® Orange (530/560) Flow Cytometry Intensity Calibration Kit (L-7307 Lot: 5864, Molecular Probes, USA). Lipopolysaccharide (LPS L-3755 Lot 84H4017 *E. coli* Serotype 026:B6 (Sigma Chemical Co. St Louis, USA). Monensin (Sigma). Nycoprep (d=1.077 mg/ml, Nycomed, Oslo, Norway). Phosphate Buffered Saline without calcium and magnesium (PBS) (Life Technologies). Penicillin/Streptomycin (Gibco-BRL, Life Technologies, Paisley, Scotland). Saponin S-7900 Lot 91H0325 (Sigma). Paraformaldehyde (Sigma). IL-1β (Genzyme, Cambridge, England), IL-6 and TNF-α (ReproTech, Rocky Hill, New Jersey).

Patients and control persons

Blood samples

For cytokine detection and peripheral blood cell count, 30 ml of EDTA peripheral venous blood (Vacutainer®, Becton Dickinson, Meylan, France) was obtained from 10 rheumatoid arthritis patients and 10 sex and age matched healthy controls.

Patient characteristics

Rheumatoid arthritis patients fulfilled the diagnostic criteria of the American College of Rheumatology for the classification of Rheumatoid Arthritis.[8] Patients were treated with non-steroidal anti-inflammatory drugs while the tests

were performed. Three men and seven women were examined and compared to 10 age and sex matched healthy volunteers.

Disease activity, bone remodelling and osteoporosis parameters

Erythrocyte sedimentation rate (ESR), peripheral white blood cell count and differentiation, and C-reactive Protein (CRP) were determined. In patients, Ritchie articular index, number of swollen joints, Visual Analogue Scale (VAS), global assessment both by physician and patient, and Health Assessment Questionnaire (HAQ) were determined. Patient's osteocalcin was determined by a RIA method (Biosource), according to the manufacturer's instructions. Patients' Z score of the lumbar spine (L2–L4) and hip were determined by a dual energy X-ray absorptiometry (DEXA) method (Lunar, type DP XL absorptiometer, Wisconsin, USA). Results were expressed as bone mineral density (BMD).

Cell separation and culture

Peripheral blood mononuclear cells (PBMCs) were separated by Nycoprep density gradient. Cells were concentrated at 5×10^6 cells/ml in AIM-V medium. PBMCs were cultured for 8 h (preliminary experiments found this to be the optimal time for maximal production of cytokines) in a 5% CO_2 humidified atmosphere at 37°C. Some of the cells were stimulated with LPS (5 μg/ml) and monensin (1 μM) in AIM-V.

Two-colour flow cytometry for cytokines

Intracellular and membrane bound IL-1β, IL-6 and TNF-α in monocytes were determined using a modified technique to measure intracellular production of cytokines.[9,10] Briefly, after stimulation, 1 ml PBS was added to the cell suspension. Cells were spun for 5 min at $1000 \times g$, and were resuspended at 1×10^7 cells/ml in PBS. To identify monocytes, the cells were incubated with anti-CD14-FITC for 15 min at room temperature. Subsequently, cells were fixed for 30 min with 4% paraformaldehyde in PBS. Cell membranes were made permeable with a 0.3% saponin solution in PBS, followed by incubation with anti-cytokine antibodies against IL-1β, IL-6 and TNF-α during 30 min at room temperature. To evaluate cell surface-associated cytokines, PBS was added instead of saponin solution. Cells were centrifuged for 10 min at $1000 \times g$ and the cell pellet was resuspended in 0.3 ml PBS.

Flow cytometric analysis

Twenty five thousand events were measured on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems), and analysed with Lysis II software. Analysis gates were set on CD14-positive cells according to FITC emission. Within this gate, intracellular cytokine production or cell membrane bound cytokines were evaluated. Results were expressed as the mean fluorescence intensity. Measurements were standardized using LinearFlow® Orange (530/560) Flow Intensity Calibration Kit. Isotype matched irrelevant antibodies were used as negative controls.

Statistical analysis

Differences in cytokine production between rheumatoid arthritis patients and controls were assessed by the Mann-Whitney U test. Correlations between cell-associated cytokine production and disease activity parameters were calculated by Spearman rank correlation. A P value less than 0.05 was considered significant.

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REFERENCES

1. Papadopoulos NG, Georganas K, Skoutellas V, Konstantellos E, Lyritis GP (1997) Correlation of interleukin-6 serum levels with bone density in postmenopausal women. Clin Rheumatol 17:162–165.

2. Campbell IK, Piccoli DS, Roberts MJ (1990) Effects of tumor necrosis factor α and β on resorption of human articular cartilage and production of plasminogen activator by human articular chondrocytes. Arthritis Rheum 33:542–552.

3. Tetta C, Camussi G, Modena V, Di Vittorio C, Baglioni C (1990) Tumor necrosis factor in serum and synovial fluid of patients with active and severe rheumatoid arthritis. Ann Rheum Dis 49: 665–667.

4. Firestein GS, Alvaro-Gracia JM, Maki R (1990) Quantitative analysis of cytokine gene expression in rheumatoid arthritis J Immunol. 144:3347–3353.

5. Manolagas SC, Jilka RL (1995) Bone marrow, cytokines and bone remodelling—Emergency insights into the pathophysiology of osteoporosis. N Eng J Med 332:305–331.

6. Zheng SX, Vrindts Y, Lopez M, De Groote D, Zangerle PF, Collette J, Franchimont N, Geenen V, Albert A, Reginster JY (1997) Increase in cytokine production (IL-1β, IL-6, TNF-α but not IFN-γ, GM-CSF or LIF) by stimulated whole blood cells in postmenopausal osteoporosis. Maturitas 26:63–71.

7. Emery P (1994) Assessment of Rheumatoid Arthritis-A Clinician's viewpoint. J Rheumatol 21: 10 supplement 42:20–24.

8. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healy LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA, Mitchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JT, Wilder RL, Hunder GG (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315.

9. Jung T, Schauer U, Heusser C, Neumann C, Rieger c (1993) Detection of intracellular cytokines by flow cytometry. J Immunol Meth 159:197–207.

10. Prussin C, Metcalfe DD (1995) Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. J Immunol Methods 188:117–128.

11. Saarinen UM, Koskelo EK, Teppo AM, Siimes MA (1990) Tumor necrosis factor in children with malignancies. Cancer Res 50:592–595.

12. Markham R, Young L, Fraser IS (1995) An amplified ELISA for human tumour necrosis factor alpha. Eur Cytokine Netw 6:49–54.

13. Lioté F, Boval-Boizard B, Weill D, Kuntz D, Wautier J-L (1996) Blood monocyte activation in rheumatoid arthritis: increased monocyte adhesiveness, integrin expression, and cytokine release. Clin Exp Immunol 106:13–19.

14. Wognum AW, van Gils FCJM, Wagemaker G (1993) Flow cytometric detection of receptors for interleukin-6 on bone marrow and peripheral blood cells of humans and rhesus monkeys. Blood 81: 2036–2043.

15. Donati D, Degiannis D, Mazzola E, Gastaldi L, Raskova J, Raska Jr K, Camussi G (1997) Interleukin-1 receptors and receptor antagonist in haemodialysis. Nephrol Dial Transplant 12:111–118.

16. Dasgupta B, Corkill M, Kirkham B, Gibson T, Panayi G (1992) Serial estimation of interleukin 6 as a measure of systemic disease in rheumatoid arthritis. J Rheumatol 19:22–25.

17. Holt I, Cooper RG, Denton J, Meager SJ (1992) Cytokine inter-relationships and their association with disease activity in arthritis. Br J Rheumatol 31:725–733.

18. Gough AKS, Lilley J, Eyre S, Holder RL, Emery P (1994) Generalised bone loss in patients with early rheumatoid arthritis. Lancet 344:23–27.

19. Gevers G, Devos P, De Roo M, Dequeker J (1986) Increased levels of osteocalcin (serum bone Gla-protein) in rheumatoid arthritis. Br J Rheumatol 25:260–262.

20. Franck H, Ittel TH, Tash O, Herborn G, Rau R (1994) Osteocalcin in patients with rheumatoid arthritis. A one-year followup study. J Rheumatol 21:1256–1259.

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INCREASED MONOCYTE MCP-1 PRODUCTION IN ACUTE ALCOHOLIC HEPATITIS

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Monocyte chemoattractant protein-1 (MCP-1) is a potent mononuclear cell-specific chemotactic protein. MCP-1 is a candidate chemoattractant for activation and hepatic infiltration of mononuclear cells in alcoholic hepatitis (AH). Blood was collected from 15 patients with AH (mean bilirubin 17.6 ± 3.5 mg/dl; normal 0.2–1.0 mg/dl) on admission and at time points for up to 6 months. Peripheral blood monocytes were isolated and MCP-1 production assessed by measuring MCP-1 concentrations in monocyte culture supernatants after overnight (20 h) incubation. Monocytes from normal subjects did not product detectable MCP-1 unless stimulated with endotoxin (LPS;5 μg/ml). The mean level of constitutive MCP-1 from AH patient monocytes was 4694 ± 2432 pg/ml 20 h on admission. The mean MCP-1 level for LPS-treated monocytes was 4903 ± 1540 pg/ml 20 h for normal subjects and was significantly elevated in AH patients to 11589 ± 3266 pg/ml/20 h. AH patient monocyte MCP-1 production was decreased in vitro when monocytes were treated with N-acetylcysteine (5 mM) and also decreased over the 6-month study as the patients improved clinically. MCP-1 plasma levels were below the detection limits of the assay used in both AH patients and normal subjects. Thus, monocytes from AH patients not only constitutively product MCP-1, but also produce higher levels of MCP-1 with endotoxin stimulation. Further studies are needed to clarify the role of MCP-1 in the activation and hepatic infiltration of mononuclear cells in alcoholic liver disease.

Alcoholic hepatitis (AH) is characterized by hepatic leukocyte infiltration that can persist, with ongoing liver injury for months despite cessation of alcohol intake.[1,2] Polymorphonuclear leukocytes (PMN) predominate the hepatic leukocyte infiltration. However, mononuclear cells are also present, including macrophages (Kupffer cells) and lymphocytes in significant numbers. Kupffer cells are major producers of cytokines and chemokines.[3–5] Studies in animals suggest that Kupffer cells and tumour necrosis factor alpha (TNF) are important mediators of alcoholic liver injury.[6–7]

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Monocyte chemotactic peptide-1 (MCP-1) is a member of the beta (C-C) chemokine family.[8] MCP-1 is secreted by a variety of cell types in response to endotoxin (lipopolysaccharide, LPS) and cytokines such as TNF and interleukin 1 (IL-1). MCP-1 activates and chemotactically attracts monocytes/macrophages.[9–11] MCP-1 gene regulation is closely associated to the oxidative stress senstive transcription factor nuclear factor kappa B (NFκB).[12,13]

Patients with AH have endotoxaemia as well as increased plasma levels of TNF and IL-1, which are potential stimuli for MCP-1 activity.[7] It is our hypothesis that in acute AH there is increased LPS-stimulated expression of MCP-1 by circulating monocytes and, presumably, cells of the liver such as hepatic Kupffer cells. If increased, MCP-1 activity may activate and facilitate hepatic influx of monocytes/macrophages and thus contribute to liver injury. The aims of this study were: (1) to measure constitutive and LPS-stimulated MCP-1 secretion by monocytes from patients with acute AH; (2) to follow monocyte MCP-1 secretion over time by monocytes from AH patients as they improved clinically with abstinence; and (3) to determine the effect of a glutathione-enhancing

TABLE 1. Alcoholic hepatitis patients' clinical laboratory profile

Serum levels	Entry	1 Month	3 Months	6 Months	Normal
Bilirubin (mg/dl)	17.6 ± 3.5	7.8 ± 3.5	3.3 ± 1.4	2.6 ± 0.9	0.2–1.0
AST (U/L)	106.5 ± 11.2	68.9 ± 9.2	79.5 ± 44.8	43.5 ± 4.9	15–40
ALT (U/L)	38.8 ± 5.8	39.9 ± 8.5	23.8 ± 2.9	25.8 ± 3.8	10–28
Albumin (g/dl)	2.2 ± 0.2	2.5 ± 0.3	3.4 ± 0.2	3.3 ± 0.2	3.5–5.0

agent N-acetylcysteine (NAC) on monocyte MCP-1 production.

RESULTS

Fifteen consenting acute AH patients with a mean age of 41.1 ± 7.3 years (9 males and 6 females) were enrolled into the study. The mean discriminant function of Maddrey (DF) for these patients was 90.7 (range 64.4 to 125.6) indicating rather severe disease.[14,15] All patients enrolled had laboratory findings typical for AH.[16] The biochemical profile of the 15 patients is summarized in Table 1 Eight patients had moderately severe AH and seven patients had severe AH. Serum transaminases were elevated in a pattern characteristic for AH with aspartate aminotransferase (AST) levels being greater than alanine aminotransferase (ALT) levels.[16] The depressed serum albumin and significantly elevated bilirubin were also typical for AH.[16] The patients' biochemical profile improved over the 6-month study period as they improved clinically. Admission plasma was assayed for MCP-1 in 10 AH patients and normal control subjects. MCP-1 plasma levels were all below the detection limits of the assay used (<5.0 pg/ml).

AH patient monocytes had constitutive MCP-1 production as assessed by MCP-1 concentrations in monocyte culture supernatants after overnight incubation. At study entry, monocytes from each of the fifteen AH patients spontaneously released MCP-1 into the culture supernatant with a mean level of 4694 ± 2432 pg/ml/20 h. However, in no case did a normal subject's monocytes spontaneously release detectable MCP-1 into the monocyte culture supernatant. The constitutive MCP-1 production by AH patient monocytes decreased over time as the patients improved clinically and biochemically with abstinence (Fig. 1).

Monocytes from both normal control subjects and AH patients produced MCP-1 when stimulated with LPS, as assessed by MCP-1 concentrations in monocyte culture supernatants after overnight (20 h) incubation. However, the AH patient monocytes had greater LPS-stimulated MCP-1 production (11589 ± 3266 pg/ml/20 h) compared with LPS-stimulated MCP-1 production by monocytes from normal subjects, 4903 ± 1540 pg/ml/20 h ($P<0.001$). Similar to the constitutive monocyte MCP-1 production, the LPS-induced monocyte MCP-1 production decreased over time as the AH patients improved clinically and biochemically with abstinence (Fig. 2).

Monocytes from nine of the fifteen AH patients were treated in vitro with NAC at the time of study entry. In these studies, NAC (5 mM) treatment decreased the constitutive production of MCP-1 by AH patient monocytes (Fig. 3). Similarly, NAC significantly decreased LPS-stimulated MCP-1 production by monocytes from both normal control subjects and AH patients (Fig. 4). The LPS-stimulated MCP-1 concentration produced by normal subjects' monocytes decreased significantly ($P<0.01$) from 4598 ± 1127 pg/ml/20 h to 1698 ± 614 pg/ml/20 h and the LPS-stimulated MCP-1 concentration produced by AH patient monocytes decreased significantly

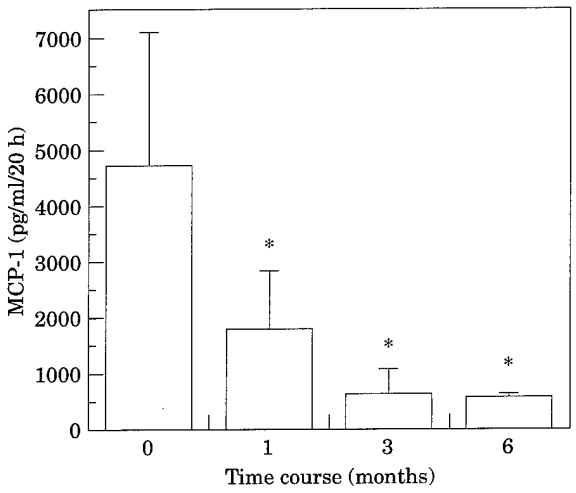

Figure 1. Monocytes from 15 patients with acute alcoholic hepatitis (AH) had constitutive MCP-1 production as assessed by MCP-1 concentrations measured in monocyte culture supernatants after overnight (20 h) incubation.

In contrast, monocytes from normal subjects did not spontaneously release detectable MCP-1 into the monocyte culture supernatant. The constitutive MCP-1 production by AH patient monocytes decreased significantly over time as the patients improved clinically and biochemically with abstinence. Values represent means ± standard deviations. *$P<0.05$ vs the time 0 value.

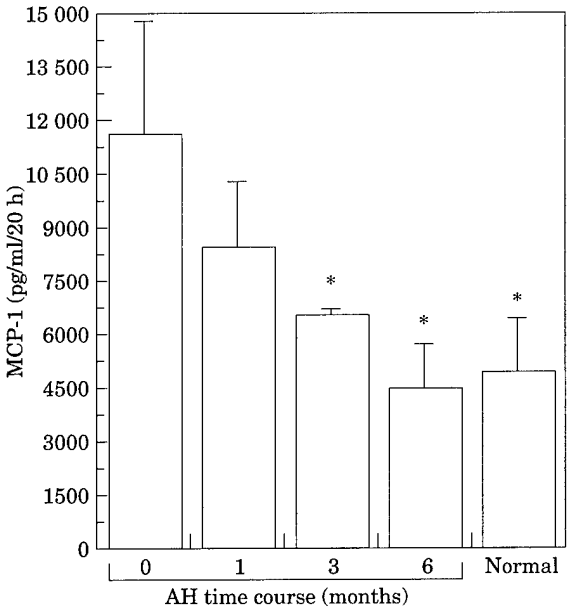

Figure 2. Monocytes from both normal control subjects (Normal) and fifteen alcoholic hepatitis (AH) patients produced MCP-1 when treated overnight (20 h) with LPS (5 μg/ml) as assessed by MCP-1 concentrations in monocyte culture supernatants.

At study entry (month 0), the 15 AH patients' monocytes had significantly greater LPS-stimulated MCP-1 production than did control monocytes. However, as the patients improved clinically and biochemically with time, the LPS stimulated monocyte MCP-1 production decreased significantly to levels that were similar to LPS production by normal subjects' monocytes. Values represent means ± standard deviations. *$P<0.05$ versus the time 0 value.

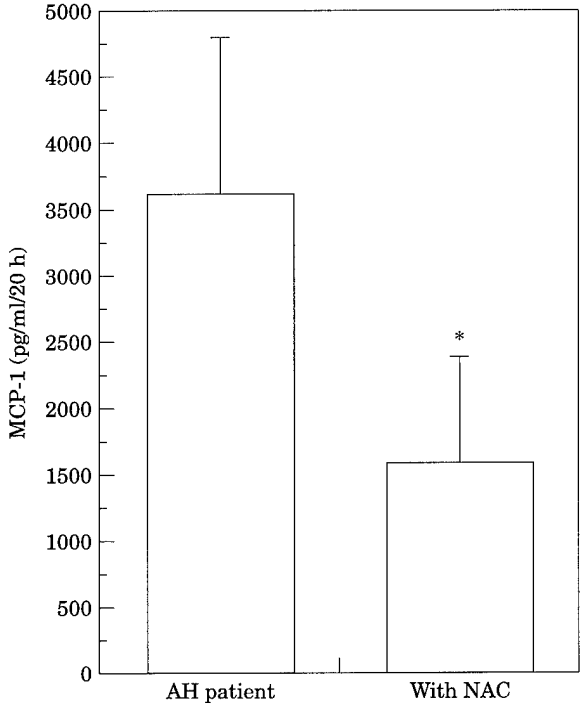

Figure 3. Treatment with N-acetylcysteine (NAC-5 mM) significantly decreased constitutive MCP-1 production as assessed by measuring MCP-1 concentrations in monocyte culture supernatants after overnight (20 h) incubation of monocyte cultures from nine acute alcoholic hepatitis (AH) patients at the time of study entry.

Values represent means ± standard deviations. *$P<0.05$.

($P<0.01$) from 11616 ± 2927 pg/ml/20 h to 6041 ± 2071 pg/ml/20 h.

DISCUSSION

AH is an inflammatory process, with many of the clinical manifestations such as fever, anorexia, leukocytosis, increased serum C reactive protein and decreased serum albumin being identical to the biological effects of cytokines.[17] These similarities prompted research into the role of cytokines in alcoholic liver disease.[18] Earlier studies had demonstrated that alcohol dependent subjects and patients with alcoholic liver disease have increased gut permeability and an increased incidence of endotoxaemia.[19,20] This suggested that LPS, derived from gut flora, may be a potential stimulus for cytokine activity in alcoholic liver disease.[7]

The initial reports of cytokine activity in alcoholic liver disease were those of IL-1 bioactivity in AH patient serum.[18] Later studies confirmed increased plasma levels and monocyte production of immunoreactive IL-1α and β.[21–23] McClain and Cohen were the first to report increased monocyte production of bioactive TNF.[17] Since their initial report, several groups have reported increased plasma levels of immunoreactive TNF and that the increased plasma TNF concentrations correlate with clinical outcome and disease severity.[21,23,24]

Endotoxin, IL-1 and TNF are all potent inducers of chemokines.[10,11] Sheron *et al.* reported dramatically increased plasma interleukin 8 (IL-8) levels in AH patients and that the degree of hepatic immunohistochemical staining for IL-8 correlated with the degree of hepatic PMN infiltration.[25] We also reported dramatically increased plasma IL-8 levels in acute AH patients and that the plasma IL-8 levels decreased over time as the patients improved clinically and biochemically while abstaining from alcohol.[26] Sheron's group later reported increased hepatic growth-regulating oncogene alpha (GROα) levels that correlated with hepatic IL-8 levels and hepatic PMN infiltration in AH patients.[27] Studies of liver samples from patients with acute fulminant and chronic liver disease including alcoholic liver disease have shown increased MCP-1 mRNA expression.[28,29] However, MCP-1 (cytokine) levels are not increased in alcoholic liver disease when determined by ELISA of liver homogenates.[27]

Animals studies have confirmed that TNF is an important mediator in several models of experimental

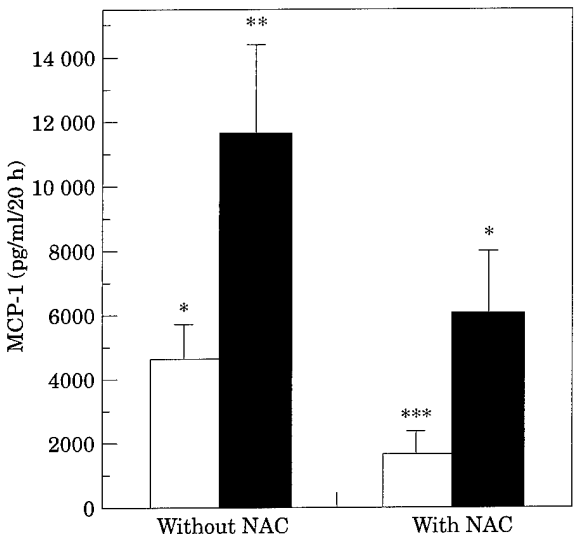

Figure 4. Treatment with N-acetylcysteine (NAC-5 mM) significantly decreased LPS (5 μg/ml) stimulated MCP-1 concentrations produced by monocytes from nine alcoholic hepatitis (AH) patients as well as normal control subjects (Control).

MCP-1 production was assessed by measuring MCP-1 concentrations in monocyte culture supernatants after overnight (20 h) LPS treatment of monocyte cultures from 9 acute alcoholic hepatitis (AH) patients at the time of study entry and 9 normal subjects. Values represent means ± standard deviations. ■, AH; □, control. * versus * was n.s.; for * versus **, * versus ***, and ** versus ***, $P<0.01$.

alcoholic liver injury.[7] When rats are chronically fed an alcohol-containing diet and then injected with LPS, plasma TNF levels increase and the animals sustain substantial liver injury.[30,31] Kupffer cells isolated from rats which have been chronically fed an alcohol-containing diet and then injected with LPS, have increased expression of TNF mRNA.[31] However, if the alcohol-fed rats are pretreated with a prostaglandin E_1 analogue, TNF production is downregulated and LPS-induced liver injury is markedly attenuated.[30] With the rat model of intragastric alcohol feeding, there is increased hepatic staining of mRNA for TNF and IL-1 as well as interleukin 6 (IL-6).[32] Further evidence that TNF can mediate liver injury comes from in vitro studies. The human HEP G2 hepatoma cell line can be sensitized using actinomycin D to direct TNF cytotoxicity.[33] Major producers of TNF in vivo are monocytes and macrophages. Thurman's group has shown that inactivation of Kupffer cells in rats using gadolinium chloride prevents early alcohol-induced liver injury.[6] Others have confirmed that endotoxin-responsive macrophages play an important role in experimental liver injury.[34] Thus, the activation and chemoattraction of monocytes/macrophages to the liver is a critical step in the pathogenesis of alcohol-induced TNF liver injury.

Acute alcohol metabolism is associated with increased oxidative stress.[35] In addition, patients with chronic alcohol abuse have decreased levels of nutritional antioxidants such as hepatic glutathione.[36] The activation of gene transcription of TNF, IL-1, IL-8 and MCP-1 are closely associated with the oxidative stress-sensitive transcription factor NFκB.[12,13,37–39] This suggests that oxidative stress-sensitive transcription factors such as NFκB may be potential sites of intervention in the treatment of inflammatory diseases, including AH, using glutathione enhancing agents such as NAC.[40]

There is not sufficient data from this study to clearly define the role of MCP-1 in alcoholic liver disease or the mechanism of increased MCP-1 production by AH patients' monocytes. However, several interesting points are raised that require further investigation. This study shows that monocytes from AH patients have constitutive MCP-1 production and increased LPS-stimulated MCP-1 production, as assessed by MCP-1 concentrations in monocyte supernatants. The increased MCP-1 production by AH patient monocytes normalized over time as the patients improved clinically and biochemically with abstinence. In addition, the increased MCP-1 production by AH patient monocytes was normalized in vitro by the addition of NAC, suggesting the possible involvement of oxidative stress-sensitive transcription factors such as NFκB. These data suggest that MCP-1 may have an important role in the pathogenesis of AH and that the increased AH patient monocyte MCP-1 production may be due to decreased nutritional free radical scavengers associated with chronic alcohol intake, such as decreased glutathione.[35,36]

Although it has been reported that both human monocytes from patients with AH and Kupffer cells isolated from rats following chronic alcohol feeding have increased TNF production, we have no data to confirm that AH patients' Kupffer cells are producers of hepatic MCP-1.[17,31,40] However, with ischaemia-reperfusion liver injury in rats, isolated Kupffer cells have increased MCP-1 production and ischaemia/oxidative stress has been implicated as a mechanism of alcoholic liver injury.[35,34] In addition, other types of liver cells such as human Ito (liver fat storing) cells and rat liver endothelial cells have been shown to produce MCP-1.[28,42] Regardless of the cell type producing MCP-1, if MCP-1 released from the liver plays a role in the chemoattraction of monocytes to the liver, we have demonstrated that plasma levels of MCP-1 are not elevated to a level that would desensitize monocytes to MCP-1 or disrupt a chemotaxis gradient of MCP-1 emulating from the liver.[43]

In addition to a potential role in the pathogenesis of liver injury, MCP-1 may play a role in the increased susceptibility to infection seen in patients with alcoholic liver disease. Not only are bacterial infections a common complication of alcoholic liver disease, but mycobacterium and fungal infections also are

increased in frequency, suggesting a defect in cell-mediated immunity.[44] Patients with alcoholic liver disease have reduced skin reactivity to common antigens, but in patients who recover from AH, the percentage of positive skin test increases.[45] Furthermore, there is a polyclonal increase in the immunoglobulins, especially IgA, in alcoholic liver disease probably due to enhanced spontaneous secretion of immunoglobulins by B-cells.[46] But the mechanisms giving rise to these changes are not well defined. Recent studies suggest that in addition to regulation of leukocyte trafficking, the C-C chemokines play a role in the development and activation of T helper cells. Naive (ThO) lymphocytes incubated with MCP-1 show Th2 lineage.[47] In addition, transgenic mice overexpressing MCP-1 are more susceptible to infection by *Listeria monocytogenes* and *Mycobacterium tuberculosis*, but in these mice the serum levels of MCP-1 were high and in our patients plasma levels of MCP-1 were not detected.[43] Thus, further studies are needed to determine if MCP-1 plays a role in the increased susceptibility to infection associated with alcoholic liver disease.

In summary, in AH there is an increased incidence of endotoxaemia and there are increased plasma levels of IL-1 and TNF which may serve as inducers of chemokines such as IL-8 and MCP-1.[40] There is increased oxidative stress associated with acute and chronic alcohol, and there is increasing evidence that the critical gene activation for these cytokines is via the oxidative stress-sensitive transcription factor NFκB.[35,40,49] We postulate that increased MCP-1 activity plays a role in the mononuclear cell activation in alcoholic liver disease. We speculate that MCP-1 is a factor in the activation, and perhaps even in the chemoattraction, of monocytes to the liver where they become major cytokine producers, playing an important role in alcoholic liver injury.[6]

MATERIALS AND METHODS

Alcoholic hepatitis patients

This research was approved by the Institutional Review Board for Human studies and all patients gave written, informed consent. Patients hospitalized who had a serum bilirubin >5.0 mg/dl and a history consistent with AH were screened for inclusion in this study. Criteria for entry into study included an ethanol consumption of at least 80 g/day for at least 1 year.[26] The patients were initially evaluated by the Gastroenterology Service to establish the aetiology of liver disease. Patients were excluded from the study if they had a history of intravenous drug abuse within the previous 2 years, another cause of liver disease, congestive heart failure, malignancy or other inflammatory disease. Following the initial screen for other types of liver disease, the diagnosis of alcoholic hepatitis was established using the criteria recommended by the International Association for the Study of the Liver.[16] Disease severity of AH was determined by the discriminant function (DF) of Maddrey.[14,15] DF was defined as 4.6 × prothrombin time (in seconds)+bilirubin (in mg/dl). A DF of 90 or more indicates severe AH and a DF of 55 to 89 indicates moderate AH. At the time of study entry and initial blood draws for cytokine studies, no patient had active alcohol withdrawal, detectable blood alcohol levels, or evidence of infection; all factors which could alter cytokine activity.[7]

Patients were hospitalized in the General Clinical Research Center (GCRC) for 28 days. After discharge, they were followed for an additional 5 months. Normal subjects included healthy and abstinent volunteer adults, who were free of known chronic or inflammatory diseases and infection, and who took no medications during the previous week. The normal subjects' complete blood counts (CBC), liver profiles, and prothrombin times (PT) were within normal limits. Blood samples were obtained and run in parallel to patient samples from the normal subjects. All blood samples were obtained between 8 and 10 am while subjects were fasting and in the University of Kentucky GCRC. The hospital clinical laboratory assayed blood samples for serum bilirubin, AST, ALT, albumin, prothrombin time and haemograms.

Monocyte culture

Sixty milliliters of heparinized (10 units/ml) blood from one AH patient and one control subject were prepared for each experiment in parallel. Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Hypaque (Sigma Chemical Company, St. Louis, MO) as previously described[17,48] and were suspended in RPMI 1640 medium (Bio Whitaker, Walkersville, MD). For MCP-1 production studies, 3.5×10^5 CD14+ (as determined by fluorescence-activated cell sorting, FACS) cells were seeded into 24-well culture plates in a total volume of 0.5 ml media and incubated at 37°C+5% CO_2 for 2 h. Non-adherent cells were removed by rinsing three times with fresh medium. Adherent monocytes were incubated in the above medium with 5% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) (GIBCO Laboratories, Grant Island NY), 2-mercaptoethanol (5×10^{-5}M) (GIBCO Laboratories). After 20 h (overnight) incubation at 37°C and 5% CO_2, additional media with lipopolysaccharide (LPS, B4:0111, Sigma Chemical Company) or vehicle phosphate buffered saline (PBS) was added (total added volume 10 μl) to the wells still containing the media from the overnight incubation, so that the final concentration of LPS was 5 μg/ml (if present). Cell-free culture supernatants were collected after 20 additional hours incubation and stored at −70°C until assayed for MCP-1. In our hands, using this system, cytokine concentrations in monocyte culture supernatants increase at the greatest rate between 4 and 8 h into the incubation. Thereafter changes are slower. Concentrations were measured at 20 h so that they were measured at a consistent timepoint as well as not being measured during a time of rapidly changing concentrations.

NAC treatment

At the time of study entry, nine of the fifteen patients also had isolated monocytes treated with NAC (N-acetyl

cysteine, Sigma Chemical Company) in the culture media. In these studies, NAC was not in the media during the 2 h monocyte-adherence period. However, NAC (5mM) was present during both the initial 20 h overnight incubation as well as the second 20 h overnight incubation with or without LPS as described above.

MCP-1 Assay

Plasma and monocyte culture supernatants were assayed by solid-phase enzyme-linked immunosorbent assay (ELISA) for secreted MCP-1 using the commercially available Quantikine Assay system (R&D systems, Minneapolis, MN). MCP-1 was measured according to the manufacturer's protocol. Samples were compared to the standard curve to determine the amount of MCP-1 present. This assay detects MCP-1 concentrations <5.0 pg/ml and its range of optimal sensitivity is 31.2–2000 pg/ml. If MCP-1 concentrations were greater than 2000 pg/ml, the samples were diluted with culture medium so that measurements were made within the range of optimal sensitivity.

Analysis

Values presented are means ± standard deviations. Statistical significance between values obtained from AH patients and normals, and AH patients at time of study entry and subsequent dates, were tested using one way analysis of variance with subsequent Newman-Keuls test, with $P<0.05$ considered to be statistically significant.

Acknowledgements

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REFERENCES

1. Paronetto F (1986) Cell mediated immunity in liver disease. Hum Pathol 17:168–178.

2. MacSween RNM (1981) Alcoholic liver disease: Morphological manifestations. Review by an International Group. Lancet 1: 707–711.

3. Shirahama M, Ishibashi Y, Tsuchiya S, Kurokawa Y, Okumura Y, Nino Y (1988) Kinetics and parameters of the induction of interleukin-1 secretion by rat Kupffer cells. J Clin Lab Immunol 27:127–132.

4. Karck U, Peters T, Decker K (1988) The release of tumor necrosis factor by endotoxin-stimulated rat Kupffer cells is regulated by prostaglandin E2 and dexamethasone. J Hepatol 7:352–361.

5. Chenuse W, Terebuh PD, Remick DG, Scales WE, Kunkel SL (1991) In vivo biologic and immunohistochemical analysis of interleukin-1α,β and tumor necrosis factor during experimental endotoxemia: kinetics. Kupffer cell expression and glucocorticoid effects. Am J Pathol 138:395–402.

6. Adachi Y, Bradford B, Gao W, Bojes H, Thurman R, Diehl AM (1994) Inactivation of Kupffer cells prevents early alcohol-induced liver injury. Hepatology 20:453–460.

7. McClain C, Hill D, Schmidt J (1993) Cytokines and alcoholic liver disease. Semin Liver Dis 13:170–182.

8. Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B (1994) Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human $CD4^+$ and $CD8^+$ lymphocytes. FASEB J 8:1055–1060.

9. Leonard EJ, Yoshimura T (1990) Human monocyte chemoattractant protein-1 (MCP-1). Immunol Today 11:97–101.

10. Kunkel SL, Standiford T, Kasahara K, Strieter RM (1991) Stimulus specific induction of monocyte chemotactic protein-1 (MCP-1) gene expression.. In Westwick J (ed.) Chemotactic Cytokines, Plenum Press, New York, pp. 65–71.

11. Brieland JK, Flory CM, Jones ML, Miller GR, Remick DG, Warren JS, Fantone JC (1995) Regulation of monocyte chemoattractant protein-1 gene expression and secretion in rat pulmonary alveolar macrophages by lipopolysaccharide, tumor necrosis factor-α, and interleukin-1β. Am J Resp Cell Mol Biol 12:104–109.

12. Ebnet K, Brown KD, Siebenlist UK, Simon MM, Shaw S (1997) *Borrelia burgdorferi* activates nuclear factor-κB and is a potent inducer of chemokine and adhesion molecule gene expression in endothelial cells and fibroblasts. J Immunol 158:3285–3292.

13. Sato M, Takashi M, Nagaya T, Murata Y, Ida N, Maeda K, Seo H (1996) Antioxidants inhibit tumor necrosis factor-α mediated stimulation of interleukin-monocyte chemoattractant protein-1, and collagenase expression in cultured human synovial cells. J Rheumatol 23:432–438.

14. Maddrey WC, Boitnott JK, Marshall S (1978) Corticosteroid therapy of alcoholic hepatitis. Gastroenterology 75:193–199.

15. Mendenhall CL, Anderson S, Garcia-Pont P (1984) Short-term and long-term survival in patients with alcoholic hepatitis treated with oxandrolone and prednisolone. N Engl J Med 311: 1464–1470.

16. Fogarty International Center Proceedings no. 22: Chapter 1: Hepatitis (571.1) Alcoholic hepatitis. In Leevy CM, Propper H, Sherlock S (eds) Diseases of the liver and biliary tract. Standardization of nomenclature, diagnostic criteria and diagnostic methodology. DHEW publications no. (NIH) 76-725 Washington, DC, 1976:6.

17. McClain CJ, Cohen DA (1989) Increased tumor necrosis factor production by monocytes in alcoholic hepatitis. Hepatology 9:349–351.

18. McClain CJ, Cohen DA, Dinarello CA, Cannon JG, Shedlofsky SI, Kaplan AM (1986) Serum interleukin-1 (IL-1) activity in alcoholic hepatitis. Life Sci 39:1479–1485.

19. Bode C, Kugler V, Bode JC (1987) Endotoxemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. J Hepatol 4:8–14.

20. Bjarnason I, Ward K, Peters TJ (1984) The leaky gut of alcoholism: possible route of entry for toxic compounds. Lancet 1:179–182.

21. Khoruts A, Stahnke L, McClain CJ, Logan G, Allen JI (1991) Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients. Hepatology 13:267–276.

22. Deviere J, Content J, Denys C, Vandenbussche P, Schandene L, Wybran J, Dupont E (1990) Excessive in vitro bacterial lipopolysaccharide-induced production of monokines in cirrhosis. Hepatology 11:628–634.

23. Felver ME, Mezey E, McGuire M, Mitchell MC, Herlong HF, Veech GA, Veech RL (1990) Plasma tumor necrosis factor α predicts decreased long-term survival in severe alcoholic hepatitis. Alcohol Clin Exp Res 14:255–259.

24. Bird GLA, Sheron N, Goka J, Alexander GJ, Williams RS (1990) Increased plasma tumor necrosis factor in severe alcoholic hepatitis. Ann Int Med 112:917–920.

25. Sheron N, Bird G, Koskinas J, Portmann B, Ceska M, Lindley I, Williams R (1993) Circulating and tissue levels of the neutrophil chemotaxin interleukin-8 are elevated in severe acute alcoholic hepatitis, and tissue levels correlate with neutrophil infiltration. Hepatology 18:41–46.

26. Hill DB, Marsano LS, McClain CJ (1993) Increased plasma interleukin-8 concentrations in alcoholic hepatitis. Hepatology 18:576–580.

27. Maltby J, Wright S, Bird G, Sheron N (1996) Chemokine levels in human liver homogenates: Associations between GRO alpha and histopathological evidence of alcoholic hepatitis. Hepatology 24:1156–1160.

28. Czaja MJ, Geerts A, Xi J, Schmiedeberg P, Ju Y (1994) Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease. J Leukoc Biol 55:120–126.

29. Brun P, Afford SC, Fear J, Burra P, Hubscher SG, Adams DH (1995) Increased expression of β chemokines in alcoholic liver disease. Hepatology 22:364a.

30. Honchel R, Ray M, Marsano L (1992) Tumor necrosis factor in alcohol enhanced endotoxin liver injury. Alcohol Clin Exp Res 16:665–669.

31. Hansen J, Cherwitz DL, Allen JI (1994) The role of tumor necrosis factor-α in acute endotoxin-induced hepatotoxicity in ethanol-fed rats. Hepatology 20:461–474.

32. Nanji AA, Zhao S, Sadradeh SM, Waxman DJ (1994) Use of reverse transcription-polymerase chain reaction to evaluate in vivo cytokine gene expression in rats fed ethanol for long periods. Hepatology 19:1483–1487.

33. Hill DB, Schmidt J, Shedlofsky SI, Cohen DA, McClain CJ (1995) In vitro tumor necrosis factor cytotoxicity in HEP G2 liver cells. Hepatology 21:1114–1119.

34. Shiratori Y, Tanaka M, Hai K, Kawase T, Shina S, Sugimoto T (1990) Role of endotoxin-responsive macrophages in hepatic injury. Hepatology 11:183–192.

35. Lieber CS (1997) Role of oxidative stress and antioxidant therapy in alcoholic and nonalcoholic liver diseases. Adv in Pharm 38:601–628.

36. Situnayake RD, Crump BJ, Thurnham DI, Davies JA, Gearty J, Davis M (1990) Lipid peroxidation and hepatic antioxidants in alcoholic liver disease. Gut 31:1311–1317.

37. Thanos D, Maniatis T (1995) NF-κB: A lesson in family values. Cell 80:529–532.

38. Mihm S, Galter D, Droge W (1995) Modulation of transcription factor NFκB activity in intracellular glutathione levels and by variations of the extra cellular cysteine supply. FASEB J 9: 246–252.

39. Tran-Thi T-A, Decker K, Baeuerle PA (1995) Differential activation of transcription factors NF-κB and AP-1 in rat liver macrophages. Hepatology 22:613–619.

40. Hill D, Shedlofsky S, McClain C, Diehl AE, Tsukamoto H (1997) Cytokines and liver disease. In Remick DG, Friedland JS (eds) Cytokines in Health and Disease. Marcel Dekker, Inc., New York, pp 401–425.

41. Yamaguchi Y, Matsumura F, Takeya M, Ichiguchi O, Kuratsu JI, Horiuchi T, Akizuki E, Matsuda T, Okabe K, Ohshiro H, Liang J, Mori K, Yamada S, Takahashi K, Ogawa M (1998) Monocyte chemoattractant protein-1 enhances expression of intercellular adhesion molecule-1 following ischemia-reperfusion of the liver in rats. Hepatology 27:727–734.

42. Marra F, Valente AJ, Pinzani M, Abboud HE (1993) Cultured human fat-storing cells produce monocyte chemotactic protein-1 regulation by proinflammatory cytokines. J Clin Invest 92:1674–1680.

43. Gu L, Rutledge B, Fiorillo J, Ernst C, Grewal I, Flavell R, Gladue R, Rollins B (1997) In vivo properties of monocyte chemoattractant protein-1. J Leuk Biol 62:577–580.

44. Darban H, Crawford GB, Watson RR (1993) Alcohol and the human immune system. In Watson RR (ed.) Alcohol, Drugs of Abuse and Immunomodulation, Pergamon Press, New York, Advances in the Biosciences, Volume 86:pp 5–9.

45. Snyder N, Bessoff J, Dwyer JM (1978) Depressed delayed cutaneous hypersensitivity in alcoholic hepatitis. Am J Dig Dis 23:353–358.

46. Berger SR, Helms RA, Bull DM (1979) Hypergammaglobulinemia: increased rates of Ig synthesis by circulating lymphoid cells. Dig Dis Sci 24:741–745.

47. Karpus WJ, Kennedy KJ (1997) MIP-1α and MCP-1 differentially regulate acute and releasing autoimmune encephalomyelitis as well as Th1/Th2 lymphocyte differentiation. J Leuk Biol 62:681–687.

48. Lukacs K, Cohen DA, Kaplan AM (1986) HLA-Dr-unrestricted accessory cell function in human neutrophils. Clin Immunol Immunopathol 38:344–349.

49. Hill DB, Awad JA (1999) Increased urinary F_2-isoprostane excretion in alcoholic liver disease. Free Radical Biology & Medicine 26:656–660.

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POLYMORPHISMS AT POSITION −308 IN THE PROMOTER REGION OF THE TNF-α AND IN THE FIRST INTRON OF THE TNF-β GENES AND SPONTANEOUS AND LIPOPOLYSACCHARIDE-INDUCED TNF-α RELEASE IN SARCOIDOSIS

Akos Somoskövi, Gernot Zissel, Ulrike Seitzer, Johannes Gerdes, Max Schlaak, Joachim Müller-Quernheim

TNF-α is a potent pro-inflammatory cytokine. Previous studies have proved that biallelic polymorphisms in the TNF-α (−308, TNFA) and TNF-β genes (intron 1, TNFB) influence TNF-α production. In sarcoidosis, a chronic granulomatous disease, as a result of an unknown in vivo activation bronchoalveolar lavage (BAL) cells release high amounts of TNF-α, spontaneously and after in vitro stimulation. Thus, sarcoidosis could serve as a model to test the in vivo effect of TNF gene polymorphisms. We determined the TNFA and TNFB polymorphisms of 44 patients with sarcoidosis and found the following allele frequencies: 0.80, 0.20, 0.38 and 0.62 for TNFA1, TNFA2, TNFB1 and TNFB2, respectively. To examine the in vivo effect of the named polymorphisms on the TNF-α production, the spontaneous and LPS-induced TNF-α release of BAL cells and peripheral blood mononuclear cells were also determined in patients with sarcoidosis. Statistical analysis did not reveal any significant difference between sarcoidosis patients with different genotypes. The results show that TNFA and TNFB polymorphisms do not determine the level of TNF-α release of mononuclear cells activated during the course of sarcoid inflammation.

Sarcoidosis is a chronic systemic granulomatous disease of unknown aetiology. Immunologic studies with inflammatory lung cells recovered by bronchoalveolar lavage (BAL) revealed that a typical feature of pulmonary sarcoidosis is an increase in the differential cell count of BAL lymphocytes and an accumulation of T-helper cells in the lung.[1] the characteristic T-helper lymphocyte/macrophage alveolitis is caused by an unknown stimulus which activates alveolar macrophages (AM) and lymphocytes, resulting in an elevated spontaneous and induced ex vivo release of immunoregulatory mediators such as tumour necrosis factor alpha (TNF-α).[2–4] TNF-α is considered to be an essential mediator in inflammation and has been shown to play a pivotal role in granuloma formation.[5] In sarcoidosis, the individual capacity of the patient to release TNF-α from AM is a phenomenon linked with progressive disease, implying that this cytokine plays a role in the pathogenesis of sarcoidosis.[2,6]

The TNF gene is located within the major histocompatibility complex (MHC) between the complement cluster region and the HLA-B gene. Since the description of biallelic polymorphisms in the TNF-α (−308, TNFA) and TNF-β (intron 1, TNFB) genes, the influence of these polymorphisms on the genetic predisposition for TNF-α production has been studied intensively.[7,8] Several authors have noted that stimulated peripheral blood mononuclear cells (PBMC) of carriers of the TNFA2 or TNFB2 haplotype show a significantly higher release of TNF-α than normal controls.[9–11]

In contrast to these findings, other studies using similar methods but also measuring plasma levels, have reported that these biallelic polymorphisms had no significant effect on TNF-α production.[8,12,13]

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Investigations performed at the molecular level using reporter gene assays describe similarly disparate results, several studies showing higher transcriptional activity of the TNF-α promoter for the TNFA2 allele and others showing no effect of this polymorphism.[12,14–16]

The −308 TNFA2 biallelic promoter polymorphism and the TNF-β intron 1 polymorphism have also been correlated with susceptibility to severe infectious (cerebral malaria, mucocutaneous leishmaniasis, severe sepsis, leprosy) and autoimmune (diabetes mellitus, inflammatory bowel diseases, celiac disease, lupus erythematosus, Löfgren syndrome) diseases, thus raising substantial speculation that genetic predisposition for TNF-α production may affect the elimination of an infectious disease or the predisposition for an autoimmune disease.[10,11,13,17–22]

To investigate whether the TNFA or TNFB polymorphisms could be linked to exaggerated TNF-α production in vivo, we analysed the spontaneous and induced TNF-α release of sarcoid mononuclear cells from BAL and peripheral blood in relation to the TNFA and TNFB genotypes.

RESULTS

Genotype analysis results of patients with sarcoidosis

The genotype analysis of 44 patients with sarcoidosis revealed that 26 (0.60) patients were homozygous for the allele TNFA1, 16 (0.37) were heterozygous and 1 (0.03) was homozygous for the allele TNFA2 (frequency of genotypes in parentheses). Concerning the TNFB gene, 6 (0.14) patients were homozygous for the allele TNFB1, 21 (0.48) were heterozygous and 17 (0.38) were homozygous for the allele TNFB2. The allele frequencies were 0.79, 0.21, 0.38 and 0.62 for TNFA1, TNFA2, TNFB1 and TNFB2, respectively. These allele frequencies do not differ from those of healthy controls.[22] As can be expected from the known gene frequencies of the investigated polymorphisms, only one patient with sarcoidosis was found to be homozygous for the allele TNFA2, which did not allow an exact statistical comparison of this genotype.

Differential cell count results of patients with sarcoidosis and controls in BAL fluid

The AM and lymphocyte differential cell counts of BAL were determined in all patients and 11 controls. In comparison with controls, patients with sarcoidosis exhibited a significant increase in BAL lymphocytes [3.3 (0–22) vs 14.0 (1.9–77); $P<0.01$] and consequently a significant decrease in AM [96.0 (78.0–99.0) vs 84.9 (20.3–98.0); $P<0.01$]. Statistical analysis did not show any significant difference in AM and lymphocyte differential cell counts between sarcoidosis patient genotypes (Table 1).

Spontaneous and LPS-induced TNF-α release of BAL cells in patients with sarcoidosis and controls

Spontaneous TNF-α release of BAL cells and PBMC were measured both in patients with sarcoidosis and in controls in order to demonstrate the in vivo activation of sarcoid BAL cells and PBMC which results in an elevated ex vivo TNF-α release in patients with sarcoidosis, compared with controls. The spontaneous TNF-α release of BAL cells was significantly higher [1297 (0–31300) pg/ml; $P<0.01$] in patients with sarcoidosis compared with controls [246 (0–959) pg/ml; Table 1]. Comparison of spontaneous TNF-α release in BAL between sarcoidosis patients with different genotypes did not reveal any statistical difference. To demonstrate the exaggerated spontaneous TNF-α release of patients with sarcoidosis, the results of a control group are given for comparison (Fig. 1).

The LPS-induced TNF-α release of sarcoid BAL cells [23 070 (1857–129 000) pg/ml] increased compared with controls, but this difference was not statistically significant [12 692 (1230–50 880) pg/ml; Table 1]. Again, the statistical test did not yield significant differences between sarcoidosis patients with different genotypes.

Spontaneous and LPS-induced TNF-α release of PBMC in patients with sarcoidosis and controls

Similar results were found for TNF-α production of PBMC. The spontaneous TNF-α release of PBMC was significantly higher [541 (0–10 100) pg/ml; $P<0.05$] in patients with sarcoidosis compared with controls [0 (0–1054) pg/ml; Table 1]. However, this difference was due to disproportionately high values of only two patients. There was no significant difference in TNF-α release between sarcoidosis patients with different genotypes. The LPS-stimulated TNF-α release of PBMC was also significantly higher [5720 (1783–15 345) pg/ml; $P<0.05$] in patients with sarcoidosis than in controls [1843 (0–11 219) pg/ml; Table 1], which was due to exaggerated values of two patients. Comparison of genotypes of patients with sarcoidosis did not yield any difference in TNF-α release.

Spontaneous and LPS-induced TNF-α release of sarcoid BAL cells and PBMC in relation to TNFA and TNFB haplotypes

TNFA2 (TNFA 1/2 and 2/2) and TNFB2 (TNFB 1/2 and 2/2) allele carrier and non-carrier (TNFA 1/1; TNFB 1/1) patients with sarcoidosis were also compared for TNF-α production and BAL differential

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TABLE 1. Differential cell count and spontaneous and LPS-induced TNF-α release in bronchoalveolar lavage (BAL) and peripheral blood mononuclear cells (PBMC) in sarcoidosis patients with different TNFA and TNFB genotypes and controls. All data are expressed as median and range. Data are results of Mann–Whitney U test for differences in LPS-induced TNF-α release of BAL cells

	BAL				PBMC	
Genotype	AM cell differential (%)	LY cell differential (%)	Spontaneous TNF-α release (pg/ml)	LPS induced TNF-α release (pg/ml)	Spontaneous TNF-α release (pg/ml)	LPS induced TNF-α release (pg/ml)
TNFA 1/1†	82.1	17.0	1532	16 306	660	6925
$n=26$	(20.3–96.8)	(1.9–77.0)	(22–31 300)	(1857–129 000)	(0–10 100)	(1855–15 345)
	$n=26$	$n=26$	$n=25$	$n=25$	$n=20$	$n=19$
TNFA 1/2*	84.9	13.7	1119	29 422	351	3792
$n=16$	(45.8–97.4)	(1.9–53.0)	(0–13 387)	(5595–59 350)	(0–2336)	(1783–14 130)
	$n=16$	$n=16$	$n=15$	$n=14$	$n=10$	$n=10$
TNFA 2/2*	92.3	3.3	1977	64 238	425	7778
$n=1$	$n=1$	$n=1$	$n=1$	$n=1$	$n=1$	$n=1$
TNFB 1/1	85.6	14.0	1201	42 679	958	6040
$n=6$	(47.1–92.3)	(3.3–52.3)	(786–4326)	(11 480–64 238)	(425–1759)	(2678–7778)
	$n=6$	$n=6$	$n=6$	$n=6$	$n=4$	$n=4$
TNFB 1/2	85.0	12.5	1184	27 060	482	3950
$n=21$	(45.8–98.0)	(1.9–53.0)	(0–23 411)	(2005–42 649)	(0–9435)	(1783–14 600)
	$n=21$	$n=21$	$n=19$	$n=18$	$n=16$	$n=15$
TNFB 2/2	81.0	17.7	2471	16 306	541	7322
$n=17$	(20.3–96.3)	(1.9–77.0)	(22–31 300)	(1857–129 000)	(0–10 100)	(1855–15 345)
	$n=17$	$n=17$	$n=17$	$n=17$	$n=12$	$n=12$
Sarcoidosis	84.9	14.0	1297	23 070	541	5720
patients	(20.3–98.0)	(1.9–77.0)	(0–31 300)	(1857–129 000)	(0–10 100)	(1783–15 345)
$n=44$	$n=44$	$n=44$	$n=42$	$n=41$	$n=32$	$n=31$
Controls	96.0	3.3	246	12 692	0	1843
$n=11$	(78.0–99.0)	(0–22.0)	(0–959)	(1230–50 880)	(0–1054)	(0–11 219)
	$n=11$	$n=11$	$n=11$	$n=11$	$n=11$	$n=11$

*TNFA2 (TNFA 1/2 and 2/2) allele carrier vs †TNFA2 (TNFA 1/1) allele non-carrier patients with sarcoidosis, $P=0.057$; after excluding the only patient with TNFA 2/2 genotype, $P=0.0936$.

cell counts. The statistical analysis did not exhibit significant differences between sarcoidosis patients with different haplotypes in TNF-α release or BAL differential cell counts. However the LPS-induced TNF-α release of BAL cells of TNFA2 allele carriers [30 337 (5595–64 238) pg/ml] was close to being significantly higher than that of TNFA2 allele non-carriers [16 306 (1857–129 000) pg/ml; $P=0.057$]. By excluding the one patient with the TNFA 2/2 genotype from the analysis, the difference became much smaller [29 422 (5595–59 350) vs 16 306 (1857–12 9000) pg/ml; $P=0.0936$].

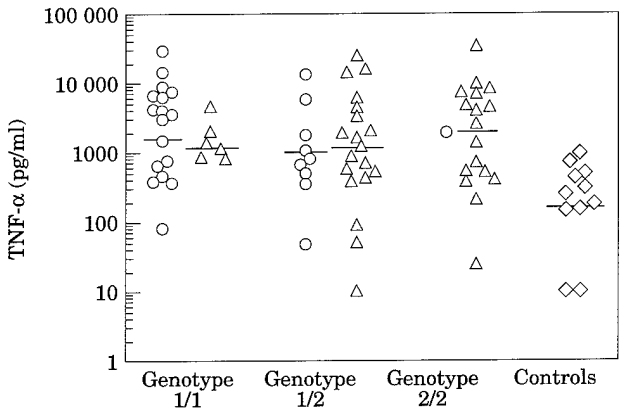

Figure 1. Spontaneous TNF-α release in bronchoalveolar lavage with different TNFA and TNFB genotypes of patients with sarcoidosis and controls.

The medians are indicated by horizontal bars. (○) sarcoidosis patients with TNFA genotype; (△) sarcoidosis patients with TNFB genotype; (◇) controls.

Discussion

In accordance with the findings of Ishihara *et al.*, we could not find any association of TNFA2 or TNFB2 with sarcoidosis.[26] It is noteworthy that in a subgroup of patients with sarcoidosis, Löfgren syndrome, such an association with TNFA2 was identified.[22] TNF-α is a potent proinflammatory cytokine and it has been shown that in sarcoidosis, as a result of an unknown in vivo activation, BAL cells release high amounts of TNF-α, spontaneously and after in vitro stimulation.[2–4,27] The results of the present study also confirm this observation. Although sarcoidosis is a systemic disease, exaggerated cytokine release is restricted to BAL cells and the corresponding cells of the peripheral blood are quiescent.[1,2,28] However, in some patients an activation of PBMC can be

observed, which results in the increase of spontaneous and LPS-induced TNF-α release observed in this study.[2] The exaggerated spontaneous and LPS-induced TNF-α release of sarcoid BAL cells is thought to be the consequence of in-situ activating processes by an unknown agent causing the disease.[1,2] This concept is supported by the observation that patients with increased TNF-α release are prone to recalcitrant or progressive disease.[6] Thus, sarcoidosis could serve as a model to test the in vivo effect of TNF gene polymorphisms.

The expression of TNF-α is regulated at both the transcriptional and post-transcriptional level, and the differences in TNF-α release may be caused by differences in promoter activity.[14,15,29] Several transfection reporter gene studies have proved that individuals with the TNFA2 or TNFB2 allele of the TNF gene have a higher ex vivo TNF-α expression upon stimulation.[9–11,14]

However, the actual in vivo biological effects of these polymorphisms are supported by only a limited number of clinical studies. McGuire *et al.* found that patients with cerebral malaria had higher TNF-α levels and were more likely to carry the TNFA2 allele, while Stüber *et al.* reported that the TNFB polymorphism is a genetic marker for patients with high circulating TNF-α response and poor prognosis in severe sepsis.[13,17]

Considering these results, we hypothesized that TNFA and/or TNFB polymorphisms may influence the high spontaneous and induced TNF-α release of sarcoid BAL cells. The absence of a correlation between the genotypes of the TNFA and TNFB polymorphisms and the amount of TNF-α revealed, indicates that in sarcoidosis the phenotype of BAL cell activation is independent of the discussed polymorphisms. Although the comparison of TNFA2 allele carrier and non-carrier patients with sarcoidosis for LPS-induced TNF-α release in BAL revealed a statistical difference close to being significant ($P=0.057$), after the exclusion of the only patient with the TNFA 2/2 genotype this difference became rather small ($P=0.0936$), casting doubt on its biological relevance. As expected from the gene frequencies, only one patient was homozygous for allele TNFA2, which precludes a statistical analysis. To the best of our knowledge this is the first report that has examined the in vivo effect of TNFA and TNFB polymorphisms on the TNF-α production of mononuclear cells activated by a disease process in vivo.

The TNF gene resides in the MHC class III region. Previous genetic studies have demonstrated that, in immune-related diseases, the increased frequency of the TNF2 allele is dependent on its association with HLA-DR17.[30,31] In Scandinavian patients with sarcoidosis, HLA-DR17 conferred a good prognosis, thus it is likely that TNFA2 in patients with Löfgren syndrome may constitute a passive component or is a component of a multifactorial mechanism with further unknown regulatory genes.[32]

Our negative observation also casts doubt on the hypothesis that the in vivo TNF-α release is generally determined by the TNFA or TNFB polymorphisms. However, before rejecting the hypothesis outright, further studies in other diseases are warranted.

In conclusion, the possibility that TNFA or TNFB polymorphisms could determine a susceptibility for sarcoidosis or play a general regulatory role in the production of TNF-α are not supported by our findings. The reported correlation between the clinical course of sarcoidosis and spontaneous TNF-α release of AM may be explained by polymorphisms in other regions of the TNF gene or by changes induced by the disease process.[2,27,33]

MATERIALS AND METHODS

Patients

The study population consisted of 44 patients with pulmonary sarcoidosis and 11 individuals who underwent bronchoscopy and were retrospectively free of any inflammatory or malignant lung disease. Sarcoidosis was diagnosed using previously defined criteria, including transbronchial biopsy.[23]

DNA extraction and typing of TNF-α and TNF-β genes

Genomic DNA was isolated by standard phenol-chloroform extraction procedures from peripheral blood taken for diagnostic purposes and surplus to requirements. Analysis of 44 unrelated patients with sarcoidosis was performed.

For PCR amplification of the TNFA and TNFB regions, 100 ng of genomic DNA was added to 100 µl of reaction mixture containing 200 ng of each primer (TNFA: 5′-AGGCAATAGGTTTTGAGGGCCAT-3′, 5′-TCCTCC CTGCTCCGATTCCG-3′; TNFB: 5′-CCGTGCTTCGTG CTTTGGACTA-3′, 5′-AGAGGGGTGGATGCTTGGG TTC-3′), 200 mM of each dNTP, 5 U Taq-Polymerase (Perkin-Elmer) in PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.1% gelatin). Cycle conditions for TNFA were as follows: 38 cycles of 1 min at 94°C, 1 min at 60°C and 1.5 min (+2s per cycle) at 72°C. The conditions for TNFB were similar, but used an annealing temperature of 70°C. Products of 107 and 782 bp were generated for TNFA and TNFB, respectively. The amplified PCR products were digested with *Ncol* and analysed on an agarose gel stained with ethidium bromide. The restriction digests of the TNFA PCR product generated fragments of 87 and 20 bp (TNFA1) and 107 bp (TNFA2). The TNFB PCR product generated fragments of 586 and 196 bp (TNFB1) and 782 bp (TNFB2).[7,13]

BAL and preparation of lung and blood mononuclear cells

BAL was performed as previously described.[24] Briefly, between 200 and 300 ml sterile saline (0.9% NaCl) was instilled into a lingula or middle-lobe segment in 25-ml aliquots. Each aliquot was immediately aspirated and the cells were washed in phosphate buffered saline. Differential cell counts were determined by counting a minimum of 200 cells in a cytocentrifuge preparation (Cytospin II; Shandon Instruments, Sewickley, PA).

PBMC were obtained from venous blood through density-gradient centrifugation as described elsewhere.[25]

Cell culture

Washed BAL cells and PBMC were cultured at a density of 1×10^6 cells/ml over a period of 24 h either without additives or with 1 μg/ml LPS (*Salmonella abortus equi*) in endotoxin-free RPMI 1640 (Seromed, Berlin, Germany) supplemented with 2% heat inactivated human serum, 20 mM HEPES (Gibco, Paisley, Scotland), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg streptomycin in 24-well tissue-culture plates in a 5% CO_2 humidified atmosphere at 37°C. At the end of the culture period the supernatants were harvested and stored at −70°C until assayed for TNF-α. The viability of the cells after culture always exceeded 95% as determined by Trypan blue exclusion.

TNF-α assay

The concentrations of TNF-α released by BAL cells and PBMC either spontaneously or after ex vivo stimulation were measured with a previously described ELISA.[2] Analysis of 44 patients with sarcoidosis, and 11 controls was performed.

Statistical analysis

Data on differential cell counts and TNF-α are expressed as median and range. Differences in differential cell counts and TNF-α concentration between patients with sarcoidosis and the control group and among sarcoidosis patients with different genotypes were evaluated with the Mann-Whitney U test. Values of P of less than 0.05 were considered significant.

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References

1. Zissel G, Müller-Quernheim J (1998) Sarcoidosis: historical perspective and immunopathogenesis (Part I). Resp Med 92: 126–140.

2. Müller-Quernheim J, Pfeifer S, Männel D, Strausz J, Ferlinz R (1992) Lung restricted activation of the alveolar macrophage/monocyte system in pulmonary sarcoidosis. Am Rev Respir Dis 145:187–192.

3. Bachwich PR, Lynch JP, Larrick J, Spengler M, Kunkel S (1986) Tumor necrosis factor production by human sarcoid alveolar macrophages. Am J Pathol 125:421–425.

4. Baughman RP, Strohofer SA, Buchsbaum J, Lower EE (1990) Release of tumor necrosis factor by alveolar macrophages of patients with sarcoidosis. J Lab Clin Med 115:36–42.

5. Kindler V, Sappino A-P, Grau GE, Piguet P-F, Vassali P (1989) The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell 56:731–740.

6. Ziegenhagen M, Benner U, Zissel G, Zabel P, Schlaak M, Müller-Quernheim J (1997) Sarcoidosis: TNFα release from alveolar macrophages and serum level of sIL-2R are prognostic markers. Am J Crit Care Med 156:1586–1592.

7. Wilson A, di Giovine FS, Blakemore AIF, Duff GW (1992) Single base polymorphism in the human tumour necrosis factor alpha (TNFα gene detectable by *Nco*I restriction of PCR product. Hum Mol Gen 1:353.

8. Messer G, Spengler U, Jung MC, Honold G, Blömer K, Pape GR, Riethmüller G, Weiss EH (1991) Polymorphic structure of the tumor necrosis factor (TNF) locus: an *Nco*I polymorphism in the first intron of the human TNF-β gene correlates with a variant amino acid in position 26 and reduced level of TNF-β production. J Exp Med 173:209–219.

9. Pociot F, Briant L, Jongeneel CV, Mölvig J, Worsaae H, Abbal M, Thomsen M, Nerup J, Cambon-Thomsen A (1993) Association of tumour necrosis factor (TNF) and class II major histocompatibility complex alleles with the secretion of TNF-α and TNF-β by human mononuclear cells: a possible link to insulin-dependent diabetes mellitus. Eur J Immunol 23:224–231.

10. Bouma G, Crusius JBA, Oudkerk Pool M, Kolkman JJ, von Blomberg BME, Kostense PJ, Giphart MJ, Schreuder GMTH, Meuwissen SGM, Peña AS (1996) Secretion of tumour necrosis factor α and lymphotoxin α in relation to polymorphism in the TNF genes and HLA-DR alleles. Relevance for Inflammatory Bowel Disease. Scand J Immunol 43:456–463.

11. Pociot F, Mölvig J, Wogensen L, Worsaae H, Dalböge H, Baek L, Nerup J (1991) A tumour necrosis factor beta gene polymorphism in relation to monokine secretion and insulin-dependent diabetes mellitus. Scand J Immunol 33:37–49.

12. Stüber F, Udalova IA, Book M, Drutskaya LN, Kuprash DV, Turetskaya RL, Schade FU, Nedospasov SA (1996) −308 tumor necrosis factor (TNF) polymorphism is not associated with survival in severe sepsis and is unrelated to lipopolysaccharide inducibility of the human TNF promoter. J Inflamm 46:42–50.

13. Stüber F, Petersen M, Bokelmann F, Schade FU (1996) A genomic polymorphism within the TNF locus influences TNF-alpha plasma levels and outcome of patients with severe sepsis. Crit Care Med 24:381–384.

14. Kroeger K, Carville K, Abraham L (1997) The −308 tumor necrosis factor-α promoter polymorphism effects transcription. Mol Immunol 34:391–399.

15. Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW (1997) Effects of a polymorphism in the human tumor necrosis factor alpha promoter transcriptional activation. Procl Natl Acad Sci USA 94:3195–3199.

16. Brinkmann BM, Zuijdgeest D, Kaijzel EL, Breedveld FC, Verweij CL (1996) Relevance of the tumor necrosis factor alpha (TNFα) −308 promoter polymorphism in TNFα gene regulation. J Inflamm 46:32–41.

17. McGuire W, Hill AVS, Allsop CEM, Greenwood BM, Kwiatkowski D (1994) Variation in the TNFα promoter region associated with susceptibility to cerebral malaria. Nature 371: 508–511.

18. Cabrera M, Shaw M-A, Sharples C, Williams H, Castes M, Convit J, Blackwell JM (1995) Polymorphism in tumor necrosis factor genes associated with mucocutaneous leishmaniasis. J Exp Med 182:1259–1264.

19. Roy S, McGuire W, Mascie-Taylor CGN, Saha B, Hazra SK, Hill AVS, Kwiatkowski D (1997) Tumor necrosis factor promoter polymorphism and susceptibility to lepromatous leprosy. J Infect Dis 176:530–532.

20. Manus R, Wilson AG, Mansfield J, Weir D, Duff G, Kelleher D (1996) TNF2, a polymorphism of the tumour necrosis-α

gene promoter, is a component of the celiac disease major histocompatibility complex haplotype. Eur J Immunol 26:2113–2118.

21. Wilson AG, Gordon C, di Giovine FS, de Vries N, van de Putte B, Emery PP, Duff GW (1994) A genetic association between systemic lupus erythematosus and tumor necrosis factor alpha. Eur J Immunol 24:191–195.

22. Seitzer U, Swider C, Stüber F, Suchnicki K, Lange A, Richter E, Zabel P, Müller-Quernheim J, Flad HD, Gerdes J (1997) Tumour necrosis factor alpha promoter gene polymorphism in sarcoidosis. Cytokine 9:787–790.

23. Müller-Quernheim J (1998) Sarcoidosis: clinical manifestations, staging and therapy (Part II). Resp Med 92:140–150.

24. Hunninghake GW, Crystal RG (1981) Pulmonary sarcoidosis: a disorder mediated by excess helper T-lymphocyte activity at sites of disease activity. N Engl J Med 305:429–434.

25. Bøyum A (1983) Isolation of human blood monocytes with Nycodenz, a new non-ionic iodinated gradient medium. Scand J Immunol 17:429–436.

26. Ishihara M, Ohno S, Ishida T, Mizuki N, Ando H, Naruse T, Ishihara H, Inoko H (1995) Genetic polymorphisms of the TNFB and HSP70 genes located in the human major histocompatibility complex in sarcoidosis. Tissue Antigens 46:59–62.

27. Müller-Quernheim J, Pfeifer S, Strausz J, Ferlinz R (1991) Correlation of clinical and immunologic parameters of the inflammatory activity of pulmonary sarcoidosis. Am Rev Respir Dis 144:1322–1329.

28. Müller-Quernheim J, Saltini C, Sondemeyer P, Crystal RG (1986) Compartmentalized activation of the interleukin-2 gene by lung T-lymphocytes in active sarcoidosis. J Immunol 137:3475–3483.

29. Sariban E, Imamura K, Luebbers R, Kufe D (1988) Transcriptional and posttranscriptional regulation of tumor necrosis factor gene expression in human monocytes. J Clin Invest 81: 1506–1510.

30. Cox A, Gonzales AM, Wilson AG, Wilson RM, Ward JD, Artlett CM, Welsh K, Duff GW (1994) Comparative analysis of the genetic associations of HLA-DR3 and tumour necrosis factor alpha with human IDDM. Diabetologia 37:500–503.

31. Wilson AG, de Vries N, Pociot F, di Giovine FS, van der Plutte LB, Duff GW (1993) An allelic polymorphism within the human tumor necrosis factor alpha promoter region is strongly associated with HLA A1, B8, and DR3 alleles. J Exp Med 177: 557–560.

32. Berlin M, Fogdell-Hahn A, Olerup O, Eklund A, Grunewald J (1997) HLA-DR predicts the prognosis in Scandinavian patients with pulmonary sarcoidosis. Am J Respir Crit Care Med 156:1601–1605.

33. Mullighan Ch, Fannin G, Chapel H, Welsh K (1997) TNF and lymphotoxin-α polymorphisms associated with common variable immunodeficiency: Role in the pathogenesis of granulomatous disease. J Immunol 159:6236–6241.

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IL-15 IS ELEVATED IN THE PATIENTS OF POSTOPERATIVE ENTEROCOLITIS

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Serum interleukin 15 (IL-15) levels were measured in 77 patients who were consecutively admitted to our intensive care unit. Postoperative enterocolitis occurred in four patients and Methicillin-resistant *Staphylococcus aureus* (MRSA), but not *Clostridium defficile*, was identified in the faecal specimens from these patients. The IL-15 levels in the patients with MRSA enterocolitis were significantly elevated compared with those of other MRSA infections without enterocolitis including pneumonia ($n=6$) and cholangitis ($n=1$), and other MRSA non-colonized patients ($n=66$) (21.2 ± 5.2 pg/ml vs 4.3 ± 0.2, 4.3 ± 0.5). Notably, an increase in serum IL-15 was observed just before clinical manifestation of severe diarrhoea. Our findings suggest that IL-15 may be associated in the pathogenesis of postoperative enterocolitis and its serum level may be a severity indicator of the disease.

Nosocomial infection is a major source of deteriorating morbidity and mortality in surgical patients. Postoperative diarrhoea is an infrequent complication. However, its prognosis is very poor once it occurs. Although *Clostridium difficile* is the most common organism of postoperative colitis, Methicillin-resistant *Staphylococcus aureus* (MRSA) enterocolitis sometimes occurs in postoperative patients after major surgery. MRSA enteritis is characterized by high fever, abdominal distention, and diarrhoea leading to severe dehydration, a decrease in peripheral blood leukocytes and shock.[1] *S. aureus* is a normal inhabitant of the gastrointestinal tract and can be found in normal bowel flora in 10% of healthy people.[1] The increase in pH in gastric juice caused by gastrectomy, administration of a histamine H_2 receptor antagonist or indwelling of naso-gastric tube is thought to be one of the aetiologic factors causing MRSA enteritis.[2,3] Furthermore, the postoperative utilization of antibiotics and paralysis of the intestine are additional risk factors in the development of MRSA enteritis. The bacteria being transmitted and resting in the stomach are not killed due to the low acidity of the gastric juice, and they move downward and grow in the lower digestive tract, resulting in the development of MRSA enteritis.[4]

A novel cytokine, IL-15, has been found to share many immunological activities with IL-2 in spite of the lack of sequence homology between these two cytokines.[5,6] IL-15 can activate T cells, B cells and NK cells through utilizing components of IL-2Rβ and γ chains and IL-15Rα for signal transduction.[7–11] In contrast to IL-2, which is exclusively produced by activated T cells, IL-15 mRNA is expressed in a variety of cell types such as placenta, skeletal muscle, kidney and activated macrophages.[6,12,13] However, it has been difficult to demonstrate IL-15 protein in the supernatant of these cells expressing IL-15 mRNA.[14] Post-transcriptional and post-translational mechanisms are present for controlling IL-15 production.[15,16] Nevertheless, this cytokine is thought to be involved in the pathogenesis of chronic inflammatory disorders with aberrant immune responses.[17] Recently, it has been reported that human intestinal epithelial cells express IL-15 mRNA[18] and that the number of IL-15-producing cells in peripheral blood is increased in patients with inflammatory bowel disease.[19]. We have also detected an increase in IL-15 in the culture supernatant of rectum biopsy samples in patients with

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TABLE 1. Summary of the Patients of MRSA Infections

Case	Age	Sex	Operative method	Site of infection	Onset	Outcome
1	71	M	Low anterior colorectal resection	Stool	Day 4	Alive
2	66	M	Hepatic right trisegmentectomy	Stool	Day 6	Dead (161 days)
3	76	M	Right hepatectomy+PD	Duodenal juice	Day 4	Alive
4	67	M	Peritoneal lavage, colostomy	Stool	Day 6	Alive
5	60	F	Patch closure of ventricular septum	Stool, sputa, decubitus	Day 10	Alive
6	63	M	PD	Nasal mucus	Day 4	Alive
7	62	M	Hepatic median lobectomy	Nasal mucus	Day 1	Alive
8	58	M	oesophagectomy	Sputa	Day 6	Alive
9	44	M	—	Bile	—	Alive
10	80	M	—	Sputa	—	Alive
11	79	M	—	Sputa	—	Alive

PD, pancreatoduodenectomy

inflammatory bowel diseases.[20] Taken together, it would thus appear that IL-15 might be associated with inflammatory bowel disease with immunological activation.

In the present study, to determine whether IL-15 is involved in the pathogenesis of postoperative enterocolitis, we examined the serum level of IL-15 in patients with MRSA enterocolitis, and compared it with that of patients with other MRSA infections.

RESULTS

We consecutively measured serum levels of IL-15 in eleven patients (10 men and 1 woman; mean age, 66.0 years old; range, 44–80) with MRSA infection. The patients' backgrounds are summarized in Table 1. Cases 1 to 4 were diagnosed as having enterocolitis with severe diarrhoea by MRSA. While *C. difficile* toxin was not examined, *C. difficile* was not detected in the culture of their faeces. Case 5–8, 10, and 11 suffered from MRSA pneumonia, and case 9 was MRSA cholangitis. In these cases, except case 5, MRSA could not be detected in their faeces. In case 5, MRSA was identified in the culture of the faeces and decubitus, but she did not suffer enterocolitis or diarrhoea. All chromosomal DNA types of cultured MRSA produce coagulase II, enterotoxin C and TSST-1.

The serum levels of IL-15 were compared between the patients with enteritis and those without enteritis on the days just before and after detection of MRSA. As shown in Figure 1, IL-15 was significantly elevated in the patients with MRSA enteritis (range 9.5–33.0 pg/ml), especially just before MRSA was identified in patient samples. Diarrhoea followed the elevation of IL-15 in all four patients. On the other hand, all of the IL-15 levels in the patients with other types of MRSA infections were less than 7 pg/ml at any stage, including the days just before and after MRSA detection. In Case 5, with MRSA in the faeces and who had no symptoms of enteritis, IL-15 levels remained at lower than 5 pg/ml. Also patients with other diseases showed a trivial level of IL-15. The changes in IL-15 of eleven patients with MRSA infections are shown in Figure 2.

Further detailed analyses on postoperative enteritis are demonstrated for each case in Figure 3.

Case 1

Low anterior colorectal resection was performed for cancer of the sigmoid colon. IL-15 was markedly elevated on postoperative day 3, and remained high until postoperative day 7. On the other hand, IL-10 was increased only on postoperative day 0 (Fig. 3A). On postoperative day 4, the patient had frequent watery diarrhoea and developed shock. MRSA was detected in the culture of this defecation. On the next day, respiratory failure from pneumonia and renal failure from hypovolaemic shock appeared. The

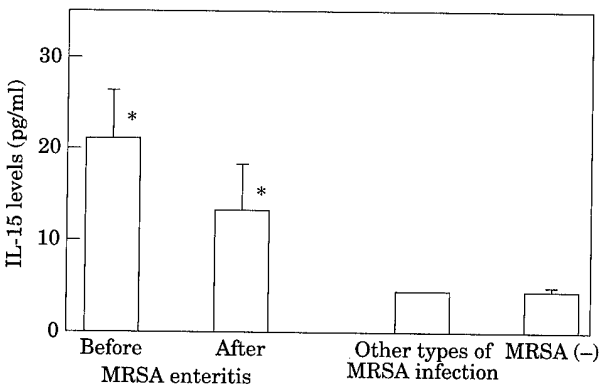

Figure 1. Serum IL-15 levels of in the patients of MRSA infections.

Serum IL-15 levels were significantly elevated in the patients with MRSA enteritis (n=4) (range 9.5–22.0 pg/ml), especially just before MRSA detection in patient samples. The elevation of IL-15 was followed by diarrhoea in all four patients. On the other hand, IL-15 levels in the patients with other types of MRSA infections (n=6) were less than 7 pg/ml at any stage, including days just before and after MRSA detection. Patients without MRSA infections (MRSA(−))(n=66) showed low levels of IL-15. Values were expressed by mean ± SE *P<0.01 vs other types of MRSA infections and MRSA (−).

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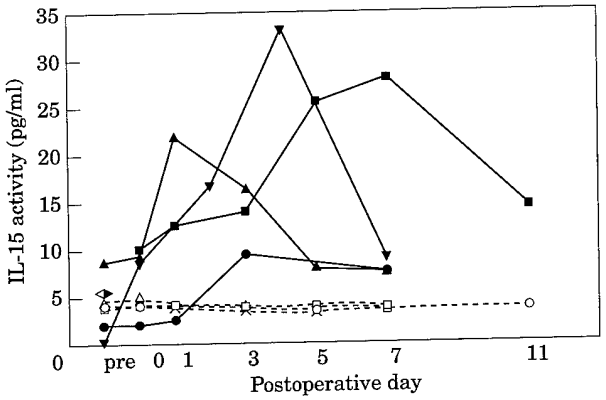

Figure 2. Changes in serum IL-15 levels in the patients of MRSA infections.

Cases 1–4 suffered from MRSA enteritis and cases 5–11 had other types of MRSA infections. (●), case 1; (■), case 2; (▲), case 3; (▼), case 4; (○), case 5; (□), case 6; (7; (), case 8; (▽), case 9; (+), case 10; (◇), case 11.

enteral administration of vancomycin prevented multiple organ failure. IL-15 remained at a high level until postoperative day 7. He was discharged on postoperative day 18.

Case 2

A 64 year-old male had hepatic right trisegmentectomy with portal vein resection for carcinoma of the hepatic hilum. IL-15 was markedly elevated but IL-10 remained at a low level on postoperative day 5. On postoperative day 6, he had severe watery diarrhoea and developed shock. MRSA was found in the faeces on day 6. IL-15 remained at a high level until postoperative day 7, then decreased on day 11 (Fig. 3B). Similar to case 1, IL-10 was only elevated just after the operation and thereafter decreased to an undetectable level. With intravenous and enteral vancomycin, diarrhoea subsided 4 days later. But after recovering from the enteritis, the patient suffered respiratory failure due to pneumonia, gastrointestinal bleeding, disseminated intravascular coagulation and hepatic and renal failure, and died on postoperative day 161.

Case 3

Right hepatectomy and pancreatoduodenectomy with portal vein resection were performed for bile duct cancer. IL-15 was elevated on postoperative day 1, and remained at a high level until postoperative day 3. (Fig. 3C). The patient had frequent vomiting on postoperative day 4 and MRSA was cultured from enteral fluid collected with an enteral tube. He had severe diarrhoea but did not develop shock. He recovered from enteritis with vancomycin administered from gastrostomy and was discharged on postoperative day 33.

Case 4

After colectomy for the carcinoma of the transverse colon, anastomosis leakage occurred. Two weeks later, colostomy, peritoneal lavage and drainage were performed (postoperative day 0). On postoperative day 5, IL-15 was elevated and severe diarrhoea that contained MRSA occurred. IL-10, that was elevated after the operation, decreased when IL-15 increased (Fig. 3D). He recovered from enteritis following intravenous vancomycin and was discharged 5 months after the colectomy.

In cases 1 to 4, changes in IL-15 level seemed to be associated with changes in IL-10. There may be a negative correlation between IL-15 and IL-10 levels in the serum. It appeared that IL-15 was elevated only when IL-10 was at a low level. Whenever serum IL-10 was elevated, IL-15 was decreased.

DISCUSSION

We here show that serum IL-15 was significantly elevated in patients with postoperative MRSA enterocolitis, compared with those with other MRSA infections. In this study, all of the patients developed enterocolitis after gastrointestinal surgery involving naso-gastric tube and during- and post-operative administration of intravenous antibiotics. Three out of four cases were pretreated by preoperative selective digestive decontamination. IL-15 elevation was evident before appearance of severe diarrhoea in these patients, suggesting that IL-15 may be involved in the pathogenesis of the disease. Serum IL-15 may be used as one of hallmarks to follow up the postoperative enterocolitis.

Takesue *et al.* reported that most of the organisms (nine out of ten) isolated from the faeces of the patients with MRSA enteritis were *Staphylococcal* enterotoxin (SE) A and C coproducing strains.[4] The analysis of the DNA pattern for MRSA in our present study also revealed that all species of MRSA produced TSST-1, coagulase II and enterotoxin C. TSST-1 and SE have been known to be representative bacterial superantigens which strongly activate T cells, expressing particularly TCRVβ, and often cause toxic shock syndrome via excessive production of T cell-derived cytokines such as IL-2 and macrophage/monocyte-derived cytokines such as interleukin 1 (IL-1) and tumour necrosis factor (TNF).[21–23] Although it remains unknown whether activated T cells can directly produce IL-15 or not, there are several lines of evidence that activated macrophages are able to produce IL-15 in humans.[15,24] We have also reported that murine macrophages infected with *Salmonella* produced IL-15, resulting in γδ T cell expansion in the infected sites of

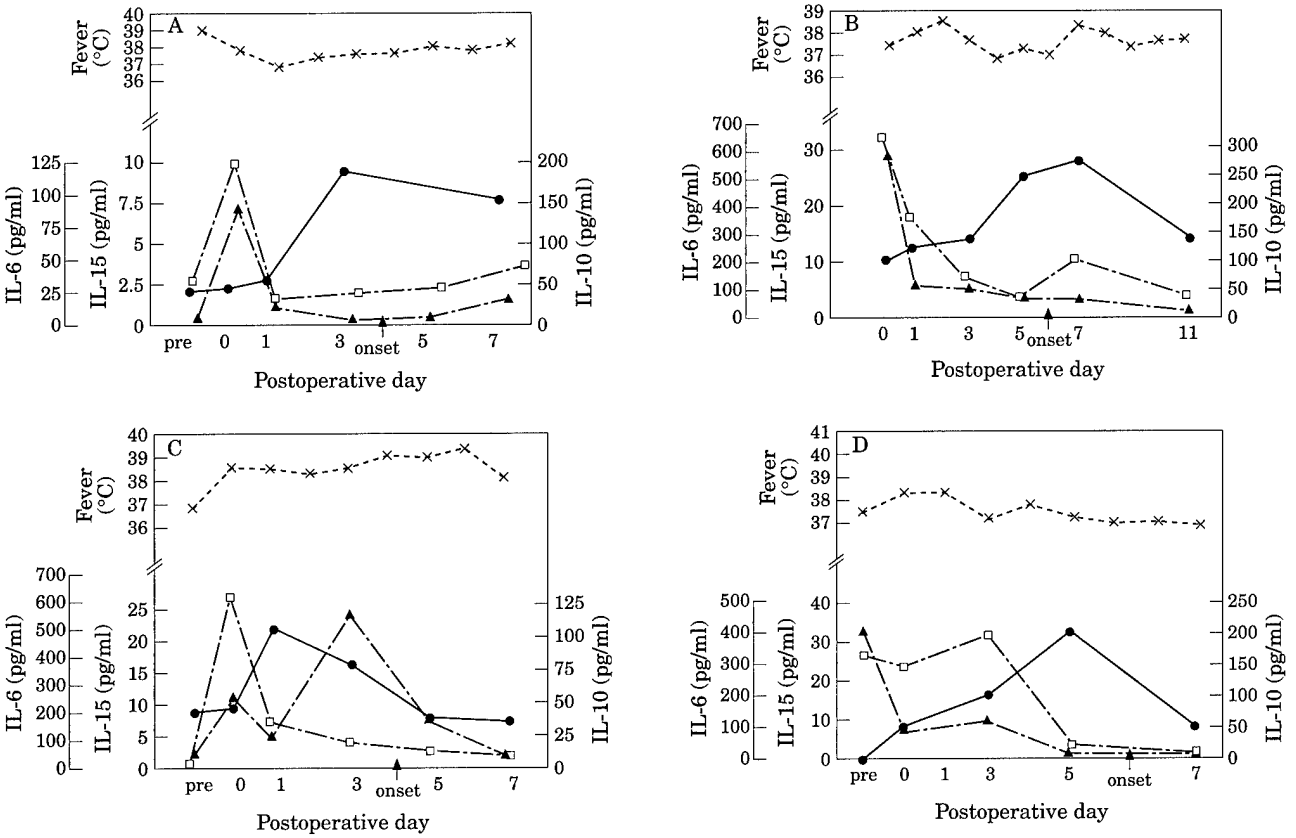

Figure 3. Changes in serum IL-15, IL-10, IL-6 and body temperature in case 1 (A), case 2 (B), case 3 (C) and case 4 (D).

In case 1, IL-10 was increased only on day 0,whereas IL-15 was increased on day 3. He suffered from severe diarrhoea and respiratory failure. MRSA was detected in the culture of faeces. After administration of perioral vancomycin, he recovered and discharged on postoperative day 18. (B) In case 2, IL-15 was increased on day 5 and frequent and severe diarrhoea occurred on postoperative day 6. IL-10 was elevated only on postoperative day 0 and remained low while IL-15 was elevated. The patient suffered from disseminated intravascular coagulation (DIC), renal failure, respiratory failure and hepatic failure which lead to multiple organ failure. He died on postoperative day 161. (C) In case 3, IL-15 was increased on postoperative day 1 when IL-10 was decreased, and vomiting and abdominal fullness appeared. MRSA was cultured in the duodenal juice. He had mild diarrhoea. (D) In case 4, on postoperative day 5, IL-15 was elevated and severe diarrhoea that contained MRSA occurred. IL-10 that elevated after the operation, decreased when IL-15 increased. (●), IL-15; (□), IL-6; (▲), IL-10; (✖), body temperature; onset, onset of enterocolitis.

mice.[13] Hence, it is possible that MRSA themselves and their toxins with superantigen activity may activate macrophages/monocytes to produce IL-15 during MRSA infection. However, elevated IL-15 levels were observed only in the patients with MRSA enterocolitis but not in the patients with other MRSA infections, suggesting that IL-15 production may be closely associated with enterocolitis. It has recently been reported that intestinal epithelial cells express IL-15 mRNA and respond significantly to exogenous IL-15.[11,18] Furthermore, Kirmann and Nielsen have shown increased numbers of IL-15-expressing cells in the peripheral blood in active ulcerative colitis.[19] We have also detected elevation of IL-15 in culture supernatants of rectal biopsy samples from patients with inflammatory bowel disease.[20] Using in situ hybridization technique, IL-15 mRNA is found to be expressed not only in infiltrating macrophages but also in epithelial cells in the intestinal mucosa of the patients.[11,20] Taken together, intestinal epithelium may be one of major sources of IL-15 and MRSA infection in the intestine may stimulate intestinal epithelial cells to produce excessive IL-15, resulting in the increase in serum IL-15 level in patients with MRSA enteritis.

We also measured other cytokines in MRSA infection. IL-10, originally described as a cytokine-synthesis inhibitory factor produced by the Th_2 subset of CD4+ T cells, is a key cytokine that regulates macrophage functions including TNF-α synthesis, MHC expression and nitric oxide production.[25] Macrophages can also produce IL-10 after stimulation such as with LPS.[26] In this study, IL-15 was elevated only when IL-10 level was low. When IL-10 was elevated, IL-15 was reciprocally decreased (Fig. 3A). Therefore IL-10 may be an inhibitory regulator of IL-15 in postoperative enterocolitis. IL-6 levels in the serum were not correlated with postoperative enterocolitis (Fig. 3). In contrast, the IL-15 level in serum reached a peak just before severe diarrhoea started and remained at a high level until the the patient recovered.

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In summary, IL-15 was produced in the patients with postoperative enterocolitis but not in the other types of MRSA infection. IL-15 may be the one of the key cytokines which are involved in postoperative enterocolitis.

MATERIALS AND METHODS

Serum IL-15 was measured in 77 patients that were consecutively admitted to our intensive care unit. MRSA were detected in 11 patients (10 men and 1 woman; mean age, 66.0 years old; range, 44–80). They were composed of: MRSA enteritis, 4; pneumonia, 6; cholangitis, 1. Backgrounds are summarized in Table 1. The patients were diagnosed as having MRSA infection, when all of the following criteria were met: (1) MRSA was cultured in the specimen of faeces, sputa, nasal mucus or bile; (2) there were objective signs of acute infection; (3) there were at least 3 of the following signs of a systemic inflammatory response: (a) hyperthermia or hypothermia (temperature >38°C or <36°C), (b) tachycardia (>90 beats per minute), (c) tachypnea (>20 breaths per minute) or $PaCO_2$ less than 32 mmHg or patients mechanically ventilated, and (d) white blood cell count greater than or equal to 12.0×10^9/l or less than 4.0×10^9/l or 0.10 or more immature neutrophils (bands). Blood was not detected macroscopically in the faeces of patients with enteritis and *Colstridium difficile* was not cultured in their faeces, excluding the possibility that the enteritis was caused by *Clostridium difficile*. In two cases (Cases 6 and 7), MRSA was detected in nasal mucus, and the diagnosis of pneumonia was supported by consolidation of chest x-ray and increased airway secretions. As controls, serum IL-15 levels were measured in 66 other patients who were admitted to our intensive care unit without MRSA infection (biliary duct carcinoma, 27; hepatic neoplasm, 18; gastric neoplasm, 14; pancreatic neoplasm, 4; rectal carcinoma, 2; oesophageal carcinoma, 1). All of the patients, except case 4, with postoperative enterocolitis received preoperative selective decontamination of the digestive tract with oral administration of 2 g/day kanamycin for 2 days. All samples were obtained with informed consent in accordance with the Helsinki Declaration.

Serum samples were collected early morning and stored at −80°C until measurements were made. Serum IL-6 and IL-10 levels were determined using human ELISA kits (R&D Systems, Minneapolis, MN for IL-6, ENDOGEN, Cambridge, MA for IL-10), which detected <3 pg/ml of human IL-6 and IL-10. Serum IL-15 was measured by a specific quantitative sandwich ELISA system that was described previously.[20] Briefly, 96-well microplates were incubated overnight with rabbit anti-human anti-IL-15 monoclonal antibody (Ab) which were kindly provided by Dr Raymond Paxon, Immunox Research and Development Corporation (Seattle, WA). After washing with 0.05% Weening/PBS, the wells were blocked by 1% BSA (Sigma, St Louis, MO) for 2 h at 37°C. Then rabbit anti-human IL-15 polyclonal Ab which was also provided by Dr Raymond Paxon, was added and incubated for 1 h at 37°C, and 50 ml of horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel, NC) was added and incubated for 1 h at 37°C. ABTS solution (Zymed, CA) was added and incubated for 15 min at room temperature. Optical density (OD) at 405 nm was measured on the ELISA reader. Serial dilution of recombinant human IL-15 was used for standards. Sensitivity of this ELISA was 5.0 pg/ml and inter-assay variation was less than 10%.[27]

Chromosomal DNA analysis by pulse field gel electrophoresis (PFGE) was performed as previously reported.[28] Briefly, chromosomal DNA was prepared as described by Smith *et al.*[29] An overnight culture was harvested, washed with saline-EDTA solution, and resuspended in Pett IV solution. The suspension was mixed with an equal volume of 1.2% low-melting-temperature agarose and allowed to solidify in a 100-ml mould. The block was incubated overnight at 37°C in a lysis solution supplemented with lysozyme and acromopeptidase. The block was incubated overnight at 50°C in ES solution supplemented with proteinase K, then treated with 1 mM phenylmethlsulfonyl fluoride in TE buffer for 4 h and washed 4 times with TE buffer. Thinly sliced sections of block were digested with 10U of *Kpn*I, *Eco*RI, *Xho*I, *Bam*HI, *Sma*I, and *Not*I for 18 h and then electrophoresed through a 0.9% agarose gel in TBE buffer at 10°C by using the contour-clamped homogeneous electric field (CHEF) system (Pulsaphor Plus; Pharmacia LKB Biotechnology, Uppsala, Sweden). The conditions for electrophoresis were 200 V for 15 h, with pulse times ranging from 5 to 45 secs for *Kpn*I, *Eco*RI, *Xho*I, and *Bam*HI digestions, and 170 V for 30 h, with pulse times ranging from 10 to 100 secs for *Sma*I, and *Not*I digestions. Then, the gels were stained with ethidium bromide, washed with distilled water, and photographed. *Saccharomyces cerevisiae* genomes or lambda DNA concatemers (Bio-Rad Laboratories, Richmond, CA), or both, were used as the size standard.

Values were expressed as mean ± SE. Statistical analysis was performed by Student's *t*-test. A P value of less than 0.05 was taken as significant.

Acknowledgements

We thank Dr R. Paxton (Immunex Research and Development Corporation, Seattle, WA) for providing IL-15 polyclonal and monoclonal antibody and IL-15 cDNA.

References

1. Christie CD, Lynch BE, Andiman WA (1988) Staphylococcal enterocolitis revisited: cytotoxic properties of *Staphylococcus aureus* from a neonate with enterocolitis. Pediatr Infect Dis J 7:791–795.

2. Suzuki K, Hori K, Ishihara H, Muramoto M, Sukurai S, Mashita K, Ishihara S, Shinagawa Y, Yura J (1990) A conculsion for the cause of MRSA enterocolitis: a clinical and experimental study. Nippon Geka Kansenshou Kenlyuu 2:262–266 (in Japanese).

3. Gunman LH, Iris ZH, Gehlbach S, Blackmon L (1976) Neonatal staphlococcal enterocolitis: association with indwelling feeding catheters and *S. aureus* colonization. J Pediatr 88:836–839.

4. Takesue Y, Yokoyama T, Kodama T, Santou T, Nakamitsu A, Murakami Y, Imamura Y, Miyamoto K, Okita M,

Tsumura H, Hirata T, Matsuura Y (1993) A study on postoperative enteritis caused by methicillin-resistant *Staphylococcus aureus*. Surg Today 23:4–8.

5. Tagaya Y, Burton JD, Miyamoto Y, Waldmann TA (1996) Identification of a novel receptor/signal transduction pathway for IL-15/T in mast cells. EMBO J 15:4928–4939.

6. Grabstein KH, Eisenman J, Shanebeck K, Rauch C, Srinivasan S, Fung V, Beers C, Richardson J, Schoenborn MA, Ahdieh M, Johnson L, Alderson MR, Watson JD, Anderson DM, Giri JG (1994) Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. Science 264(5161):965–968.

7. Giri JG, Ahdieh M, Eisenman J, Shanebeck K, Grabstein K, Kumaki S, Namen A, Park LS, Cosman D, Anderson D (1994) Utilization of the beta (β) and gamma (γ) chains of the IL-2 receptor by the novel cytokine IL-15. EMBO J 13:2822–2830.

8. Giri JG, Kumaki S, Ahdieh M, Friend DJ, Loomis A, Shanebeck K, DuBose R, Cosman D, Park LS, Anderson DM (1995) Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. EMBO J 14:3654–3663.

9. Carson WE, Giri JG, Lindemann MJ, Linett ML, Ahdieh M, Paxton R, Anderson D, Eisenmann J, Grabstein K, Caligiuri MA (1994) Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J Experi Med 180:1395–1403.

10. Armitage RJ, Macduff BM, Eisenman J, Paxton R, Grabstein KH (1995) IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. J Immunol 154:483–490.

11. Inagaki-Ohara K, Nishimura H, Mitani A, Yoshikai Y (1997) Interleukin-15 preferentially promotes the growth of intestinal intraepithelial lymphocytes bearing γδ T cell receptor in mice. Eur J Immunol 27:2885–2891.

12. Doherty TM, Seder RA, Sher A (1996) Induction and regulation of IL-15 expression in murine macrophages. J Immunol 156:735–741.

13. Nishimura H, Hiromatsu K, Kobayashi N, Grabstein KH, Paxton R, Sugamura K, Bluestone JA, Yoshikai Y (1996) IL-15 is a novel growth factor for murine gamma delta T cells induced by Salmonella infection. J Immunol 156:663–669.

14. Onu A, Pohl T, Krause H, Bulfone-Paus S (1997) Regulation of IL-15 secretion via the leader peptide of two IL-15 isoforms. J Immunol 158:255–262.

15. Tagaya Y, Kurys G, Thies TA, Losi JM, Azimi N, Hanover JA, Bamford RN, Waldman TA (1997) Generation of secretable and nonsecretable interleukin 15 isoforms through alternate usage of signal peptides. Proc Natl Acad Sci USA 94:14444–14449.

16. Nishimura H, Washizu J, Nakamura N, Enomoto A, Yoshikai Y (1998) Translational efficiency is up-regulated by Alternative Exon in Murine IL-15 mRNA. J Immunol 160:936–942.

17. McInnes I, Leung B, Sturrock R, Field M, Liew F (1997) Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-alpha production in rheumatoid arthritis. Nat Med 3:189–195.

18. Reinecker HC, MacDermott RP, Mirau S, Dignass A, Podolsky DK (1996) Intestinal epithelial cells both express and respond to interleukin 15. Gastroenterology 111:1706–1713.

19. Kirman I, Nielsen OH (1996) Increased numbers of interleukin-15-expressing cells in active ulcerative colitis. Am J Gastroenterology 91:1789–1794.

20. Sakai T, Kusugami K, Nishimura H, Yamaguchi T, Ohsuga M, Enomoto A, Kimura Y, Yoshikai Y (1998) Interleukin 15 activity in the mucosa of inflammatory bowel disease. Gasteroenterology 114:1237–1243.

21. Beezhold DH, Best GK, Bonventre PF, Thompson M (1987) Synergistic induction of interleukin-1 by endotoxin and toxic shock syndrome toxin-1 using rat macrophages. Infect Immun 55:2865–2869.

22. Jupin C, Anderson S, Damais C, Alouf JE, Parant M (1988) Toxic shock syndrome toxin 1 as an inducer of human tumor necrosis factors and gamma interferon. J Experi Med 167:752–761.

23. Marrack P, Kappler J (1990) The staphylococcal enterotoxins and their relatives. Science 248 (4956):705–711.

24. Alleva DG, Kaser SB, Monroy MA, Fenton MJ, Beller DI (1997) IL-15 functions as a potent autocrine regulator of macrophage proinflammatory cytokine production: evidence for differential receptor subunit utilization associated with stimulation or inhibition. J Immunol 159:2941–2951.

25. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR (1993) Interleukin-10. Annu Review Immunol 11:165–190.

26. Arai T, Hiromatsu K, Kobayashi N, Takano M, Ishida H, Nimura Y, Yoshikai Y (1995) IL-10 is involved in the protective effect of dibutyryl cyclic adenosine monophosphate on endotoxin-induced inflammatory liver injury. J Immunol 155:5743–5749.

27. Kakumu S, Okumura A, Ishikawa T, Yano M, Enomoto A, Nishimura H, Yoshioka K, Yoshikai Y (1997) Serum levels of IL-10, IL-15 and soluble tumor necrosis factor-alpha (TNF-α) receptors in type C chronic liver disease. Clin Exp Immunol 109:458–463.

28. Ichiyama S, Ohta M, Shimokata K, Kato N, Takeuchi J (1991). Genomic DNA fingerprinting by pulsed-field gel electrophoresis as an epidemiological marker for study of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus*. J Clini Microbiol 29:2690–2695.

29. Smith CL, Econome JG, Schutt A, Kloo S, Cantor CR (1987). A physical map of the *Escherichia coli* K12 genome. Science 236 (4807):1448–1453.

Seventh Annual Conference

of the

International Cytokine Society

PROGRAM AND ABSTRACTS

Hilton Head, South Carolina

December 5-9, 1999

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SHELDON WOLFF PRIZE IN CYTOKINE RESEARCH

Andrew Bowie

HONORARY LIFETIME MEMBERSHIP AWARD

Tada Taniguchi

Tadatsugu Taniguchi has made landmark discoveries in cytokine research with an impact that reverberates through biology and medicine. His first major discovery was published in a japanese journal in 1979, shortly after he returned to Tokyo from training in Charles Weissman's laboratory in Zurich; this paper announced the first cDNA cloning of an interferon gene, beta. He has continued to make breakthrough discoveries in the interferon field, describing the molecular basis of activation of the interferon genes and in turn of the genes they induce. These studies led to discovery of two transcriptional regulators, IRF-1 and -2, and the subsequent finding that IRF-1 has a key tumor suppressor function in that it cooperates with p53 in recognizing DNA damage. He has also made major discoveries in IL-2 research, having been the first to clone the cDNA in 1983. He then turned to the IL-2 receptor and made important findings in identifying its components and associated kinases.

The scope of these many contributions is remarkable in its intellectual power and energy. The impact on medicine of creating recombinant IL-2 and interferon is well known. In recognition of these contributions Tadatsugu Taniguchi has been given the Achievement Award from the International Cytokine Society.

HONORS AND AWARDS COMMITTEE
Ruth Neta, Chair

Awards will be presented by Members of the Awards Committee at the Wednesday evening Banquet.

SEVENTH ANNUAL CONFERENCE
INTERNATIONAL CYTOKINE SOCIETY

Hyatt Regency Hilton Head
Hilton Head Island, South Carolina
December 5-9, 1999

SUNDAY, DECEMBER 5, 1999

1:00 - 8:00 pm **Registration**

2:30 - 5:30 pm **COUNCIL MEETING**

6:00 - 7:00 pm **KEYNOTE ADDRESS**

8:30 - 10:00 pm **WELCOME RECEPTION**

MONDAY, DECEMBER 6, 1999

7:00 am - 6:00 pm **Registration**

8:00 - 9:00 am **Continental Breakfast, Exhibit and Poster Viewing I**

9:00 - 11:40 am **SYMPOSIUM I: Gene Induction**, Papers 1-2
Chairs: Jan Vilcek, New York University, New York and
Warren Leonard, National Institutes of Health, Bethesda, Maryland

9:00 am To be Announced
Richard Flavell
Yale University School of Medicine, New Haven, Connecticut

9:30 am **Signal Transduction Through the Cytokine Receptor Superfamily**
James Ihle
St. Jude Children's Research Center, Memphis, Tennessee

10:00 am **T Helper Subset-Specific Transcription Factors**
Lori Glimcher
Harvard School of Public Health and Medicine, Boston, Massachusetts

10:30 am **TGF Beta Signaling Through Smads**
Rik Derynck
University of California, San Francisco

Sheldon Wolff Prize in Cytokine Research: Andrew Bowie

11:00 am **SalF9R and SalF15R are Potential Viral Antagonists of IL1 and Toll Signalling (#1)**
Andrew Bowie
Trinity College, Dublin, Ireland

11:30 am **Cloning, Expression Analysis, and Functional Characterization of a Novel Class I Cytokine Receptor Expressed in Cells of Lymphoid Origin (#2)**
Angie Hammond
ZymoGenetics, Inc., Seattle, Washington

11:40 a.m. - 1:30 pm **POSTER SESSION I, Papers 3-90, 257-258**

Cancer, Papers 3-14
Chemokines, Papers 15-29
Receptor-Ligand Interactions, Papers 30-35, 257
Posttranslational Regulation, Papers 36-37
Knockout and Transgenic Mice, Paper 38
Infectious Diseases, Papers 39-45

Novel Cytokines and Cytokine Function, Papers 46-57
Diagnostics, Papers 58-59
Interferons, Papers 60-64
Signal Transduction, Papers 65-79, 258
Gene Regulation, Papers 80-88
Antagonists, Papers 89-90

1:30 - 3:00 pm **MINISYMPOSIUM I: Signal Transduction I**, Papers 91-96
Chairs: James Ihle, St. Jude Children's Research Center, Memphis, Tennessee and Carl Ware, La Jolla Institute for Allergy and Immunology, La Jolla, California

Outstanding Scholar Award: Deborah Alpert

1:30 pm **Evidence for p38-Dependent and p38-Independent Mechanisms of NF-κB Inhibition by Sodium Salicylate (#91)**
Deborah Alpert
New York School of Medicine, New York

1:45 pm **Vitamin C Inhibition of TNF-Stimulated IKK Activity Via p38 MAP Kinase (#92)**
Andrew Bowie
Trinity College, Dublin, Ireland

2:00 pm **The Death Domain of the Interleukin 1 Receptor Associated Kinase (IRAK) is Crucial for IL-1 Signaling (#93)**
Michael Martin
Medical School Hannover, Germany

Postdoctoral Investigator Award: Yoshinori Kawazoe

2:15 pm **SSI/SOCS Family Proteins are Involved in the Negative Regulation for Various Signal Transduction Pathway (#94)**
Yoshinori Kawazoe

Young Investigator Award: Silvia Bufone-Paus

2:30 pm **The IL-15 Receptor-α Chain Signals Through Association with Syk Kinase (#95)**
Silvia Bufone-Paus
Free University, Berlin, Hermany

2:45 pm **Tyrosine Phosphatase SHP-1 Regulates Cytoskeletal Proteins in Murine Macrophages: Lack of SHP-1 Leads to Degranulation of Cytoskeletal Organization in Macrophages of Motheaten (me/me) Mice (#96)**
Maya Kozlowski
University of Ottawa, Ottawa, Canada

1:30 - 3:00 pm **MINISYMPOSIUM II: Chemokines**, Papers 97-102
Chairs: Kouji Matsushima, University of Tokyo Medical School, Tokyo, Japan and Gary Johnson, National Jewish Medical Research Center, Denver, Colorado

1:30 pm ***S. aureus* Down-Regulates CXCR1 and CXCR2 Expression on Human Neutrophils Through TNF-Mediated Pathway (#97)**
Ilia Tikhonov
University of Wisconsin, Madison, Wisconsin

1:45 pm **Induction of Functional IL-8 Receptors by IL-4 and IL-13 in Human Monocytes (#98)**
Silvano Sozzani
Mario Negri Institute, Milan, Italy

2:00 pm **Chemokine Redundancy Ensures Local Neutrophil Recruitment (#99)**
Daniel Remick
University of Michigan, Ann Arbor, Michigan

2:15 pm **IL-1-Mediated Stabilization of GRO-A mRNA Depends on Sequences in both 5' and 3' Untranslated Regions (#100)**
Thomas Hamilton
Cleveland Clinic Foundation, Cleveland, Ohio

Postdoctoral Investigator Award: Matthew Park

2:30 pm **Targeted Disruption of the MIG Gene Reveals a role for MIG in Antibody Production (#101)**
Matthew Park
National Institutes of Health, Bethesda, Maryland

2:45 pm **Regulation of Chemokine Receptor Expression in Human NK Cells (#102)**
Carla Paganin
Mario Negri Institute, Milan, Italy

1:30 - 3:00 pm **MINISYMPOSIUM III: Gene Regulation I**, Papers 103-108
Chairs: Richard Flavell, Yale University Medical School, New Haven, Connecticut and Lori Glimcher, Harvard School of Public Health and Medicine, Boston, Massachusetts

1:30 pm **Functional IRF4 is Expressed in Macrophages and Regulates IL-1β Gene Expression (#103)**
Matthew Fenton
Boston University School of Medicine, Boston, Massachusetts

1:45 pm **Differential Activation of Type 1 Interferon Genes by Interferon Regulatory Factor 3 and 7 (#104)**
Rongtuan Lin
McGill University, Montreal, Canada

2:00 pm **Transcriptional Regulation of the MGSA Chemokine Gene by the Immediate Upstream Region (IUR) (#105)**
Chaitanya Nirodi
Vanderbilt University School of Medicine, Nashville, Tennessee

2:15 pm **An *NFκB* Element Flanking Sequence of the IL-8 Promotor is Involved in Both Transcriptional Repression and Induction by IL-1 (#106)**
Michael Kracht
Medical School Hannover, Germany

2:30 pm **A Novel 3'-UTR Element in the TNF-α Gene Activates PKR to Enhance mRNA Splicing without Impeding Translation (#107)**
Raymond Kaempfer
The Hebrew University Hadassah Medical School, Jerusalem, Israel

2:45 pm **Analysis of Signaling Factors Involved in IFN-γ Induction of IDO (#108)**
Milton Taylor
Indiana University, Bloomington, Indiana

3:15 - 4:45 pm **MINISYMPOSIUM IV: Signal Transduction II**, Papers 109-114
Chairs: Nancy Ruddle, Yale University School of Medicine, New Haven, Connecticut and Raymond Kaempfer, The Hebrew University Hadassah Medical School, Jerusalem, Israel

3:15 pm **Oncogenic Effects of Dysregulation of M-Ras (#109)**
John Schrader
University of British Columbia, Vancouver, Canada

3:30 pm **Cytokine-Induced mRNA Stabilization Via the p38 MAP Kinase Pathway: A Role for MAP Kinase-Activated Protein Kinase 2 and AU-Rich Cis-Elements (#110)**
Helmut Holtmann
Medical School Hannover, Germany

3:45 pm **P38 MAPK Regulates Pro-Inflammatory mRNA Stability Via MAPKAPK2 (#111)**
Andy Clark
Kennedy Institute of Rheumatology, London, United Kingdom

4:00 pm **IL-1 Activates Ras and Rap in EL4.NOB-1 Cells: A Role for Ras in p38 MAPK Activation by IL-1 (#112)**
Eva Palsson
Trinity College, Dublin, Ireland

4:15 pm **Role of MAP Kinases in IL-1β Synthesis Induced by Engagement of CD11b and CD11c β2 Integrins on Human Monocytes (#113)**
Roger Rezzonico
University Hospital, Geneva, Switzerland

4:30 pm **An Inhibitory Role for the Pseudokinase Domain in Regulation of the Jak2 Tyrosine Kinase (#114)**
Pipsa Saharinen
University of Helsinki, Finland

3:15 - 4:45 pm **MINISYMPOSIUM V: Chemokines and AIDS**, Papers 115-120
Chairs: Dan Littman, New York University Medical Center and
Amanda Proudfoot, Serono Pharmaceutical Research Institute, Geneva, Switzerland

3:15 pm **Redox Regulation of Chemokine Receptor Expression (#115)**
Antonio Sica
Mario Negri Institute, Milan, Italy

3:30 pm **CCR5, CXCR4 and CD4 are Clustered and Localized on Microvilli of Human Macrophages and T-Cells Via Immuno-EM (#116)**
Irwin Singer
Merck Research Labaoratories, Rahway, New Jersey

3:45 pm **Molecular Modeling and Mutational Analysis of CCR5 in Chemokine Binding and HIV-1 Entry* (#117)**
Naiming Zhou
Thomas Jefferson University, Philadelphia, Pennsylvania

4:00 pm **CD62L$^-$ and CD62L$^+$ Memory CD4$^+$ T Cells Differentially Support M-Tropic HIV Replication (#118)**
Manuela Mengozzi
Stanford University, Stanford, California

4:15 pm **Semi-Allogeneic Cell Hybrids Stimulate HIV-1 Envelope-Specific Cytotoxic T Lymphocytes (#119)**
Edith Grene
National Institutes of Health, Bethesda, Maryland

4:30 pm **Polymorphism in Control Regions of Chemokine and Cytokine Genes (#120)**
Jay Bream
National Cancer Institute, Frederick, Maryland

3:15 - 4:45 p.m. **MINISYMPOSIUM VI: Gene Regulation II**, Papers 121-126
Chairs: Matthew Fenton, Boston University School of Medicine, Boston, Massachusetts and Tadasugu Taniguchi, University of Tokyo, Japan

3:15 pm **The *NFκB* Subunit P50 Transactivates While P65 Represses Expression of C-Reactive Protein (CRP) (#121)**
Irving Kushner
MetroHealth Medical Center, Cleveland, Ohio

3:30 pm **Analysis of Interferon Type I Receptor Activation Using Cytokine Receptor Chimeras (#122)**
Xaveer Van Ostade
Univeristy of Ghent, Belgium

Outstanding Scholar Award: Caroline Jefferies

3:45 pm **Rac1 Regulates IL1-Induced *NFκB* Activation in an IκB-Independent Manner by Enhancing the Ability of the P65 Subunit to Transactivate Gene Expression (#123)**
Caroline Jefferies
Trinity College, Dublin, Ireland

4:00 pm **Interferons Inhibit Activation of Stat6 by Interleukin-4 in Human Monocytes by Inducing *SOCS1* Gene Expression (#124)**
Harold Dickensheets
Food and Drug Administration, Bethesda, Maryland

4:15 pm **Characterization of the Kinase Activity Implicated in the Activation of the IRF-3 Transcription Factor (#125)**
Marc Servant
McGill University, Montreal, Canada

4:30 pm **Novel Post-Translational Regulation of Interferon Regulatory Factor-4 Activity by the Immunophilin FKBP52 (#126)**
John Hiscott
McGill University, Montreal, Canada

5:00 - 7:30 pm **SYMPOSIUM II: Receptor Recycling and Signaling**, Papers 127-128
Chairs: Alice Dautry-Varsat, Institut Pasteur, Paris, France and
John Schrader, University of British Columbia, Vancouver, Canada

5:00 pm **Endocytosis and Intracellular Fate of Interleukin-2 Receptors**
Alice Dautry-Varsat
Institut Pasteur, Paris, France

5:30 pm **Regulation of Signaling Receptors by Ubiquitin**
Linda Hicke
Northwestern University, Evanston, Illinois

6:00 pm **Rab GTPases and the Coupling of Receptor Signaling and Membrane Trafficking**
Philip Stahl
Washington University School of Medicine, St. Louis, Missouri

6:30 pm **Tlr4: Sole Gateway to LPS Responses**
Bruce Beutler
Howard Hughes Medical Institute, Dallas, Texas

7:00 pm **CXCR2 Carboxyl-Terminus Leucine-Leucine and Isoleucine-Leucine Motifs are Involved in Receptor Internalization and Chemotaxis (#127)**
Guo-Huang Fan
Vanderbilt University School of Medicine, Nashville, Tennessee

7:15 pm **IL-16/CD4 Activation of T Cells Desensitizes to SDF-1/CXCR4 Chemotaxis (#128)**
William Cruikshank
Boston University School of Medicine, Boston, Massachusetts

7:30 - 9:00 pm **Dinner Break**

9:00 - 10:00 pm **Entertainment**

TUESDAY, DECEMBER 7, 1999

7:00 a.m. - 4:00 pm **Registration**

8:00 - 9:00 am **Continental Breakfast and Exhibit Viewing**

9:00 - 11:30 am **SYMPOSIUM III: Chromatin Regulation of Gene Expression**, Papers 129-131
Chairs: Kathrin Muegge, National Cancer Institute, Frederick, Maryland and
Gerald Crabtree, Stanford University Medical School, Stanford, California

9:00 am **Transcriptional Control: Chromatin, Corepressors and Coactivators**
Alan Wolffe
National Institutes of Health, Bethesda, Maryland

9:30 am **Signaling to the SWI/SNF-Like Chromatin Remodeling Complex in Lymphocyte Development and Activation**
Gerald Crabtree
Stanford University Medical School, Stanford, California

10:00 am **Gene Regulation in the Immune Response**
Anjana Rao
Harvard Medical School, Boston, Massachusetts

10:30 am **Reduced Accessibility at the TCR Gamma Locus for Rag Mediated Cleavage in IL- 7R -/-Thymocytes (#129)**
Kathrin Muegge
National Cancer Institute, Frederick, Maryland

10:50 am **A Disease Protective and Immune Suppressive Role of the TNF in Models of Systemic and Organ-Specific Autoimmunity (#130)**
George Kollias
Hellenic Pasteur Institute, Athens, Greece

11:10 am **Retroviral Cross-Talk: HTLV-II Downregulates HIV-1 Replication from PBMC of Co-Infected Individuals Via MIP-1α (#131)**
Guido Poli
San Raffaele Scientific Institute, Milan, Italy
Toho University, Funabashi, Japan

FREE AFTERNOON

5:00 - 7:30 pm **SYMPOSIUM IV: Chemokines**, Papers 247-249
Chairs: Ann Richmond, Vanderbilt University School of Medicine, Nashville, Tennessee and Joost Oppenheim, National Cancer Institute, Frederick, Maryland

5:00 pm **Chemokine Receptors - Multi-Faceted Targets**
Amanda Proudfoot
Serono Pharmaceutical Research Institute, Geneva, Switzerland

5:30 pm **Chemokine Receptors in Development and in HIV Disease**
Dan Littman
New York University Medical Center, New York

6:00 pm **Targeted Disruption of MEKK1 and MEKK2 Define Their Role in Regulating Cytokine Production in Response to Antigens and Cellular Stress**
Gary Johnson
National Jewish Medical Research Center, Denver, Colorado

6:30 pm **The Neutrophil Chemoattractant GCP-2 Selectively Antagonizes the Interaction of IP10 with CXCR3 (#247)**
Youmin Weng
Merck Research Laboratories, Rahway, new Jersey

6:50 pm **Lymphoid Tissue Homing Chemokines are Expressed in Chronic Inflammation (#248)**
Nancy Ruddle
Yale University School of Medicine, New Haven, Connecticut

7:10 pm **IFN-γ Attenuates IL-2 Induced CXCR3 Function in Primary Human Lymphocytes: Implications for Th1 Responses (#249)**
Venugopal Gangur
National Cancer Institute, Frederick, Maryland

7:30 - 9:00 pm **Dinner Break**

8:30 - 10:00 pm **Entertainment**

WEDNESDAY, DECEMBER 8, 1999

7:00 am -12:00 pm **Registration**

8:00 - 9:00 am **Continental Breakfast, Exhibit and Poster Viewing II**

9:00 - 11:30 am **SYMPOSIUM V: Structural Biology**, Papers 250-252
Chairs: Stephen Sprang, Howard Hughes Medical Institute, Dallas, Texas and
Robert Stroud, University of California, San Francisco, California

9:00 am To be Announced
Robert Stroud
University of California, San Francisco, California

9:30 am **A Death Domain Complex from Flies**
Stephen Sprang
Howard Hughes Medical Institute, Dallas, Texas

10:00 am **Learning about Function of Apoptosis Proteins from NMR Structures**
Gerhard Wagner
Harvard Medical School, Boston, Massachusetts

10:30 am **Interaction of Receptor Binding Epitopes of IL-6 Type Cytokines: The Specificity of Site III Depends on Site I (#250)**
K.-J. Kallen
University of Mainz, Germany

10:50 am **Cytomegalovirus Harbors Its Own Unique IL-10 Homolog (#251)**
Sergio Kotenko
UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey

11:10 am **Cellular Distribution of a Binding Partner for CLF-1 (#252)**
Greg Elson
Centre D'Immunologie Pierre Fabre, St. Julien-en-Genevois, France

11:30 am - 1:00 pm **POSTER SESSION II**, Papers 132-216

Autoimmunity, Papers 132-137
Disease Roles of Cytokines, Papers 138-144
Hematopoiesis, Papers 145-146
Reproduction, Papers 147-150
TH1/TH2 Cytokines, Papers 151-157
Neurobiology, Paper 158
Inflammation, Papers 159-181
Immunoregulation, Papers 182-192
AIDS, Papers 193-199
Apoptosis, Papers 200-204
Genomics, Papers 205-206
Cytokine Interactions, Papers 207-216

1:30 - 3:00 p.m. **MINISYMPOSIUM VII: Disease, Papers 217-223**
Chairs: Elizabeth Kovacs, Loyola University Chicago, Maywood, Illinois and
Marc Feldman, Kennedy Institute of Rheumatology, London,
United Kingdom

Outstanding Scholar Award: Terry Means

1:30 pm **The CD14 Ligands Lipoarabinomannan and LPS Differ in Their Requirement for Toll-Like Receptors (#217)**
and
Human Toll-Like Receptors Mediate Cellular Activation by *M. Tuberculosis* (#218)
Terry Means
Boston University School of Medicine, Boston, Massachusetts

1:45 pm **Differential *Ex Vivo* Response to IFN-β and IL-12 in Cutaneous Leishmaniasis Patients (#219)**
Johan Van Weyenbergh
LIMI-Oswaldo Cruz Foundation, Salvador, Brazil

2:00 pm **Constitutive Expression of GP-130-Binding Cytokines in Bone Marrow: Implications for Myeloma Pathology (#220)**
Beverly Barton
UMDNJ-New Jersey Medical School, Newark, New Jersey

2:15 pm **Evidence for a Key Role of IL-6 in Antibody-Mediated Experimental Myasthenia Gravis Pathogenesis (#221)**
Premkumar Christadoss

2:30 pm **Arteritis in Mice Lacking IL-1RA (#222)**
Martin Nicklin
University of Sheffield, United Kingdom

2:45 pm **Anti-Interleukin-6 Antibody Treatment Restores Some but Not All Immune Functions after Thermal Injury (#223)**
Elizabeth Kovacs
Loyola University Chicago, Maywood, Illinois

1:30 - 2:45 pm **MINISYMPOSIUM VIII: Inflammation**, Papers 224-228
Chairs: Jeremy Saklatvala, Kennedy Institute, London, United Kingdom and
Jean-Michel Dayer, University Hospital, Geneva, Switzerland

1:30 pm **Roles of Interferon Consensus Sequence Binding Protein and PU.1 in Regulating IL-18 Gene Expression in Mouse Macrophages (#224)**
Yong-Man Kim
Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea

1:45 pm **Expression of the Novel Cytokine IL-B30 in Transgenic Mice Induces a Multi- Organ Inflammatory Disease (#225)**
Maria Wiekowski
Schering-Plough Research Institute, Kenilworth, New Jersey

2:00 pm **HMG-1 Induces TNF Synthesis in Human Peripheral Blood Mononuclear Cells (#226)**
Haichao Wang
The Picower Institute for Medical Research, Manhasset, New York

2:15 pm **Distinct and Overlapping Roles of Lymphotoxin and TNF in Development of Lymphoid Tissues: Correlations with Gene Expression Pattern (#227)**
Sergei Nedospasov
National Cancer Institute, Frederick, Maryland

Postdoctoral Investigator Award: De Yang

2:30 pm **Human β-Defensins Use CCR6 as a Receptor to Chemoattract Dendritic and T Cells (#228)**
De Yang
National Cancer Institute, Frederick, Maryland

3:15 - 4:45 pm **MINISYMPOSIUM IX: Immunoregulation**, Papers 229-234
Chairs: Alberto Mantovani, Mario Negri Institute, Milan, Italy and
Anjana Rao, Harvard Medical School, Boston, Massachusetts

3:15 pm **Identification of a Novel Four-Transmembrane Domain Protein on TH1 Lymphocytes (#229)**
C. Venkataraman
Tularik, Inc., South San Francisco, California

3:30 pm **Cloning and Characterization of Mouse and Human TIF, A New IL-10-Related Cytokine (#230)**
Laure Dumoutier
Ludwig Institute for Cancer Research, Belgium

3:45 pm **BlyS, A Novel Member of the Tumor Necrosis Factor family and Potent B Lymphocyte Stimulator (#231)**
Judith Giri
Human Genome Sciences, Inc., Rockville, Maryland

4:00 pm **Analysis and Functionality of Isoforms of Naturally Occurring IL-18 Binding Protein (IL-18BP) (#232)**
Soo-Hyun Kim
University of Colorado Helath Science Center, Denver, Colorado

4:15 pm **Interferon-Gamma Induced Desensitization (IGID) for House Dust Mites: Modulation of Immune Status from TH2 to TH1 Using Inteferon-Gamma as a New Therapeutic Concept for Atopic Dermatitis (#233)**
Geunwoong Noh
Sungkyunkwan University School of Medicine, Seoul, Korea

4:30 pm **Xanthine Oxidase as a Mediator of Cytokine Action in Arthritis and Experimental Autoimmune Encephalomyelitis (#234)**
Pietro Ghezzi
Mario Negri Institute, Milan, Italy

3:15 - 4:45 pm **MINISYMPOSIUM X: Cancer**, Papers 235-240
Chairs: Michael Lotze, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania and Robert Strieter, The University of Michigan Medical Center, Ann Arbor, Michigan

3:15 pm **Transformation byt TNF-α in BALB/3T3 Cells and Overexpression of IL-1 Gene in the Transformants (#235)**
Masami Suganuma
Saitama Cancer Center Research Institute, Saitama, Japan

3:30 pm **Immunotherapy of Renal Carcinoma Suppresses NF-κB Response and Increases Susceptibility to Cytotoxic Effectors (#236)**
Udo Junker
University of Jena, Germany

Young Investigator Award: Jean-Christophe Renauld

3:45 pm **CCR8-Dependent Activation of the Ras/MAP-Kinase Pathway Mediates Anti-Apoptotic Activity of I-309 and vMIP1 (#237)**
Jean-Christophe Renauld
Ludwig Institute for Cancer Research, Brussels, Belgium

4:00 pm **The Mechanism of MGSA/GROα Mediated Transformation in Mouse Melanocytes (#238)**
DingZhi Wang
Vanderbilt University School of Medicine, Nashville, Tennessee

4:15 pm **Differential Activity of IL-1 Alpha and IL-1 Beta in Tumor Development (#239)**
E. Voronov
Ben-Gurion University of the Negev, Beer-Sheva, Israel

Postdoctoral Investigator Award: Xuedong Liu

4:30 pm **Interaction of the Ski Oncoprotein with Smad3 Regulates TGF-β Signaling (#240)**
Xuedong Liu
Whitehead Institute for Biomedical Research, Cambridge, Massachusetts

3:15 - 4:45 pm **MINISYMPOSIUM XI: Apoptosis**, Papers 241-246
Chairs: David Wallach, The Weizmann Institute of Science, Rehovot, Israel and Anneliese Schimpl, University of Würzburg, Germany

3:15 pm **Overexpression of the Bcl-2 Family Member A1 in CD4$^+$ T Cells from IL-2 Deficient Mice (#241)**
Sabine Wagner
University of Würzburg, Germany

3:30 pm **Multiple Mechanisms of Tumor Lysis by Activated T Cells (#242)**
Thomas Sayers
National Cancer Institute, Frederick, Maryland

3:45 pm **Human and Murine Cellular Models for Caspase Independent Death Induced by TNF or dsRNA (#243)**
Michael Kalai
University of Ghent, Belgium

4:00 pm **Molecular Mechanisms Involved in the Lithium-Induced Sensitization of Tumor Cells to TNF-Induced Apoptosis (#244)**
Rudi Beyaert
University of Ghent, Belgium

4:15 pm **Interleukin (IL)-7 Induces Rapid Activation of Pyk2 Which is Bound to Jak1 and IL-7Rα (#245)**
Naima Benbernou
National Cancer Institute, Frederick, Maryland

4:30 pm **Transient Infiltration of Neutrophils into the Thymus in Association with Apoptosis Induced by Whole Body X-Irradiation (#246)**
Yoshiro Kobayashi

5:00 - 7:30 pm **SYMPOSIUM VI: Clinical**, Papers 253-254
Chairs: Charles Dinarello, University of Colorado Health Sciences Center, Denver and Elizabeth Kovacs, Loyola University Chicago, Maywood, Illinois

5:00 pm **Novel Cytokines in the Treatment of Cancer: IL-17 and Beyond**
Michael Lotze
University of Pittsburg Cancer Institute, Pittsburgh, Pennsylvania

5:30 pm **CXC Chemokines In Angiogenesis**
Robert Streiter
The University of Michigan Medical Center, Ann Arbor, Michigan

6:00 pm **Infection Susceptibility and How to Overcome it: Interferon Gamma and Its Receptor**
Steven Holland
National Institutes of Health, Bethesda, Maryland

6:30 pm **Interleukin-1 Genes and Artery Disease and Artery Disease (#259)**
Gordon Duff
University of Sheffield, United Kingdom

6:50 pm **Cytokine disregulation in the Rheumatoid Synovium: Mechanisms of TNFα Regulation and Disease Chronicity (#253)**
Marc Feldmann
Kennedy Institute of Rheumatology, London, United Kingdom

7:10 pm **Molecular Cloning of a Novel Macrophage-Derived Cytokine (SMAF-1) and Its Immuomodulating Capacities (#254)**
Lucie Franzen
Innogenetics, Ghent, Belgium

8:00 - 9:00 pm. **Banquet**

9:00 - 10:00 pm **AWARD PRESENTATIONS**

ICS LIFETIME HONORARY MEMBERSHIP AWARD LECTURE
Regulation of the Interferon Systems in Innate and Adaptive Immune Responses
Tadatsugu Taniguchi
University of Tokyo, Japan

10:00 - Midnight **Entertainment**

THURSDAY, DECEMBER 9, 1999

8:00 - 9:00 am **Continental Breakfast**

9:00 - 11:30 a.m. **SYMPOSIUM VII: Apoptosis**, Papers 255-256, 260
Chairs: Bruce Beutler, Howard Hughes Medical Institute, Dallas Texas and
Scott Durum, National Cancer Institute, Frederick, Maryland

9:00 am To be Announced
Douglas Green
La Jolla Institute of Cellular and Molecular Immunology, San Diego, California

9:30 am **Death and Transcription Regulation by Receptors of the TNF/NGF Family**
David Wallach
The Weizmann Institute of Science, Rehovot, Israel

10:00 am To be Announced
Craig Thompson
University of Chicago, Illinois

Postdoctoral Investigator Award: Annette Khaled

10:30 am **IL-7 Withdrawal Induces a Rapid Rise in Intracellular pH Causing: A) Bax Translocation to Mitochondria and B) Transient Mitochondrial Hyper-Polarization (#255)**
Annette Khaled
National Cancer Institute, Frederick, Maryland

10:50 am **An Interferon-Induced Protein of the Bcl-2 Family Has Two Independent Activities: It Promotes Apoptosis and Synthesizes 2-5 (A) (#256)**
Ganes Sen
The Cleveland Clinic Foundation, Cleveland, Ohio

11:10 am **Normal Homeostasis, Lack of Autoimmunity, but Severely Impaired IL-2R Function by Mature T Cells after Thymic Expression of the IL-2R β Chain in IL-2R β-Deficient Mice (#260)**
Tom Malek
University of Miami School of Medicine

1

SALF9R AND SALF15R ARE POTENTIAL VIRAL ANTAGONISTS OF IL1 AND TOLL SIGNALLING. A.G. Bowie†, E. Kiss-Toth§, S.K. Dower§, J. A. Symons¶, G.L. Smith¶ and L.A.J. O'Neill†, †Dept. of Biochemistry, Trinity College Dublin, Ireland, §University of Sheffield, U.K. and ¶Sir William Dunne School of Pathology, University of Oxford, U.K.

The genome of vaccinia virus (VV) contains genes that encode proteins that interfere with host immune responses. We previously cloned two novel vaccinia open reading frames (ORFs) with sequence homology to the cytoplasmic signalling domain of the IL1-R1 and human Toll-like receptors (hTLRs). These ORFs are termed SalF9R (or A46R) and SalF15R (or A52R). Here we have analysed the sequences of the ORFs and characterised the effect of the gene products on IL1 and TLR signalling. SalF9R has a region strongly related to the YDAYILY region of the IL1-R1, while SalF15R has significant homology to the Heguy region of the IL1-R1. Neither ORFs have homology to a third defined region of the signalling domain, shown to be important for both IL1 and TLR4 signalling. Epitope-tagged versions of both ORFs were shown to be expressed in 293 cells, although SalF15R was expressed more strongly. Overexpression of proteins important in IL1 and TLR4 signalling induced the expression of an NFκB-dependent reporter gene. The effects of SalF9R and SalF15R on these inductions was determined, and compared to the effect of a truncated dominant-negative form of MyD88, a pivotal molecule in both IL1 and TLR4 signalling. SalF15R and the mutant MyD88 strongly inhibited both IL1 and TLR signalling, while SalF9R was less potent. The results suggest that the ORFs are novel viral mechanisms of antagonising IL1/TLR signalling. They are likely to be useful tools in defining the functional relevance of different regions of the IL1/TLR cytoplasmic signalling domain.

2

CLONING, EXPRESSION ANALYSIS, AND FUNCTIONAL CHARACTERIZATION OF A NOVEL CLASS I CYTOKINE RECEPTOR EXPRESSED IN CELLS OF LYMPHOID ORIGIN. A Hammond, J Parrish, S Presnell, D Conklin, C Sprecher, S Schrader, W Xu, K Madden, C Brandt, J Gross, J Johnston, S Mudri, A Nelson, S Dillon, K Hambly, H-P Ren, F Raymond, T Whitmore, M Maurer, and D Foster, ZymoGenetics, Inc., Seattle, WA

We have identified a novel Class I cytokine receptor, zalpha11, which is expressed in lymphoid tissues. This receptor contains the hallmark motifs of its class, including two pairs of conserved cysteine residues and a conserved "WSxWS" motif in its extracellular domain, and conserved Box I and Box II signaling motifs in its intracellular domain. Northern analysis indicates low-level expression in spleen, thymus, lymph node, bone marrow, and peripheral blood lymphocytes; expression appears to be upregulated in a Burkitt lymphoma cell line (Raji). RT/PCR and flow cytometry suggest expression of zalpha11 on resting and activated B cells and on activated T cells. Immunohistochemistry using a polyclonal antibody raised against soluble zalpha11 localizes the receptor to a subset of cells in spleen and thymus. In order to assess the signaling capability of zalpha11, a chimeric receptor was constructed which consisted of the extracellular and transmembrane domains of Mpl, which forms a homodimer in the presence of thrombopoietin (TPO), fused with the cytoplasmic domain of zalpha11. This construct was transfected into the murine IL-3-dependent cell line Baf3, and proliferative capacity was measured in the presence of TPO without IL-3. Cells transfected with the chimeric receptor were able to proliferate in response to TPO, indicating that homodimeric zalpha11 is capable of delivering a proliferative signal to Baf3 cells. Given the lymphoid expression pattern and signaling capabilities of zalpha11, its ligand can be expected to be a potent lymphopoietic factor.

3

INTERLEUKIN (IL)-13 RECEPTORS AND SIGNALING IN HUMAN NON-NEOPLASTIC ASTROGLIAL CELLS: ALTERATION WITH MALIGNANT PROGRESSION. BP Barna, H. Liu, J. Liu, B. Jacobs, GH Barnett, and ML Estes. MetroHealth Medical Center, Cleveland, OH 44109; and Cleveland Clinic Foundation, Cleveland, OH 44195.

IL-4 mediates G_1 growth arrest of human astroglial cells in a receptor-dependent manner. Because the IL-4 receptor is a complex that may contain IL-13 binding elements, we speculated that IL-13 might also affect astroglial proliferation. IL-13 receptor components and sensitivity were investigated in 13 glial cell lines derived from non-neoplastic cerebral cortex, low grade astrocytoma, anaplastic astrocytoma, and glioblastoma multiforme. Unlike peripheral blood lymphocytes (PBL), glial cells did not express IL-2 receptor gamma chain (IL-2Rγc). IL-13 receptor alpha-1 (IL-13Rα1) however, was present in 11/13 glial lines and PBL. Deficient cell lines were glioblastoma-derived. IL-13 receptor alpha-2 (IL-13Rα2) was found in all 8 anaplastic astrocytoma and glioblastoma lines but not in other glial lines or PBL. In non-neoplastic, low grade, and anaplastic astrocytoma, IL-13 reduced DNA synthesis, an effect reversible with antibody to IL-4Rα. Glioblastoma cells were uniformly insensitive and failed to phosphorylate STAT6 after IL-13 challenge. Data suggest that IL-13 receptors and sensitivity are characteristics of non-neoplastic astroglia and that these characteristics become altered with malignant progression. Supported by NIH grant RO1NS-33932.

4

CYTOKINES IN DEPOT FORMULATIONS ARE ADJUVANTS F.W. Falkenberg, O.C. Krup, M. Peters, R. Schröder and S. Wasmuth. Abteilung für Medizinische Mikrobiologie, Ruhr-Universität Bochum, 44780 Bochum, Germany.

The induction of an immune response is a strictly local process, involving local resident cells, locally deposited antigen and signaling by locally released cytokines. The cytokine molecules released attract other cells to the site which themselves release other cytokines, and so forth. As a consequence, a complete immune reaction resulting in systemic immunity is induced. This process has been succesfully mimicked by applying cytokine gene-transfected cells in a local depot. However, once a transfected cell has been injected, it is very difficult to control its actions. We have approached this problem by applying cytokines in depot formulations, e.g. encapsulated into liposomes or simply adsorbed to adsorbent materials. As a result we found that strong humoral and cellular immune responses can be induced when soluble or cellular antigens are applied mixed with a cytokine/cytokines in a depot formulation ("Cytokine-Depots"). Applying "Cytokine-Depot Tumor Vaccines" we were able to induce not only prophylactic protective but also therapeutic curative tumor-specific cytotoxic immune responses in tumor-bearing animals. Applying this concept, immunity to a variety of murine tumors has been induced with different cytokines in different depot formulations.

5

TNF-α / IL-1 AS ESSENTIAL CYTOKINES IN TUMOR PROMOTION. H. FUJIKI, M. Suganuma, S. Okabe, E. Sueoka and M.W. Marino[1]. Saitama Cancer Center Res. Inst. Saitama 362-0806, Japan and [1]Memorial Sloan-Kettering Cancer Center, New York, N.Y. 10021

We previously demonstrated that okadaic acid induces TNF-α/IL-1 in the target tissues of tumor promotion, and that TNF-α stimulated transformation of BALB/3T3 cells initiated with 3-methylcholanthrene. To examine our hypothesis that TNF-α is an essential cytokine in carcinogenesis, we conducted two-stage carcinogenesis experiments with DMBA + okadaic acid, and DMBA + TPA, on the skin of TNF-α deficient mice. Tumorigenesis in TNF-α $^{-/-}$ mice was significantly depressed compared with TNF-α $^{+/+}$ mice, although some tumor development was observed in the late weeks of tumor promotion, suggesting that IL-1 might be responsible for play the role in this residual tumor promoting activity. Since TNF-α $^{-/-}$ mice develop normally and have no gross structural or morphological abnormalities, we think that this response to a tumor promoter in TNF-α $^{-/-}$ mice reflects innate cancer susceptibility. This correlated well with evidence that most cancer preventive agents, such as tamoxifen and sulindac, commonly inhibited both TNF-α release from the cells and TNF-α gene expression in the cells induced by okadaic acid. Thus, inhibition of TNF-α release by such agents is a key element in their inhibition of tumor development.

6

REGULATION OF CD44 EXPRESSION BY IL4 AND IL13 IN B CELL LINES. K. Gee, M. Kozlowski, M. Kryworuchko, S. Aucoin, F. Diaz-Mitoma and A. Kumar. Department of Biochemistry, Microbiology and Immunology, and Research Institute, Children Hospital of Eastern Ontario, University of Ottawa, Ottawa, Canada.

CD44, an adhesion molecule, plays a critical role in lymphocyte homing, cell migration and tumor metastasis. We have previously shown that IL4 enhances CD44 expression and hyaluronan (HA) binding ability in an Epstein Barr-virus (EBV) positive Burkitt's lymphoma (BL) B cell line, BL30/B95-8, but not in an EBV transformed lymphoblastoid B cell line (B-LCL), MK3.31. IL13, a cytokine sharing similar functions with IL4, did not influence CD44 expression or HA binding in either cell line. To understand the molecular mechanism for the differential regulation of CD44 expression by IL-4 and IL-13, we investigated activation of JAK/STAT signaling pathways in BL and B-LCL cell lines. JAK-1, JAK-3 and STAT-6 were phosphorylated in response to IL-4 in both cell lines suggesting that the JAK/STAT signalling pathway alone may not account for the differential CD44 expression. In contrast, IL13 failed to induce the phosphorylation of JAK1, JAK2, JAK3, Tyk2 and STAT-6 proteins in BL30/B95-8 cells, indicating possible loss of receptor expression or function. Studies on a panel of BL cell lines by RT-PCR analysis revealed loss of expression of IL-13 receptor subunits α1 and α2 in all the BL cell lines examined. In addition, we have investigated the role of EGR-1, the transcription factor implicated in B cell activation, in the regulation of CD44 expression. The results show that PMA induces phosphorylation of EGR-1 in BL30/B95-8 and MK3.31 cells. However, DNA binding ability as determined by the gel mobility shift assay was induced in BL30/B95-8 cells but not in MK3.31 cells. While loss of IL13 receptor explains the inability of BL30/B95-8 cells to respond to IL13, the results suggest a role for EGR-1 in the regulation of CD44 expression in BL30/B95-8 and MK3.31 cells.

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CD40L INDUCES MMP-9 BUT NOT TIMP-1 IN CERVICAL CARCINOMA CELLS

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Matrix-metalloproteinases (MMPs) are essential for tumor cell invasion and metastasis. Production of proMMPs is regulated on transcriptional level, while activation of the pro-enzymes is tightly controlled by proteolytic cleavage and enzyme activity is blocked by specific tissue inhibitors of MMPs (TIMPs). We show that activation of CD40, a receptor highly upregulated on cervical carcinomas, strongly induces MMP-9 in these cells, while having no effect on TIMP-1 production, the complementary inhibitor of MMP-9 activity. Transcription factors like NF-κB, AP-1 and STAT are potential targets of CD40 signaling, NF-κB and AP-1 being involved in MMP-9 regulation, whereas AP-1 and STAT3 are supposed to regulate the TIMP-1 promoter. Both, NF-κB and AP-1, were activated by CD40L in cervical carcinoma cells. Induction of MMP-9 was largely suppressed by antioxidants and inhibitors of NF-κB activity, while retinoic acid suppressing AP-1 activity had only little effect. These data indicated an important role of NF-κB. In fact, transient expression of the CD40-inducible NF-κB subunit p65 was sufficient for MMP-9 induction. In contrast, CD40 did not activate STAT 3 and failed to stimulate TIMP-1 production. Our data suggest that CD40-mediated signaling in cervical carcinoma cells shifts the balance between MMP-9 and TIMP-1 towards proteolytic activity and may thereby enhance the invasive potential of the tumor.

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ANTI-INFLAMMATORY CYTOKINE ANTIBODIES ARE DIFFERENTLY AFFECT MMP-9 SECRETION BY SKOV-3 CELLS. M. Huleihel, B. Piura, A. Rabinovich, M. Wolfson, G. Holcberg, M. Mazor. Ben-Gurion University of the Negev, Beer-Sheva, Israel.

The invasion and spread of metastasizing tumors is known to be mediated partly by the action of matrix metalloproteinase (MMP) family. In the present study we have examined the effect of anti-inflammatory cytokines on the capacity of ovarian cell line (SKOV-3) to secrete MMP-9. SKOV-3 cells were cultured overnight in 0.1% FCS –containing media. In the next day SKOV-3 cell cultures were washed and 5% FCS-cntaining media or serum free media were added to the cultures in the presence or absence of anti-IL-1α, anti-IL-1β, anti-IL-6 or ant-TNF-α antibodies for 6-72 hours. Our results showed that SKOV-3 cells produce MMP-9 constitutively and addition of FCS to the cultures increased their capacity to secrete MMP-9, and maximal levels were detected 24 hours.after the incubation Addition of anti-IL-1α or anti-IL-1β to SKOV cell cultures which grew in serum free media increased their capacity to secrete MMP-9 only in the first 6 hours of incubation. However, addition of anti-IL-6 or anti-TNF-α to these cultures decreased their capacity to secrete MMP-9. On the other hand, SKOV cells which cultured in 5% FCS containing media showed decrease in the secreted MMP-9 levels 72 hours after incubation. Addition of anti-IL-1α or anti-IL-1β to these cultures increased their capacity to secrete MMP-9 only after 72 hour of incubation. However, addition of anti-IL-6 or anti-TNF-α decreased their capacity to secrete MMP-9 in the first 24 hours and increased their capacity to secrete MMP-9 after 72 hours of incubation.

In conclusion, our results may suggest the involvement of factors in the serum and cytokines in the regulation of MMP-9 secretion by SKOV-3 cells. Thus, cytokines may be involved in regulation the metastatic capacity of this tumor cell line.

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EFFECT OF ANTI-INFLAMMATORY CYTOKINE ANTIBODIES IN THE SECRETION OF IL-1β AND IL-6 BY SKOV-3 CELLS. M. Huleihel, B. Piura, A. Rabinovich, M. Wolfson, G. Holcberg, M. Mazor. Ben-Gurion University of the Negev, Beer-Sheva, Israel.

Cytokines such as IL-1, IL-6 and TNF-α may control the local growth and tumorigenicity of cancerous cells through autocrine/paracrine effects or as angiogenesis-promoting factors. In the present study we have examined the effect of anti-inflammatory cytokines on the capacity of ovarian cell line (SKOV-3) to secrete IL-1β or IL-6. SKOV-3 cells were cultured overnight in 0.1% FCS – containing media. In the next day SKOV-3 cell cultures were washed and 5% FCS-containing media or serum-free media were added to the cultures in the presence or absence of anti-IL-1α, anti-IL-1β, anti-IL-6 or ant-TNF-α antibodies for 6-72 hours. Our results show that SKOV-3 cells produce IL-1β and IL-6 constitutively and addition of FCS to these cultures increased this capacity. Addition of anti-IL-1α or anti-IL-6 antibodies to SKOV cell cultures, which grew in serum-free media or in 5% FCS-containing media, did not affect their capacity to secrete IL-1β. However, addition of anti-TNF-α antibodies to these cultures increased their capacity to secrete IL-1β. On the other hand, addition of anti-IL-1α or anti-IL-1β antibodies to SKOV cell cultures, which grew in 5 % FCS-containing media, did not affect their capacity to secrete IL-6. However, addition of anti-TNF-α antibodies to these cultures increased their capacity to secrete IL-6. On the other hand, addition of anti-IL-1β or anti-TNF-α antibodies to SKOV cell cultures, which grew in serum-free media, did not affect their capacity to secrete IL-6, but addition of anti-IL-1α antibodies decreased their capacity to secrete IL-6 after 72 hours of incubation. Our results may suggest the involvement of different regulatory factors, with paracrine/autocrine effects, on the capacity of SKOVE cells to secrete cytokines. These results should be considered in future immunotherapy strategies.

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URINARY CYTOKINE LEVELS IN CANCER PATIENTS UNDERGOING RADIOTHERAPY. RM JOHNKE, JM Edwards, CJ Kovacs, BM Daly, MJ Evans, TK Lee, UL Karlsson, H Arastu, K Christie, MR Salehpour. East Carolina University, Greenville, NC 27858.

It has been hypothesized that a disregulation of the body's normal cytokine homeostasis may be responsible for the development of the late complications sometimes associated with radiotherapy (xRT). In order to test this hypothesis, we initiated studies investigating the expression of proinflammatory (TNF-α, IL-6, IL-α & IL-β) and profibrotic (TGF-β) cytokines in the urine of prostate cancer patients undergoing fractionated, pelvic xRT. Patients studied (n=20) were selected on the basis of the requirements established for RTOG protocols 94-08 & 94-13. Urine samples were collected from patients prior to xRT, weekly during xRT, and at follow up visits and analyzed for cytokine content using ELISA procedures. The results obtained demonstrated that urinary TGF-β was readily detectable in all patients, and its levels underwent two distinct waves of elevation during the course of xRT in over half (12) the patients studied. Detectable levels of IL-1α & IL-1β were also found in almost all patients, and 7 patients showed a pronounced peak in IL-1α during xRT. Most patients demonstrated no significant fluctuation in IL-1β levels, however. Additionally, 10 patients had detectable IL-6 levels in their urine, of which 6 patients demonstrated significant IL-6 fluctuations during xRT. In contrast, levels of urinary TNF-α were below detectable limits in all patients throughout the time course of this study. Supported by Grant # CA75948 from NIH.

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CYCLIC ENHANCEMENT OF PLASMA CYTOKINES ASSOCIATED WITH THE INFLAMMATORY RESPONSE IN PROSTATE PATIENTS RECEIVING PELVIC IRRADIATION (XRT). C.J. Kovacs, B.M. Daly, M.J. Evans, and R.M. Johnke. East Carolina University School of Medicine, Greenville, NC 27858

While the gastrointestinal response to xRT is traditionally viewed as a depletion of potentially mitotic cells, increasing evidence suggests that cytokines constitute a humoral component of the radiation response resulting in acute periods of inflammation followed by the late development of fibrosis. In order to determine whether plasma cytokines have prognostic value for these normal tissue reactions, inflammatory (IL-1α/β; TNFα/β; IL-6; and M/GM-CSF) and fibrotic (TGFβ) cytokines were measured in the plasma of patients (n=30) receiving a course of pelvic + prostate boost xRT for prostate cancer. Characteristic of a majority (25/30)of the patients were two cycles of enhanced plasma levels of IL-1α, M-CSF and IL-6, separated by a wave of elevated TGFβ. The magnitude of the cytokine waves of IL-1α, M-CSF, and IL-6 within individual patients ranged from 175-411%, 146-321%, and 142-286% of pretreatment values, respectively, while the TGFβ values ranged from 195-360% of pretreatment values. A significant ($p < 0.05$) correlation existed between the temporal appearance of these enhanced cytokine waves and the integral xRTdose (ID). IL-1α, M-CSF and IL-6 were elevated after IDs of ~ 9 Gy and ~ 43. 2 Gy, respectively, In several patients (n=3), a second wave of TGFβ was first observed during the xRT boost and was maintained for months during the followup to the completion of xRT. Corresponding clinical response data for these patients will be presented. Supported by Grant CA75948 from NIH, DHHS.

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MULTIPLE MYELOMA: AN OVERVIEW REGARDING RESULTS OBTAINED BY THE USE OF INTERFERON ALPHA. V.M. LAUTA, Department of Biomedical Sciences and Human Oncology - Section of Internal Medicine and Clinical Oncology, University of Bari, Medical School: Italy.

The effectiveness of the interferon in clinical trials including multiple myeloma patients has its source in a large number of in vivo and in vitro studies wich use either myeloma cell lines or bone marrow plasma cells. Clinical trials conducted on IFN in multiple myeloma can be divided into three categories: a) previously untreated patient; trials of IFN alone and IFN plus CT; b) patients in relapse or refractory to previous treatment; trials of IFN alone and IFN plus CT; c) patients responding to conventional chemoterapy; randomized studies of IFN as maintenance therapy. The treatment of IFN alone as induction therapy in untreated patients is not usefull because the response rate obtained is lower than in chemotherapy treated patients. There is not a homogeneity of results on the use of combined chemotherapy plus interferon versus chemotherapy or interferon used either as single agent or in combination with chemotherapy. Results of prospective randomized studies suggest that interferon may be usefull as maintenance therapy in patients achieving a "minimal disease" with standard chemotherapy.

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CONTRASTING ANTI-TUMOR ACTIVITY OF IL-1 ALPHA AND IL-1 BETA IN CYTOKINE-GENE-TRANSFER APPROACHES .
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IL-1 consists of a family of two proteins, namely IL-1α and IL-1β, which overlap in their biological activities, in their recombinant form, and bind to the same receptors. Their differential localization in the producing cell that may determine their physiological functions; IL-1α is active as a cytosolic precursor and as a membrane-associated form, whereas IL-1β is active only as a secreted product. Previously, we have shown the anti-tumor and therapeutic potential of fibrosarcomas transfected with the precursor of IL-1α. In this study the same violent fibrosarcomas were transfected with IL-1β-encoding cDNA constructs; the mature form of IL-1β and the mature form of IL-1β linked to a signal peptide to allow active secretion through the ER-Golgi pathway. To our surprise, IL-1β did not lose their tumorigenicity and were even more malignant than the violent parental tumor cells. This is in contrast to the IL-1α transfectants, derived from the same parental fibrosarcoma cells which completely regress. This marked differences in the tumorigenicity of IL-1α versus IL-1β transfectants stem form differences in the immunogenicity of the IL-1 expressing cells; tumor cell-associated IL-1α is a strong adjuvant, whereas IL-1β expression by the malignant cells does not affect their low immunogenicity. In addition, tumor cell-associated IL-1α and IL-1β differentially affected molecules that are involved in tumor progression. Thus, IL-1α expression in the fibrosarcoma cells down-regulated metalloproteinase (MMP)-9 and MMP-2 expression, whereas in IL-1β transfectants these genes were unregulated, as compared to the violent cells. The complex effects of IL-1α and IL-1β on the tumor cells and the tumor microenvironment are being elucidated in our lab.

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DEFINING A ROLE FOR INTERFERON-γ IN TUMOR IMMUNITY AND AUTOIMMUNITY RESULTING FROM DNA IMMUNIZATION.
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Xenogeneic DNA immunization with melanosomal differentiation antigens is an effective means of generating both tumor immunity and autoimmune depigmentation. We have previously shown that immunization of mice with human tyrosinase-related protein-2 (hTRP-2) leads to protection from syngeneic tumor challenge and treatment of established tumor as well as autoimmune depigmentation via a mechanism requiring both CD4+ and CD8+ T-cells. Based on the above data implicating a Th-1 response, we investigated the importance of IFN-γ in tumor immunity and autoimmunity by immunizing IFN-γ knockout ($IFN\gamma^{-/-}$) mice by gene gun with hTRP-2 DNA. No protection from syngeneic tumor challenge with B16 melanoma was observed in $IFN\gamma^{-/-}$ animals, in comparison to wild type mice and $IL4^{-/-}$ mice where >80% protection has been routinely found. Interestingly, the number of lung metastases present in untreated $IFN\gamma^{-/-}$ mice was at least 50% greater than in untreated wild type mice, reinforcing the importance of IFN-γ in endogenous tumor surveillance mechanisms. Rapid and extensive autoimmune depigmentation of coat occurred in wild type and $IL4^{-/-}$ mice, whereas relatively little depigmentation was seen in the $IFN\gamma^{-/-}$ animals. Injection of IFN-γ protein following the completion of immunization did not restore tumor immunity or autoimmunity, suggesting that IFN-γ is necessary during the immunization phase.

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TNF AND PDGF INDUCE CHANGES IN ACCESSIBILITY AND ASSEMBLY OF THE MCP-1 PROMOTER.
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The murine monocyte chemoattractant protein-1 (MCP-1) is transcriptionally regulated by a variety of cytokines such as tumor necrosis factor (TNF), platelet-derived growth factor-α (PDGF-BB), and interferon-γ. Proper regulation of MCP-1 is important for normal inflammatory responses. Induction of MCP-1 by TNF requires two regions, a proximal and a distal regulatory region, which are separated by 2.3 kb of DNA. Both regions are unoccupied prior to the expression of the gene. Using a novel Sp1 mutant cell line, data are presented that show that Sp1 is required for expression and that Sp3, which can bind to the site can provide a partial level of activity. We also show that the Sp1 binding site is required for PDGF induction and that treatment of cells with trans-retinoic acid, which partially inhibits PDGF induction of MCP-1, functions by blocking the binding of Sp1 to its site in vivo. The distal region requires two NF-κB binding sites, both of which are required for TNF but not PDGF induction. TNF induction also requires the Sp1 site in the proximal region; however, unlike PDGF, retinoic acid does not inhibit TNF-induced expression or accessibility of factors to the DNA. These results were suggestive of the requirement for a coactivator for function. To determine if the CBP/p300 coactivator complex was required, experiments were performed with mutant and wild-type forms of E1A, a viral protein known to block CBP/p300 function. These results show a clear requirement for coactivator function and suggest that the assembly of factors and the activation of transcription of MCP-1 requires multiple steps including chromatin accessibility, factor assembly, and coactivation of transcription.

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ANALYSIS OF CCR4 KNOCKOUT MICE IN MURINE MODELS OF INFLAMMATION.
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CCR4, a high affinity receptor for the CC chemokines TARC and MDC, is expressed in thymus and spleen, and also by peripheral blood T cells (predominantly Th2), macrophages, platelets and basophils. To study the in vivo role of CCR4, we have generated CCR4-deficient mice (CCR4-/-) by gene targeting. CCR4-/- mice developed normally and no microscopic changes were evident in the thymus or spleen. The role of CCR4 in T cell differentiation as well as in allergic airway inflammation was analysed using the CCR4-/- mice. Our study demonstrated unexpected results for a role of CCR4 in Th2-mediated lung inflammation as well as in innate immunity.

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CCR8 Expressed by Monocytes or Monocyte Derived Dendritic Cells is a receptor for I309, TARC and LEC

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Earlier studies of lymphoid cells observed that virally encoded chemokines and I309, but not TARC were high affinity ligands for CCR8 In this study we investigated the ligands using CCR8 in primary myeloid cells and CCR8/HEK transfectants. We observed that human monocytes and immature monocyte derived dendritic cells (DCs) express CCR8, but not CCR4 and migrate in response to TARC, I309 and LEC. Competitive binding studies using monocytes or CCR8/HEK cells showed the affinity order to be I309≈TARC > LEC. LEC induced maximum migration of primary myeloid cells and CCR8/HEK transfectants at 83 nM and CCR8/HEK-293 cell adhesion at 10.4 nM. The molar concentration of LEC required to induce maximum cell migration was 200 fold greater than that for I309, but cell adhesion was induced by equal concentrations of both chemokines, suggesting that LEC may be a more effective inducer of cell adhesion than cell migration. TARC induced maximum migration at 12.5 nM. Thus, capacity of CCR8 to interact with certain ligands appears to be cell type dependent. Furthermore, TARC and LEC as well as I309 participate in CCR8 mediated trafficking of monocyte and DCs.

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TGFβ1 SELECTIVELY UP-REGULATES CCR1 EXPRESSION IN PRIMARY MURINE ASTROCYTES

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Chemokine receptors dictate the cellular responses to chemokines on target cells. Therefore, the regulation of expression of chemokine receptors is likely a crucial point for the regulation of chemokine action. TGFβ has potent effects on the proliferation, function, or survival of both neurons and glia and is involved in pathogenesis of some CNS disorders. The purpose of this study is to investigate the capability of TGFβ1 to regulate chemokine receptor expression in primary murine astrocytes using RNAse protection, immunofluorescence flow cytometry and cell migration assays. Our results demonstrate that expression of CCR1 by primary mouse astrocytes is increased after TGFβ1 stimulation. TGFβ1 increased CCR1 mRNA accumulation and CCR1 protein expression and augmented chemotactic responses to a physiological ligand, MIP-1α. Further, we provide evidence that TGFβ1-induced CCR1 mRNA accumulation occurs at the transcriptional level and is cell-type specific. This is the first evidence that TGFβ1 may modulate central nervous system inflammation in part by affecting chemokine receptor expression on astrocytes.

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FRACTALKINE AND CX_3CR1 mRNA EXPRESSION IN ISCHEMIC BRAIN INJURY AND EFFECT OF FRACTALKINE ON RELEASE OF PROINFLAMMATORY CYTOKINES BY MICROGLIA.

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To characterize the putative involvement of fractalkine and its receptor in brain inflammation, the mRNA expression and the effect of fractalkine on the release of proinflammatory cytokines by microglia was analyzed. Quantitative Northern blot analysis revealed, that the expression of fractalkine and CX_3CR1 was increased in the lesioned hemisphere after transient middle cerebral artery (MCA) occlusion in mice. While fractalkine message was significantly increased at 1 and 6 h after lesion, CX_3CR1 levels were significantly increased 3-14 days after the lesion. A slight increase of CX_3CR1 message in the non-lesioned hemisphere was also observed. In contrast to transient ischemia, no increased levels of fractalkine and CX_3CR1 were detectable after permanent MCA occlusion. Exposure of rat primary microglia to fractalkine results in a dose-dependent (10-300 nM) release of TNFα, Il-1β, and IL-6. Fractalkine-induced cytokine release was not inhibited by vMIP-II, rather tended to be increased, while Ca^{2+}-mobilization in rat CX_3CR1-transfected cells was effectively inhibited. Exposure of microglia to MIP-1α and MCP-1 (1-100 nM) did not result in release of cytokines. These data indicate that fractalkine and CX_3CR1 are upregulated in a model of transient ischemic brain injury. Moreover, fractalkine induces release of proinflammatory cytokines from microglia, the exact mechanism of which remains to be elucidated. Fractalkine may play an important role in the pathophysiology of brain inflammation.

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Total Chemical Synthesis of Fluorescently-labeled Chemokines

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Chemical protein synthesis is a powerful tool that enables the *site-specific* labeling of proteins with a variety of biophysical probes. Peptide segments are synthesized by highly optimized chemistry, specific residues are *selectively* de-protected and labeled. Labeled peptides are then combined to form a full-length polypeptide via native chemical ligation. Once folded, these proteins are invaluable reagents for biophysical measurements where precise labeling is required. The study of protein-protein interactions by fluorescence resonance energy transfer and fluorescence anisotropy is greatly facilitated with these well defined labeled proteins. We have applied this labeling strategy to the synthesis of fluorescent and luminescent chemokines and other larger proteins.

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DIFFERENCES IN RECEPTOR BINDING AND SIGNALING-TRANSDUCING PROPERTIES OF IP-10, MIG AND I-TAC REVEALED THROUGH THE CHARACTERIZATION OF AMINO-TERMINAL TRUNCATION MUTANTS OF CXCR3

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IP-10, MIG and I-TAC are three structurally related CXC chemokines bind to and activate CXCR3, a receptor implicated in the preferential recruitment of activated T-cells to sites of inflammation. The elaboration of CXCR3 chemokines by diverse cell types such as keratinocytes, astrocytes and monocytes is interferon γ – inducible, but the level of IP-10 protein can also selectively and differentially be upregulated by other inflammatory stimuli like bacterial lipopolysaccharide. The selective production of IP-10 in some *in vivo* host defense situations suggests that this ligand may have unique binding or signaling properties at CXCR3 not shared with MIG and I-TAC. In an effort to dissect out potential differences in binding interactions between CXCR3 and its various ligands, we generated, expressed and characterized three mutant CXCR3 receptors (Δ15, Δ29, Δ37), each with incrementally longer truncations in the receptor's amino terminus. We found that whereas the binding affinity of IP-10 for these N-terminal truncation mutants was virtually identical to its affinity for the full length receptor, progressive removal of residues in the amino terminus of CXCR3 resulted in progressive and ultimately profound losses in binding affinity for both MIG and I-TAC. We also found that whereas in transfected RBL cells the Δ15 receptor mutant was equivalently competent to the wild type receptor in its ability to mobilize calcium in response to the different CXCR3 ligands, the Δ37 or Δ29 CXCR3 truncations were uniformly signal incompetent, unable even to support calcium mobilization mediated by IP-10 despite the high binding potency it exhibits for these receptors. Therefore residues downstream of the amino acid at position 15 in the amino terminus appear to be required by the receptor to transduce chemokine- mediated signals. As the Δ29 and Δ37 truncations eliminated respectively one or both of two potential N-linked glycosylation sites in the amino terminus of CXCR3, we explored the effects of N-glycosylation to chemokine binding and CXCR3 mediated signal transduction by characterizing full length receptors where these asparagines were converted to alanines. Collectively the data suggest that covalent modifications to the amino terminus of CXCR3 impact differentially on the ability of this promiscuous receptor to bind ligands other than IP-10, but globally on its ability to transduce some chemokine mediated signals.

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DIFFERENTIAL GLYCOSYLATION OF THE NEUTROPHIL CXC-CHEMOKINE RECEPTOR 2: HIGH GLYCOSYLATION IS VITAL FOR MAINTAINANCE OF ITS SURFACE-EXPRESSION

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The chemokine receptor CXCR-2 mediates activation of neutrophils by CXC-chemokines such as neutrophil-activating peptide 2 (NAP-2). CXCR-2 is a heptahelical G protein-coupled receptor containing three sits for potential N-glycosylation. In this study we demonstrate intracellular and surface expression of differentially glycosylated CXCR-2 in neutrophils by means of immunoprecipitation using our monoclonal antibody to CXCR-2 (mAb RII115). Surface-expressed CXCR-2 was analysed following its crosslinking to 125I-labeled NAP-2 on intact neutrophils. After subsequent immunoprecipitation a single 64 kD receptor-ligand complex was identified by electrophoresis and autoradiography. Treatment with N-glycosidase F shifted its apparent molecular weight to 46 kD, indicating that surface-expressed CXCR-2 carries N-linked carbohydrates of 18 kD. To address surface-expressed and intracellular CXCR-2 the immunoprecipitated receptor itself was visualized using biotinylated mAb RII115. A 56 kD protein, and three additional smaller proteins were recognized. Upon treatment with N-glycosidase F all precipitated proteins migrated at 38 kD indicating that heterogeneity was due to differential N-glycosylation of CXCR-2. However, in contrast to the less glycosylated proteins, only the highly glycosylated 56 kD receptor is expressed on the cell surface as shown by selective precipitation of surface-expressed receptors. When glycosyl residues of surface-expressed CXCR-2 were removed by incubating intact cells with N-glycosidase F the receptor was downregulated from the cell surface and the cellular response to NAP-2 was reduced. Both effects did not occur in the presence of PMSF indicating that high glycosylation of surface-expressed CXCR-2 is relevant for its resistance to PMSF-sensitive neutrophil proteases. Thus by preventing degradation of surface-expressed CXCR-2 its high glycosylation may help to maintain neutrophil responsiveness to CXC-chemokines in inflammatory lesions.

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Expression and Release of Chemokines Associated with Apoptotic Cell Death in Human Promonocytic U937 Cells and Peripheral Blood Mononuclear Cells

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To further characterize mechanisms which may determine the fate of apoptotic cells, we investigated chemokine expression in apoptotic U937 cells or PBMC. Exposure of U937 cells to the anti-cancer drug etoposide (VP-16) or the nitric oxide (NO) donor DETA-NO, both inducers of apoptosis in these cells, was associated with increased expression of the chemokines IL-8 and MIP-1α. Upregulation of IL-8 mRNA expression by VP-16 or DETA-NO was observed as early as 4h or 6h respectively, after onset of treatment and was still detectable in the viable cell fraction after 19h of exposure. In accordance with data obtained using U937 cells, we observed that incubation with the chemotherapeutic drug 2-chlorodeoxyadenosine (CdA) upregulated release of IL-8 from adherent PBMC in parallel to induction of apoptosis. In these cells a modest but significant induction of TNFα release by CdA was also detected. Moreover, CdA augmented release of IL-8 from whole blood cultures. By facilitating adequate recruitment of phagocytes to sites of cell death, stress-induced upregulation of chemokines during cellular pertubations associated with apoptosis may contribute to mechanisms aiming at efficient removal of apoptotic cells.

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THE LD78β ISOFORM OF MIP-1α IS THE MOST POTENT HIV-1 INHIBITING CHEMOKINE AND CCR5 AGONIST

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LD78α and LD78β are two highly related non-allelic genes which code for different isoforms of the human CC chemokine macrophage inflammatory protein-1α (MIP-1α). LD78α and LD78β proteins only differ in three amino acids. Two natural forms of LD78β (7,778 Da and 7,793 Da) were purified from conditioned media of stimulated peripheral blood mononuclear cells. The chemotactic potency of the two LD78β variants was 100-fold higher on mouse lymphocytes compared to that of LD78α. On the contrary, LD78α was only 2-fold less efficient than LD78β in chemotaxis assays on human lymphocytes and monocytes. Both molecular forms of LD78β proved to be much more potent than LD78α in inducing an intracellular calcium rise through CC chemokine receptor 5 (CCR5). This preferential binding of LD78β, compared to LD78α and RANTES, to CCR5 resulted in a 10 to 50-fold higher potency in inhibiting infection of peripheral blood mononuclear cells by CCR5-using (R5) HIV-1 strains. In conclusion, LD78β is more potent than any other chemokine in inhibiting HIV-1 infection and can be considered as a potentially important drug candidate for the treatment of infection with R5 HIV-1 strains.

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CHEMOKINES AND THEIR RECEPTORS IN THE OVARIAN TUMOUR MICROENVIRONMENT.
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We have studied CC-chemokines and their receptors in human ovarian cancer and ascites. mRNA for five CC-chemokines (MCP-1, 2, MIP-1α, β, and RANTES) was detected in a majority of samples. Chemokine protein was found at significant levels in ascitic fluid, with MCP-1 present at the highest concentration. MCP-1 was also the predominant chemokine in the solid tumour, with levels of expression correlating with the extent of the $CD8^+$ and $CD68^+$ infiltrate. mRNA for the corresponding CC-chemokine receptors (CCR1, 2, 3, 4, and 5) was strongly expressed by cells in the tumour ascites. However CCR1 was the only chemokine receptor consistently found in solid tumours, and CCR2b, the main receptor for MCP-1, could not be detected. *In situ* hybridisation to CCR1 mRNA localised the receptor to infiltrating cells within the tumour. We suggest that MCP-1 may attract peripheral blood cells to the solid tumour, where the microenvironment downregulates CCR2b and other CC-chemokine receptors. CCR1 may then be responsible for trafficking of infiltrating cells within the tumour.

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QUANTITATION OF MRNA LEVELS FOR CCR2 AND T CELL AND MONOCYTE SPECIFIC MARKERS AT SITES OF INFLAMMATION.
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In order to follow the migration of lymphoid cells into sites of inflammation we used the ABI PRISM 7700 Sequence Detection System to quantify relative mRNA levels for T cell (CD3 and CD25) and monocyte/macrophage specific (CD14 and CD68) markers and for the chemokine receptor CCR2. The delayed hypersensitivity response (DH) was used as a model of inflammation. Mice were sensitized i.v. with sheep red blood cells (SRBC) followed 4 days later by SRBC challenge in a footpad. Swelling was measured 24 hours later by mercury displacement plethysmography and immediately thereafter foot pads were collected and quickly frozen in liquid nitrogen. RNA was extracted, mRNA purified and quantitative PCR measurements were carried out. Significant (3-20 fold) increases were observed depending on the mRNA species tested. Treatment of mice with indomethacin during the 24 hour period of the secondary challenge reduced footpad swelling by 70-80% without a significant reduction in mRNA levels. These results indicate a recruitment of monocytes and T cells to the site of inflammation in this DH model and possibly the up regulation of CCR2. Quantitative PCR analysis offers a number of advantages over other approaches such as flow cytometry and microscopy to study cell trafficking. It is sensitive, quantitative and allows for the relatively rapid analysis of a large number of samples. Furthermore, tissues from which cells can not be recovered easily can be analyzed.

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THE BIOLOGICAL ROLE OF CHEMOKINE RECEPTOR CXCR2 ON ENDOTHELIAL CELLS.
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CXCR2 is a seven-transmembrane G protein-coupled receptor that mediates signals in response to IL-8, MGSA/GRO and other ELR^+ CXC chemokines. Several reports indicate that CXCR1 and CXCR2 are present on endothelial cells (ECs) within the tumor environment. The biological role of CXCR2 in ECs remains to be understood. In the present study, we have investigated the expression and functional role of CXCR2 in ECs *in vitro*. Flow cytometry experiments with monoclonal antibody against CXCR2 demonstrated no expression of CXCR2 on two cultured EC cell lines, ECV304 and HMEC-1. To explore the role of CXCR2 in ECs, ECV304 was stably transfected with CXCR2 (ECV304/CXCR2). We demonstrate ligand induced receptor internalization in EC304/CXCR2. We also show that the CXCR2-expressing EC304 cells chemotax well and mobilize calcium in response to both IL-8 and MGSA/GRO. Chemotaxis as well as calcium flux were blocked by CXCR2 inhibitors, SB225002 and Antileukinate. These data indicate that the CXCR2-transfected EC304 cell line could serve as an excellent model system to explore receptor function and ligand induced CXCR2 mediated signal transduction in endothelial cells. It could also be used to investigate the role of CXCR2 in angiogenesis.

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EXPRESSION OF FUNCTIONAL CCR6 IN CYTOKINE-STIMULATED HUMAN NEUTROPHILS.
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It has been reported that neutrophils can acquire features characteristic of antigen-presenting dendritic cells (DC). In the present study, we found that cytokine-stimulated human neutrophils can be induced to express CCR6, the receptor for liver and activation-regulated chemokine (LARC), also known as macrophage inflammatory protein-3α. CCR6 mRNA was not detectable in freshly isolated neutrophils, whereas CCR6 mRNA was readily detectable in neutrophils cultured with cytokine-rich culture supernatant of phytohemagglutinin-stimulated peripheral blood mononuclear cells. This induction was partially inhibited by antibody against either TNF-α or IFN-γ. Incubation of neutrophils with recombinant TNF-α or IFN-γ also induced the expression of CCR6 mRNA dose-dependently with the peak at 3 to 6 h after stimulation. When neutrophils were incubated with both TNF-α and IFN-γ, these cytokines synergistically increased the expression of CCR6 mRNA. In contrast, GM-CSF did not induce the expression of CCR6 mRNA. However, GM-CSF augmented the expression of CCR6 mRNA induced by TNF-α and IFN-γ. The binding of ^{125}I-labeled LARC to stimulated neutrophils could be inhibited by unlabeled LARC in a dose-dependent manner. By chemotaxis assay, cytokine-stimulated neutrophils also migrated to LARC in a dose-dependent manner. These data clearly indicate that neutrophils express functional CCR6 upon stimulation with cytokines such as TNF-α and IFN-γ. Since CCR6 is highly expressed on immature DC, CCR6 expression may be one of the phenotypic characteristics neutrophils acquire in the process of developing properties of DC.

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SYNTHETIC PEPTIDES DERIVED FROM THE N-TERMINUS OF THE VIRAL CHEMOKINE VMIP-II DISPLAY DISTINCT INTERACTIONS WITH CXCR4

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The viral Macrophage Inflammatory Protein-II (vMIP-II) encoded by Kaposi's sarcoma-associated herpesvirus is unique among all known chemokines in that vMIP-II shows a broad-spectrum interaction with both CC and CXC chemokine receptors including CCR5 and CXCR4. To elucidate the mechanism of this promiscuous receptor interaction, synthetic peptides derived from the N-terminus of vMIP-II were studied. A peptide corresponding to residues 1-21 of vMIP-II was shown to interact with CXCR4 but not CCR5. This peptide selectively prevented CXCR4 signal transduction and coreceptor function in mediating the entry of T- and dual-tropic HIV-1 isolates, but not those of CCR5. In addition, this peptide strongly induced the internalization of CXCR4. Further studies of this peptide and other analogs revealed different sites on CXCR4 for binding and internalization. Such results led to a model for the chemokine-receptor interaction.

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LIF AND OSM CONTACT LIF-R VIA THE IG-LIKE DOMAIN AND THE MEMBRAN DISTAL CYTOKINE BINDING DOMAIN

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IL-6 type cytokines influence a multitude of biological functions. IL-6 signals via a gp130 homodimer after binding to the IL-6 receptor (IL-6R), whereas leukemia inhibitory factor (LIF) and oncostatin M (OSM) directly induce a gp130 and LIF receptor (LIFR) heterodimer. The LIFR has two cytokine binding domains (CBD) linked by an Ig-like domain (Ig-D), whereas gp130 lacks the second CBD.

Chimeras of hu LIFR and hu IL-6R were constructed in which either the membrane distal (R ΔI) or membrane proximal (R ΔII) CBD of LIFR were substituted by the CBD of the IL-6R. In additional constructs, the membrane distal (L ΔI) or the membrane proximal domain (L ΔII) of LIFR were completely deleted.

Upon expression in COS-7 cells only radioactively labeled R ΔII and L ΔI could be immunoprecipitated with sepharose-coupled LIF, suggesting that the Ig-D of the LIFR is responsible for binding of LIF. The constructs were also transfected into Baf/3 cells expressing human gp130. Only transfection with RΔII, but not the other constructs, yielded proliferating Baf/3 clones and activated membrane complexes after stimulation with LIF and OSM. Baf/3-gp130-RΔII also could be stimulated with IL-6 and IC-7, a recently developed chimera of IL-6 and CNTF that first binds to the IL-6R, but signals via a gp130/LIFR heterodimer. Substitution of both CBDs and the Ig-D of the LIFR by the IL-6R CBD (RΔI+II) resulted in Baf/3-gp130-RΔI+II clones inducable by IL-6, but not the other cytokines

Our results suggest that the membrane proximal CBD of LIFR only serves as a spacer which brings the membrane distal CBD and the Ig-D into the right positon for forming an activated receptor complex. The Ig-D of the LIFR appears to be sufficient for LIF-binding, but not for mediating LIF-bioactivity.

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FUNCTIONAL EXPRESSION OF FAS LIGAND BY HUMAN PANCREATIC CARCINOMAS: A POTENTIAL MECHANISM OF TUMOR-INDUCED SUPPRESSION OF IMMUNE SURVEILLANCE

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CD95 ligand (CD95L/FasL) is a pro-apoptotic protein whose extracellular domain can be released from the cell surface by proteolytic cleavage. Cell-associated CD95L induces apoptotic cell death by engaging its cognate receptor (CD95/Fas receptor/Apo-1) on the target cells, followed by recruitment and activation of intracellular enzymes of the caspase family, which initiate and execute the apoptotic process. High levels of CD95L expression have been observed in some solid malignancies including hepatocellular, colon, and pancreatic carcinomas. Tumor-associated CD95L has been considered as part of the "counterattack" of tumor cells against immune effector cells. In the present study, we investigated the relative contribution of secreted CD95L expressed by pancreatic carcinoma cells to killing of target T cells in vitro. We demonstrated that (1) media conditioned by pancreatic carcinoma cells caused apoptosis of Jurkat cells, (2) soluble, tumor derived CD95L contributed significantly to this effect and, in contrast to Jurkat cells, pancreatic carcinoma cells themselves were resistant to induction of apoptosis by autocrine, soluble CD95L. Moreover we found that progression of pancreatic carcinomas in tumor patients is associated with increased serum levels of both, soluble forms of CD95L and its receptor, CD95. These results suggest that tumor-derived FasL may represent a potential mechanism for local and peripheral deletion of tumor-reactive T-cell clones.

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IL-2 RECEPTOR-γ CHAIN (γ_c)-DEFICIENT MACROPHAGES EXHIBIT DIMINISHED RESPONSES TO IL-4 BUT NOT IL-13.

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The IL-4Rα chain can heterodimerize with either the IL-2Rγ chain (γ_c) to generate type-I IL-4R complexes or with IL-13Rα1 to generate type-II IL-4R complexes. IL-4 can signal through either type-I or type-II IL-4R, whereas IL-13 signals exclusively through type-II IL-4R. To determine if macrophages utilize one or both types of IL-4R for IL-4-induced signalling, we examined the role of γ_c in mediating IL-4-induced signal transduction in macrophages derived from normal and γ_c knockout mice. Pretreatment of normal macrophages with anti-IL-4Rα antibodies blocked induction of STAT6 activity by both IL-4 and IL-13, demonstrating that IL-4Rα is an essential component of both type-I and type-II IL-4R. In contrast, anti-γ_c antibodies inhibited activation of STAT6 by IL-4- but not IL-13. Analysis of IL-4- and IL-13-induced responses in macrophages from control (γ_c+) and γ_c knockout mice showed that lack of γ_c expression results in markedly reduced IL-4 responsiveness. In contrast, IL-13-induced signalling was equivalent in γ_c^+ and γ_c^- macrophages. STAT activation by other cytokines, including IFN-γ, GM-CSF, and IL-10, was similar in γ_c^+ and γ_c^- macrophages demonstrating that the absence of γ_c does not globally inhibit signal transduction by all cytokines. These findings demonstrate that although macrophages express both type-I and type-II IL-4R, IL-4-induced responses are primarily mediated via type-I IL-4R.

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NATURAL SPLICING OF EXON 2 OF THE HUMAN INTERLEUKIN-15 RECEPTOR ALPHA CHAIN mRNA RESULTS IN A SHORTENED FORM WITH DISTINCT PATTERN OF EXPRESSION. S. DUBOIS, F. Magrangeas, P. Lehours, S. Raher, J. Bernard, O. Boisteau, S. Leroy, S. Minvielle, A. Godard, Y. Jacques. Unité INSERM 463, Institut de Biologie, 9 Quai Moncousu, 44035 Nantes Cedex 01, France

We report the existence of 8 different IL-15Rα transcripts resulting from exon splicing mechanisms within the IL-15Rα gene. Two main classes of transcripts can be distinguished which contain or not (Δ2 isoforms) the exon 2 coding sequence. Both classes were expressed in numerous cell lines and tissues (including peripheral blood lymphocytes) at comparable levels, could be transcribed in COS-7 cells and the proteins expressed at the cell surface. Both receptor forms displayed numerous glycosylation states, reflecting differential usage of a single N-glycosylation site as well as extensive O-glycosylations. Whereas IL-15Rα bound IL-15 with high affinity, Δ2IL-15Rα was unable to bind IL-15, thus revealing the indispensable role of the exon-2 encoded domain for cytokine binding. A large proportion of IL-15Rα was expressed at the nuclear membrane with some intranuclear localization, supporting a potential direct action of the IL-15/IL-15Rα complex at the nuclear level. In sharp contrast, Δ2IL-15Rα was only found in the non-nuclear membrane compartments, indicating that the exon-2 encoded domain (which is shown to contain a potential nuclear localization signal) plays an important role in receptor post-translational routing. Together, our data indicate that exon-2 splicing of human IL-15Rα is a natural process which might play regulatory roles at different levels.

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DIFFERENCES IN THE ABILITY OF THE p75 AND p55 TNF SOLUBLE RECEPTORS TO INHIBIT SPONTANEOUS AND TNF-INDUCIBLE IL-8 AND IL-1 RECEPTOR ANTAGONIST PRODUCTION IN HUMAN WHOLE BLOOD CULTURES.
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IL-8 production is primarily under the control of IL-1 and TNF and therapeutic successes of IL-1 and TNF blockade in patients with rheumatoid arthritis are, in part, likely due to reduction in IL-8 production. We employed whole human blood cultures from healthy volunteers to assess the efficacy of the p75-Fc and truncated p55-TNF receptor constructs to inhibit spontaneous and TNFα-induced IL-8 and IL-1 receptor antagonist (IL-1Ra) production. After 6, 24 and 48 hours at 37°C, the whole blood cultures were assayed for IL-8 and IL-1Ra. At 5 μg/mL, the p75-Fc construct induced an unexpected increase (212%, p=0.026) in the spontaneous production of IL-8 whereas the p55 construct had no effect. In cultures of TNFα-inducible IL-8, the p55 construct exhibited a dose dependent inhibition of IL-8 production (84% at 5 μg/mL). In contrast, the p75-Fc construct exhibited a U-shaped dose response curve (86% inhibition at 640 ng/mL but only 35% at 5 μg/mL, p<.01). A monomeric form of p75 also unexpectedly increased TNFα-induced IL-8 (at 2.5 μg/mL 166% increase, p<0.05). TNFα-induced IL-1Ra production was reduced more significantly by the p75-Fc construct (83%) compared to the p55 molecule (56%, the difference in % inhibition p75-Fc vs p55; p<0.01). In contrast to these results in whole blood cultures, the p75-Fc construct exhibited a straight line dose-response inhibition of TNFα-induced IL-8 production from A549 human lung epithelial cell line. Thus, the human whole blood assay reveals a difference in the ability of these two soluble TNF receptor constructs to inhibit natural and recombinant TNFα-inducible pro- and anti-inflammatory cytokines and may have clinical relevance.

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STUDY OF CYTOKINES BINDING TO HL-60 CELLS
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Human promyelocytic line HL-60 cells were shown to express specific receptors for IFN-α, IFN-β, IFN-γ, IL-1β, IL-1α, IL-2, IL-6, TNF-α. For each of listed proteins kinetic characteristics of binding to HL-60 cell receptors were determined (K_d of ^{125}I-labeled ligand/receptor complex and density of the receptors). It was found that during the process of HL-60 cell differentiation by granulocyte pathway (regardless of what kind of stimulus was used) high-affinity binding of [^{125}I]HuIL-1β (K_d = 0,32 nM), [^{125}I]HuIFN-γ (K_d = 0,28 nM) and [^{125}I]HuIL-6 (K_d = 0,075) was transformed to a low-affinity one (K_d values for differentiated cells were 13,3, 10,0 and 2,1 nM, respectively). At the same time, differentiated HL-60 cells displayed 2,4-fold increase in [^{125}I]HuTNF-α receptors density comparing to non-differentiated cells. Thus, while HL-60 cells differentiate by granulocyte pathway their sensitivity strongly decreases in the case of IL-1β, IFN-γ and IL-6 and increases in the case of TNF-α. The character of [^{125}I]HuIFN-α, [^{125}I]HuIFN-β and [^{125}I]HuIL-2 binding to HL-60 cells was not altered during the differentiation process. On the whole, the results obtained together with previously reported findings that IL-1β, IL-6, TNF-α and IFN-γ expression is elevated in differentiating HL-60 cells, suggest that these proteins and their receptors participate in the process of cell differentiation.

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MANNOSE-6-PHOSPHATE / INSULIN-LIKE GROWTH FACTOR-II RECEPTOR MEDIATES INTERNALIZATION AND DEGRADATION OF LEUKEMIA INHIBITORY FACTOR, BUT NOT SIGNAL TRANSDUCTION. L. DUPLOMB, F. Blanchard, S. Raher, P. Vusio, B. Hoflack, Y. Jacques, Anne Godard. Unité INSERM 463, Institut de Biologie, 9 Quai Moncousu, 44035 Nantes Cedex 01, France

Leukemia Inhibitory Factor (LIF) is a multifunctional cytokine belonging to the Interleukin-6 subfamily of cytokines, all of which use the gp130 subunit for signal transduction. The specific receptor for LIF, gp190, binds this cytokine with low affinity and is also required for signal transduction. We have recently reported that glycosylated LIF produced by transfected Chinese Hamster Ovary (CHO) cells also binds to a lectin-like receptor, the Mannose-6-Phosphate/Insulin-like Growth Factor II receptor (Man-6-P/IGFII-R)[J Biol Chem 273, 20886]. The present study shows that (i) man-6-P containing LIF is naturally produced by a number of normal and tumor cell lines; (ii) other cytokines in the interleukin-6 family do not bind to Man-6-P/IGFII-R; (iii) another unrelated cytokine, (M-CSF) is also able to bind Man-6-P/IGFII-R in a man-6-P sensitive manner. No functional effects or signal transduction mediated by this lectin-like receptor were observed in various biological assays after LIF binding, and man-6-P containing LIF was as active as non-glycosylated LIF. However, man-6-P sensitive LIF binding resulted in rapid internalization and degradation of the cytokine on numerous cell lines, suggesting that Man-6-P/IGFII-R could be an important regulator of LIF metabolism and bioavailability.

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TYROSINE PHOSPHORYLATION AND THE CLEAVAGE AND SECRETION OF ANGIOTENSIN-CONVERTING ENZYME. I. SEN, R. Sadhukhan, and K.R. Santhamma, Cleveland Clinic Foundation, Cleveland, OH 44195.

In this study, we show that enhancement of tyrosine phosphorylation of cells by vanadium compounds, which are potent inhibitors of phosphotyrosine phosphatases, induced a rapid time- and concentration-dependent increase in the cleavage and secretion of angiotensin-converting enzyme (ACE). Pervanadate-induced tyrosine phosphorylation and proteolysis occur in ACE89 cells, a mouse epithelial cell line permanently transfected with the testicular isozyme of ACE (ACE_T) and in primary cultures of rabbit renal proximal tubule epithelial cells expressing endogenous pulmonary ACE (ACE_P). Increased secretion was completely abolished by treatment with Compound 3, a hydroxamic acid-based inhibitor of a specific class of metalloproteases. We have shown previously that phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), enhances proteolytic cleavage of ACE_T. Pretreatment of ACE89 cells with a specific inhibitor of PKC (bisindolylmaleimide GF 109203X) completely abolished PMA-induced cleavage and secretion of ACE_T, but had no effect on pervanadate-stimulated ACE_T cleavage, indicating that pervanadate's action is independent of the action of PKC. Moreover, unlike pervanadate treatment, PMA treatment was not accompanied by any significant increase in cellular tyrosine phosphorylation. Thus, pervanadate and PMA employ different mechanisms to enhance the cleavage and secretion of ACE, but both pathways converge to a metalloprotease-dependent step.

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SCID-LIKE LYMPHOCYTE DECREASE IN SSI-1/SOCS-1/JAB TRANSGENIC MICE. Minoru Fujimoto, Tetsuji Naka, Reiko Nakagawa, Akihiro Tateishi, Yoshinori Kawazoe, Yoshiaki Morita, Masashi Narazaki, Hiromi Fujiwara, Takashi Nagasawa and Tadamitsu Kishimoto, Osaka Univ. Medical School, OSAKA, 565-0871, JAPAN

Previous experiments in vitro suggest that STAT-induced STAT inhibitor (SSI) -1 functions as a negative feedback regulator of cytokines by inhibiting JAKs. Being SSI-1 predominantly expressed in lymphoid organs, we generated 2 types of SSI-1 transgenic mice, Ick-SSI-1 and Eμ-SSI-1, which target the expression of SSI-1 in T and B cell lineage, respectively. Lymphocytes over-expressing SSI-1 showed reduced response to cytokine stimuli such as IFNγ, IL-6 and IL-7, suggesting that SSI-1 can inhibit cytokine signalings in primary lymphocytes. Furthermore, In Ick-SSI-1 Tg mice, T cells were reduced due to the developmental defect of early thymocytes and increased apoptosis of peripheral T cells. In Eμ-SSI-1, bone marrow B cells were decreased especially at pre-B stage. These phenotypes resemble that of mice lacking JAK3 or γc, model animals for human SCID, suggesting that SSI-1 inhibits JAKs also in vivo. Further studies are underway to characterize some phenotypic differences between SSI-1 Tg mice and those SCID models.

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IMMUNOMODULATING PROPERTIES OF THE PsaA ANTIGEN OF *YERSINIA PESTIS*

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PsaA protein (pH6 antigen) of *Yersinia pestis* is a virulence factor whose gene, *psaA*, is positively regulated at the transcriptional level by low pH. PsaA protein is a novel bacterial Fc-receptor with limited species specificity. The protein forms immune complexes with IgG1, IgG2 and IgG3, but cannot be bound to human IgG4 and rabbit, mouse and sheep IgG. We studied immunomodulating activities of recombinant PsaA protein of *Y.pestis*. It was established that PsaA did not influence the IL-2 synthesis and secretion by mouse splenocytes. Suppression of the proliferative activity of IL-2 took place after addition of PsaA to the culture of ConA-induced T-blasts of mouse splenocytes. Mouse and human tumour cells demonstrate their sensitivity to PsaA action, which is manifested in suppression of the proliferative response. The experiments using mouse tumour cells indicate that EL-4, a line of T-cell origin, is the most sensitive one to the presence of PsaA. In experiments using human tumour T- and B-cell lines, the most sensitive lines to PsaA are CCRF (T cell line) and Daudi (B-cell line). PsaA protein significantly increase the level of TNFα mRNA. in HL-60 cells. It was shown that PsaA protein causes activation of TNFα synthesis in HL 60 cells. These results allow speculating that cytotoxicity of PsaA may be mediated via TNFα.

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COMPARATIVE STUDY OF BIOLOGICAL ACTIVITY OF HUMAN IL-1β AND CAPSULAR SUBUNIT PROTEIN CAF1 OF *Y. PESTIS*

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Previously we identified a statistically significant level of similarity between the capsular subunit protein Caf1 of *Y.pestis* and IL-1α, IL-1β and IL-1ra - central cytokines of the immune system. To compare immunomodulating properties of recombinant Caf1 of *Y.pestis* and human IL-1β, the influence of these proteins on proliferation of mouse thymocytes stimulated by PHA and ConA at submitogenic doses (1,0-1,5 μg/ml) was studied. Proteins were purified using Pharmacia FPLC system. It was shown that IL-1β and Caf1 did not contain LPS. CBA line mouse at the age of 10-12 weeks were used in the experiments. The DNA synthesis (proliferation) was measured by ^{3}H-thymidine incorporation. Capsular subunit protein Caf1 was shown to stimulate the PHA- and ConA-dependent proliferation of the thymocytes at doses of 2,4 ng/ml. The level of cell proliferation in the presence of Caf1 was 3,5-8 times higher in comparison with the control ones. It was shown that the dimeric form of capsular protein Caf1 of *Y. pestis* (obtained after boiling of Caf1 polymer) interacts with IL-1β but not with IL-1α or IL-ra.

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INDUCTION OF ANTI-IL-9 AUTO-ANTIBODIES REVEALS A KEY ROLE FOR IL-9 IN INTESTINAL PARASITE INFECTIONS.

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The *in vivo* production of neutralizing anti-IL-9 antibodies was induced in mice by immunization with mouse IL-9 coupled to ovalbumin. Mice developed a long lasting anti-IL-9 response with inhibitory titers of 10^{-3} to 10^{-5}, irrespective of the mouse strains. This treatment completely abrogated the elevation of mast cell protease MMCP-1 levels as well as eosinophilia consecutive to implantation of an IL-9-secreting tumor. We took advantage of this model to assess the role of IL-9 during infections by parasites such as *Trichuris muris*, as IL-9 production has been reported to correlate with resistance against this nematode. C57BL/6 mice that are resistant to T. muris became susceptible to the infection when they were subjected to anti-IL-9 immunization, demonstrating that IL-9 plays a critical role in this model. Neutralization of IL-9 also inhibited blood eosinophilia induced by the parasite but did not affect intestinal mast cell numbers, seric MMCP-1 levels nor the anti-parasite antibody response. Taken together, our data provide us with a new potent strategy to antagonize IL-9 activity in vivo and demonstrate that this cytokine plays a major role for resistance against *T. muris* infection.

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THE CONFORMATIONAL PROPERTIES OF IL-1-LIKE CAPSULAR PROTEIN CAF1 OF *Y.PESTIS* AND HUMAN IL-1β.

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Structural properties of two homologous proteins capsular protein Caf1 from *Yersinia pestis* and human interleukin 1β (huIL-1β) were compared, using such spectroscopic methods as circular dichroism (CD), fluorescence (ANSF) and infrared spectroscopy (FTIR). The FTIR spectra reflects the most important finding, that these two proteins have very close (almost identical) secondary structure. The CD spectrum of huIL-1β is typical of the β-structural proteins, whereas Caf1 is characterized by very unusual far UV CD spectrum. One more important point should be emphasized, that is the structure of Caf1 is practically independent of the pH. Both near and far UV spectra of this protein do not change with the decrease in pH from 7.5 to 2.0. Neither huIL-1β, nor Caf1 bind ANS. In other words, these proteins do not have the appropriate sites for the interaction with hydrophobic fluorescent probe.

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EFFECT OF BACTERIOPHAGES ON TNF-α, IL-6 AND IFN PRODUCTION BY HUMAN PERIPHERAL BLOOD CELLS (PBC).

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The role of bacteriophages in stimulation of cytokines has not been investigated. The aim of this study was to examine „in vitro" ability of Staphylococcus, Escherichia and Pseudomonas phages to induce release of TNF-α, IL-6 and IFN by PBC of healthy volunteers. Human whole–blood cultures were incubated 20 h with suspension of bacteriophages and purified one. Supernatants were assayed for TNF-α, IL-6 and IFN. The measurement of cytokines activity was performed using bioassays. We showed that levels of TNF-α, IL-6 and IFN (~10 U/ ml) were low after stimulation of blood cells by purified phages. The lysates of bacteriophages induced a little higher (~20 U/ml) release of these cytokines. In summary, we demonstrated that phages induced weak immunological response apart from bactericidal properties.

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INFLUENCE OF PSEUDOMONAS AERUGINOSA EXOPOLISACCHADYD ON RELEASE OF IL-6 AND IL-10 BY HUMAN PERIPHERAL BLOOD-CELLS (PBC).

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Pseudomonas aeruginosa is one of important pathogens for compromised hospitalized patients. This microorganism produces an exopolisaccharide mucoid substance (alginate). The aim of this study was to examine „in vitro" ability of alginate antigen Pseudomonas aeruginosa to induce release of IL-6 and IL-10 from PBC of human. The whole-blood cultures were incubated 20h (37°C, 5% CO_2) with 50μg, 100μg and 200μg of exopolisaccharide Pseudomonas aeruginosa. The measurement of IL-6 activity was performed using bioassays. The level of IL-10 was determined by immunoenzymatic test. The extracellular antigen induced synthesis of IL-6 by PBC. The highest levels were detected by using doses of 100μg and 200μg of crude extract. The synthesis of IL-10 by PBC after induction of exopolisaccharide antigen was depended on doses. The low spontaneous production of IL-10 by blood cells cultures was elevated significantly (5 or 6 times) after stimulation of antigen. The level of IL-10 varied inversely as dose of antigen. The lowest activity of IL-10 was observed after stimulation of cells by 200μg/ml of antigen.

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CD40-CD40L INTERACTIONS MODULATE DEFENSE AGAINST DISSEMINATED CANDIDIASIS THROUGH ENDOGENOUS TNF

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CD40/CD40L are members of the tumor necrosis factor (TNF)/TNF-receptor family of molecules. Their interaction modulates synthesis of TNF and interleukin-1 (IL-1), cytokines important for the defense against Candida albicans. Infection with $5x10^5$ CFU C. albicans resulted in a significantly higher mortality in CD40L knock-out (CD40L-/-) mice (88%) than in control CD40L+/+ mice (22%, $p<0.05$). While the outgrowth of the microorganisms in the kidneys of CD40L-/- and CD40L+/+ mice did not differ significantly on day 8 (4.7 ± 0.5 vs. 5.3 ± 0.3 log CFU/g, $p>0.05$), there was a significantly increased yeast load in the kidneys of CD40L-/- mice on day 15 of infection (7.2 ± 0.7 vs. 5.7 ± 0.6 log CFU/g, $p<0.05$). The peak TNF plasma concentrations were measured on day 3 of infection, when plasma TNF levels were significantly lower in the CD40L-/- mice compared with control CD40L+/+ (40 ± 12 vs. 92 ± 23 pg/ml, $p<0.05$). Plasma concentrations of IL-1α and IL-1β were below detection limit in all samples. An increased susceptibility to disseminated candidiasis was also observed when wild-type mice were treated with blocking antibodies against CD40L. In conclusion, lack of CD40-CD40L interactions results in increased susceptibility to disseminated infection with C. albicans. Because endogenous TNF is instrumental for the defense against Candida, and CD40L-/- mice responded with lower production of this cytokine, the observed effects were probably mediated through inadequate production of endogenous TNF.

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NEUTRALIZATION OF INTERLEUKIN-18 REDUCES NEUTROPHIL TISSUE ACCUMULATION AND PROTECTS AGAINST LETHAL ENDOTOXEMIA

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Interleukin-18 (IL-18) main function is to induce IFNγ synthesis in the context of co-stimulation with microbial products or IL-12. In addition, IL-18 has direct proinflammatory effects by inducing synthesis of the proinflammatory cytokines tumor necrosis factor (TNF), IL-1β, and the chemokines IL-8 and macrophage inflammatory protein-2 (MIP-2). Therefore, we hypothesized that IL-18 may be involved in the pathogenesis of lethal endotoxaemia. Indeed, IL-1β converting enzyme (ICE) deficient mice, lacking both mature IL-18 and IL-1β, appeared completely resistant to Escherichia coli LPS (100% survival). In contrast, both wild type and IL-1β-/- mice were equally susceptible to the lethal effects of LPS (0% survival). Treatment of mice with anti-IL-18 antibodies completely protected the animals against a lethal injection of LPS (100% vs. 0% in the placebo-treated controls, $p<0.01$). The increased survival in anti-IL-18 treated animals was accompanied by decreased levels of IFNγ (92% inhibition, $p<0.01$) and MIP-2 (52%, $p<0.05$). TNF concentrations were similar in treated and control mice. In addition, anti-IL-18 treatment reduced neutrophil infiltration and protected against tissue damage induced by endotoxin, as measured by decreased myeloperoxidase levels in the lungs (51%, $p<0.05$) and liver (79%, $p<0.05$) of IL-18-treated mice. In conclusion, IL-18 is a major mediator during lethal endotoxemia, and its mechanism of action probably involves neutrophil-mediated tissue damage.

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THE ROLE OF MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) IN CYTOTOXIC T LYMPHOCYTE DEVELOPMENT. R. ABE, J. Sailors, T. Peng, C. Metz, and R. Bucala, The Picower Institute for Medical Research, Manhasset, NY 11030.

Previously we reported that anti-MIF mAbs blocked antigen-specific T cell activation (Bacher et al. 1996) and suppressed tumor growth in vivo (Chesney et al. 1999). In this study, we investigated whether this effect is mediated through the regulation of cytotoxic T lymphocyte (CTL) activity. Initially, we revealed that spleen cells obtained from antigen (OVA)-immunized mice secrete high levels of MIF following re-stimulation of antigen in vitro. We observed an increase in CTL activity when spleen (responder) cells from mice primed by injection of EG.7 (OVA expressing) cells were stimulated in vitro with irradiated EG.7 (stimulator) cells in the presence of anti-MIF mAbs. Examination of the culture supernatants obtained from the co-culture of responder and stimulator cells in the presence of anti-MIF mAbs showed elevated levels of IFNγ, but not TNFα, IL-2, or IL-12. Using an in vivo CTL activity model, the administration of anti-MIF mAbs to tumor-bearing mice slowed tumor growth when compared to control mAb-treated animals. Histological examination of these tumors revealed an accumulation of T cells and apoptotic tumor cells within the mass, suggesting an induction of CTL activity in animals treated with anti-MIF mAbs. These data indicate that MIF plays an important immunoregulatory role in the development CTL and anti tumor immunity.

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INDUCTION OF INTERACTIONS BETWEEN CD44 AND HYALURONIC ACID BY A SHORT EXPOSURE OF HUMAN T CELLS TO DIVERSE PRO-INFLAMMATORY MEDIATORS. Amiram Ariel, Rami Hershkoviz, Liora Cahalon, Betty Schwartz, and Ofer Lider, Weizmann Institute of Science, and Hebrew University, Rehovot, Israel.

Migration of T cells into extravascular sites of inflammation is mediated by cell-cell and cell-matrix adhesion receptors, including the hyaluronan-binding glycoprotein, CD44. The biochemical nature of variants of CD44 and the ligand specificity, function, and the regulation of activation of CD44 expressed on various cell types have been extensively studied. However, little is known about the short-term influence of cytokines and chemokines on activation of CD44 on human T cells. We studied the involvement of inflammatory mediators in regulation of human peripheral blood T cell adhesion to hyaluronan. We found that the CD44-dependent adhesion of T cells to hyaluronan requires T cell activation of 2-3 hours and is regulated by cross-linking of CD3, certain cytokines (IL-2 and TNFα), and certain chemokines (MIP-1β, IL-8, and RANTES). The CD44-dependent T cell adhesion required active protein synthesis and modification (sulfation and O- and N-linked glycosylation) of the CD44 molecule. This T cell adhesion was manifested by polarization, spreading, co-localization of cell surface CD44, and by the re-arrangement of cytoskeleton in hyaluronan-bound T cells. Thus, cytokines and chemokines present at sites of immune reactions are capable of rapidly activating CD44 molecules expressed on T cells.

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A NOVEL GENE IN THE INTERLEUKIN-1 CLUSTER.

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We report cloning of a novel member of the interleukin-1 (IL-1) family, IL-1L1. Its cDNA was isolated by a combination of solution phase hybridisation with a YAC clone covering the IL-1 cluster, cDNA library screening and 5' RACE. The gene, which has a similar intron-exon structure to the IL-1ra gene, is situated between the IL-1β and the IL-1ra genes. Its cDNA contains a 550bp open reading frame encoding a 17kDa protein that shares 47% amino acid identity with IL-1ra but lacks a signal sequence and glycosylation sites. Comparison of human and mouse IL-1L1 shows a high degree of conservation in the coding region. IL-1L1 mRNA shows restricted expression and was detected in human placenta, peripheral blood mononuclear cells, and in the trophoblastic cell line, JEG-3. A 17kDa protein was specifically immunoprecipitated from metabolically labelled JEG-3 cell lysates with rabbit anti-IL-1L1 serum. Recombinant IL-1L1, tested up to 0.1 μM, failed to stimulate IL-6 production from fibroblasts or endothelial cells, or to block significantly the activity of 0.1 nM IL-1β or interleukin-1α. Control IL-1ra, produced in parallel, was fully biologically active. We conclude that IL-1L1 is not an IL-1RI ligand, nor does it have agonistic IL-1-like effects through other receptors on fibroblasts or endothelial cells.

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INTERFERON INDUCING AND ANTIVIRAL ACTIVITIES OF PROTHYMOSIN α, PEPTOFERON α AND ALBEFERON

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The possibility to use compounds of peptide nature - prothymosin α (ProTα), peptoferon α and albeferon as interferon inductors was studied. Intraperitoneal injection of ProTα to rabbits in the dose of 3 mg/kg increased the level of serum interferon in hour after introduction, which reached its maximum by the end of the second hour and was equal to 128 (IU/ml). 48 hours later the second peak of interferon activity was observed and was equal to the first one. Introduction of peptoferon α caused gradual increase in the level of serum interferon in rabbits for 24 hours. The maximal serum activity was observed in 24 hours. In experiments *in vitro* using osteosarcoma cells MG-63 it was found that albeferon had direct antiviral activity. Treatment of embryonal human lung fibroblasts with ProTα or peptoferon α dose-dependently stimulates antiviral activity of these cells.

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MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST THE PORCINE INTERLEUKIN 12 MOLECULE

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IL-12 is a heterodimeric glycoprotein that is composed of unrelated 35kDa and 40 kDa subunits. The p40 subunit is constituatively expressed, however both units are necessary for biological activity. The array of activities of the IL-12 cytokine suggest clinical uses as an anti-tumor and anti-viral agent, in restoring immunity in immune supressed individuals and in combating opportunistic infections in immune supressed patients. The analysis of IL-12 may show an important role in xenotransplantation. Porcine rIL-12 was used to immunize mice and rabbits for the production of antibodies. As a result, seven porcine IL-12 specific monoclonal and four IL-12 polyclonal antibodies were produced. Each monoclonal and polyclonal was further screened by western blot. Reactivity was seen against porcine IL-12 at 70 kDa (whole molecule), as well as at 40 kDa (p40 subunit). A preliminary ELISA pair, polyclonals 1335 and 1502, was found to be specific in the detection of recombinant and natural IL-12 . The high standard of the assay, 2 ng/ml, gave an OD of 0.940 and the low standard of the assay, 31 pg/ml, gave an OD of 0.162. Sensitivity was found to be less than 30 pg/ml. Stimulated samples (*in vivo* LPS presentation) gave values at time 0 of 0 pg/ml, yet at approximately 1000 pg/ml at time 24 hours. These antibodies are shown to be excellent tools in research involving porcine IL-12 .

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MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST THE PORCINE INTERLEUKIN 1β MOLECULE

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IL-1β is a 17 kDa protein that has a wide range of biological activities involved around mounting and maintaining an inflamatory response. Most clinical efforts around IL-1β are based on inhibiting IL-1 action. Porcine rIL-1β was used to immunize mice and rabbits for the production of antibodies. As a result, 22 porcine IL-1β specific monoclonal and two IL-1β polyclonal antibodies were produced. A monoclonal fusion was performed using antigen presented mice. Spleens from mice with high serum antibody titers against IL-1β were fused with SP2/0 cells. Growing clones were screened via direct ELISA against porcine rIL-1β. Each monoclonal and polyclonal antibody was further screened by western blot. Reactivity was seen against porcine IL-1β at approximately 17 kDa. A preliminary ELISA using a polyclonal antibody designated as 1337, was found to be specific in the detection of recombinant and natural IL-1β . The high standard of the assay, 2 ng/ml, gave an optical density of 2.12 and the low standard of the assay, 31 pg/ml, gave an optical density of 0.448. Sensitivity was found to be less than 30 pg/ml. Stimulated samples (*in vivo* LPS presentation) gave values at time 0 of 0 pg/ml, yet at ~ 450 pg/ml at time 24 hours. One would expect these trends as the action of IL-1β has been shown to increase with the presentation of LPS. These antibodies are shown to be excellent tools in research involving porcine IL-1β.

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REGULATION OF BLYS EXPRESSION IN MONOCYTES AND DENDRITIC CELLS. B. Nardelli, O. Belvedere, H. Olsen, V. Roschke, S. Sosnovtseva, A. Garcia, E. V. Cochrane, D. K. Morahan, J. Giri and D. M. Hilbert, Human Genome Sciences Inc., Rockville, MD 20850

BLyS (B Lymphocyte Stimulator) is as a recently described B cell growth factor expressed in myeloid cells. The aim of the present study was to investigate the regulation of BLyS expression in monocytes and dendritic cells in response to cytokines. On monocytes, FACS analysis indicated that constitutive membrane BLyS expression was not significantly changed by pro-inflammatory cytokines, such as TNF-α or IL-1β, but was strongly enhanced by IL-10 and IFN-γ. IL-4 and IL-13 were potent inhibitors of IL-10-induced upregulation of BLyS, but were less effective in inhibiting IFN-γ-induced upregulation. Analysis of BLyS gene expression indicated that treatment with IFN-γ increased BLyS mRNA levels. On dendritic cells, IFN-γ but not IL-10 appeared to upregulate cell surface BLyS expression. In summary, we have identified cytokines acting as positive or negative regulators of BLyS expression. IFN-γ was the most potent regulator, increasing cell surface protein levels on both monocytes and dendritic cells. The regulation of BLyS expression on antigen-presenting cells by type II interferon together with the selective binding of BLyS to B cells are suggestive of an important role for this molecule in immune processes.

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VIRUS-INDUCED PROIL-18 PROCESSING BY CASPASES-1 AND -3 IN HUMAN MACROPHAGES.
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Macrophages play a significant role in host's defense system. Activated macrophages produce a number of cytokines and one of the key cytokines released during virus infection is IL-18. We have studied IL-18 mRNA and protein expression in primary human macrophages in response to influenza A and Sendai virus infections. Monocyte-derived, GM-CSF-differentiated macrophages expressed constitutively IL-18 mRNA and proIL-18 protein but only virus-infected cells could release biologically active, mature IL-18. Similarly to IL-1β, activation of IL-18 requires cleavage of its proform by caspase-1 enzyme. Pro-IL-18 is also cleaved by another, apoptosis-linked caspase: caspase-3. Both caspase-1 and caspase-3 mRNA and protein expression was induced as a result of virus infections. To examine this association of virus infection, caspase activation, and IL-18 production, we treated virus-infected macrophages with specific caspase-1 and caspase-3 inhibitors. The inhibitors had crucial effect on virus-induced IL-18 expression. ELISA analysis and measurement of IFN-γ-inducing activity of macrophage supernatants revealed that caspase inhibitors blocked the release of biologically active IL-18 from macrophages. Western blotting analysis of cell extracts confirmed that the decreased IL-18 production was due to inhibited processing of proIL-18 into mature IL-18.

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MITOGENIC ACTIVITY INDUCED WITH γ-IRRADIATION IN $CD4^-CD8^-$THYMIC CELL LINE. V. Shichkin, V. Talaev, E.Protsak, A. Yarilin, Institute of Immunology, Moscow 115478, Russia.

A thymic cell line was induced in BALB/c mice by injections of human IL-2. The cell line had phenotype of $CD4^-CD8^-$ T-precursors and secreted a mitogenic activity (THGF) in response to γ-irradiation. THGF directly stimulates action proliferation of nonactivated thymocytes. We established that γ-irradiation at doses of 10-12 Gy strongly increased synthesis and secretion the factor, which was not initially secreted spontaneously. However, the spontaneous level and induced secretion were practically equivalent once the cell line was established. Like thymocytes, the mitogenic activity also appeared when spleen or lymphatic node cells were used as target cells. Only 1h incubation of thymocytes in presence of the factor was enough to induce proliferative response in 5-day test culture. However, the maximal effect was obtained after 24 h incubation of thymocytes with the factor. Thymocytes, which were selected by γ-irradiation at doses of 10-50 Gy, could respond with increased proliferation to THGF, but not to IL-2. However, thymocytes preincubated with THGF acquired capacity to respond also to IL-2 without additional comitogenic stimulus. Analysis of cell populations responding to the factor showed that thimic THGF-sensitive cells are radioresistant $SC\text{-}1^+PNA^+CD25^+CD4^-CD8^-$ thymocytes. In spleen responding cells were presented in both Ig^+ and Ig^- populations. Moreover, the factor stimulated formation of haemopoietic spleen colonies, if administered alone or together with $Ig^-SC\text{-}1^-Thy\text{-}1^-$ bone marrow cells to lethally irradiated mice. Thus, induction by γ-irradiation of mitogenic activity, which is probably an autocrine growth factor for radioresistant stem cells of thymus and acts also as colony stimulating factor for bone marrow and splenic precursors, reflects an evolved mechanism aimed at restoration of immune system after damaging action of radiation.

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THE INTRACELLULAR ROLE OF PRECURSOR IL-1 ALPHA (pIL-1α). A. Werman, R. M. White, M. Dobkin, E. Voronov, R. Venkert, [2]C.A. Dinarello and R. N. Apte. Ben-Gurion University, Beer-Sheva, Israel and [2]University of Colorado, Denver, CO, USA.

The IL-1 family, of which IL-1α and IL-1β are the agonist members, is a family of pleiotropic cytokines involved mainly in initiation and propagation of the inflammatory response. IL-1α is expressed in a wide array of cells under homeostatic conditions, and when produced is rarely secreted. In most cells it remains intracellular and as such it has been linked to several biological phenomena, such as gene regulation, differentiation, cell cycle arrest and promotion of cell senescence. A mechanism for any of these intracellular roles is lacking. We demonstrate here that IL-1α translocates into the nucleus in cells that secrete it (macrophages) and cells that do not secrete it (fibroblasts) under similar stimuli. In addition, translocation in macrophages is inhibited by exogenous IL-1Ra. Nuclear translocation is associated with modulation of gene expression (collagenase and MMP9 in fibroblasts). We used the yeast GAL4 system in order to assess a possible role of intracellular IL-1α in transcription; in this system the potential of a sequence to activate transcription can be assessed and quantified. pIL-1α showed a 100-fold activation over control levels. A two-hybrid screen was next used to identify IL-1α binding proteins. We screened 2,500,000 clones and identified three genes encoding: (1) HAX-1- an HS-1 (Src TK member)-interacting protein. It is found in the mitochondria and nucleus, two transcriptionally active sites and shares homology with members of the Bcl2 anti-apoptosis family; (2) Cyclin G1 associated protein- a protein with unknown function. Amino-acid analysis suggests this protein to be nuclear associated; (3) A novel protein not related to known genes. It possesses a zinc-finger, suggesting that it acts as a DNA binding protein.
Elucidating the nature of IL-1α interaction with these proteins is pivotal to a better understanding of the nature of IL-1α as an intracellular effector molecule distinct from IL-1β.

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DETECTION OF INTERLEUKIN 18 FAMILY EXPRESSION IN RAT BRAIN BY RT-PCR. R.D. Wheeler, M.D. Hall*, N.J. Rothwell, G.N. Luheshi, School of Biological Sciences, University of Manchester, Manchester, UK and *Parke-Davis Research Institute, Cambridge, UK.

Interleukin 18 (IL-18) is the most recent interleukin to be identified and is a key mediator in the peripheral immune system. It has been implicated in pathological conditions such as diabetes and multiple sclerosis but little is known about its function within the central nervous system. The IL-18 family now includes the IL-18 receptor (IL-18R), accessory protein (AcPL) and binding protein (IL-18BP), all of which were recently discovered in peripheral tissue but have not to date been detected in the brain. We have previously described the widespread expression of IL-18 in rat brain and have now extended these studies to investigate the presence of the other members of this family by using reverse transcription polymerase chain reaction. Various rat brain regions were studied, especially hypothalamus. These studies demonstrate, for the first time, IL-18R, AcPL and IL-18BP expression. These results should help to elucidate the functional role of IL-18 within the central nervous system.

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The role of sIL-2R in evaluation of immunocorrecting therapy efficacy in kidney allotransplantat recipients.

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In standard insignificant quantity of IL-2R present in blood serum. However, production of IL-2R is change at some pathologies. For example, the IL-2R concentration in serum is very high in recipients with corpseal kidney in the cries moment. The inclusion in therapy anti T-lymphocient globulin (ALG) result in decrease level of this cytokine in serum. So, in the purpose control efficacy of immunocorrecting therapy in recipients with corpseal kidney we were determined sil-2R concentration. In all patients have been established a cries.
15 patients was investigated both in the cries moment and after anti T- lymphocient globulin (ALG) therapy. It was showed that after ALG therapy in 50 % of cases the level of sIL-2R was significantly lowered. The additional immunocorrectind therapy with Tactivin in other 50% of patients resulted in decrease of serum s IL-2R concentration only in 35% of them and correlated with clinical improve.
However, some patients (15%) unfavorable decease prognosis had steadfast high level s IL-2R.
Thus, the definition of s IL-2R concentration in steroidresistent patients with kidney allotransplantant can be used as criteria of immunocorrecting therapy efficiency.

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PROINFLAMMATORY CYTOKINES IN ACUTE INVASIVE GASTROENTERITIS

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Involvement of proinflammatory cytokines during first 72 h of acute invasive gastroenteritis (AIGE) was studied in 31 subjects (6 m-6y) and in7aged matched controls. Physical examination, history, stool and blood cultures, WBC were correlated to serum cytokine (IL-1ß,IL-6,IL-8, TNFα) levels before/after treatment. Extremely high IL-6 levels (49.4pg/ml) were detected only in bacterial AIGE($p<0.0001$). Increased IL-1ß (3.1pg/ml) and IL-8 (62.3pg/ml) in all patients($p<0.002$) with a tendency for higher levels in bacterial etiology ($p<0.07$) and higher TNFα levels (61.4pg/ml) only in the non-bacterial group, were found. Positive correlations between IL-8 and stools/day (r-0.62, $p<0.04$) and between WBC and % neutrophils in the bacterial group to elevated cytokine levels were found. In conclusion, while IL-1ß and IL-8 are elevated in bacterial AIGE, IL-6 discriminated best between bacterial/non-bacterial etiology.

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ANTI-INFLAMMATORY EFFECT OF IFN-ß BY TWO CONCURRENT PATHWAYS ON HUMAN LYMPHOCYTES AND MONOCYTES, RESPECTIVELY. **D. BURGER, F. Brunner, C. Modoux, and J.-M. Dayer, Division of Immunology & Allergy, University Hospital, CH-1211 Geneva 14, Switzerland.**

Direct contact between T lymphocytes (T) and monocyte-macrophages (Mo) is one of the hallmarks of inflammatory lesions. To date, this appears to be the principal endogenous mechanism that triggers production and release of large amounts of pro-inflammatory cytokines in Mo. We have attempted to answer the question whether IFN-ß modulates this pro-inflammatory mechanism through T, Mo, or both. The effect of rhIFNß (Ares-Serono) was assessed on: (i) co-coltures of isolated Mo or T in double chambers, stimulated by phyto-hemagglutinin (PHA), (ii) the induction of monocyte-activating factors on stimulated T, and (iii) isolated Mo activated by plasma membranes from stimulated T. IFN-ß inhibited by 80 to 90% the induction of tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) ß production, but only when both T and Mo were in direct cellular contact. This was due to the inhibition of expression of monocyte-activating factors in plasma membrane of stimulated T lymphocytes, thus leading to a decrease. Concomitantly, IFN-ß increased twofold the production of IL-1 receptor antagonist (IL-1Ra) at protein and mRNA levels by acting directly on Mo when activated by contact with stimulated T. In conclusion, IFN-ß modulates T signalling of Mo by both diminishing the expression of monocyte-activating factors on stimulated T and directly increasing IL-1Ra production by Mo. This could account for the efficacy of IFN-ß in the treatment of patients suffering from autoimmune diseases such as relapsing-remitting multiple sclerosis.

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SUCCESSFUL INTERFERON-ALPHA THERAPY IN ATOPIC DERMATITIS OF BESNIER'S PRURIGO PATTERN WITH NORMAL SERUM IGE AND BLOOD EOSINOPHIL FRACTION: RANDOMIZED CASE-CONTROLLED STUDY. G.W. NOH and K.Y. Lee, Sungkyunkwan Univ. Sch. Med., Seoul, Korea 100-380 and Yonsei Univ. Col. Med., Seoul, Korea 120-749.

Interferon-alpha therapy has been tried with variable and even conflicting results. A randomized case-controlled study was tried for interferon-alpha therapy in atopic dermatitis of Besnier's prurigo pattern with normal serum IgE and normal blood eosinophil fraction. Interferon-alpha therapy was conducted on 14 non-responders to interferon-gamma and subsequent thymopentin therapy among 100 atopic dermatitis. In a total of 44 Besnier's prurigo patients, 13 Besnier's prurigo patients were treated by interferon-alpha therapy, 10 by interferon-gamma, 10 by thymopentin, and the remaining 11 were untreated as the control group. Among 100 atopic dermatitis, 14 patients were unresponsive to interferon-gamma and subsequent thymopentin therapy. Eight of them improved significantly by interferon-alpha therapy. Interferon-alpha responders showed characteristic skin lesions of the Besnier's prurigo pattern, normal serum IgE, and normal blood eosinophil fraction. For the randomized prospective case-controlled study, 44 patients who fulfilled the above characteristics were selected. By interferon-alpha therapy, 11 out of 13 Besnier's prurigo patient with normal IgE and normal blood eosinophil fraction improved their condition significantly, and 2 out of 10 improved by interferon-gamma therapy, and none improved by thymopentin therapy or in the untreated control group. Conclusively, interferon-alpha therapy may be effective on atopic dermatitis of Besnier's prurigo pattern with normal serum IgE and normal blood eosinophil fraction.

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PROTEOME ANALYSIS REVEALS UBIQUITIN CONJUGATING ENZYMES TO BE A NEW FAMILY OF IFN-α-REGULATED GENES. Tuula A. Nyman, Sampsa Matikainen, Timo Sareneva, Ilkka Julkunen, and Nisse Kalkkinen, Turku Centre for Biotechnology, BioCity 5th floor, P.O.Box 123, FIN-20521 Turku, FINLAND.

The proteins expressed by a genome or tissue are called "proteome". Unlike the genome, the proteome is not a fixed feature of an organism but is a subject to changes by translational control, posttranslational modifications or cellular proteolytic activity. Two-dimensional gel electrophoresis (2-DE) is currently the only analytical and preparative separation method capable of separating all, or at least the majority of the proteins in a proteome. We have used metabolic labeling and 2-DE followed by mass spectrometry and database searches to identify potentially new IFN-α -induced protein products in human T cells. With this analysis we show that IFN-α induces the expression of ubiquitin cross-reactive protein (ISG15) and two ubiquitin conjugating enzymes, UbcH5 and UbcH8. Northern blot analysis showed that IFN-α rapidly enhanced mRNA expression of UbcH5, UbcH6 and UbcH8 in T cells. These genes were also induced in macrophages stimulated with IFN-α or IFN-γ or infected with influenza A or Sendai viruses. Ubiquitin conjugation is a rate limiting step in antigen presentation and therefore it may be that the upregulation of UbcHs by IFNs contributes to the enhanced antigen presentation by macrophages.

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RAPID CONSTRUCTION OF ARTIFICIAL GENE CODING EQUINE INTERFERON ALPHA-1 FROM SHORT DNA OLIGOMERS BY A NEW METHOD.
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In spite of many advantages, artificial genes for cytokines have seldom been prepared due to the difficulty of the construction of long gene from short DNA oligomres. To improve the method for the construction of gene, a new method by which long gene is easily, rapidly constructed, was developed. Using plus gene and minus gene both as template and primer that have 20-40 bases of cohesive ends, a double stranded DNA is synthesized by the reaction of polymerase. We named this as "SPR method".

First, the gene sequence of equine interferon alpha-1 was designed under consideration of the creation of restriction site and the adaptation to the yeast codon usage. Then 11 of DNA short oligomers (each is 80mer) containing 5 of plus genes and 6 of minus genes, were synthesized by the DNA synthesizer. Each set of plus and minus gene was mixed and reacted with rTaq DNA polymerase. The analysis of electrophoresis showed that each product was 140bp of double stranded DNA. Then, similar reactions were repeated with the sets of these products. Final product was about 600bp of length, indicated that the full-length gene should be prepared. As the result of DNA sequencing, it was shown that this artificial gene had complete sequence of equine interferon alpha-1.

Within only a week, about 600bp of artificial gene was easily, rapidly, and completely constructed. More than 1000bp of gene will be able to prepare using SPR method.

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DE NOVO PROTEIN ALBEFERON SPECIFICALLY INTERACTS WITH MONOSIALOGANGLIOSIDES AND WITH HIGH-EFFICACY SUPRESSES THE PROLIFERATION OF CELLS
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It has been established that rhIFN-α2 and albeferon (ABF, *de novo* protein albebetin (ABB) with the grafted sequence 130-137 of hIFN-α2) are able to specifically bind to gangliosides. Binding of ^{125}I-rhIFN-α2 to Gm1,2,3 is characterised by K_d 45 nM, 12 nM and 78 nM, respectively. Binding is inhibited by unlabelled rhIFN-α2 and not inhibited by unlabelled rhIL-2 and rhIFN-γ. Unlabelled ABF competitively inhibited specific binding of ^{125}I-rhIFN-α2 to Gm1,2,3. K_i were 0.2 nM, 0.6 nM and 1.3 nM, respectively. Unlabelled ABB did not have inhibiting properties. One can conclude that the sequence 130-137 of hIFN-α2 participates in binding of hIFN-α2 and ABF to gangliosides. ABF supresses the proliferation of T-lymphocytes of human peripheral blood and lymphoblastoma MT-4 cells with the same efficacy as rhIFN-α2. It has been established that at a concentration of 50 nM Gm2 completely inhibits ^{125}I-rhIFN-α2 and ^{125}I-ABF ability to bind to T-lymphocytes and to abolish PHA-induced blast transformation of the cells. The results obtained suggest that a ganglioside-like component is a part of hIFN-α2 receptor on these cells.

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POSSIBLE ROLE FOR PROTEIN KINASE B IN THE ANTI-APOPTOTIC ACTION OF PROLACTIN. K.A. Al-Sakkaf, L. Mooney, P.R.M. Dobson, & *B.L. Brown. Oncology & Cellular Pathology and *Institute of Endocrinology, Sheffield Univ. Med. Sch., Sheffield, UK.

Prolactin (PRL) is a pleiotropic cytokine which acts as a mitogen and survival factor for a number of cell types including Nb2 lymphoma cells. However, the intracellular signalling pathways by which PRL promotes survival are unknown. We have previously shown that PRL caused the activation of Phosphatidylinositol 3-kinase (PI3-kinase) and the phosphorylation and activation of protein kinase B (PKB), a downstream component of the PI3-kinase pathway. We now show, using confocal microscopy, translocation of PKB to the membrane of Nb2 cells in response to PRL, an effect which was blocked by the PI3-kinase inhibitor LY294002 (10μg/ml). We have also examined the role of PKB in PRL-induced survival of Nb2 cells. Apoptosis was induced by dexamethasone (Dex: 100nM), staurosporine (0.1-1.0μM) or ionising radiation (10Gy). PRL (100ng/ml) inhibited apoptosis induced by Dex and ionising radiation; this effect of PRL was reversed by the addition of LY294002, which by itself induced apoptosis of Nb2 cells. In contrast, PRL had no effect on staurosporine-induced apoptosis. Some of these effects may be cell-specific since, although staurosporine induced apoptosis in MCF7 and T47D cells, dexamethasone and LY294002 did not. Interestingly, staurosporine induced DNA fragmentation and PARP cleavage in MCF7 cells in the absence of caspase-3 expression. However, even in these cells PRL did not rescue from staurosporine-induced apoptosis. These results suggest that the PI3-kinase/PKB pathway may mediate the anti-apoptotic effect of PRL, at least in Nb2 cells.

We are grateful to Yorkshire Cancer Research for funding.

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CHARACTERIZATION OF INTERLEUKIN-2 INDUCED IMMEDIATE-EARLY GENES

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Interleukin-2 plays a pivotal role in directing the proliferation, survival and functional differentiation of T lymphocytes. To elucidate further the mechanisms of IL-2 action, we have utilized a sulfhydryl-labeling and affinity purification method to identify IL-2-induced immediate-early genes from human T cells. Two of the cytokine-responsive (CR) genes, CR1 and CR6, exhibit distinct kinetics of IL-2-induced expression, with the former transiently induced from 2-4 hr, and the latter expressed throughout G1 and decreasing at the onset of S phase. The CR1 gene encodes a member of the regulator of G protein signaling (RGS) family, RGS16, and it inhibits signaling by Gq and Gi-linked seven transmembrane receptors. The CR6 gene encodes a small acidic protein which has high homology to GADD45 and MyD118, and regulates the cell cycle through induction of p21. These results indicate that IL-2 signals diverse T cell functions through the induction of specific target genes.

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REGULATION OF INTERLEUKIN-1β-INDUCED PDGF RECEPTOR-α EXPRESSION IN LUNG MYOFIBROBLASTS BY p38 MAP KINASE.

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The potential role of p38 mitogen-activated protein (MAP) kinase in platelet-derived growth factor receptor-α (PDGF-Rα) gene expression was investigated using cultured rat pulmonary myofibroblasts. p38 MAP kinase was constitutively expressed in myofibroblasts and activated by interleukin (IL)-1β. A pyridinylimidazole compound, SB203580, completely inhibited the ability of p38 MAP kinase activity to phosphorylate Phas-1 substrate. SB203580 inhibited IL-1β-induced up-regulation of PDGF-Rα mRNA and protein in a concentration-dependent manner. Moreover, the IL-1β-induced increase in the number of [^{125}I]PDGF-AA binding sites at the cell surface was reduced >70% by pretreatment with SB203580. Accordingly, an enhancement of PDGF-AA-stimulated DNA synthesis caused by IL-1β-induced up-regulation of PDGF-Rα was blocked >70% by SB203580. Treatment of cells with SB203580 following inhibition of mRNA synthesis by Actinomycin D significantly increased the degradation rate of PDGF-Rα mRNA that was up-regulated by IL-1β. These data indicate that IL-1β-activated p38 MAP kinase functions to stabilize PDGF-Rα mRNA, which contributes to increased cell-surface PDGF-Rα and enhanced PDGF-stimulated mitogenesis following IL-1β treatment.

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CONSTITUTIVE ACTIVATION OF STATs UPON HIV INFECTION.

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Cytokines are relevant for the pathogenesis and the therapy of HIV disease in that they are both targets for virus-induced immunologic dysregulation and important determinants of HIV replication in T lymphocytes and mononuclear phagocytes. In recent years, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway has been demonstrated to be a very rapid membrane-to-nucleus signaling system adopted by most cytokines, based on the activation of the STATs by JAK-dependent tyrosine phosphorylation. STATs ultimately dimerize and translocate from the cytosol to the nucleus where activate cytokine-inducible gene transcription. Although profound dysregulation of the cytokine network has been reported in HIV disease, very little is known on whether HIV infection can directly affect the JAK/STAT pathway. Aim of our study was to investigate the state of activation of the JAK/STAT pathway both in HIV infected individuals and during *in vitro* acute HIV infection. We have observed that approximately 75% of randomly selected HIV infected individuals are characterized by constitutive activation of the JAK/STAT pathway, mostly accounted for by STAT1 and C-terminal truncated variants of STAT5 (STAT5Δ). STAT5Δ retains binding but lacks the transactivating domain, as demonstrated by EMSA on their PBMC in the absence of *in vitro* stimulation. Furthermore, STAT5Δ is activated preferentially in $CD4^+$ T cells. Finally, an increased constitutive activation of STAT was detectable in resting PBMC acutely infected *in vitro* with either the HIV-1 X4/IIIB or R5/BaL strains compared to uninfected cells. Because truncated STAT5 isoforms may act as transdominant DNA binding molecules, their constitutive activation may have implications for both pathogenesis and immune reconstitution of the infected individuals. The effect of antivirals and immunotherapy on STAT activation is under evaluation.

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ANALYSIS OF INTERLEUKIN-1 SIGNAL TRANSDUCTION PATHWAYS IN RAT STRIATAL PRIMARY CULTURES. S. L. DUNN, M. D. Hall, S. McNulty and E. A. Hammond, Parke-Davis Neuroscience Research Centre, Cambridge University Forvie Site, Robinson Way, Cambridge, United Kingdom CB2 2QB.

We have used striatal primary cultures to investigate features of the IL-1 intracellular signalling cascade, with particular attention given to a MAP kinase pathway. Firstly we have shown that the cultures consist primarily of neuronal cells (>65%) using calcium imaging and immunocytochemistry (ICC) techniques. We have demonstrated, using western blotting, that p38 MAP kinase is phosphorylated in response to treatment with IL-1, and that differences exist between stimulation by the two forms of IL-1, IL-1α and IL-1β. Stimulation of p38 occurred after only 5 minutes incubation with IL-1α, and was maintained for a prolonged period, up to 90 minutes. IL-1β-induced p38 phosphorylation occurred later, after 40 minutes, and was maintained until 90 minutes. The receptor antagonist IL-1ra blocked p38 phosphorylation by both forms. The cellular localisation of p38 phosphorylation was investigated using ICC with cell-type specific fluorescent-labelled antibodies. Lastly we investigated IL-1 stimulated gene activation using quantitative PCR. Cultures were stimulated with IL-1α or IL-1β for 0.5, 2, 4, 6, 12, 18 and 24 hours, and the level of mRNA of a number of IL-1–responsive genes was assessed at each timepoint. This study therefore gives a wide-ranging representation of the effects of IL-1, from receptor to gene expression, in a neuronal background, and is the first time that IL-1 signalling pathways have been studied in striatal primary cultures.

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ANTIPROLIFERATIVE EFFECT OF INTERLEUKIN 1 (IL-1) IS MEDIATED BY P38MAP KINASE IN HUMAN MELANOMA CELLS A375: CONTRIBUTION OF ERK1/2

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The role of p38- and classical (ERK1/2) - MAPK in IL-1-induced growth inhibition was investigated using IL-1 sensitive human melanoma A375-6 or A375-C2-1 cells. In the cells, p38MAPK was activated by IL-1 stimulation. A selective inhibitor for p38MAPK, SB203580 almost completely recovered the IL-1-induced growth inhibition. Down-regulation of ornithine decarboxylase (ODC) activity as well as its protein level has been shown to be essential for IL-1-induced growth inhibition. SB203580 also reversed the IL-1-induced down-regulation of ODC activity and intracellular polyamine levels without affecting ODC mRNA level in cells. On the other hand, classical MAPKs (ERK1/2) were constitutively activated in the cells and IL-1 treatment did not alter their activation. MEK1/2 specific inhibitors, PD98059 inhibited the constitutive ERK1/2 activation and almost completely rescued the IL-1 induced cell growth inhibition. Unlike SB203580, IL-1-induced down-regulation of ODC activity was not affected by PD98059 treatment. These findings demonstrate that p38MAPK plays a crucial role in IL-1-induced growth inhibition in A375 cells through down regulating ODC activity. In addition to p38MAPK activation, ERK1/2 or other PD98059 sensitive kinase(s) may contribute to the antiproliferative effect of IL-1 in an ODC degradation independent manner.

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MODULATION AND TERMINATION OF INTERLEUKIN-6-SIGNALLING

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Interleukin-6 (IL-6) and the related long chain α-helical bundle cytokines IL-11, LIF, OSM, CNTF and CT-1 signal through the Jak/STAT pathway, which involves the dimerization of the signal transducers gp130, LIF-R or OSM-Rβ, their tyrosine phosphorylation, recruitment of STAT factors and SHP-2, translocation of tyrosine- and serine-phosphorylated STAT dimers to the nucleus and binding of STATs to enhancer sequences of respective target genes resulting in transcriptional activation. In the first part of the paper structure-function studies using gp130 point (Y_{190}, F_{191} and V_{252}) and domain (D1, D4, D5, D6) deletion mutants will be presented. In the second part of the presentation mechanisms of termination and modulation of IL-6 signalling will be discussed, i.e. (i) the role of the tyrosine-phosphatase SHP-2 counteracting acute phase protein (APP) induction (ii) the role of SOCS feedback inhibitors in APP synthesis (iii) the importance of different half-lives of the signalling molecules gp130, Janus kinases, SHP-2, STATs and SOCS (iv) internalization of the receptor complexes (constitutive internalization of gp130 due to a di-leucine motif within the cytoplasmic tail). Finally, new data on the inhibitory effect of MAP kinases on IL-6-induced STAT activation will be presented. It is also shown that SOCS-3 mRNA is increased in cells transfected with a gp130 $Y_{759} \rightarrow F$ mutant and that both SOCS-3 and SHP-2 interact with a phospho-tyrosine Y_{759} peptide of gp130.

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INTERLEUKIN-18 ACTIVATES STAT3 IN NK92 CELLS, AUGMENTS CYTOTOXIC ACTIVITY AND MEDIATES IFN-γ PRODUCTION BY $P42^{ERK-1}$AND $P42^{ERK-2}$ D. Kauschat, N. Koyama, D. Hoelzer, O.G. Ottmann and U. Kalina, Univ. Frankfurt, Dpt. Hematology Frankfurt/Main, Germany 60590.

Interleukin-18 (IL-18), is a regulator of natural killer (NK) cell function which has been shown to activate the IRAK signaling pathway and may be involved in other not yet characterized signaling pathways. Here we evaluated the IL-18 signaling pathways in the human NK92 cell line. NK92 cells were shown by RT-PCR to express all three receptor chains (IL-18R, AcPL, IL-18BP) described to bind IL-18. Tyrosine phosphorylation of STAT3 and of MAP kinase $p42^{erk-1}$and $p42^{erk-2}$ was strongly enhanced following stimulation of NK92 cells by IL-18 for 5 minutes. In contrast, STAT5 was not activated. NK92 lytic activity against K562 target cells, which was augmented in a dose dependent manner by IL-18 (0.5-10ng/ml) in the presence of 5U/ml IL-2, was suppresed by a specific inhibitor of MAPK pathway (PD 98059). IL-18 did not stimulate proliferation of NK92 cells, either alone, or in combination with IL-2 or IL-12 (^{3}H-TdR uptake). While the IL-2 and IL-12 induced proliferation of NK92 cells was not significantly altered by the MAPK pathway inhibitor, the janus kinase/STAT pathway inhibitor AG490 suppressed proliferation by 80%. The stimulatory effect of IL-18 given in combination with IL-2 on interferon-γ (IFNγ) protein production was counteracted by inhibiton of MAPK. IL-18 alone failed to stimulate IFNγ protein production despite inducing expression of IFNγ mRNA, which was not effected by inhibition of MAPK. IL-2 alone failed to stimulate the IFNγ mRNA expression and IFNγ protein production. This suggests, that the translation of IL-18 induced IFNγ mRNA involves MAPK activation and costimulatory factors activated by IL-2. MAPK also participates in the IL-18 induced cytolytic effect and does not alter the proliferation of NK92 cells.

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CONSTITUTIVE EXPRESSION OF CIS1 CONFERS RESISTANCE TO ANTI PROLIFERATIVE EFFECTS OF ONCOSTATIN-M IN HUMAN A375 MELANOMA CELLS

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Oncostatin-M (OSM) is a member of a cytokine subfamily that includes interleukin-6, ciliary neurotrophic factor, interleukin-11, cardiotrophin-1 and leukemia inhibitory factor (LIF). It inhibits the growth of human A375 melanoma cells through specific receptor complex, containing OSMRß and the common signaling subunit, gp130. OSM binding to its specific receptor leads to activation of JAK1, JAK2 and TYK2 and subsequent recruitment and activation of STAT3 and STAT5b. Recently, a family of negative regulators of JAK/STAT signaling pathway referred as suppressor of cytokine signaling (SOCS) or cytokine-inducible SH2 protein (CIS) has been identified. These proteins are rapidly induced in response to cytokine stimulation and they act through interaction with either phosphorylated cytokine receptor or activated JAK. To address whether one of the CIS family member, CIS1, can inhibit OSM signaling we established an A375 cell line that constitutively expresses CIS1 protein. Our results show that forced expression of CIS1 inhibits antiproliferative effect of OSM and allows G1 to S cell cycle transition. Furthermore CIS1 expression partially abolishes STAT5 activation, but has no effect on STAT3 activation in response to OSM. In conclusion, these data show that CIS1 can inhibit OSM signaling and suggest that deregulated CIS1 expression could play a role in melanoma progression.

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PLATELET-DERIVED GROWTH FACTOR (PDGF)-INDUCED ACTIVATION OF STAT5 IS MEDIATED BY PDGF β-RECEPTOR. K. Paukku[1], S. Valgeirsdòttir[2], P. Saharinen[1], M. Bergman[3], C-H. Heldin[2], and O. Silvennoinen[1,4]. [1]Haartman Institute, Department of Virology, P.O. Box 21, FIN-00014, University of Helsinki, Finland, [2]Ludwig Institute for Cancer Research, Biomedical Center, Uppsala, Sweden, [3]Division of Biochemistry, Department of Biosciences, Helsinki, Finland, [4]Institute of Medical Technology, Tampere, Finland.

Several growth factors activate Signal transducers and activators of transcription (Stats) but the mechanism of Stat activation in receptor tyrosine kinase signaling has remained elusive. Ligand binding activates the platelet-derived growth factor receptors (PDGFRs) but leads also in activation of cytoplasmic Src and Jak kinases. Co-expression experiments in insect and mammalian cells demonstrated that both PDGF β-R and Jak1, but not c-Src, induced activation of Stat5. Furthermore, immune complex-purified PDGF β-R was able to directly phosphorylate Stat5. Experiments with overexpressed kinase negative (KN) and wild type Jak and c-Src kinases showed that both Src-KN and wild type c-Src similarly reduced the PDGF β-R-induced activation of Stat5 while Jak1-KN or Jak2-KN had no effect. Activation of both Src and Stat5 is dependent on the same tyrosine residues Tyr579 and Tyr581 in PDGF β-R; therefore the observed inhibition by Src is likely to result from competition for binding of Stat5 to the receptor. Finally, fibroblasts derived from $Src^{-/-}$ and $Fyn^{-/-}$ mice showed normal pattern of PDGF-induced tyrosine phosphorylation of Stat5. Taken together these results indicate that Stat5 is a direct substrate for PDGF β-R and that the activation of Stat5 does not require Jak1, Jak2, c-Src or Fyn tyrosine kinases.

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EXPRESSION OF TNF RECEPTOR ASSOCIATED FACTORS (TRAFs & cIAPs) IN HUMAN AND MURINE PULMONARY EPITHELIAL CELLS. G. Pryhuber, H. Huyck and G. Reichlen. Univ. Rochester, Rochester, NY 14642

TNF-α receptors (TNFRs), type II transmembrane receptors, signal by recruiting secondary signaling proteins including TNF receptor associated factors, TRAF 1 and 2. TRAF1 is required for the association of TNFRs with inhibitor of apoptosis proteins (IAPs). In the current study, ribonuclease protection and Western assays demonstrate TNF-α dose and time dependent induction of TRAF1 and cIAP2 in H441 and A549 pulmonary adenocarcinoma cell lines, that was blocked by the inhibitor of NFkB activation, MG132. Expression of TRAF1 in the U937 monocyte cell line was constitutive and not induced by TNF-α. The protein kinase C agonist, TPA, induced TRAF1 gene expression in H441 and U937 cells but not A549 cells, suggesting PKC isotype dependent regulation. TRAF2 and cIAP1 genes were consistently expressed in each cell and not altered by TNF-α. Transfection of A549 cells with TRAF1 antisense oligodeoxynucleotides inhibited TRAF1 induction and markedly increased sensitivity to TNF-α induced apoptosis. In order to test the relevance of cell culture data to whole lung function, C57Bl/6J mice were treated with recombinant murine TNF-α (5μg, IT) 24 hrs prior to lung isolation. TRAF1 was detected by IHC in TNF-α but not saline treated mice, in cells morphologically consistent with alveolar Type II cells and macrophages. TRAF1 protein was also detected in human neonatal autopsy lung specimens in a pattern consistent with vascular smooth muscle cells, bronchiolar and alveolar epithelium and macrophages. These studies suggest clinically relevant, cell-type specific regulation of TRAF1 and cIAP2 in pulmonary epithelial cells by TNF-α. We speculate that TRAF1 induction is NFkB activation dependent and may also be activated by a TNFR independent, protein kinase C mediated pathway. One likely function of TRAF1 in lung cells is to protect from TNF-α induced apoptosis. Supported by NIH KO8 HL 03318-5 and the American Cancer Society. Oligonucleotides kindly supplied by Dr. R. Case, Midland Certified Reagent Company, Midland TX.

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REGULATION OF THE IL-12 P40 PROMOTER BY HUMAN TOLL4 RECEPTOR.

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Toll-like receptors (TLRs) are transmembrane immune receptors that interact directly or indirectly with bacterial products. TLRs are homologs of Drosophila Toll protein which is involved in dorsal-ventral development and antifungal defense in adult flies. Human TLRs, such as hTLR4, activate proinflammatory cytokine genes IL-1, IL-6, IL-8 and IL-12. IL-12 is a heterodimeric cytokine containing p40 and p35 subunits. In our co-transfection experiments, overexpressed mCD4/hTLR4 fusion protein activates an IL-12 p40 promoter-CAT reporter construct in murine macrophages in the absence of LPS. Because p40 transcription requires, minimally, the induction of Rel, C/EBP and AP-1 family members, hTLR4 must transmit at least 3 distinct signals. As a first step towards determining the point at which these signals diverge from the hTLR4, we have systematically mutated the hTLR4 cytoplasmic domain. The mutants are being tested for their effect on the activation of Rel, C/EBP and AP-1 proteins, as well as on their effect on receptor dimerization and Myd88 binding.

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TRAF6 IS REQUIERED FOR IL-17-INDUCED ACTIVATION OF NUCLEAR FACTOR-κB AND INDUCTION OF PRO-INFLAMMATORY CYTOKINES

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IL-17 is secreted by activated CD4+ T cells, mainly from the Th-0 and Th-1 subtype, and signals through its wildly distributed cell surface receptor to enhances the transcription of genes encoding proinflammatory molecules. Although it has been well documented that IL-17 activates the transcription factor NF-κB, the upstream signaling events are largely unknown. Here we report the requirement of TRAF-6-(TNF receptor-associated factor 6) in IL-17-induced NF-κB activation. In embryonic fibroblasts derived from TRAF-6 knock-out mice, IL-17 failed to activate the IκB kinases complex (IKKs) and, hence, the activation of NF-κB. Consequently, IL-17-induced IL-6 secretion and cell surface expression of ICAM-1 were abolished. Lack of TRAF-6 is likely the sole defect responsible for the observed unresponsiveness to IL-17, because TRAF-6-independent signaling pathways were intact and the levels of IL-17 receptor on the TRAF-6-deficient mouse embryonic fibroblasts (MEFs) were comparable to those on the wild type control cells. Furthermore, transient transfection of TRAF-6 expression plasmid into the TRAF-6-deficent MEFs restored IL-17-induced NF-κB activation confirmed by luciferase reporter gene activation assays. Defects in IL-17 response was not observed in TRAF-2-deficient MEFs. Together, these results indicate that TRAF-6, but not TRAF-2, is a crucial component in IL-17-induced signaling events leading to NF-κB activation and proinflammatory responses.

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GENERATION OF MUTANT CELL LINES RESISTANT TO THE INHIBITORY ACTION OF SALICYLATE ON TNF SIGNALING. P. SCHWENGER and J. Vilcek, NYU School of Medicine, New York, NY 10016.

Recent studies have shown that nonsteroidal anti-inflammatory drugs such as sodium salicylate (NaSal) have novel cyclooxygenase-independent effects. Some of these effects involve interference with cytokine signal transduction pathways. In previous work, we demonstrated that in normal human FS-4 fibroblasts NaSal inhibited TNF-induced activation of two subfamilies of MAP kinases (MAPKs), the ERKs and JNKs. This inhibitory action of NaSal was selective for TNF because activation of these MAPK isoforms by growth factors and other cytokines, such as EGF or IL-1, was much less affected by NaSal. Unexpectedly, NaSal by itself activated a third MAPK family member, p38 MAPK, and induced apoptosis in FS-4 cells in a p38 MAPK-dependent manner. In addition, we showed that NaSal by itself activated JNK in some cells. Others have shown that NaSal inhibited the activation of the transcription factor NF-κB by preventing the inducible phosphorylation and degradation of its inhibitory IκB subunit. We demonstrated that inhibition of TNF-induced IκB phosphorylation and degradation by NaSal required NaSal-induced p38 MAPK activation. Applying a genetic approach to the analysis of NaSal-induced signaling pathways, we generated mutant COS-1 cell lines unresponsive to NaSal. COS-1 cells were subjected to mutagenesis with the frameshift mutagen compound ICR-191 and then selected for survival in high-dose NaSal. Clones resistant to NaSal-induced killing were then grown in non-selective medium, and subsequently screened for their response to NaSal based on several parameters. Several clones were identified in which NaSal no longer inhibited TNF-induced IκB phosphorylation and degradation. These clones may be useful for identification of the precise molecular mechanisms whereby NaSal inhibits TNF signaling, and could help to reveal novel cellular targets for anti-inflammatory drug action.

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DIFFERENTIAL ROLES FOR PI3-KINASE, S6 KINASE AND MAP KINASES IN INTERLEUKIN-1 ACTION.

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The relative roles of protein kinases in the action of interleukin-1 (IL-1) in different cell types have not been fully elucidated. The aim of these studies was to delineate the roles of these kinases in lymphoid and epithelial cells. We now show that IL-1 activated PI-3 kinase in Jurkat T cells and T47D epithelial cells with maximal stimulation at 60pM and with similar kinetics. However, IL-1 activated S6 kinase only in the T cells, an effect which was inhibited by the PI-3 kinase inhibitor, LY294002. Thus, IL-1 activated PI3-kinase leads to S6 activation in T cells but not in T47D cells. The effects of IL-1 on MAP kinases in T47D cells and T cells (in this case, EL4 cells) were also studied. ERK1/2 activity was stimulated by IL-1 (max. 60pM) in T47D cells at 30-60 mins., returning to basal levels thereafter, whereas there was no response in EL4 cells until 4-24 hr. IL-1 elicited rapid and transient increases (5-10 min.) in JNK and p38 kinase activity in EL4 cells, but a slower response (30-60 min.) in T47D cells. Also, the dose of IL-1 required to activate p38 was lower in EL4 cells than in T47D. LY294002 partially inhibited ERK1/2 activity in T47D cells and abrogated the JNK response to IL-1 in EL4 cells, but had no effect on JNK activation in T47D cells or p38 activation in either cell type. It appears that PI3-kinase may be involved in linking IL-1R activation to some downstream kinases in T cells. In T47D cells, PI3-kinase may only be responsible for some, but interestingly not all, of the IL-1 induced ERK activity. Thus. significant quantitative, qualitative and/or dynamic differences in the activation of protein kinases by IL-1 occur in distinct cell types.

We are grateful to Yorkshire Cancer Research for funding.

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THE TRANSCRIPTION FACTORS INTERACTING WITH THE BI-ALLELIC -308 TNF-α PROMOTER REGION.

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The TNF2 promoter allele is associated with both susceptibility to some inflammatory and autoimune diseases as well as elevated serum TNF levels. Previously, we have demonstrated that the G/A -308 polymorphism affects both binding of transcription factors and expression from the TNF promoter linked to a reporter gene. Our current aims are to identify and characterise the transcription factors binding to the region. Linker scanning across the -308 region showed that an extended region of at least 27 bp was necessary for expression of the TNF2 phenotype and for the formation of a TNF2-specific complex (called complex E) in EMSA. Consistent with these data, DNase I footprinting showed an extended region of protection. In addition, the TNF2 sequence shows a unique protection pattern. Supershift analysis established that the major EMSA complex C that formed on both allelic sequences was Sp1. A potential ets binding site near -308 was also assessed for its ability to influence expression of the -308 polymorphism. Although weak binding was apparent, overexpression of ets1 or ets2 in cotransfection experiments, using wild-type and mutated ets site constructs, showed that the ets site was probably not functional. However, overexpression of ets1 or ets2 served to differentially increase TNF2 expression compared to TNF1 and may indicate that the downstream ets site interacts with the -308 enhancersome. Characterisation of the allele-specific EMSA complex E indicated that it was a homomeric complex composed of a single 56 kD protein. Using mutant oligonucleotides as competitors enabled the definition of the Complex E DNA binding specificity as not related to known transcription factor binding sites. We believe that the complex E protein may represent a novel binding activity.

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CURCUMIN REGULATES CYTOKINE EXPRESSION DURING HEMORRHAGIC SHOCK AND RESUSCITATION IN RATS. J. P. Gaddipati, J. Calemine, P. Seth, G. S. Sidhu and R. K. Maheshwari. Center for Combat Casualty Care and Life Sustainment Research, Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814.

Proinflammatory cytokine cascades are initiated following hemorrhagic shock and are widely implicated in organ dysfunction. Significant hemorrhage carries considerable death rate regardless of intervention. Curcumin (diferuloymethane), a natural compound with anti-inflammatory, anti-oxidative and anti-carcinogenic activities has been shown to have pleiotropic biologic activities including inhibition of neutrophil activation, suppression of mononuclear cell proliferation. We have tested the efficacy of curcumin for the prevention of hemorrhagic shock injury and data indicate a significant survival advantage by pretreatment with curcumin. We have compared the cytokine gene expression in various organs during hemorrhagic shock and resuscitation and the response to curcumin pretreatment. Shock was initiated in anesthetized rats by bleeding of 30 ml/ kg body weight from the femoral artery. After one hour, the rats were resuscitated with 2X volume of lactated Ringer's solution. The animals were sacrificed 2 h post-resuscitation and liver, intestine, kidney, lung, brain, heart and spleen were harvested. Total RNA was extracted and cytokine mRNA (IL-1 alpha, IL-1 beta, IL-2, IL-6, IL-10 and TNF-alpha) was analyzed by semi-quantitative RT-PCR and Northern hybridization. The results demonstrate that pretreatment with curcumin is effective in inhibiting some of these proinflammatory cytokines. The data also show that the hemorrhage induced cytokine profiles are quite different between organs.
This work was supported by ONR grant G174HV.

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STAT5 BUT NOT STAT3 BINDS SPECIFICALLY TO THE HUMAN INTERLEUKIN-18 PROMOTER U.KALINA, K. BALLAS, N. KOYAMA, D. KOELZER, and O.G. OTTMANN, University Frankfurt, Dpt. Hematology Frankfurt/Main, Germany 60590.

The human interleukin-18 (IL-18) is a key regulator of interferon-γ production and T-cell differentiation. IL-18 stimulates Th1 cells, augments the cell-mediated immunity against foreign and tumor antigens and is involved in inflammatory and infectious diseases. Here we report the complete genomic structure of the human IL-18 gene including the transcriptional start site and putative promoter regions. Spanning a region of 19,5 kb on Chromosome 11q, the human IL-18 gene is composed of five translated exons and four introns. In contrast to the murine gene there is only one untranslated exon in the human IL-18 gene and a sequence differing by one nucleotide from the consensus TATA box is present 67 nucleotides upstream of the translation initiation codon ATG. Analysis of the two putative promoter regions upstream of the first untranslated and the second translated exons revealed multiple consensus sequences for factors that regulate either basal transcription or gene expression during cell differentiation and proliferation. GATA boxes, also present in the mouse gene, were located upstream of the transcription initiation point. In the first promoter a number of binding sites for signal transducer and activator of transcription factors (STATs) were identified. In DNA-protein binding experiments we have shown that activated STAT5 but not STAT3 binds specifically to distinct regions of the human IL-18 promoter. Elucidation of the genomic structure and promoter sequences of IL-18 will permit analysis of its transcriptional regulation in the context of immune responses, T-cell differentiation and cytotoxicity, and inflammatory processes.

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SUPPRESSION OF ACUTE TERMINAL DIFFERENTIATION OF PRIMARY MOUSE B CELLS BY BCR- OR CD40 LIGATION AND IL-4: REVERSAL BY IL-2, IL-5 AND RETROVIRALLY TRANSDUCED BLIMP-1. M. Knödel, D. Schliephake, A. W. Kuss, I. Berberich and A. Schimpl, University of Würzburg, D 97078 Versbacherstr. 7, Würzburg, Germany (e-mail: schimpl@uni-wuerzburg.de)

Following stimulation, primary B cells either directly undergo terminal differentiation to IgM secreting plasma cells or enter the memory pathway, characterized by affinity maturation and isotype switching before high rate secretion is turned on or cells revert to a resting memory phenotype. Which of the various fates is adopted by the B cells is likely determined by the strength and duration of the antigenic signal and the availability of T cell help in the germinal center milieu. High rate secretion has been correlated with and can be caused by high expression of Blimp-1. Using cultures of resting primary mouse B cells stimulated in vitro with LPS and, in various combinations, with IL-4, anti-μ F(ab')2 and anti CD40, we show that IgM secretion and the expression of Blimp-1 induced by LPS is strongly suppressed by BCR or CD40 ligation and by IL-4. Suppression of IgM and IgG1 secretion is reverted by IL-2 and IL-5, which induce Blimp-1 expression, or directly by retroviral transduction of Blimp-1. On the other hand, the drastic increase in membrane $IgG1^+$ cells with time in cultures treated with LPS and IL-4 is greatly diminished in Blimp-1 expressing cells.
We conclude that extended BCR and CD40 ligation in the presence of IL-4 facilitate the entrance into the memory pathway by delaying Blimp-1 expression and terminal differentiation which then can be induced by IL-2 and IL-5.

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CYCLIC AMP RESPONSIVE ELEMENTS ARE INVOLVED IN THE TRANSCRIPTIONAL ACTIVATION OF THE IL-10 GENE IN MONOCYTIC CELLS. C. Platzer*, E. Fritsch, T. Elsner, H.-D. Volk and S. Prösch, *Institute of Anatomy II, Friedrich Schiller University, D-07740 Jena, email: cplatzer@mti-n.uni-jena.de

IL-10 plays an important role in the regulation of immune response. We and others have demonstrated recently that cyclic adenosine monophosphate (cAMP) elevating substances up-regulate monocytic IL-10 expression *in vitro* and *in vivo*. Computer analysis of the IL-10 promoter/enhancer region localized four putative cAMP responsive elements (CRE1-4) with homology to the CRE consensus motif. In electrophoretic mobility shift assays (EMSA) CRE1 and CRE4 bound protein complexes consisting of transcription factors CREB-1 and ATF-1, while CRE3 bound only marginal amounts of CREB-1/ATF-1 in combination with unknown protein(s). CRE2 showed no protein binding activity. *In vitro* mutation of CRE1 and CRE4 reduced the level of cAMP-stimulated transactivation in reporter gene assays in comparison to the wild type promoter by 20% and 50%, respectively, while mutation of CRE3 had no effect. The main action of CRE4 on cAMP-dependent stimulation is probably based on its adjacent localization to the TATA box and its sequence comprising a perfect half site. Experiments with double and triple mutants and with deleted promoter fragments indicated the participation of additional elements beside the CRE motifs in the cAMP-dependent stimulation.

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MOLECULAR ANALYSIS OF INTERLEUKIN-10 EXPRESSION BY ACTIVATED B CELLS. T.W. Redford, A.K. Yi, and A. M. Krieg. Colleges of Pharmacy and Medicine University of Iowa, Iowa City, IA 52242.

IL-10 is a potent autocrine factor for proliferation and differentiation of B cells and is a known inhibitor of IL-12, IL-2 and γIFN proinflammatory proteins associated with a Th1-type or cell mediated immune effector response. We initially reported IL-10 expression in B cells by the vaccine adjuvant, CpG DNA. Additional studies have found that B cell receptor (BCR) stimulation also increases IL-10 expression and demonstrates synergistic IL-10 promoter activation with CpG DNA. Truncation analysis of the IL-10 promoter revealed a 100 bp region approximately 175 bp from the transcriptional start site that is critical in BCR stimulation of IL-10. The human IL-10 promoter was cloned upstream of the luciferase reporter gene and transiently transfected into the RAMOS B cell line. BCR stimulation of the IL-10 luciferase truncation constructs demonstrated 10 times the activity over background and was similar to PMA stimulation. BCR stimulation was abrogated when the promoter was truncated to 175 bp from the transcription start site whereas PMA stimulation was not affected. The region upstream from 175 contains a GGAAA consensus sequence for NF-AT and BCR stimulation of IL-10 was diminished by the calcineurin and NF-AT inhibitor, cyclosporine A. This analysis will provide insight into the regulation of IL-10 particularly in the context of antigen and adjuvant stimulation present in vaccine response. (This work was supported by the Burroughs Wellcome Fund and the American Foundation for Pharmaceutical Education.)

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DIFFERENTIAL REGULATION OF IFN-γ GENE EXPRESSION BY IFN-α OR IL-12 IN NK AND T CELLS. T. SARENEVA, A. Paananen, T. Timonen, I. Julkunen, and S. Matikainen. National Public Health Institute, Helsinki, Finland.

In many virus infections interferons (IFN)-α/β, but not interleukin (IL)-12, are secreted by macrophages. IFN-α/β are potent inducers of NK cell activity, and these cells are important contributors to innate defence against viral infections. We have previously reported that influenza A virus-infected human macrophages did not produce IL-12. Instead, macrophages secreted IFN-α and IL-18, which synergistically enhanced IFN-γ gene expression in activated human T cells. In this study, we demonstrate that the combined stimulation of resting NK cells with IFN-α and IL-18 also leads to the efficient secretion of IFN-γ protein. In addition, we directly compared the kinetics of either IFN-α or IL-12 plus IL-18–induced IFN-γ gene activation in NK and T cells. IFN-α in combination with IL-18 induces a rapid IFN-γ mRNA synthesis, which is down-regulated soon after induction. In contrast, IL-12 plus IL-18 induces long-lasting transcriptional activation of IFN-γ gene, and high amount of IFN-γ protein is produced. Furthermore, we demonstrate that both IFN-α and IL-12 are able to induce Stat4 DNA binding to the IFN-γ promoter GAS element, but IL-12–induced Stat4/DNA complex is more stable compared to the IFN-α–induced complex. Thus the kinetic difference in IFN-γ production by IFN-α or IL-12 may depend on different Stat4 stability.

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KINETICS OF IL-6 MRNA TRANSCRIPTION IN LIPOPOLYSACCHARIDE-INDUCED MOUSE MACROPHAGES. S.W. Van Arsdell, K.P. Murphy, C. C. Pazmany, D. Erickson, and M.D. Moody, Endogen, Inc., Woburn, MA 01801.

Interleukin-6, a pleiotropic cytokine which plays an important role in inflammation and activation of the immune response, is secreted by macrophages in response to host invasion or after exposure to various inducing agents such as lipopolysaccharide (LPS). We have investigated the kinetics of IL-6 mRNA transcription by mouse macrophage cell line J774A.1 after treatment with LPS. The amount of IL-6 mRNA produced in the macrophages was measured using the Xplore™ assay, a novel mRNA quantification system. This microtiter plate-based signal amplification system is rapid (less than 6 hours), sensitive (1 attomole of mRNA can de detected in 0.5 μg of total RNA), and quantitative over three orders of magnitude. The target sequence is not amplified during the assay, so the risk of false positives from contamination is greatly reduced compared to methods such as RT-PCR. Using this assay, we determined that the steady state level of IL-6 mRNA increased from a basal level of 50 molecules per cell before LPS treatment to a peak level of 1200 molecules per cell at 7 hours, and then decreased to 450 molecules per cell at 24 hours post-induction. The amount of IL-6 protein secreted into the cell culture medium, as measured by ELISA, increased from basal levels of 3 pg/ml to 16 ng/ml at 7 hours, and then continued to increase, reaching 27 ng/ml at 24 hours post-induction.

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IFNα SELECTIVELY ENHANCES IL-6 INDUCTION BY dsRNA BUT NOT BY IL-1β. J.L. VanBergen and M.K. Offermann, Emory University, Atlanta GA 30322.

Double stranded RNA (dsRNA) is an intermediate that is common during viral infection and directly induces the expression of IL-6 in HeLa cells. Although there are some elements of the IL-6 promoter that are involved in responsiveness to both cytokines and dsRNA, there are a number of differences that affect both the kinetics and magnitude of induction. We have previously shown that IFNα does not directly induce the expression of IL-6, yet pretreatment of cells with IFNα dramatically enhances IL-6 expression in response to dsRNA but not to IL-1β. We have generated IL-6 promoter-CAT constructs containing variable regions of the IL-6 promoter and have used site-directed mutagenesis to determine the role of specific elements in these responses. Deletion of the region between -200 and -160 almost completely ablates the high level of CAT reporter activity that occur in response to IFNα pretreatment followed by PIC. This critical region contains the multiple response element I (MRE I) that includes an ATF/CREB site, whereas the NF-κB and NF-IL6 binding sites are downstream of this region. Mutation in the ATF/CREB binding element eliminates responsiveness to both dsRNA and IL-1β. IFNα prior to dsRNA restores responsiveness of this mutant to levels that are greater than activity of wild-type reporter in response to dsRNA alone (5-fold higher) but less than the activity of the wild type promoter in response to IFNα followed by PIC (30% of wild-type activity). In contrast, IFNα pretreatment does not restore responsiveness of the mutant reporter to IL-1β. These studies indicate IFNα pretreatment can in part overcome the consequences of disruption of the ATF/CREB site selectively for dsRNA but not for IL-1β.

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HUMAN INTERLEUKIN-1 RECEPTOR ANTAGONIST: LARGE-SCALE EXPRESSION IN *Bacillus brevis* 47-5Q.

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Interleukin 1 receptor antagonist (IL-1ra) has been used as a tool to study the biological activity of IL-1 and as a possible therapeutic substance for inflammatory disease. To perform in *vivo* study, however, large quantities of IL-1ra are required. It is well known that *Bacillus brevis* (*B. brevis*) strains secrete large amounts of proteins but little protease into the medium. Using *B. brevis* 47-5Q, we developed a large-scale expression system of human IL-1ra (hIL-1ra). The bacteria secreted hIL-1ra into the culture medium at very high levels, approximately 200 milligrams per liter. The protein was isolated in one-step purification with monoclonal antibody against hIL-1ra. The IL-1ra molecule was functionally active by the inhibiting assay of human IL-1 induced cell proliferation in a mouse T cell line D10N4M.

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Inhibition of RANTES/CCR1 Mediated Chemotaxis by Novel Cosalane Compounds

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Chemokines and their receptors play a desirable role in immune function. However, they also contribute to chronic inflammation and auto-immune disease. RANTES through its interaction with CCR1, CCR3, or CCR5 has been implicated in asthma, rheumatoid arthritis, and respiratory injury. Earlier studies showed that cosalane (a polyanionic compound) and its analogs have immuno-modulatory effects. We therefore examined the ability of cosalane and several analogs to inhibit monocyte chemotaxis induced by various chemokines. We observed that cosalane and *meta*-carboxybenzyloxy analogs significantly inhibited RANTES-induced migration of human monocytes but did not inhibit migration induced by MIP1α or MIP1β. Further investigation using single receptor HEK-293 transfectants showed that RANTES-induced migration of CCR1-HEK transfectants was inhibited. The one-half effective concentration was at the sublethal micromolar range for all cosalane analogs. Our data suggests that cosalane can selectively interfere with RANTES/CCR1 interaction by binding RANTES.

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EVIDENCE FOR p38-DEPENDENT AND p38-INDEPENDENT MECHANISMS OF NF-κB INHIBITION BY SODIUM SALICYLATE.

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Many effects of TNF are mediated by the transcription factor NF-κB. Sodium salicylate (NaSal) inhibits NF-κB activation by blocking phosphorylation of the NF-κB inhibitor IκBα. We have implicated p38 MAP kinase activation by NaSal in the inhibition of IκBα phosphorylation, and demonstrated that p38 activation may play a more general role in NF-κB inhibition. To determine the target of p38-mediated NF-κB inhibition in TNF signaling, we overexpressed various TNFR-1 signaling intermediates along with the p38 activator MKK6b(E) and analyzed (i) phosphorylation of cotransfected IκBα and (ii) NF-κB-dependent reporter activity. MKK6b(E) coexpression inhibited IκBα phosphorylation and NF-κB activation by TNFR-1 signaling intermediates upstream of the kinases NIK and MEKK1, while NaSal inhibited these effects for all TNFR-1 intermediates examined. This suggests that p38-mediated inhibition of NF-κB occurs upstream of NIK and MEKK1, and that NaSal may also exert p38-independent inhibitory effects further downstream. NaSal has been shown to inhibit the IKKβ component of the IKK complex. We observed that treatment of intact cells with NaSal inhibited IKK activity induced by TNF, but not by IL-1. In contrast, *in vitro* treatment of immunoprecipitated IKK with NaSal comparably inhibited IKK activity from TNF- and IL-1-treated cells. Whereas the p38 inhibitor SB203580 reversed the inhibition of TNF-induced IKK activity by NaSal in intact cells, SB203580 failed to reverse the inhibition of immunoprecipitated IKK by NaSal *in vitro*. We conclude that direct inhibition of the IKK complex *in vitro* may not entirely reflect the inhibitory mechanism of NaSal in intact cells.

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VITAMIN C INHIBITION OF TNF-STIMULATED IKK ACTIVITY VIA p38 MAP KINASE.

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High dose vitamin C has been suggested as an anti-viral agent and as a therapy in a range of diseases including cancer and atherosclerosis. The transcription factor NFκB regulates the expression of a number of genes, some of which have roles in apoptosis and the development of atherosclerosis, as well as having a role in the replication of several viruses. Here, millimolar doses of vitamin C inhibited activation of NFκB by a range of stimuli, including interleukin-1 (IL1) and tumour necrosis factor (TNF), in a number of cell types. Although some antioxidants have been shown to block NFκB activation, the inhibition observed here was not primarily due to an antioxidant effect, since redox-insensitive pathways to NFκB were also blocked by vitamin C. At concentrations that inhibited NFκB, vitamin C was not toxic to cells, did not inhibit another transcription factor, Oct-1, and had no effect on the DNA binding of NFκB. Rather, degradation and phosphorylation of IκBα were specifically blocked, due to inhibition of IKK activity. Surprisingly, treatment of cells with vitamin C led to a rapid and sustained activation of p38 MAP kinase. In the case of TNF-, but not IL1-mediated NFκB activation, the specific p38 inhibitor SB203580 reversed the inhibitory effect of vitamin C on IKK activity, IκBα phosphorylation and NFκB activation. The results demonstrate that vitamin C is a good general inhibitor of NFκB. In the case of TNF, inhibition is mediated by a sustained activation of p38 MAP kinase, as has been previously demonstrated for sodium salicylate inhibition of TNF-mediated NFκB (Schwenger et al, (1998) *Mol. Cell. Biol.* **18**, 78-84). The results may also suggest a rationale for the reported anti-viral and other beneficial effects of high dose vitamin C.

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THE DEATH DOMAIN OF THE INTERLEUKIN-1 RECEPTOR ASSOCIATED KINASE (IRAK) IS CRUCIAL FOR IL-1 SIGNALING. J.Knop and M.MARTIN, Pharmacology, Medical School Hannover, 30623 Hannover, Germany

Interleukin-1 (IL-1) stimulates the transient association of the IL-1 Receptor Associated Kinase (IRAK) with the heterodimer of IL-1RI and IL-1RAcP via the adapter protein MyD88. In the receptor complex IRAK becomes heavily phosphorylated by auto- or cross-phosphorylation. By homology search an N-terminal death domain (DD) and a central Ser/Thr kinase motif could be identified. while the C-terminus did not show any known homologies. We generated a series of IRAK deletion mutants and tested them for their ability to serve as substrate for IRAK's kinase activity. Coprecipitation studies showed that the DD of IRAK – but not the kinase domain or the C-terminus - can interact with full length IRAK and becomes heavily phosphorylated by IRAK *in vitro*. Upon IL-1 stimulation, IRAK's death domain is thought to transiently interact with the upstream adaptor MyD88. As phosphorylated IRAK fails to interact with MyD88, our observation may give an explanation on how IRAK leaves the receptor complex after IL-1 stimulation. Next we asked, which domains of IRAK are essential for IL-1 signaling. While expression of full length or kinase-inactive IRAK clearly enhanced IL-1 stimulated NFkB activation, a mutant lacking the C-terminus functioned as a dominant negative inhibitor. Additionaly, the DD alone potently inhibited IL-1 signaling, while IRAK mutants lacking the DD failed to interfere with IL-1 stimulated NFkB activity. In conclusion IRAK needs both, the N-terminal DD and its C-terminus but not it's kianse activity to transduce signals from the IL-1 receptor complex.

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SSI/SOCS FAMILY PROTEINS ARE INVOLVED IN THE NEGATIVE REGULATION FOR VARIOUS SIGNAL TRANSDUCTION PATHWAY. Yoshinori Kawazoe, Tetsuji Naka, Koichi Okumura, Minoru Fujimoto, Masashi Narazaki, Yoshiaki Morita, Reiko Nakagawa and Tadamitsu Kishimoto, Osaka Univ. Medical School, OSAKA, 565-0871, JAPAN

STAT-induced STAT inibitor (SSI)-1 was first identified as a negative feedback regulator in the JAK-STAT pathway of cytokine signaling, and so far, at least eight members of SSI family gene (SSI-1~7 and CIS) have been isolated. Among these, SSI-1 and SSI-3 bind to JAK kinases, CIS binds to cytokine receptors, and consequently inhibit further signaling processes. But, little is known about the nature of other family member genes. To explore SSI associating protein, we performed two-hybrid screening with SSI-1, 2, 3, and 5 as a bait. Interestingly, almost positive clones appeared both receptor or non-receptor type kinase, for example, FAK and MDK1 for SSI-2, CaMK and guanylate kinase for SSI-5. Furthermore, in the course of confirmming the binding of these kinases to the SSI family protein in 293T cells, we found SSI-5 bound to the Insuline receptor. As the SSI-1 null mice showed the low blood sugar level, we examined the effect of SSI family in insuline signaling. We found SSI-1, but not SSI-5 repressed the phosphorylation of IRS-1 after insulin stimulation in L929 cells. Therefore, it was speculated SSI-1 inhibited insuline signaling pathway, further studies that prove the inhibition mechanism are now underway. These results suggested SSI family protein might be a negative regulator for the broad range of signal transduction as well as JAK-STAT pathway.

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THE IL-15 RECEPTOR-α CHAIN SIGNALS THROUGH ASSOCIATION WITH SYK KINASE. S. BULFONE-PAUS, V. Budagyan, and E. Bulanova, Univ. Hospital Benjamin Franklin, Free Univ., Berlin, Germany.

Interleukin 15 (IL-15) binds to and induces proliferation of lymphoid cells coexpressing the IL-15 receptor (IL-15Rα)-, IL-2Rβ-, and γ-chain. The IL-15Rα chain is the IL-15-specific, high affinity receptor, which is widely expressed, including on activated B cells and lymphoma cell lines. The IL-2Rβ and γ chains are known to transduce signals through the activation/association of kinases belonging to the JAK and the src kinase families. It is still controversial whether the α chain by itself can induce a signal upon ligand binding. Here, we have explored this further in Raji cells, a human B-lymphoblastoid cell line which expresses the IL-15Rα and γ chains, but lacks the β chain. Stimulation of Raji cells with IL-15 induces their proliferation and rescues them from C2-ceramide-induced apoptosis. By immunoprecipitation and Western blotting, we show that stimulation of Raji cells by IL-15, but not IL-2, selectively coprecipitates Syk kinase with the IL-15Rα chain. Upon association, the activated Syk kinase phosphorylates the IL-15Rα chain (at tyrosine residue 227) and the phospholipase Cγ, which also coprecipitates with Syk. Mutation of tyrosine 227 located in the intracellular part of the IL-15Rα chain inhibits Syk co-immunoprecipitation. Furthermore, treatment of Raji cells with dexamethasone downmodulates IL-15Rα expression and induces loss of its association with Syk. Our data suggest that Syk kinase physically and functionally associates with the IL-15Rα chain in B cells and that Syk plays a key role in mediating the distinct functional consequences of IL-15 versus IL-2 binding to B cells.

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TYROSINE PHOSPHATASE SHP-1 REGULATES CYTOSKELETAL PROTEINS IN MURINE MACROPHAGES: LACK OF SHP-1 LEADS TO DEGRANULATION OF CYTOSKELETAL ORGANIZATION IN MACROPHAGES OF MOTHEATEN (me/me) MICE. M. Kozlowski, H. Yang, F. Lee, O. Zoueva*, A. Ridsdale, G. Graziani-Bowering and A. Kumar. Health Canada, Therapeutic Products Program and Dept. of Biochem. Microbiol. and Immunol. U. of Ottawa, Ontario, Canada.

The SH2 domain-containing tyrosine phosphatase, SHP-1, regulates antigen/cytokine receptor-driven proliferative pathways. However, the role of SHP-1 in regulating the differentiation process is not understood. To explore this potential function of SHP-1, we have taken advantage of macrophages from SHP-1 deficient me/me mice and studied the effect of granulocyte-monocyte colony stimulating factor (GM-CSF) on their morphology and induction of tyrosine phosphorylated proteins. We purified tyrosine phosphorylated proteins from me/me macrophages by using SHP-1SH2 domains-glutathione-S-transferase fusion proteins and determined their sequences. We show for the first time that SHP-1 interacts with two cytoskeletal proteins F-actin and gelsolin, which are constitutively tyrosine phosphorylated in me/me mice and are activated by GM-CSF. Their level of tyrosine phosphorylation is markedly increased in me/me cells as compared to controls, and correlates with the differential organization and rearrangements of cytoskeletal fibers as demonstrated by confocal microscopy. In addition, the proto-oncoprotein VAV interacts with both SHP-1 and actin and the level of VAV-actin binding is enhanced in me/me macrophages. Our results suggest that SHP-1 acts as an important regulator of cytoskeletal rearrangements involving a multimeric protein complex including actin, gelsolin and VAV. The hyper-phosphorylation of these proteins in the absence of SHP-1 may explain at least in part, the hyperproliferation and differentiation of monocytes/ macrophages in me/me mice.

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S. aureus DOWN-REGULATES CXCR1 AND CXCR2 EXPRESSION ON HUMAN NEUTROPHILS THROUGH TNF-MEDIATED PATHWAY. I.TIKHONOV, T.Doroshenko, Y.Chaly, V.Smolnikova, D. Horejsh, and N.Voitenok.
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Cellular response to chemokines may be regulated at the level of receptor expression on target cells. We suggested that bacterial products down-regulate leukocyte chemokine receptors during sepsis and this mechanism may account for impairment of leukocyte functions in septic syndrome. Previous studies indicated that endotoxin down-regulates the expression of CC and CXC receptors on leukocytes. Now, we have examined the effect of *S. aureus* on expression of the IL-8 receptors CXCR1 and CXCR2 in PMN in the whole blood and purified PMN fraction. *S. aureus* down regulated the cell surface expression of both CXCR1 and CXCR2 and the effect was abrogated by antibody to TNF-alpha. LPS-induced inhibition of CXCR1 and CXCR2 expression was independent of TNF. Endogenously produced IL-8 played no role in CXCR1 and CXCR2 modulation since the effect was not prevented by neutralizing antibody to IL-8.

Exogenous TNF-alpha down-regulated the cell surface expression of both CXCR1 and CXCR2 on human PMN and inhibited both IL-8 binding and chemotactic responses to IL-8. The addition of a mixture of protease inhibitors prevented the decrease of CXCR1 and CXCR2 expression on PMNs, as assessed by flow cytometry, but was unable to prevent the decrease of ligand binding. TNF-alpha caused profound decrease of CXCR1 and substantial decrease of CXCR2 mRNA levels.

The data imply that TNF-alpha produced during S. aureus septicemia inhibits CXCR1 and CXCR2 expression on PMN and plays a role in the impairment of leukocyte functions.

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INDUCTION OF FUNCTIONAL IL-8 RECEPTORS BY IL-4 AND IL-13 IN HUMAN MONOCYTES

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Monocytes migrate to some CC but not to CXC chemokines. Chemokine receptors can be modulated by inflammatory and anti-inflammatory cytokines. This study investigates the effect of interleukin(IL)-13 and IL-4 on chemokine receptor expression. IL-13/IL-4 did not alter CCR mRNA levels, but strongly increased CXCR1 and CXCR2 expression in human monocytes and in two related cell types, macrophages and dendritic cells. The effect was rapid, starting at 4 h, and concentration dependent (EC50=6.2 and 8.3 ng/ml for CXCR1 and CXCR2, respectively) and due to new transcriptional activity. IL-13/IL-4-treated monocytes showed increased CXCR1 and CXCR2 membrane expression. In IL-13/IL-4 treated monocytes, IL-8 induced activation of chemotaxis and superoxide release. The effect of IL-8 was comparable to that observed with other monocyte active chemokines. Macrophages and dendritic cells present in biopsies from Omenn's syndrome and atopic dermatitis patients, two Th2 skewed pathologies, expressed IL-8 receptors by immunohistochemistry. This study shows that monocytes exposed to IL-13/IL-4 become responsive to IL-8 and that this mechanism may contribute to formation of the mononuclear infiltrate that characterize Th2 responses and late phase reaction. Furthermore, it suggests that the selectivity of chemokines for their target cells may be altered by the cytokine context present at the inflammatory sites.

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CHEMOKINE REDUNDANCY ENSURES LOCAL NEUTROPHIL RECRUITMENT. D. Remick, L.Green, D. Newcomb, S. Garg, G. Bolgos, and D. Call, U of Michigan, Ann Arbor, MI 48103

Elevated plasma levels of IL-8 decrease neutrophil (PMN) recruitment to sites of local inflammation. We tested if IL-8 would reduce local PMN influx. Mice transgenic for human IL-8 were separated into IL-8 pos (plasma>90 ng/ml) and IL-8 neg (IL-8< 1 ng/ml) mice. Peripheral blood PMN from IL-8 pos and neg mice exhibited equal chemotaxis towards recombinant KC or MIP-2. IL-8 pos mice did not have improved survival or decreased peritoneal PMN recruitment in the cecal ligation and puncture model of sepsis. In an acute lung injury model induced by the intratracheal injection of acid, the IL-8 pos mice had equivalent alveolar PMN recruitment as the IL-8 neg mice. Additionally, there was no difference in the local recruitment of PMN when thioglycollate (thio) or glycogen was injected intraperitoneally. However, after thio injection the IL-8 pos mice had increased peritoneal levels of the murine CXC chemokines KC and MIP-2 compared to the IL-8 neg mice. These levels were nearly 3 fold higher in the IL-8 pos mice. Our data demonstrate that even with high plasma levels of IL-8, PMN are still recruited to local sites of inflammation. This influx is probably regulated by other, redundant CXC chemokines.

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IL-1-MEDIATED STABILIZATION OF GRO-A MRNA DEPENDS ON SEQUENCES IN BOTH 5' AND 3' UNTRANSLATED REGIONS. T. A. Hamilton, R. Kishore, M. Kolosov, J. A. Major, and J. M. Tebo, Cleveland Clinic Fdn., Cleveland, OH 44195

The mouse KC gene encodes a CXC family chemokine which, like human IL-8, recruits neutrophils to sites of inflammation. Both IL-1 and TNF can stimulate KC gene transcription in BALB/c 3T3 cells but mRNA accumulates to significantly higher levels in IL-1 treated cells. This results from IL-1 induced stabilization of KC mRNA. The effects of IL-1 on stabilization of KC mRNA are observed in multiple cell types including fibroblasts, endothelial cells, and epithelial cells. In order to determine if IL-1-mediated mRNA stabilization depends upon specific nucleotide sequence(s) within KC mRNA, the 5' and 3'UTRs of KC mRNA were placed in front of and behind the chloramphenicol acetyl transferase (CAT) gene in the pCATcontrol plasmid. Following transfection in BALB/c 3T3 cells, CAT activity and mRNA stability were examined following stimulation with IL-1. Inclusion of either the 3' or 5' untranslated regions of the KC gene did not alter the expression of CAT mRNA or protein. In contrast, plasmids which contained both the 5' and 3'UTRs produced modified CAT mRNA which decayed more rapidly than control CAT mRNA and this enhanced decay was prevented by treatment with IL-1. These findings indicate that IL-1 acts to stabilize specific mRNAs via nucleotide sequence motifs found in both the 5' and 3' UTR of sensitive mRNAs and further suggest that this response to IL-1 involves a signaling pathway distinct from that utilized to activate NFκB.

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TARGETED DISRUPTION OF THE MIG GENE REVEALS A ROLE FOR MIG IN ANTIBODY PRODUCTION. M. Park, D. Amichay, E. Wick, P. Love, A. Grinberg, H. Westphal, A. Iwasaki, B. Kelsall, R. Rabin, K. Elkins, and J. Farber. NIH, FDA, Bethesda, MD 20892.

MIG(CXCL9) is an IFNγ-inducible CXC chemokine that is known to function as a chemotactic factor for human T cells, particularly following T cell activation, and is presumed to be involved in the recruitment of T cells to inflammatory sites. To investigate a possible role for MIG in host defenses, we produced mice with a targeted disruption of the *mig* gene and challenged them with the bacterial pathogen *Franciscella tularensis*. Surprisingly, and in multiple experiments, the MIG knockout (MKO) mice showed significant reductions of 50-80% in anti-*Franciscella* antibody titers as compared with wild-type controls. In contrast, the MKO mice showed no deficiencies in antibody responses to standard T-dependent or T-independent antigens, demonstrating no intrinsic abnormality in B cell function. These results suggested that MIG played a role in T cell-B cell interaction and activation during the Th1-type response to this replicating pathogen. As in humans, we found that mouse $CD4^+$ and $CD8^+$ T cells could migrate to MIG. Although MIG had no effects on resting B cells, MIG induced both calcium signals and chemotaxis in B cells that had been activated through antigen receptors. Consistent with these findings, mRNA for CXCR3, the receptor for MIG, was dramatically upregulated in B cells following activation. In addition, IFNγ could induce the expression of *mig* in B cells, $CD4^+$ and $CD8^+$ T cells, and lymphoid and myeloid dendritic cells (DC). Together, our data suggest that MIG and CXCR3 may be important not only in trafficking of T cells to peripheral inflammatory sites, but also in the aggregation of activated T cells and DC and T cells and B cells within lymphoid organs that are essential for antibody responses to pathogens.

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REGULATION OF CHEMOKINE RECEPTOR EXPRESSION IN HUMAN NK CELLS

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To explore a possible role of chemokines in migration of NK cells the expression of chemokine receptors on human NK cells was investigated and the biological responses to chemokines were tested by chemotaxis assay. Freshly isolated NK cells expressed mRNA for CCR1, CCR2 , CCR5, CCR6 and CCR7, CXCR3 and CXCR4 and CX3CR1; whereas CCR3, CCR4, CCR8, CXCR1, CXCR2 and CXCR5 were absent. Interestingly, expression of CCR4 mRNA was undetectable although NK cells respond to MDC and TARC, ligands for CCR4. IL-2 activation of NK cells upregulated CCR1, CCR2, CCR5 and CCR6 and the effect was already observed after two hours of exposure. In contrast, IL-2 negatively regulated the expression of CCR7. Human NK cells express a series of inhibitory or activating receptors which regulate NK activity. We extended our studies to understand whether surface molecules that are involved in NK cell triggering can affect chemotactic response of NK cells. We have observed that exposure of NK cells to anti-CD16 and anti NKp46 down-modulates the expression of CCR1 and CCR5; the expression of other chemokine receptors is under investigation. In conclusion our data demonstrate that chemokine receptor expression on NK cells is regulated in different manner by activating soluble factors (e.g. IL-2) and by activating surface molecules (e.g. NKp46). These results will help clarifying the mechanism underlying extravasation of NK cells into various tissue.

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FUNCTIONAL IRF4 IS EXPRESSED IN MACROPHAGES AND REGULATES IL-1β GENE EXPRESSION. S. Marecki and M. J. Fenton. The Pulmonary Center and Department of Pathology, Boston Univ. Sch. Med., Boston, MA 02118.

Interferon regulatory factor 4 (IRF4) and interferon consensus sequence binding protein (ICSBP) are members of the interferon regulatory factor (IRF) family. ICSBP expression is restricted to lymphoid and myeloid cells, whereas IRF4 expression has been reported to be lymphoid-restricted. We present evidence that primary murine and human macrophages express IRF4, thereby extending its range of expression to myeloid cells. We also found that these IRF proteins can form specific complexes with the Ets-like protein PU.1, and can activate transcription via binding to PU.1/IRF composite sequences. EMSA analysis revealed that murine macrophages contained both IRF4/PU.1 and ICSBP/PU.1 complexes. Over-expression of either IRF4 or ICSBP in both macrophages and fibroblasts suppressed transcription of the PU.1-independent $H\text{-}2L^d$ MHC class I promoter. In macrophages, over-expression of IRF4 could activate an IL-1β reporter plasmid, whereas over-expression of ICSBP only weakly activated this promoter. Furthermore, both IRF4 and ICSBP could synergize with PU.1 to activate transcription of the IL-1β promoter in fibroblasts. Synergistic activation of this promoter was found to require PU.1 serine 148. A regulatory element that mediates IRF/PU.1 synergy was mapped to a region within the LPS- and cytokine-inducible enhancer element of the IL-1β gene. Together, these data demonstrate that IRF4 and ICSBP are dichotomous regulators of transcription in macrophages.

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Differential Activation of Type 1 Interferon Genes by Interferon Regulatory Factor 3 and 7, **Rongtuan Lin, Pierre Genin,Yael Mamane and John Hiscott,** Lady Davis Institute for Medical Research, and Depts. of Microbiology and Immunology and Medicine, McGill University, Montreal CANADA H3T 1E2

Recent studies implicate the interferon regulatory factors (IRF) - IRF-3 and IRF-7 - as key activators of the Type I interferon genes, as well as the RANTES chemokine gene. Because of the common and distinct biological features of IRF-3 and IRF-7, we sought to examine the differential regulation of several members of the Type I IFN gene family (IFNA and IFNB) by the interferon regulatory factors 3 and 7. Structure-function studies with IRF-7 identified a bipartite activation domain composed of a constitutive transactivation domain located between aa150 to aa246 and a negative regulatory domain between aa246 and aa416. A constitutively active form of IRF-7 was also generated by substitution of the Ser-477 and Ser-479 residues with the phosphomimetic Asp. IRF-7 - particularly IRF-7(D477/D479) - was a strong transactivator of type I interferon and RANTES chemokine gene expression. Unlike wild type IRF-3, IRF-7 overexpression was able to stimulate IFN gene expression in the absence of virus infection. Using tagged versions of IRF-7 and IRF-3, formation of homo- and heterodimers was detected by co-immunoprecipitation. Analysis of protein-DNA interactions revealed that recombinant IRF-3 and IRF-7 proteins selectively bound to different regions of the IFNB promoter; IRF-3 associated preferentially to the PRDIII domain of the IFNB promoter, while IRF-7 interacted exclusively with the adjacent PRDI domain. Consistent with protein-DNA binding, IFNB, IFNA1 and IFNA2 promoters were activated by co-expression of either IRF-3 or IRF-7, whereas IFNA4, IFNA7 and IFNA14 were exclusively activated by IRF-7. but not by IRF-3. These results demonstrate that both IRF-3 and IRF-7 transcription factors possess unique functional characteristics and share complementary rather than redundant roles in the activation of the Type I interferon genes.

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TRANSCRIPTIONAL REGULATION OF THE MGSA CHEMOKINE GENE BY THE IMMEDIATE UPSTREAM REGION (IUR). C. NIRODI and A. Richmond, Vanderbilt Univ. Sch. Med., Nashville, TN 37232 and Dept. of Veterans Affairs, Nashville, TN 37212.

Constitutive expression of the chemokine, Melanoma Growth Stimulatory Activity (MGSA/GRO), is transcriptionally regulated through several positive cis-acting elements including NF-κB, SP1, HMGI(Y), and IUR. The IUR element (-97 to -117 bp) lies immediately upstream of the NF-κB element and is necessary for basal and cytokine-induced transcription of the MGSA/GRO gene. We have purified and identified two proteins which specifically bind the IUR element, a 115 kDa poly ADP ribose polymerase (PARP) and a 170 kDa protein, the CCAAT displacement protein (CDP/Cut). Both anti-PARP and anti-Cut antibodies eliminated the IUR specific complexes in electrophoretic mobility shift analysis. In co-transfection experiments, over-expression of the Cut protein repressed MGSA promotor activity by 50%, while co-transfection with an anti-sense Cut construct led to a 5-6 fold increase in promotor activity. The data indicate that Cut represses MGSA/GRO expression by binding to the IUR element. We show that purified PARP can bind the IUR element in a sequence specific manner. Inhibition of PARP self-ADP ribosylation by 3-amino benzamide elevated MGSA mRNA levels up to four fold. The data indicate that PARP may relieve Cut-mediated repression by interactions with the IUR element. Altogether, these data suggest a mechanism of MGSA/GRO transcriptional regulation in which the IUR element is the locus of dynamic interations involving Cut and PARP.

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AN NFκB ELEMENT FLANKING SEQUENCE OF THE IL-8 PROMOTOR IS INVOLVED IN BOTH, TRANSCRIPTIONAL REPRESSION AND INDUCTION BY IL-1. M.Nourbakhsh[1], S.Eickemeier[2], H.Hauser[1], K.Resch[2], and M.Kracht[2], [1]Department of Gene Regulation and Differentiation, GBF-National Institute for Biotechnology, D-38124 Braunschweig and [2]Institute of Molecular Pharmacology, Medical School Hannover, D-30625 Hannover, Germany

Interleukin-8 (IL-8), a prototypic chemokine, can be rapidly induced by the pro-inflammatory cytokine IL-1, but is barely detectable in non-induced cells. Very little is known about the *cis*-elements and *trans*-acting factors involved in silencing of the IL-8 promotor and how these may interact with known positive transcriptional regulators of IL-8, such as NFκB. By sequence comparison with the IFN-β promotor we found a negative regulatory element (NRE) in the IL-8 promotor overlapping partially with the NFκB response element. The novel NFκB repressing factor NRF is present in the nucleus of untreated HeLa cells and binds in *vitro* and in *vivo* to the IL-8 promotor NRE. Inducible overexpression of an NRF antisense RNA leads to spontaneous IL-8 gene expression, which is compatible with NRF functioning as a transcriptional repressor. Mutation of two basepairs of the NRE immediately adjacent to the NFκB site significantly reduces IL-8 transcription induced by the coexpressed protein kinases MEKK1 and TAK1 or by IL-1 treatment. *In vitro*, this NRE mutation abolishes DNA binding of NRF, but importantly, does not affect nuclear translocation, DNA binding and transcriptional activity of NFκB p65. These data provide evidence for a dual role of the NRE in IL-8 transcription. While in the absence of stimulation it binds the repressor NRF and is involved in transcriptional silencing, in IL-1-induced cells it is required for full induction of the IL-8 promotor, in addition to NFκB. We thus identify a hitherto unrecognized molecular mechanism of IL-8 gene regulation.

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A NOVEL 3'-UTR ELEMENT IN THE TNF-α GENE ACTIVATES PKR TO ENHANCE mRNA SPLICING WITHOUT IMPEDING TRANSLATION

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We show here that the human TNF-α 3'-UTR contains a *cis*-acting element that renders splicing of precursor transcripts dependent upon the activation of PKR. When this element, designated 2-APRE, is present, splicing becomes sensitive to inhibition by the PKR inhibitor, 2-aminopurine, or by co-expression of transdominant-negative mutant PKR. Conversely, splicing is enhanced 20-fold when wild type PKR is overexpressed, showing that activation of PKR is not only necessary for splicing of mRNA when precursor transcripts contain the 2-APRE but also makes it more efficient. Thus, PKR responds as *trans*-acting factor to the 2-APRE. Deletion of the 2-APRE, or its replacement by TNF-β 3'-UTR sequences, frees splicing from a dependence on PKR activation, while its insertion into the TNF-β 3'-UTR leads to gain of this control. 2-APRE RNA forms a stable, 17-bp stem-loop structure and strongly activates PKR in vitro, inducing eIF2α phosphorylation. We show that despite its ability to activate PKR during nuclear splicing, the 2-APRE does not noticeably affect the translation efficiency of the resulting TNF-α mRNA in the cytoplasm. PKR and the 3'-UTR thus regulate mRNA splicing to permit a more efficient expression of the TNF-α gene.

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ANALYSIS OF SIGNALING FACTORS INVOLVED IN IFN-γ INDUCTION OF IDO. M.Du, W. Sotero, and M.W. Taylor Indiana University, Bloomington, IN. 47405.

IFN-γ treatment of the human cervical carcinoma cell line ME180 results in cell death due to the induction of indoleamine 2,3 dioxygenase (IDO) and resulting starvation for tryptophan. A mutant cell line 3B6A derived from ME180 was resistant to IFN due to loss of IDO activity. Cotransfection of an IDO promoter-CAT construct with IRF-1 resulted in induction of CAT activity in both ME180 and 3B6A cells even in the absence of IFN-γ. However, IRF-1 expression was not able to restore IDO activity suggesting a possible repressor site outside of the IDO promoter. STAT-1 transfection restored both CAT and IDO activities in 3B6A cells following IFN-γ treatment. 3B6A doubly treated with IFN-γ and IFN-α or β, but not TNF-β or all-trans-retinoic acid (ATRA), restored IDO activity although neither cytokine on its own could induce IDO. Western blot analysis showed that both constitutive expression and induction of STAT-1 by IFN-γ were much reduced in 3B6A cells and double treatment of IFN-γ with IFN-α or β restored the expression level of STAT-1. These data indicated that the defect in 3B6A cells was reduced expression of STAT-1 and that IRF-1, NF-kB, and PKR were all involved to some extent in the induction of IDO following IFN-γ treatment.

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ONCOGENIC EFFECTS OF DYSREGULATION OF M-RAS

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M-Ras is a newly described, highly conserved member of the Ras superfamily. Available data suggest that M-Ras is activated in parallel with p21 Ras by cytokines and other stimuli but engages a distinct set of downstream effectors. Dominant-negative Ras mutants and the monoclonal antibody Y13-259, fail to discriminate between M-Ras and p21 Ras, mandating re-evaluation of data on p21 Ras. Expression of the constitutively active mutant M-Ras G22V, in the IL-3 dependent hemopoietic cell-line R6-X, increased survival and growth in the absence of IL-3 but *decreased* growth in the presence of IL-3. In syngeneic animals, R6-X cells expressing M-Ras G22V proliferated rapidly, replacing the hemopoietic cells of the bone-marrow, entering the blood and infiltrating spleen, lung, liver and kidneys. The parental cell-line R6-X is bipotential and, when grown *in vitro* in IL-3, generates mast cells and megakaryocytes, but not platelets. However, when injected in syngeneic mice, R6-X cells expressing M-Ras G22V gave rise to platelets. M-Ras thus has positive and negative effects on the growth of hemopoeitic cells and may regulate differentiation of megakaryocytes.

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CYTOKINE-INDUCED mRNA STABILIZATION VIA THE p38 MAP KINASE PATHWAY: A ROLE FOR MAP KINASE-ACTIVATED PROTEIN KINASE 2 AND AU-RICH CIS-ELEMENTS.

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Stabilization of mRNAs contributes to the strong and rapid induction of genes in inflammation. The signaling mechanisms involved in mRNA stabilization were investigated by determining the half lives of mRNAs expressed using a tetracycline regulatable promoter system. After transcriptional shut-off the mRNAs of interleukin (IL-)6 and IL-8 rapidly disappeared in untreated HeLa cells, indicating a high basal degradation rate. mRNA half lives were markedly increased in cells expressing the catalytic domain of the MAP kinase kinase kinase MEKK1. Destabilization and signal-induced stabilization was transferred to the stable ß-globin mRNA by AU-rich region-containing fragments of IL-8 and IL-6 mRNAs, and also by defined AU-rich elements (ARE) of the c-*fos* and GM-CSF mRNAs. Of the different signaling pathways activated by MEKK1, no significant effects on mRNA degradation were observed for the SAPK/JNK, ERK and NF-κB pathways. In contrast, selective activation of the p38 MAP kinase pathway by MAP kinase kinase 6 induced mRNA stabilization, whereas a dominant negative mutant of p38 MAP kinase interfered with MEKK1 as well as with IL-1-induced stabilization. Furthermore, an active form of the p38 MAP kinase-activated protein kinase MAPKAPK2 (MK2) induced mRNA stabilization, whereas a negative interfering MK2 mutant interfered with MAP kinase kinase 6-induced stabilization. These findings indicate that the p38 MAP kinase pathway contributes to cytokine/stress-induced gene expression by stabilizing mRNAs via MK2 and an ARE-targeted mechanism.

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p38 MAPK REGULATES PRO-INFLAMMATORY mRNA STABILITY VIA MAPKAPK2.

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Inflammatory stimuli (for example IL-1, TNF and LPS) all activate the p38 MAPK cascade (MKK6 → p38 MAPK → MAPKAPK2 → hsp27). The function of this p38 kinase cascade in inflammation is uncertain, however studies using pharmacological inhibitors of transcription and of p38 activity implicate the cascade in the regulation of stability of pro-inflammatory mRNAs such as cyclooxygenase 2 (Cox 2). Here we describe an mRNA stability assay in which a chimeric β-globin/Cox 2 mRNA is expressed under the control of a tetracycline-responsive ("tet-off") promoter. We demonstrate that the 2.5 kb-long 3' untranslated region (3' UTR) of Cox 2 mRNA contains p38-responsive destabilising elements. Chimeric β-globin/Cox 2 mRNAs are inherently unstable, but can be stabilised by constitutively-active MKK6 and MAPKAPK2. Stabilisation can be reversed by blockade of the p38 pathway using dominant negative MAPKAPK2, or the p38-specific inhibitor SB203580. The p38 response resides within a short (120 nucleotide) stretch which contains only 6 of the 22 copies of the AUUUA "instability motif" present within the 3' UTR. These results confirm our previous observations made with pharmacological inhibitors. They provide the first direct evidence that p38 regulates Cox 2 mRNA stability through the 3' UTR, and that this regulation is mediated by the kinase MAPKAPK2.

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IL-1 ACTIVATES RAS AND RAP IN EL4.NOB-1 CELLS: A ROLE FOR RAS IN p38 MAPK ACTIVATION BY IL-1.

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In order to investigate the role of small G proteins in IL-1 signal transduction, we have utilised two approaches. The first of these involved lethal toxin from *Clostridium sordellii*, which specifically inactivates Ras, Rap, Rac, and Ral. This toxin inhibited p38 MAP kinase activation by IL-1 in EL4.NOB-1 cells. The target for the toxin was likely to be a Ras family member since Toxin B, from *Clostridium difficile*, which inhibits Rac, Cdc42, and Rho was without effect. To explore these phenomena further, we used the second approach, which involved dominant negative and constitutively active versions of Ras and Rap. Dominant negative RasN17 inhibited the p38 MAPK activation by IL-1, while active RasVHa activated p38 MAPK. Intriguingly, the opposite was true for Rap, whereby dominant negative Rap1AN17 activated p38 MAPK with active Rap1AV12 inhibiting IL-1 mediated activation. This result is consistent with Rap antagonising Ras function. IL-1 also activated Ras and Rap in EL4.NOB-1, the activation of Rap occurring at a later time point to Ras.
Our studies therefore provide clear evidence for Ras as a signalling component in the activation of p38 MAPK by IL-1, with Rap having an inhibitory effect.

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ROLE OF MAP KINASES IN IL-1β SYNTHESIS INDUCED BY ENGAGEMENT OF CD11b AND CD11c β2 INTEGRINS ON HUMAN MONOCYTES.

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β2 integrins are involved in the recruitment of leukocytes to inflammatory sites and in cellular activation. We demonstrate that ligation of CD11b (Mac-1, CR3) or CD11c (p150, CR4) alpha chains of β2 integrins by mAbs (clones ICRF44 or 44 and BU15 or 3.9 respectively) or soluble chimeric CD23 (obtained from Glaxo), on human monocytes rapidly induces high level of interleukin-1β production. This induction takes place at the transcriptional level and is regulated by MAP kinases family members. Indeed, stimulation of monocytes through engagement of CD11b or CD11c by mAbs or sCD23 fusion proteins, results in the phosphorylation and activation of the ERK1, ERK2 and p38 MAP-related kinases. U0126 and PD98059, inhibitors of the upstream activator of ERK1/2, the MEK1/2, suppress IL-1β mRNA expression in a dose dependent fashion showing the implication of this pathway in the transcriptional control of IL-1β production. On the other hand, inhibition of the p38/SAPK2 by SB203580 indicates that this pathway seems rather to be involved in the control of IL-1β production at a pre-translational level. Together these data demonstrate that engagement of CD11b and CD11c β2 integrins by mAbs or sCD23 lead to the activation of distinct MAP kinases family members that subsequently control IL-1β synthesis at different levels.

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AN INHIBITORY ROLE FOR THE PSEUDOKINASE DOMAIN IN REGULATION OF THE JAK2 TYROSINE KINASE.

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Hematopoietic cytokine receptors utilize the Jak/STAT pathway to mediate signals from ligand bound receptor to nucleus. The Jak2 tyrosine kinase is critical in signal transduction through several cytokine receptors such as EPO and IFN-γ receptors. Jak kinases are characterized by a tandem kinase domain structure. A tyrosine kinase domain is located in the C-terminus and preceded by a pseudokinase domain. The pseudokinase domain has sequence similarity to kinase domains, but its function is currently unknown. To analyze the roles of the different protein domains of Jak2 we constructed a series of Jak2 deletion constructs. These constructs were expressed in 293T cells, where activation of Jak2 is ligand independent. Deletion of the pseudokinase domain activated Jak2 markedly, whereas deletions in the N-terminal region did not significantly affect the activity of Jak2. Deletion of the pseudokinase domain of Jak2 resulted also in enhanced activation of Stat5. As predicted, deletion of the kinase domain resulted in catalytically inactive Jak2. This deletion co-immunoprecipitated with and inhibited the activity of the co-expressed kinase domain, and this inhibition required the pseudokinase domain. Furthermore, expression of a Jak2 mutant lacking the pseudokinase domain in a Jak2 deficient cell line deregulated IFN-γ signaling and caused constitutive ligand-independent activation of Stat1. These results suggest that the pseudokinase domain plays an important regulatory role in activation of Jak2.

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REDOX REGULATION OF CHEMOKINE RECEPTOR EXPRESSION

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Cytokines and reactive oxygen intermediates (ROI) are frequent companions at sites of acute inflammation. We have previously shown that in human monocytes LPS, IL-1 and TNF-α, induce a rapid downregulation of the Monocyte Chemotactic Protein-1 receptor (CCR2). These stimuli also induce production of ROI. Here we investigated the influence of antioxidants and/or ROI on chemokine receptors expression. In human monocytes, the antioxidant pyrrolidine dithiocarbamate (PDTC) rapidly inhibited CCR2 and CCR5 mRNA expression, by strongly decreasing transcript stability. This inhibitory activity included also CXCR4, but not CXCR2 receptor and, although to a lesser extent, was shared by the antioxidants N-Acetyl-L-cysteine (NAC) and β-mercaptoethanol. In contrast, the ROI-generating system Xanthine/Xanthine Oxidase (X/XO) increased CCR5 and CXCR4 mRNA expression, and this effect was counteracted by PDTC. Accordingly, H_2O_2 and the GSH depleting drug Buthionine Sulfoximine (BSO) increased to different extent CCR2, CCR5 and CXCR4 mRNA expression. The PDTC-mediated inhibition of CCR5 and CXCR4 mRNA expression was associated with decreased chemotactic responsiveness and with a marked inhibition of surface receptor expression. These results suggest that the redox status of cells is a crucial determinant in the regulation of chemokines system with potential relevance in HIV infection and inflammatory diseases.

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CCR5, CXCR4, AND CD4 ARE CLUSTERED AND LOCALIZED ON MICROVILLI OF HUMAN MACROPHAGES AND T-CELLS VIA IMMUNO-EM.

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The chemokine receptors CCR5 and CXCR4 act synergistically with CD4 in an ordered multi-step mechanism to allow the binding and entry of HIV-1. The efficiency of such a coordinated mechanism depends on the spatial distribution of the participating molecules on the cell surface. Immuno-EM was performed to address the subcellular localization of the receptors and CD4 at high resolution. Cells were fixed, cryo-processed, frozen, and 80 nM cryosections generated that were double labeled with combinations of CCR5, CXCR4, and CD4 antibodies, and then stained with immunogold. Surprisingly, CCR5, CXCR4, and CD4 were found predominantly on microvilli, and appeared to form homogeneous micro-clusters in all cell types examined, including macrophages and T-cells. Further, while mixed micro-clusters were not observed, micro-clusters of different molecules were frequently separated by distances less than the diameter of an HIV-1 virion. Homogeneous clusters of chemokine receptors were observed in small trans-Golgi vesicles implying that such clusters were organized shortly after synthesis. These results strongly suggest that CCR5, CXCR4, and CD4 are distributed in a manner which facilitates cooperative receptor interactions during HIV-1 adsorption to, and penetration of human leukocytes. Moreover, since selectins, some integrins, and actin are also located on/in the microvillus, this organelle has many of the major elements required for chemotaxis.

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MOLECULAR MODELING AND MUTATIONAL ANALYSIS OF CCR5 IN CHEMOKINE BINDING AND HIV-1 ENTRY*

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The human CC chemokine receptor 5 (CCR5) is the receptor for several chemokines such as macrophage inflammatory protein 1 β (MIP-1β) and the coreceptor for the entry of human immunodeficiency virus type 1 (HIV-1). An approach combining protein structure modeling and site-directed mutagenesis was used to probe the structure of CCR5 and its interactions with chemokine ligands and HIV-1. Hypothetical three-dimensional structures were proposed for the CCR5-MIP-1β complex, which served as a useful vehicle to rationalize data from genetic and molecular biological experiments. These models were further tested by site-directed mutagenesis of the amino-terminal (Nt) region and the second extracellular loop (ECL2) of CCR5. These structural and mutational studies provided valuable information about the structural basis for CCR5 activity in chemokine binding and HIV-1 viral entry and may guide the design of novel inhibitors.

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CD62L$^-$ AND CD62L$^+$ MEMORY CD4$^+$ T CELLS DIFFERENTIALLY SUPPORT M-TROPIC HIV REPLICATION. M. MENGOZZI, S.C. De Rosa, E. Vicenzi, G. Poli, M. Malipatlolla, L.A. Herzenberg, L.A. Herzenberg and M. Roederer, Stanford Univ., Stanford, CA 94305-5318, San Raffaele Scientific Institute, 20132 Milano, Italy and Univ. of California at San Francisco, CA 94143-0422.

CCR5, the major coreceptor for M-tropic (R5) HIV-1 strains, is expressed by ~50% of memory CD45RA$^-$CD62L$^-$ CD4 T (M1) cells and by only a small fraction of memory CD45RA$^-$CD62L$^+$ (M2) cells. We studied the ability of M1 and M2 to support R5 HIV replication upon CD28 costimulation. M1 and M2 cells were sorted, infected with HIV-1 BaL and stimulated either with antiCD3/B7.1 or with antiCD3/CD28-coated beads. After antiCD3/B7.1 stimulation, total CD4 and both M1 and M2 efficiently supported HIV replication, indicating that the low CCR5 levels on M2 are sufficient for infection to occur. However, when antiCD3/CD28 beads were used as a stimulus, only M1 were able to efficiently support HIV replication; only very low levels of HIV RT activity could be detected in sorted M2 or even in bulk CD4 (comprised of M1, M2, and naïve T cells)–apparently, bulk CD4 T cells could inhibit viral replication that occurs in purified M1 cells. This inhibition was also observed by adding M2 or naïve T cells (that make very low levels of β-chemokines) to M1 cells. June *et al.* had previously described that antiCD3/CD28 stimulation induces a state of resistance to R5 HIV in bulk CD4, due to decreased CCR5 expression and increased β-chemokine production. However, antiCD3/CD28-stimulated M1 cells produced *higher* levels of MIP-α, MIP-1β and RANTES than M2 and bulk CD4. Moreover, inhibition of HIV replication in co-cultures did not correlate with β-chemokine production. Our data demonstrate that other mechanisms (than downregulation of CCR5 and expression of β-chemokines) exist that can induce resistance to R5 HIV strains in CD4 T cells, and that these inhibitory mechanisms are selectively expressed by naïve and M2 CD4 T cells.

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SEMI-ALLOGENEIC CELL HYBRIDS STIMULATE HIV-1 ENVELOPE-SPECIFIC CYTOTOXIC T LYMPHOCYTES.

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Several studies suggest that immunity to human leukocyte antigens (HLA) may protect against HIV infection, and raise the possibility that stimulation of the allogeneic T helper (Th) response could be used in the immune-based therapy to drive HIV-specific cytotoxic T cells (CTL). To enhance the simultaneous interaction of allogeneic Th, HIV-specific CTL, and antigen presenting cells (APC), we prepared semi-allogeneic cell hybrids from the fusion of HIV-positive donor PBMC with the FO1-12 allogeneic melanoma cell line. These hybrids were used as APC for HIV envelope peptide-specific cytotoxic assays. The hybrids express HLA class I and II antigens from both parental cells. HIV–specific CTL activity was generated when patients' PBMC were stimulated with a pool of envelope peptides plus semi-allogeneic hybrids, but not with either envelope peptides or hybrid cells alone. Thus, the semi-allogeneic hybrids enhanced HIV-specific CTL. Irradiated, semi-allogeneic cell hybrids engineered for individual AIDS patients provide efficient and simultaneous co-recognition of HLA allogeneic and viral antigenic determinants presented by self-HLA molecules on the same APC that can result in the generation of enhanced HIV-specific CTL activity.

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POLYMORPHISM IN CONTROL REGIONS OF CHEMOKINE AND CYTOKINE GENES. J. H. Bream, M. Carrington, M. Martin, A. Ping, C. Winkler, M. Smith, H. D. Shin, S. J. O'Brien, H. A. Young, LEI and LGD, NCI-FCRDC, Frederick, MD 21702-1201.

The identification of polymorphic loci in cis-acting portions of genes, offers a potential mechanism to explain at least in part, interindividual differences in gene expression and disease pathogenesis. Relative to some disease states, this may be particularly true for genes involved in the control of immune responses. Our group has recently focused on this hypothesis in two ways: 1) relating novel as well as known polymorphisms in cytokine and chemokine genes to different disease outcomes, and 2) screening for functional differences between alleles. Thus far, novel or previously known single nucleotide polymorphisms (SNP) in the promoters of CCR5, and IL-10, were found to associate with progression rates of HIV-1 infection. We have indications that there may be functional differences between the wild type (WT) and variant alleles as evidenced by protein expression and/or DNA protein interactions. Likewise, SNPs in the proximal human IFN-γ and IL-1β promoters have differential DNA-binding activities between WT and variant sequences that for IL-1β, is cell-type, and stimulus specific. By gel-shift analysis, differential binding patterns between IL-1β alleles have been demonstrated in nuclear extracts from LPS-stimulated fresh monocytes, but not PMA + ionomycin-stimulated monocytes or T cells. A rare SNP in the proximal human IFN-γ promoter creates a new DNA-binding site with sequence homology to AP-1. Nuclear extracts from fresh human T cells stimulated with P/I form a unique DNA-protein complex on the variant sequence as compared to WT. Currently we are trying to identify signal transduction pathways leading to functional differences in gene expression between WT and variant alleles in hopes of better understanding disease pathogenesis.

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THE NFκB SUBUNIT P50 TRANSACTIVATES, WHILE P65 REPRESSES EXPRESSION OF C-REACTIVE PROTEIN (CRP). A. Agrawal, C. Hyunjoo, D. Samols and I. KUSHNER, Case Western Reserve Univ. and MetroHealth Med. Center, Cleveland, OH 44109.

IL-6 activates transcription of the acute phase protein CRP in Hep 3B cells while IL-1β, which alone has no effect, is markedly synergistic in combination with IL-6. Previous studies of 123 bp of the CRP promoter showed that C/EBP isoforms and STAT3 were major mediators of the IL-6 effect. The transcription factors responsible for IL-1β synergy are unknown. Since IL-1β activates the NFκB system and nonconsensus potential κB sites appear to flank the C/EBP binding site and to overlap the STAT3 binding site in the proximal CRP promoter, we explored the effects of rel family members on CRP expression. Overexpression of the NFκB subunit p50 transactivated a series of 7 deletion constructs of the CRP promoter extending from -904 to -86, an effect greatly enhanced by both IL-6 and IL-1β. In contrast, overexpressed p65 inhibited the response of -157 CRP-luciferase to cytokines, overexpressed p50, overexpressed C/EBPβ, and their combinations. To determine if p50 was associated with the CRP promoter, we performed electrophoretic mobility shift assays employing a 24-bp probe containing the C/EBP binding site at position -50 to -55 of the CRP promoter. We found p50, but not p65, present in complexes with C/EBPβ and C/EBPδ following IL-6 treatment. The p50 binding site was positioned in a polypyrimidine tract just 3' to the C/EBP binding site. We conclude that p50, but not p65, can act as a positive effector of cytokine responses on the CRP gene. We speculate that p50 homodimers stabilize C/EBP isoform binding to the CRP promoter and that IL-1β synergy is due to increased availability of p50 after IL-1β exposure. The inhibitory effect of overexpressed p65 may be attributed to its ability to bind p50 and prevent formation of p50 homodimers. (Supported by NIH grant AG02467)

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ANALYSIS OF INTERFERON TYPE I RECEPTOR ACTIVATION USING CYTOKINE RECEPTOR CHIMERAS. X. VAN OSTADE, E. PATTYN, L. SCHAUVLIEGE, A. VERHEE, M. KALAÏ, J. VANDEKERCKHOVE AND J. TAVERNIER, Department of Medical Protein Research, Flanders Interuniversity Institute for Biotechnology, University of Ghent, Faculty of Medicine, K.L. Ledeganckstraat 35, 9000 Gent, Belgium

We constructed chimeric receptors, comprising the extracellular domains of the Epo receptor or the interleukin-5 receptor α (IL-5Rα) or β (βc) subunits, fused to the transmembrane and intracellular domains of the interferon receptors IFNaR1 and IFNaR2-2. Cells transfected with either the IL-5$R\alpha_{extra}$/IFNaR1_{intra} + βc_{extra}/IFNaR-2_{intra} or the reciprocal IL-5$R\alpha_{extra}$/IFNaR-2_{intra} + βc_{extra}/IFNaR1_{intra} combinations could sustain IL-5 induced activation of the IFN-inducible 6-16 promoter, indicating that a certain degree of flexibility is allowed for IFNaR heterooligomerisation. In addition, expression of only EpoR_{extra}/IFNaR-2_{intra} activated the 6-16 promoter upon Epo treatment, thereby demonstrating that IFNaR2-2 homodimerisation is sufficient for activation of at least part of the IFN signaling pathway. However, since IFNaR-2_{intra} homodimerisation is not functional in the case of IL-5Rα/IFNaR2-2 + βc/IFNaR2-2 or the reciprokal combination, these results suggest that a correct positioning of the IFNaR2-2 intracellular domains is required. Analysis of Epo vs. IFNα induced transcription of genes, involved in antiviral protection, was comparable up to 24 hrs. after stimulation. At later time points a sharp decrease in mRNA levels was observed, only in the case of Epo induction. Since, in contrast to IFNα, Epo showed no or a reduced protection against VSV or EMCV infection respectively, our results imply a role for IFNaR1 in the induction of sustained mRNA levels that are likely required for optimal antiviral activity.

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RAC1 REGULATES IL1-INDUCED NFκB ACTIVATION IN AN IκB-INDEPENDENT MANNER BY ENHANCING THE ABILITY OF THE P65 SUBUNIT TO TRANSACTIVATE GENE EXPRESSION. C.A.Jefferies and L.A.J.O'Neill, Department of Biochemistry and Biotechnology Institute, Trinity College, Dublin 2.

Nuclear factor-κB is a key pro-inflammatory transcription factor which is activated on stimulation with the cytokine interleukin-1 (IL-1). While the pathway leading to phosphorylation and subsequent degradation of the NFκB inhibitory subunit IκBα in response to IL1 has been well characterised, NFκB activity is also regulated by a separate but less well characterised pathway leading to enhanced transactivation activity, possibly via phosphorylation of the p65 subunit of NFκB. We have examined the involvement of the low molecular weight G protein Rac1 in both of these pathways in the murine thymoma cell line EL4.NOB-1. We have found that IL-1 activates Rac1 in EL4 from 1 minute. Using constitutively active RacV12 and dominant negative RacN17 mutants we found that RacN17 inhibits IL1-induced κB-dependent reporter gene expression but not IκBα degradation while RacV12 potentiates κB-dependent reporter gene expression in response to IL1 but has no effect on its own. This suggested a role for Rac1 in p65-mediated transactivation of NFκB, independent of IκBα regulation. In support of this we show that IL1 activates a pathway leading to increased p65 transactivation activity and that RacV12 alone can drive this response. In addition, RacN17 inhibits IL1-driven p65-mediated transactivation, suggesting that Rac1 participates in this pathway. Using specific inhibitors of p38, p42/p44 and JNK mitogen activated protein kinase pathways we propose that both p38 and p42/p44, but not JNK, lie downstream of Rac1 on the IL1 pathway leading to enhanced transactivation by p65. Our study therefore identifies Rac1 as an important G protein in IL-1 signalling which via p38 and p42/p44 MAP kinase activation plays a critical role in the transactivation of gene expression by of NFκB.

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INTERFERONS INHIBIT ACTIVATION OF STAT6 BY INTERLEUKIN-4 IN HUMAN MONOCYTES BY INDUCING *SOCS1* GENE EXPRESSION. Dickensheets, H., C. Venkataraman*, U. Schindler*, and R. Donnelly. FDA, Bethesda, MD 20892; and *Tularik, Inc. South San Francisco, CA 94080.

Interferons inhibit induction by IL-4 and IL-13 of multiple genes in human monocytes. However, the mechanism by which IFNs mediate this inhibition has not been defined. IL-4 activates gene expression by inducing tyrosine phosphorylation, homodimerization and nuclear translocation of the latent transcription factor, STAT6 (signal transducer and activator of transcription-6). STAT6-responsive elements are characteristically present in the promoters of IL-4-inducible genes. Because STAT6 activation is essential for IL-4-induced gene expression, we examined the effects of type-I and type-II IFNs on induction of STAT6 activity by IL-4 in primary human monocytes. Pretreatment of monocytes with IFN-β or IFN-γ, but not IL-1, IL-2, macrophage colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, IL-6, or transforming growth factor β suppressed activation of STAT6 by IL-4. This inhibition was associated with decreased tyrosine phosphorylation and nuclear translocation of STAT6, and was not evident unless cells were preincubated with IFN for at least one hour before IL-4 stimulation. Furthermore, inhibition by IFN could be blocked by cotreatment with actinomycin D and correlated temporally with induction of the gene for the JAK/STAT inhibitory protein, *SOCS-1*. Forced expression of *SOCS-1* in a macrophage cell line, RAW264, markedly suppressed trans-activation of an IL-4-inducible reporter as well as IL-6 and IFN-γ-induced reporter gene activity. These findings demonstrate that IFNs inhibit IL-4-induced activation of STAT6 and STAT6-dependent gene expression, at least in part, by up-regulating expression of *SOCS-1*.

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CHARACTERIZATION OF THE KINASE ACTIVITY IMPLICATED IN THE ACTIVATION OF THE IRF-3 TRANSCRIPTION FACTOR. Marc J. Servant, Christophe Heylbroeck, Hakju Kwon, Benjamin ten Oever, Rongtuan Lin and John Hiscott. Molecular Oncology Group, Lady Davis Institute, McGill University, Montreal, Quebec, Canada, H3T 1E2.

Infection of host cells by viruses leads to the activation of multiple transcription factors that are involved in the host response to virus infection. Among these factors is the interferon regulatory factor 3 (IRF-3) transcription factor which is post translationally modified as a result of virus infection; phosphorylation of latent cytoplasmic IRF-3 on serine and threonine residues in the C-terminal region leads to dimerization, cytoplasmic to nuclear translocation, association with the p300/CBP coactivator and stimulation of DNA binding and transcriptional activities. The rate limiting step in the activation of IRF-3 is therefore its phosphorylation by an unknown kinase. In an effort to identify this kinase, we conducted a series of molecular and biochemical experiments. First, the use of pharmacological inhibitors demonstrated that intracellular calcium and tyrosine kinase(s) are both implicated in the pathway leading to IRF-3 phosphorylation. In addition, IRF-3 phosphorylation seems to be essentially dependent on virus infection, since stimulation of target cells with growth factors, stress inducers or cytokines did not induce IRF-3 phosphorylation. Second, expression of dominant negative form of kinases known to be activated by virus infection demonstrated that MEKK-1, COT, NIK, IKKα/β and PKR are not implicated directly or indirectly in the phosphorylation of IRF-3. Partial biochemical purification (by gel exclusion chromatography (Superose 12)) however indicates the kinase likely exists as a monomeric protein or a protein complex with an approximative molecular weight of 150 kDa. Further characterization is underway to identify the virus activated kinase that phosphorylates IRF-3.

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NOVEL POST-TRANSLATIONAL REGULATION OF INTERFERON REGULATORY FACTOR-4 ACTIVITY BY THE IMMUNOPHILIN FKBP52. **Y. Mamane, S. Sharma, L. Petropoulos, R. Lin and John Hiscott** Molecular Oncology Group, Lady Davis Institute, and Dept. of Microbiology, McGill University, Montreal, CANADA, H3T 1E2.

The lymphoid restricted transcription factor Interferon Regulatory Factor-4 (IRF-4) plays a critical role in immunoregulatory gene expression in B and T lymphocytes and also is highly expressed in cells infected with the human T cell leukemia virus type 1 (HTLV-1). In a yeast two hybrid screen, a novel interaction between IRF-4 and the FK506 binding protein 52 (FKBP52), a member of the immunophilin family with peptidyl-prolyl isomerase activity (PPIase) was observed. Using a combination of affinity chromatography and co-immunoprecipitation, the IRF-4 proline rich domain (aa150-237) was identified as the minimal domain required for interaction with the tetratricopeptide repeat domain of FKBP52. In EMSA, IRF-4-FKBP52 association inhibited IRF4/PU.1 binding to its natural target, the Immunoglobulin (Ig) light chain enhancer Eλ2-4, by blocking IRF-4 but not PU.1 binding. Inhibition of IRF-4 DNA binding required functional PPIase activity since the PPIase inhibitor ascomycin restored IRF-4 binding. Furthermore, FKBP52 inhibited the synergistic transactivation of the IRF-4/PU.1 dependent Eλ2-4 promoter in co-transfection assays. Finally, FKBP52 association with IRF-4 resulted in a structural modification of IRF-4 detectable by immunoblot analysis as a more rapidly migrating IRF-4 form, an effect that was also dependent on functional PPIase activity. These results suggest that FKBP52 induces a conformational change in IRF-4 by cis-trans prolyl isomerization that interferes with IRF-4 DNA binding and transcriptional activity. The direct involvement of the immunophilin FKBP52 in the regulation of IRF-4 function demonstrates a novel post-translational modification controlling IRF-4 dependent gene expression.

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CXCR2 CARBOXYL-TERMINUS LEUCINE-LEUCINE AND ISOLEUCINE-LEUCINE MOTIFS ARE INVOLVED IN RECEPTOR INTERNALIZATION AND CHEMOTAXIS. G. H. FAN, W. Yang, X. J. Wang, and A. Richmond, Vanderbilt Univ. Sch. Med., Nashville, TN 37232 and Dept. of Veterans Affairs, Nashville, TN 37212.

Agonist treatment of the seven transmembrane G protein-coupled chemokine receptor CXCR2 induces receptor internalization, but the mechanism underlying the endocytosis is still poorly understood. In the present study, we demonstrate that HEK-293 cells expressing a transfected mutant form of CXCR2 with the C-terminal phosphorylation sites truncated (CXCR2-331T) still undergo internalization upon agonist stimulation. The association of this mutant receptor with beta-arrestin is greatly reduced, suggesting that the C-terminal serine/threonine residues are not necessary for the endocytosis of CXCR2. Using point mutation, we show that the leucine-leucine and isoleucine-leucine motifs in the C-terminus proximal to the serine residues targeted for phosphorylation play an important role in the agonist-induced internalization of CXCR2 and CXCR2-331T in HEK-293 cells. Mutation of L-320 and L-321 or L-323 and L-324 to A causes little change in surface expression and ligand binding. However, agonist-induced internalization is remarkably reduced in L320/321A, and I323A/L324A double mutants, and greatly impaired in the L320A/L321A/I323A/L324A quadruple mutant, as measured by radioligand binding and immunofluorescence. Furthermore, agonist-induced chemotaxis is significantly decreased in the cells stably expressing the above mutants, indicating that C-terminal leucine-leucine and isoleucine-leucine motifs involved in agonist-induced internalization of CXCR2 are also required for chemotaxis. We postulate that the leucine repeats may associate with a second adaptor protein which facilitates internalization.

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IL-16/CD4 ACTIVATION OF T CELLS DESENSITIZES TO SDF-1/CXCR4 CHEMOTAXIS. W. CRUIKSHANK, A. Jenkins, L. Ledwich, M. Vallen, W. Brazer, and D. Center. Pulmonary Center, Bost Univ Sch Med, Boston, MA., 02118.

We have recently reported that stimulation of T cells by IL-16 via CD4 and MIP-1β via CCR5, results in reciprocal receptor cross-desensitization and inhibition of chemotaxis. The kinase activity of p56lck activation was required for chemotactic deactivation. The cross desensitization was selective for CD4/CCR5 as chemotactic signals through CCR1,2,3 were unaffected. We now report on the chemotactic relationship between CD4 and CXCR4. Human T cells were incubated with IL-16 prior to SDF-1α-induced chemotaxis. IL-16 (10^{-10}M) pretreatment blocked >95% of SDF-1α-induced migration at all concentrations used. This effect was not due to loss of CXCR4 expression. Unlike CD4/CCR5 desensitization, the CD4/CXCR4 effect was not dependent on p56lck enzymatic activity as treatment with herbimycin A did not inhibit IL-16-induced desensitization. However, association of CD4 with p56lck was required as IL-16 stimulation of cells expressing mutated CD4 unable to associate with lck did not desensitize SDF-1α migration. Also, unlike the CD4/CCR5 relationship, CD4/CXCR4 cross desensitization was not reciprocal as pretreatment with SDF-1α was not inhibitory for IL-16-induced migration. These studies demonstrate that IL-16 via CD4 can desensitize CXCR4 signaling which is dependent on an adaptor function but not the enzymatic activity of p56lck and imply a different relationship between CXCR4 and CD4 than that observed for CCR5 and CD4.

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REDUCED ACCESSIBILITY AT THE TCR GAMMA LOCUS FOR RAG MEDIATEDCLEAVAGE IN IL-7R-/- THYMOCYTES K.Muegge, S.K. Durum, and M.Schlissel, National Cancer Institute, Frederick, MD, 21701 and Univ. of California, Berkely, CA, 94720.

Defects in the IL-7 signal transduction pathway lead to severe immunedeficiency in man or mice. In IL-7R-/- mice, lymphoid precursors show a reduced survival rate and V(D)J recombination is arrested in the TCR gamma locus, aberrant in the IgH locus and delayed in the TCR beta locus. Here we analyse the recombination defect of the TCR gamma locus. Using ligation mediated PCR we sought intermediates of the recombination process. In the absence of the IL-7 signal, no initiation of recombination of the TCR gamma locus was observed, whereas, recombination intermediates at the TCR beta locus could be detected. Thus, the failure to rearrange the TCR gamma locus is due to a failure to initiate cleavage rather than a failure to religate broken DNA ends. VDJ recombination was previously thought to begin at the pro-T2 stage of T cell development after the arrest of IL-7R-/- thymocytes at the pro-T1 stage. However here we show that both TCR gamma and beta recombination intermediates are readily detectable in normal pro-T1 cells, but only TCR beta intermediates were detected in IL-7R-/- pro-T1 cells, supporting a mechanistic role for IL-7 in TCR gamma locus rearrangement. Since reduced rag gene expression has been reported in the absence of the IL-7 signal we directly tested whether the TCR gamma locus is accessible to cleavage by recombinant Rag proteins in vitro. We found a sixfold reduction in chromatin accessibility for Rag mediated cleavage in IL-7R-/- thymocytes in comparison with wild type. Thus IL-7 controls recombination at the TCR gamma locus by regulating locus accessibility.

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A DISEASE PROTECTIVE AND IMMUNE SUPPRESSIVE ROLE OF TNF IN MODELS OF SYSTEMIC AND ORGAN-SPECIFIC AUTOIMMUNITY. G. KOLLIAS, G. Kassiotis and D. Kontoyiannis. Hellenic Pasteur Institute, Athens 115-21, Greece

The pathogenic capacity of tumor necrosis factor (TNF) has been classically attributed to its pro-inflammatory activities. Recent experimental evidence however, have suggested that TNF can act as an immune modulating factor and thus reduction in the levels of this cytokine may prove equally pathogenic. Moreover, anti-TNF treatment of several human autoimmune diseases has unexpectedly led to immune activation and increased disease activity. Because at excessive TNF levels the immune suppressive and disease protective effects of TNF may be masked by its pro-inflammatory properties, we examined the effect of TNF deficiency in two models of systemic and organ specific autoimmune disease, namely the (NZBxNZW) F1 hybrid mouse as a model for human systemic lupus erythematosus (SLE) and experimental autoimmune encephalomyelitis (EAE) as a model for human multiple sclerosis (MS). We report here that an engineered TNF deficiency in an otherwise disease-free NZB hybrid background is associated with the early spontaneous development of antinuclear autoantibodies and autoreactive B cells, followed by the appearance of lupus-like glomerulonephritis. We further show that TNF deficiency enables the development of sustained myelin basic protein (MBP)-specific T cell responses, which effectively convert MBP-resistant mice into a state of susceptibility to a chronic form of EAE. Together these results indicate that TNF deficiency prolongs and intensifies pathogenic autoimmune responses in diverse autoimmune models. The opposing pro-inflammatory and immune suppressive properties of TNF allow better understanding of autoimmune disease pathogenesis and promise a more effective design of anti-TNF therapeutic approaches.

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RETROVIRAL CROSS-TALK: HTLV-II DOWN-REGULATES HIV-1 REPLICATION FROM PBMC OF CO-INFECTED INDIVIDUALS VIA MIP-1α. G. POLI, E. Vicenzi, A. Cimarelli, G. Magnani, P. Ciancianaini, E. Cattaneo, P.P. Dall'Aglio, U. Bertazzoni, and C. Casoli. San Raffaele Scientific Institute, Milano, Italy, and University of Parma and University of Verona, Italy.

Coinfection by the human T-cell leukemia/lymphoma types II (HTLV-II) and human immunodeficiency virus (HIV) occurs in 10-20 % of Italian intravenous drug users. Yet, the potential reciprocal influence of these two human retroviruses is poorly understood, although it is reported anecdotally that coinfection by HTLV-II may slow down HIV disease progression. Primary PBMC cultures from mono and co-infected individuals were established in the presence of IL-2. The kinetics of HIV-1 replication were inversely correlated to the *in vivo* HTLV-II proviral load and its replication in *in vitro* cultures. In the same co-infected PBMC cultures, endogenous IL-2 was not produced, whereas IL-6 and TNF-α were secreted at levels compatible with their ability to upregulate HIV-1 expression. Of interest, the kinetics and concentrations of the HIV suppressive CC-chemokines RANTES, MIP-1α, and MIP-1β were inversely related to those of HIV-1 replication. Furthermore, $CD8^+$ T-cells or PBMC from HTLV-II mono-infected individuals were cocultivated with $CD4^+$ T-cells from HIV-1 mono-infected individuals separated by a semipermeable membrane in the presence or absence of anti-chemokine neutralizing antibodies. These experiments indicated that HTLV-II can interfere with the replicative potential of HIV-1 by upregulating viral suppressive CC-chemokines and, in particular, MIP-1α. Thus, HTLV-II downregulates HIV replication *in vitro* via upregulation of CC-chemokines.

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mRNA MONOKINE EXPRESSION & REGULATION IN MS DISEASE Graham A, LG Filion, D Matusevicius, MA Freedman, Univ. of Ottawa, Ottawa ON K1H 8M5

Multiple Sclerosis (MS) is a chronic, autoimmune, inflammatory disease of the central nervous system. We hypothesized that monokines that favor Th1 development are involved in this process. In our study, healthy controls (HC), MS patients either Relapsing Remitting (RR) or Secondary Progressive (SP)were included. Enriched monocytes were obtained and were cultured for 24 hours in medium alone, IL-10 and IFNβ-1a. Total mRNA was obtained from the cells and the Riboquant RNase protection assay (IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1Ra, IL-6, and IFNγ) was performed. SP and RR patients showed significantly higher levels of IL-1β as compared to HC levels. SP patients showed significantly higher levels of IL-1α, IL-1Ra, and IL-6 as compared to HC. SP patients showed significantly higher levels of IL-10, IL-1α, IL-1β, IL-1Ra, and IL6 as compared to RR patients, and significantly higher levels of all cytokines under study as compared to RR patients undergoing therapy. 24 hr culture with IL-10 and IFNβ-1 significantly reduced levels of IL1-Ra, IL-6, and IFNγ in HC samples. Similar trends were noted for RR patients undergoing therapy where reduction in levels of IL1-Ra, IL6, and IL-10 approached significance. Levels of all monocytic cytokines, excluding IL-12, were significantly reduced in both RR and SP patients following culture with IL-10. Importantly, patient cytokine levels following IL-10 culture did not significantly differ from HC levels. Interestingly, IFNβ-1a, which is presently used as a MS therapy, only appeared to approach significance in the reduction of IFN-γ levels in RR patients, and IL-1Ra and IL-6 levels in SP patients. In conclusion, increased monocytic cytokine levels such as IL-1α, IL-1β, and IL6 correlate with increased disease severity as compared to HC levels. These results suggest a role for these cytokines in the pathogenesis of MS.

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UP-REGULATED IL-6 IN SYSTEMIC LUPUS ERYTHEMATOSUS (SLE): NOT JUST ANOTHER IMMUNE ANOMALY? M. Linker-Israeli, Cedars-Sinai Research Institute and UCLA, Los Angeles, CA 90048.

Because human SLE can be studied only at the stage of overt disease, the primary factors involved in its pathogenesis have yet to be sorted out. Previously, we found that SLE monocytes and B cells express IL-6 constitutively, that endogenous IL-6 supports production of autoantibodies by SLE PBMC and, in follow-up studies, that marked reduction of IL-6 levels was seen in sera of patients that responded to treatment. Next, we identified several factors that could contribute to IL-6 up-regulation in SLE. IL-6 mRNA decay was significantly delayed in SLE PBMC ($p<0.01$), likely due to endogenous TNF-α and IL-1β, since blocking these cytokines destabilized IL-6 transcripts. In contrast, exogenously added IL-10, IL-4 and TGFβ_1 down-regulated LPS-induced IL-6 expression. Accordingly, using RNase Protection Assays, we found that whereas TNF-α and IL-1β expression in SLE PBMC coincided with that of IL-6 (1-4h), IL-10 was expressed at 4- 6h. By PCR- genotyping 146 SLE and 139 controls (Caucasian and African-American), we found strong association between IL-6 minisatellite alleles and SLE ($p=0.001$), and high IL-6 levels ($p<0.05$). Transient transfections with a luciferase reporter gene construct suggest that the SLE-associated alleles can modulate gene expression. Together, these studies show that, although the response to up- and down- regulating signals for IL-6 production is intact in SLE, the availability and kinetics of cytokines providing these signals, as well as the genetic susceptibility that could be conferred by SLE-associated IL-6 alleles, may contribute to the deregulated expression of IL-6 in lupus.

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EXPLANATION FOR THE PROTECTIVE EFFECT OF ENDOGENOUS IFN-γ IN THE PATHOGENESIS OF EXPERIMENTAL AUTOIMMUNE DISEASES
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In several autoimmune disease models, the outcome of IFN-γ ablation procedures has been supportive of a protective rather than a disease-promoting role. Such examples are experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveitis (EAU) and collagen-induced arthritis (CIA). The protective effect of IFN-γ in these models contrasts with apparent disease-promoting role in transgenic mice overexpressing IFN-γ and in spontaneous lupus nephritis and diabetes. We initiated the present study, as we noted that all three models in which endogenous IFN-γ exerts a distinct protective role, rely on the use of the killed mycobacteria-containing adjuvant, CFA, for immunisation of the animals. With the use of the CIA model, we present evidence that the protective role of IFN-γ is completely ascribed to the presence of mycobacterium. In fact, if mycobacterium is omitted in the induction of CIA, endogenous IFN-γ acts as a disease-promoting cytokine. Killed mycobacterium appears to stimulate a de novo Mac-1^+ splenic cell population, by myelopoiesis that is profoundly inhibited by endogenous IFN-γ, and that predominates over the proinflammatory role of IFN-γ in the pathogenesis of autoimmune arthritis by an increased cellular (DTH) response.

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DOUBLE-BLIND STUDY OF THE CLINICAL EFFECT OF ANTI-INTERFERON-γ IN RHEUMATOID ARTHRITIS (RA)
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In previous work we found that polyclonal anti-IFN-γ abs in the treatment of acute RA gave results comparable to or better than anti-TNF-α abs or anti-IFN-α abs—remission of more than 2 years in some patients (clinical observation and *Int J Immunotherapy* (1) 23-32 (1998)). A double-blind, randomized study was conducted in 23 patients with acute RA refractory to anti-inflammatory drugs and at least one DMARD. Polyclonal abs to IFN-γ and to TNF-α and a placebo were administered to separate groups of patients. All preparations were given daily IM for 5 successive days. Results were evaluated on days 7 and 28. In both treatment groups, on day 7 after start of treatment, a significant improvement in pain, morning stiffness, fatigue, no. of swollen and tender jts., c-reactive protein, and grip strength in both hands was observed. In the placebo group, only pain, morning stiffness, and fatigue dropped. A 20% improvement in Paulus was observed on day 28 in 2 patients in each of the 2 treatment groups. By days 7-10, in 7 of the 8 patients in the placebo group, an expression of acute RA occurred, requiring supplementary therapy. The present work, the first stage of a larger double blind study of anti-cytokines in RA patients, is objective confirmation of the therapeutic effect of IFN-γ abs in this disease and permits consideration of IFN-γ abs or IFN-γ abs and TNF-α abs combined as possible new therapies. In future, humanized monoclonal abs must be used to allow longer treatment.

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CLINICO-IMMUNOLOGICAL EFFECTS OF A SHORT COURSE OF ANTI-INTERFERON-γ ANTIBODIES IN SECONDARY PROGRESSIVE MULTIPLE SCLEROSIS
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Dysregulation of cytokine production was proposed as a mechanism of autoimmune disease in 1974 by one of us (*Nature*, 247:551, 1974). Reduction of pro-inflammatory cytokine levels is believed to be a method of treatment of multiple sclerosis (MS). We conducted a randomized, placebo-controlled trial of purified polyclonal anti-tumor necrosis factor-α (TNF-α) and anti-interferon-γ (IFN-γ) antibodies (IgG) in 45 patients (15 in each group) with secondary progressive MS (5 daily IM injections of abs or placebo). Clinical observation, data on evoked potentials, MRI, and immunological parameters, including serial determination of TNF-α, IFN-γ, and TGF-β levels in supernatants of short term blood cell cultures were recorded. After 6-12 months of observation, only patients who received anti-IFN-γ showed stabilization of the disease course as shown by MRI with a decrease in TNF-α and an increase in TGF-β levels in contrast to the placebo and anti-TNF-α groups. Thus even a short course of anti-IFN-γ may in some MS patients induce slowing of disease progression for at least 6 months. To study potential of achieving sustained improvement with anti-IFN-γ abs, humanized monoclonal abs must be used for a longer period of time.

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PATIENTS WITH RHEUMATOID ARTHRITIS HAVE ELEVATED INTERLEUKIN-15-LEVELS IN SERUM
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Elevated IL-15 levels have been previously demonstrated in synovial fluids of RA patients where they were found partly responsible for T-cell chemoattraction and proliferation. The aim of this study was to discover whether IL-15 can be demonstrated in sera of RA patients and patients with other arthritides. Detectable IL-15 levels were found in sera of 30/38 (79%) of RA patients in contrast to 3/17 (18%) of OA patients, 11/26 (42%) of patients with inflammatory arthritides and no detectable levels in all healthy controls. IL-15 mean (± SE) level in sera of RA patients (87.8 ± 22.1 pg/ml) was significantly elevated as compared with mean level in sera of OA patients (6.2 ±3.2 pg/ml, P = 0.012) and sera of patients with inflammatory arthritides (16.7 ± 7.7 pg/ml, P = 0.013). In 11 of 12 samples that were obtained from the same patients after an interval of 3-8 months from the time of the first sample, IL-15 levels were maintained in the same range. This may indicate that in RA patients there is a continuous systemic production of IL-15. IL-15 levels did not correlate with clinical parameters of RA (number of active or effused joints, existence of eroded or deformed joints, duration of morning stiffness or duration of the disease). Continuous systemic production of anti-apoptotic factor such as IL-15 might interfere with the normal elimination of auto-reactive T cells and thus contribute to the articular and systemic manifestations of RA.

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THE EFFECT ON IMMUNOSUPPRESIVE DRUGS ON IL-15-INDUCED LYMPHOCYTE PROLIFERATION IN A MIXED LYMPHOCYTES KIDNEY-CELLS CULTURE
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Elevated Interleukin-15 (IL-15) mRNA levels strongly correlate acute rejection of kidney allografts. To study the interaction between lymphocytes and kidney cells we co-cultured PBMC with human tubular epithelial cells (TEC). Lymphocyte proliferation was enhanced in the presence of TEC and increased further by pretreatment of PBMC-with OKT3 (anti-CD3). Lymphocyte proliferation in co-culture was blocked by antibodies to IL-15 but remained unaffected by anti-IL-2 antibodies. TEC pretreatment with interferon-γ increases IL-15 production and at the same magnitude lymphocyte proliferation. We tested the effect of immunosuppressive drugs on lymphocyte proliferation in this system. Cyclosporin-A (CsA) dexamethasone (Dex) and rapamycin (Rapa) had no effect on IL-15 production by TEC. Dex and Rapa but not CsA significantly impaired IL-15 induced lymphocyte proliferation and lymphocyte proliferation co-cultured with TEC.
We conclude that TEC-derived IL-15 is the major mediator for lymphocyte proliferation in this co-culture. This data strongly suggest IL-15 as an important mediator in kidney allograft rejection and that certain failure of immunosuppressive drugs to prevent rejection may be explained by their incompetence to inhibit IL-15 production and activity.

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ARTHRITIS IN MALE AND FEMALE TUMOR NECROSIS FACTOR-α KNOCKOUT (TNF-$\alpha^{-/-}$) AND MEMBRANE-BOUND TNF-α TRANSGENIC (TNF-α^{TgA86}) MICE INJECTED WITH *MYCOPLASMA PULMONIS* OR *MYCOPLASMA ARTHRITIDIS*.
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Experimental infection with *Mycoplasma* in rodents results in a chronic arthritis histologically resembling human RA. Objectives: (1) characterize the *Mycoplasma*-induced arthritis in control mice, (2) study the roles that either the soluble and/or membrane-bound forms of TNF-α (sTNF-α/mTNF-α) play(s) in *Mycoplasma*-induced disease in novel TNF-α Tg and KO mice, and (3) determine if inhibiting sTNF-α or mTNF-α with PEG sTNF-RI affects progression. TNF-$\alpha^{-/-}$, TNF-α^{TgA86} (overexpressing a non-cleavable mTNF-α, but *no* sTNF-α), or control mice (age 4-24 wks, SPF housed) were injected iv with veh. or with viable *M. pulmonis* or *M. arthritidis* (10^{10} CFU) and followed for several weeks both clinically and histopathologically. TNF-$\alpha^{-/-}$ mice inj. with *M. pulmonis* develop severe arthritis with extensive PMN/Mϕ infiltration and bone/cartilage destruction. By d+25, lesion incidence (INC) and severity (SEV) is greater in male than female mice (100% vs 40% INC; 1.6 vs 0.6 SEV). Disease was more pronounced with *M. pulmonis* than *M. arthritidis*. TNF-α^{TgA86} also develop arthritis. Injection of *M. pulmonis* enhanced the lesions. *M. pulmonis* infected TNF-$\alpha^{-/-}$ mice treated with PEG sTNF-RI had a non-significant reduction in disease. TNF-α^{TgA86} mice treated with PEG sTNF-RI had a >50% reduction in SEV compared to vehicle-controls. These results demonstrate that PEG sTNF-RI inhibits membrane-bound TNF-α-induced arthritis.

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ABNORMAL RESPONSES TO IL-6 IN OLIGODENDROCYTES LACKING SHP-1.
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Il-6 is a proinflammatory cytokine released by activated cells during viral infections as part of the immediate innate immune response. We are interested in the regulation of Il-6 activities during central nervous system(CNS) viral infections where Il-6 is known to be highly expressed. It is proposed that genetically altered regulation of Il-6 activities on oligodendrocytes may be responsible for pathology of these cells in virus-induced demyelinating disease. We have found that the protein tyrosine phosphatase SHP-1 is expressed by and regulates pathological changes in oligodendrocytes in response to Il-6 exposure. Oligodendrocytes isolated in pure cultures from motheaten(me/me) mice lacking functional SHP-1 genes display increased STAT3 activation, abnormal morphological transformation, and cell death in response to Il-6 not seen in oligodendrocytes of normal littermates. A gene dosage effect is seen in heterozygous me/+ mice demonstrating functional haploinsufficiency in controlling these Il-6 activities. Me/me mice also lack SHP-1 in the CNS in vivo and rapidly succumb to virus-induced demyelinating disease including extensive white matter inflammation and corresponding spastic limb paralysis. Thus, interactions between Il-6 and SHP-1 activities appear to have a direct pathophysiological effect on oligodendrocytes and possibly demyelinating disease.
This work was supported by a grant from the National Multiple Sclerosis Society.

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DIFFERENTIAL SUSCEPTIBILITY OF MICE LACKING TUMOR NECROSIS FACTOR-α AND/OR LYMPHOTOXIN-α FOR LIPOPOLYSACCHARIDES DERIVED FROM DIFFERENT GRAM-NEGATIVE BACTERIA

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Neutralization studies have shown that tumor necrosis factor-α (TNF) and lymphotoxin-α (LT) mediate many of the deleterious effects induced by the lipopolysaccharide (LPS) components of Gram-negative bacteria. However, recent studies performed in TNF-deficient mice have failed to demonstrate a central role for this cytokine during lethal endotoxemia. We investigated this apparent discrepancy by assessing the susceptibility of TNF knock-out (TNF-/-) and TNF/LT double knock-out mice (TNF-/-LT-/-) to lethal endotoxaemia induced by LPS derived from various Gram-negative microorganisms. Whereas TNF-/- mice were as susceptible as TNF+/+ controls to Salmonella typhimurium LPS (10 vs. 0% survival, $p>0.05$), they were significantly more resistant to lethal endotoxemia induced by Escherichia coli (100 vs. 20%, $p<0.01$) or Klebsiella pneumoniae LPS (100% vs. 10%, $p<0.01$). These effects were even more pronounced in the TNF-/-LT-/- mice. The difference in lethality was accompanied by decreased interleukin-1 (IL-1α and β) and interferon (IFN)-γ production in TNF and LT deficient mice after in vivo stimulation with E. coli LPS. In contrast, there was a more than 4-fold up-regulation of IFNγ and IL-1 synthesis in TNF-/- and TNF-/-LT-/- mice after S. typhimurium LPS. In conclusion, the role of TNF and LT is central in lethal endotoxaemia, but is dependent on the bacterial source of the LPS.

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REGULATION OF CHOLESTEROL TRAFFICKING IN MACROPHAGE DERIVED FOAM CELLS BY GAMMA INTERFERON. C.G. Panousis, and S.H. Zuckerman, Lilly Research Labs, Indianapolis, IN 46250

The Th1 derived cytokine gamma interferon, IFN-γ, is present within the microenvironment of an atheromatous lesion and likely contributes to lesion progression through macrophage activation. While the inflammatory effects of IFN-γ are well known, the role of this cytokine in cholesterol metabolism in macrophage derived foam cells is unclear. In the present study, the incubation of foam cells with IFN-γ resulted in the reduction of HDL3 mediated cholesterol efflux. The decrease in cholesterol efflux was not observed with other macrophage activating factors as colony stimulating factors failed to demonstrate a similar effect. The reduction in cholesterol efflux was independent of apo E synthesis or SR-BI expression and was associated with a redistribution of intracellular cholesterol with an increase in cholesterol ester accumulation. The increase in the esterified pool, primarily in cholesterol eicosaprentadenoate, dococapentaenoate, arachidonate and linoleate was associated with a 2 fold increase in Acyl-CoA:Cholesterol-O-Acyltransferase, ACAT, activity and message without any change in neutral cholesterol ester hydrolase activity. While CD36 message was reduced in IFN-γ treated foam cells, the ability to reverse the decrease in efflux by the ACAT inhibitor A58035 in a dose dependent manner suggests that the IFN-γ effect on efflux is primarily through the modulation of ACAT expression. Therefore, in addition to its inflammatory effects, IFN-γ can contribute to the progression of an atherosclerotic lesion by altering the pathway of intracellular cholesterol trafficking in macrophage derived foam cells.

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CASPASE-1-INDEPENDENT FAS/FAS LIGAND-MEDIATED IL-18 SECRETION FROM MACROPHAGES. H. Tsutsui,* N. Kayagaki, K.† Kuida,§ H. Yagita,† H. Okamura,* and K. Nakanishi,* *Hyogo College of Medicine, Nishinomiya 663-8501 Japan, †Juntendo Univ. Tokyo 113-8421, Japan, and §Vertex Pharmaceuticals Inc., 02139-4242 MA.

IL-18, produced as bioinactive precursor, is processed by caspase-1 in LPS-activated macrophages. In this study, we investigated caspase-1-independent processing of IL-18 in Fas ligand (FasL)-stimulated macrophages and its involvement in liver injury. Administration of *Propionibacterium acnes* (*P. acnes*) upregulated functional Fas expression on macrophages in an IFN-γ-dependent manner, and these macrophages became to secrete mature IL-18 upon stimulation with FasL. This was also the case for caspase-1-deficient mice. Administration of recombinant soluble FasL (rFasL) after *P. acnes*-priming induced comparable elevation of serum IL-18 in parallel with elevated serum liver enzyme levels. However, liver injury was not observed in IL-18-deficient mice even after rFasL administration. These results indicate caspase-1-independent pathway of mature IL-18 secretion from FasL-stimulated macrophages and its critical involvement in FasL-induced liver injury.

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MULTIPARAMETER ANALYSIS OF CELLULAR RESPONSES BY PBMC FROM DONORS IMMUNIZED WITH INFLUENZA VACCINE

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There is great interest in applying multiple immunological assays to the analysis of the cellular responses of PBMC that are stimulated with antigen *in vitro*. ELISA and flow cytometric techniques have especially been useful for investigating the immunological competence of individuals. In this study we investigated the response of eight immunized and two non-immunized donors to a commercial influenza vaccine antigen *in vitro*. Different doses of antigen were used to determine an optimal concentration for the stimulation of donor PBMC. The levels of IL-2, IFN-γ and TNF-α secreted by cultured cell populations from each donor were measured by ELISA. Cellular proliferation in response to antigenic stimulation was determined through the analysis of ^{3}[H]-thymidine, MTT and/or BrdU incorporation. The results showed a marked increase in the cellular proliferation and cytokine production by cell populations from most immunized donors as compared to the responses of the two non-immunized individuals. Furthermore, the frequency of antigen-induced cytokine-producing cells was determined using fluorescent anti-cytokine antibodies and flow cytometric analysis in an intracellular staining procedure. Lymphocytes producing IFN-γ and/or TNF-α were detected in immunized donors up to even 22 months post immunization. BrdU incorporation and subsequent immunofluorescent staining was found to show increases in DNA synthesis in PBMC from immunized versus non-immunized donors following *in vitro* stimulation. This study demonstrates the efficacy of using ELISA and flow cytometric techniques for the quantitation of the cellular responses of PBMC to antigenic stimulation with influenza antigen.

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BONE MARROW STROMA OF CYTOTOXIC DRUG-TREATED MICE PRESERVES ITS FUNCTIONS: GM-CSF PRODUCTION AND HEMATOPOIESIS STIMULATION.

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Our aim was to study stroma's supportive functions of hematopoiesis after Ara-C treatment, parallel to chemotherapy preBMT. Results:**A**)Ara-C impact on BM stroma:decreased CFU-F stem cells - day 2, recovery - day 4. Pre - CFU-F gave rise to huge colonies(0.5 - 2 mm) - day 1, increased - day 2 and decreased - day 4. **B**) GM-CSF levels in CM of stroma from Ara-C mice and controls were in the same low range (5 pg/ml). IL-3, IL-4, IFNγ were undetectable, High pretreatment IL-6 levels decreased on days 1,2 and increased-day 4. **C**) The significant decrease of total BM cells on day 1 and the gradual increase through day 4 correlates inversly to the incidence of marrow blast cells at days 1,2 decreasing on day 4. This finding indicated BM regeneration starting soon (day 1) after Ara-C.**D**). Hematopoietic stimulation by stromal layer expressed by HPPC clonogenicity increases above normal level on day 1, peaks on day 2 and decreases to normal level on day 4, despite transient decrease of CFU-F and marked decrease of IL-6. We conclude a transitory BM stromal dysfunction post Ara-C. Despite initial CFU-F stem cells decrease and low levels of stromal IL-6, Ara-C' stroma stimulated hematopoiesis of target cells in coculture.

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GENE EXPRESSION AND PRODUCTION OF IFN-γ BY HUMAN'S ERYTHROID CELLS.

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Previous research showed the ability of mouse erythroid blast cells to suppress the immune response both *in vivo* and *in vitro*. It was demonstrated that the protein synthesis is needed for this effect of suppression, and the effect is mediated by the soluble factor. Mouse erythroid cells separated from spleen with erythroid hyperplasia showed the IFN-γ gene expression. The effect of the soluble factor was blocked by adding neutralizing anti-mouse-IFN-γ antibodies. We have studied IFN-γ gene expression and IFN-γ production by human erythroid blast cells. Human erythroid blast cells population was isolated from fetal liver (15-20 weeks) by percoll density gradient and subseqient removal of adherent cells and 72 hours cultivation with presence of Epo. Isolation purity evaluated morphologically was more than 98%. Expression level was estimated by RT-PCR, and cytokine production measured by electrochemiluminescent method using poly- and monoclonal antibodies. Conditional media of erythroid cells ($5x10^6$ cell/ml) was obtained after 24 hour cultivation without Epo. IFN-γ mRNA was detected by RT-PCR in erythroid blast cells cultivated both with and without Epo. IFN-γ concentration in conditional media of erythroid cells cultivated without Epo varied from 0 to 150 pg/ml, while increased up to 42631pg/ml if Epo was added into the cultivation media, comparable to PBMC IFN-γ production after mitogenic stimulation taking the cell concentration the same. Increasing production of IFN-γ after added Epo can carry out a negative feedback at regulation of erythroid proliferation.

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THE IL-11 SYSTEM AND SPERMATOGENESIS.

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IL-11 is a pleiotropic hemopoietic growth factor which also exhibit regulatory activities of adipogenesis and bone development. Functional IL-11 receptor (IL-11R) results from the dimerization of the common IL-6R family subunit, gp 130, with a transmembrane subunit able to bind specifically IL-11 with low affinity. Recently, 2 genes encoding a 99% homologous IL-11 binding receptor subunit have been isolated in mice, they differ by their 5'end untranslated region and exhibit different tissue expression patterns. Interestingly, while the first gene appeared ubiquitous, the expression of the second locus was restricted with a particular abundance in the testis. As IL-11 has also been shown to be strongly expressed within the murine testis, this prompted us to study IL-11 and IL-11R expression in this organ. For practical reasons – quantity of material available, ability to isolate a great number of testicular cells with high purity – the rat model was chosen. We have first characterized the rat transcript encoding IL-11-R by northern blot and sequenced most of it. The restriction map analysis of the gene has not revealed any polymorphism which could confirm the existence of 2 genes in the rat, as shown previously in mice. We are currently investigating the 5'end untranslated région of IL-11-R mRNA in the testis and the spleen. IL-11-R was found to be stably expressed in the rat testis during embryogenesis, from 13.5 days pc until birth, during puberty and adulthood. RT-PCR analysis has revealed the presence of the transcript in Sertoli cells, peritubular cells and in meiotic germ cells. Moreover, we were able to study testis sample from 3 different individuals using northern blot and RT-PCR analysis, it shows that human testis express both IL-11R and its ligand. Altogether, our results establish the existence of an IL-11 system in the testis of all mammalian species tested which strongly suggests a role for this cytokine in the regulation of the testicular function.

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INTERLEUKIN-1 RECEPTOR ANTAGONIST PRODUCTION BY SERTOLI CELLS.

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Interleukin-1 has been suggested to be involved in the cell-cell cross talk within the testis. It is produced by Leydig cells, Sertoli cells, germ cells and testicular macrophages. To identify the testicular cell source and factors involved in the regulation of IL-1ra production, mouse Sertoli-cells were isolated, purified, cultured and examined for IL-1ra levels. IL-1ra levels were determined in the supernatant and the lysates of primary Sertoli cells cultures under normal and infection stimulatory in-vitro conditions. Immunohistochemical staining for IL-1ra was performed on Sertoli cell cultures with or without stimulation.

Sertoli cells from the testes of 15-day old Balb/c mice were isolated by subsequential enzymatic digestion. On day 3 they were purified by hypotonic shock treatment and incubated on day 4 for 24h in the presence of FSH or lipopolysaccharides (LPS). Supernatants and lysates of Sertoli cells were examined for IL-1ra by ELISA. Sertoli cell-germ cell cocultures were established on 4-chamber culture slides and incubated with or without LPS. Slides were immunohistochemically stained using IL-1ra antibodies.

Our results show that large amounts of immunoreactive IL-1ra could be detected in lysates of unstimulated Sertoli cells. IL-1ra levels in the supernatants were below detection level. LPS, IL-1α, IL-1β, and FSH were found to stimulate the production of IL-1ra by Sertoli cells, and maximal levels were examined after 8 hours of stimulation. Immunohistochemical staining confirmed the presence of IL-1ra in the cytoplasma of Sertoli cells. The expression of IL-1ra was demonstrated by RT-PCR analysis. Our results may indicate the involvement of IL-1ra in the autocrine and paracrine regulation of testicular cell function and thus to affect male fertility.

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DIFFERENT EXPRESSION OF IL-1α, IL-1β and IL-1Ra IN TESTICULAR CELLS OF MATURE AND IMMATURE MICE. M Huleihel, E Lunenfeld, D Zeyse, I Prinsloo, G Potashnik and M Mazor. Faculty of Health Sciences Ben-Gurion University of the Negev, Beer-Sheva, Israel and Christian-Albrechts-University of Kiel Medical School, Kiel, Germany.

The aim of the study was to characterize the cellular source(s) and the levels of IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra) in the testis of mature and immature mice under normal and pathological conditions.

Mature (8 weeks) and immature mice (2 weeks) were injected (i.p.) with saline or LPS (10μg/mouse). Mice were killed after 4, 16 and 24 hours, following which their testicular tissues were examined by immunohistochemical staining for cell source and level of expression of IL-1α, IL-1β and IL-1ra. Formaline- and/or B5-fixed paraffin-embedded testicular tissues were stained using polyclonal rabbit anti-mouse IL-1α, or anti-mouse IL-1β, or anti-mouse IL-1ra primary antibodies. As negative controls, the primary antibodies were pretreated with the relevant peptide and tissues were stained with the secondary antibody only.

Testicular tissues from mature and immature mice highly expressed IL-1α in both seminiferous tubule cells and Leydig cells. However, low expression of IL-1β (compared to the expression of IL-1α) was found in seminiferous tubule cells of mature and immature mice. IL-1β was also expressed in Leydig cells of mature mice but not in immature mice. IL-1ra was shown to be highly expressed in seminiferous tubule cells but less than in Leydig cells of mature and immature mice. In addition, prominent expression of IL-1ra was observed in the Golgi apparatus of tubular and Leydig cells. The levels of IL-1α, IL-1β and IL-1ra in testicular tissues from mature and immature mice were not affected by LPS injection.

Our results may suggest the involvement of IL-1 system in the physiological functions of testicular tissue and the process of spermatogenesis. Thus, IL-1 may be involved in male fertility.

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REDUCTION OF NF-κB PROTEIN ASSOCIATED WITH INTERLEUKIN-1β DEFICIENCY DETERMINES A HIGHER SENSITIVITY TO THE ANTI-INFLAMMATORY EFFECTS OF PROGESTERONE. L. L. Reznikov, S-H. Kim, G. Fantuzzi, J. Frishman, B. D. Shames, C. H. Selzman, and C. A. Dinarello, Univ. Colorado Health Sciences Center, Denver, CO 80262.

We have reported that IL-1β$^{-/-}$ mice exhibit decreased LPS-induced uterine and splenic cytokine production in pregnancy compared to similarly challenged pregnant wild-type mice. This decrease in LPS-inducible cytokines in pregnant IL-1β$^{-/-}$ mice was associated with a reduction in the constitutive level of the NF-κB p65 protein. In the present study we observed that splenocytes from non-pregnant mice exhibited decreased LPS-induced TNFα and MIP-1α production when primed *in vitro* with progesterone. This suppression was 25% greater ($p<0.05$) in IL-1β$^{-/-}$ than in IL-1β$^{+/+}$ mice. The *in vitro* blockade of IL-1 activity with IL-1 receptor antagonist (IL-1Ra) in splenocytes cultured from non-pregnant IL-1β$^{+/+}$ mice also significantly increased immunosuppressive properties of progesterone ($p<0.05$) bringing levels of LPS-induced TNFα and MIP-1α production down to those observed in splenocytes cultured from IL-1β$^{-/-}$ mice. Associated with this suppression, we observed a reduction in NF-κB p65 by Western blotting after 24 hours incubation of IL-1β$^{+/+}$ splenocytes with IL-1Ra plus progesterone. Therefore, the relative reduction of NF-κB p65 protein associated with IL-1β deficiency or with *in vitro* blocking of IL-1β activity determines a higher sensitivity of LPS-challenged murine splenocytes to the anti-inflammatory effects of progesterone. This finding implicates a role of IL-1β in the steady state level of p65 protein and subsequently in the development of immunosuppressive effects in pregnancy.

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IL-18 BINDING PROTEIN UPREGULATES SPONTANEOUS AND IL-1-INDUCED PGE_2 PRODUCTION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC). L. L.REZNIKOV[1], S-H. Kim[1], J. Y. Westcott[2], J. Frishman[1], G. Fantuzzi[1], D. Novick[3], M. Rubinstein[3], C.A. Dinarello[1], [1]Univ. Colorado Health Sciences Center, Denver, CO 80262, [2]National Jewish Medical and Research Center, Denver, CO 80206, and [3]Weizmann Institute of Science, Rehovot, Israel.

IL-18 binding protein (IL-18BP) binds IL-18 and prevents its biological activities. Because of similarities of IL-18 and IL-1 signaling pathways, the role for IL-18 and IL-18BP in PGE_2 production in human PBMC was studied. Although IL-1β induced a 12-fold increase in PGE_2 production in PBMC after 48 hours compared to resting cells ($p<0.01$), no induction of PGE_2 synthesis nor cyclooxygenase-2 gene expression were observed in PBMC-stimulated with IL-18. Incubation of human blood macrophages with IL-18 for 48 hours resulted in production of the chemokine IL-8 ($p<0.05$) whereas induction of PGE_2 synthesis was not detected. In PBMC IL-18 synergistically enhanced IL-1β-induced IL-8 production, but suppressed IL-1β-induced production of PGE_2. The suppressive effect of IL-18 on PGE_2 production in PBMC appeared to be mediated by IFNγ as revealed by anti-IFNγ-antibody. Similarly, IL-12 augmented IL-1β-induced IFNγ but suppressed IL-1β-induced PGE_2 by 75% ($p<0.05$). Blocking of endogenous IL-18 by an addition of IL-18BP to PBMC increased IL-1β-induced PGE_2 production (1.8 fold-increase; $p<0.05$). Unexpectedly, treatment with IL-18BP also increased the spontaneous PGE_2 production (2.3 fold-increase; $p<0.05$). This study suggests that endogenous and exogenous IL-18 exerts a suppressive effect on PGE_2 synthesis in PBMC via IFNγ production. Since the immunosuppressive properties of PGE_2 in Th1 responses are known, the ability of IL-18BP to increase spontaneous and inducible PGE_2 may offer an advantage in treatment of Th1-mediated autoimmune diseases as IL-18BP reduces IFNγ production while avoiding suppression of PGE_2.

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CONDITION OF CYTOKINE SYSTEM OF THE PERSONS UNDERGONE THE INFLUENCE OF RADIATION. L.V. GRISHINA, Yu.A. Semikova, V.V. Temchura, S.V. Semikov, V.A. Kozlov, Institute of Clinical Immunology, 630099, Yadrintsevskaya str., 14. Novosibirsk, Russia. E-mail: ici@online.nsk.su

Gene expression of cytokines IL-1β, IL-6, TNF-α in peripheral blood mononuclear cells and serum levels of IL-1β, IL-6, TNF-α, IL-4, IFN-γ of the persons, who were exposed to radiation resulted from action at Semipalatinsk nuclear range were measured by the RT-PCR and the electrochemiluminescence method. The increased gene expression of IL-1β, IL-6, TNF-α in peripheral blood intact mononuclear cells was observed as well as the increased serum levels of proinflammatory cytokines IL-1 and TNF-α. The increase of their concentration higher than threshold level (for IL-1β - 3000 pg/ml, for TNF-α - 2000 pg/ml) resulted in change of other cytokines serum levels, particularly Th1 and Th2 cytokines – IFN-γ and IL-4 which largely determine immunopathogenesis features of a radiation defeat. These changes depended of the equivalent doze of territory radioactive pollution. The concentration of serum cytokines were higher at women, than at men. The increase in expression and production of proinflammatory cytokines are due to patogenetic influence of the radiation on a cytokine network, and result in increase of serum T-helper type 1 and 2 cytokines level.

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IL-2, IFN-γ AND IL-4 SERUM PROFILES IN HEPATITIS C AND MIXED C+B INFECTION. D.H.KURAMSHIN, N.P.Tolokonskaya*, A.N.Silkov, S.V.Sennikov, V.A.Kozlov
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A group of 132 patients of hepatitis C and mixed hepatitis C+B studied both in acute and chronical form, serum of healthy donors was used as a control. Serum levels of Th1 (IL-2 and IFN-γ) and Th2 (IL-4) cytokines were measured and analysed in relation to the results of PCR and anti-NS tests. IL-4 serum levels was significantly higher in patients (all subgroups of C and C+B hepatitis) compared to controls. Maximum IFN-γ and IL-2 levels were detected in acute monohepatitis C subgroup with positive RNA HCV PCR results(216 and 46 pg/ml respectively). In acute monohepatitis C subgroup with negative PCR IFN-γ and IL-2 levels were lower (77 and 9 pg/ml), while in chronic monohepatitis and all mixed hepatitis subgroups figures were comparable to controls despite the PCR test results. In acute monohepatitis C subgroup with positive anti-NS test IFN-γ and IL-2 levels were 207 and 42 pg/ml, while in acute monohepatitis C subgroup with negative anti-NS test as well as all mixed hepatitis subgroups serum IFN-γ and IL-2 concentrations were at control level. According to our data positive PCR and anti-NS test results in monohepatitis C patients are assosiated with marked increase in Th1 cells secretory activity.

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IL-18 HAS POTENTIAL TO STIMULATE IL-4 PRODUCTION BY BASOPHILS BUT WITH IL-12 ONLY EXHIBITS ITS ANTI-ALLERGIC ACTION IN VIVO.
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Mast cells and basophils play a key role in allergic inflammation. Upon cross-linkage of FcεR1, they release various chemical mediators and cytokines. For this reason, we regard it important to examine the effect of IL-12 and IL-18 on the function of these cells. Here we show that basophils derived by culture of bone marrow cells with IL-3 for 10 days express IL-18Rα chain and produce large amounts of IL-4 and IL-13 in response to stimulation with IL-3 and IL-18. Injection of a mixture of IL-12 and IL-18 inhibits IgE production in helminth infected mice and abolishes the capacity of their basophils to produce IL-4 and IL-13 in response to stimulation either with IL-3 and IL-18 or with FcεR cross-linkage. By contrast, this combination of cytokines actually increases IgE levels in helminth infected IFNγ-/- mice and enhances IL-4 and IL-13 production by their basophils. Injection of IL-18 alone enhances basophil production of IL-4 and histamine both in wild type and IFNγ-/- mice. Thus, IL-18, although anti-allergic when administered with IL-12, stimulates IL-4 and histamine release by basophils in vivo.

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STAPHYLOCOCCUS EPIDERMIDIS-INDUCED IFN-γ IN WHOLE BLOOD: THE ROLE OF ENDOGENOUS IL-18, IL-12, IL-1, and TNF. R. Stuyt[1], M. Netea[1], S-H. Kim[1], D. Novick[2], M. Rubinstein[2], C.A. Dinarello[1], [1] Univ. of Colorado Health Sciences Center, Denver, CO 80262 and [2] Dept. of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel

S. epidermidis is a Gram-positive bacterium known to induce IFN-γ, TNF-α and IL-1. These cytokines are important in the Th1/Th2 paradigm. IL-18 is a co-stimulus of IFN-γ production in the context of IL-12, IL-2 or microbal agents. IL-12 and IL-18 act synergistically to increase IFN-γ production. Recently, IL-18 binding protein (IL-18BP) was described as a constitutive modulator of the Th1 cytokine response. Since IL-18BP binds IL-18 and blocks its biological activity, it may play an important role in the immune response. We stimulated whole human blood with heat-killed *S. epidermidis* in the presence of various cytokine antagonists in order to investigate the cytokine cascade leading to IFN-γ production. To assess the importance of IL-18, IL-12, IL-1 and TNF we used specific antibodies/ antagonists such as IL-18BP, anti-IL-12, IL-1 receptor antagonist (IL-1Ra) and TNF binding protein (TNF-BP). IFN-γ was measured in these whole blood cultures. IL-18BP dose-dependently inhibited IFN-γ production by 50% at 100 ng/mL and at the highest concentration tested (5 μg/mL) reduced *S. epidermidis*-induced IFN-γ production by 80%. IFN-γ inhibition in these cultures was also observed with anti-IL-12 (77%). IL-1Ra (10 μg/mL) marginally reduced IFN-γ production, non-statistically significant. TNF-BP (10 μg/mL) significantly reduced IFN-γ levels by 36%. This study shows that endogenous IL-18 is essential for *S. epidermidis*-induced IFN-γ production.

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THE RECOMBINANT AGLYCOSILATED IL-4δ2 PARTICIPATES IN THE REGULATION OF IFN-γ AND IL-4 ACTIVITIES.
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An alternative splice variant of IL-4 in which the second of four exons (residues 22-37) is omitted, has been recently discovered and designated as IL-4δ2. The protein has been previously described as a potential naturally occurring antagonist of IL-4, that is preferentially expressed in the thymus and airways. In this work we investigated the effects of aglycosilated recombinant human (rh)IL-4δ2 on the activities rhIL-4 and rhIFN-γ. The IL-4δ2 protein inhibited IL-4-stimulated proliferation of human thymocytes. The ability of IFN-γ (high doses) to inhibit the proliferation of mitogen-stimulated thymocytes was diminished by IL-4δ2 but not IL-4. Labelled [^{125}I]-IFN-γ was bound to two types of sites of thymic epithelial cells (TEC): those of high-affinity, $Kd_1=2{,}2x10^{-11}$ M and medium-affinity, $Kd_2=1{,}9x10^{-9}$ M. The pre-incubation of TEC with IL-4 but not IL-4δ2 or IL-4 + IL-4δ2 decreased medium-affinity IFN-γ sites expression.

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TH1/TH2 CYTOKINE PRODUCTION DURING THE STRESS OF BREAST CANCER DIAGNOSIS. L. WITEK-JANUSEK and H. L. Mathews. Loyola University of Chicago, Maywood, IL 60153.

Psychological stress can lead to changes in immune function. In this study, the experience of diagnostic breast biopsy was evaluated for its' effect, as a naturalistic stressor, on NK cell activity (NKCA) and cytokine production. A within group design in which women experiencing heightened psychological stress (pre biopsy) followed by alleviation of stress (post-biopsy) was employed. Psychological stress was measured by assessing mood disturbance, perceived stress, and anxiety. Most women experienced heightened perceived stress, anxiety, anger, depression, and tension prior to biopsy with alleviation post biopsy. Additionally, most women experienced decreased vigor and increased fatigue pre biopsy with alleviation post biopsy. Overall, these psychological effects corresponded with a reduction in NKCA pre-biopsy and an increase in NKCA post biopsy. Women with continued high mood disturbance and high stress post biopsy had even further reductions in NKCA. Women with high mood disturbance and high fatigue also had decreased capacities to produce interferon γ. Further, Th1/Th2 ratios of IL-2 or interferon γ : IL-10 showed that women with heightened perceived stress and fatigue had reduced ratios of Th1 to Th2 production of cytokines. These observations demonstrate this paradigm of human stress to result in not only an acute reduction in immune function at the time of breast biopsy but also a sustained reduction in NKCA and Th1 cytokine production with continued stress.

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THE EFFECT OF EXCITOTOXIC DAMAGE ON ENDOGENOUS IL-1β EXPRESSION IN THE RAT BRAIN. LC Parker, GN Luheshi, NJ Rothwell & SM Allan. University of Manchester, M13 9PT, UK.

The cytokine interleukin-1β (IL-1β) has been proposed to mediate several forms of acute neurodegeneration. Co-injection of the glutamate receptor agonist S-AMPA and IL-1β in the striatum of the rat brain results in severe distant injury in the cortex, which is not seen when S-AMPA is injected alone. The objective of the present study was to investigate the role of endogenous IL-1β in this phenomenon. This was achieved by measuring IL-1β mRNA expression, at several sites in the rat brain, using competitive RT-PCR. IL-1β mRNA expression was significantly increased at the site of injection (striatum) by insertion of the injection cannulae (sham operated animals) compared to untreated animals, probably due to mechanical tissue damage. Injection of vehicle, S-AMPA, IL-1β or IL-1β+S-AMPA caused no further increases in IL-1β mRNA expression in the striatum. In the cortex there was a significant increase in IL-1β mRNA expression in sham operated rats when compared to naïve animals, and injection into the striatum of hrL-1β or IL-1β+S-AMPA caused a further significant increase in IL-1β mRNA expression when compared to sham, vehicle or S-AMPA treated groups. This increase in endogenous IL-1β in the cortex may contribute to the cell death that occurs in this area when IL-1β is co-injected with S-AMPA in the striatum. In the hypothalamus, an important target of cytokine actions in the brain, there was a significant increase in IL-1β mRNA expression only in response to striatal injection of IL-1β or IL-1β+S-AMPA (compared to untreated, sham, vehicle or S-AMPA treated animals). This result may indicate that IL-1β injection in the striatum upregulates its own expression in different remote brain regions, through the activation of specific neuronal pathways, utilising important brain regions such as the hypothalamus. Further work will investigate the nature of these pathways.

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INDUCIBLE EXPRESSION OF THE LONG PENTRAXIN PTX3 IN THE CENTRAL NERVOUS SYSTEM

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PTX3, a prototypic long pentraxin, is induced by primary proinflammatory signals in various cell types, most prominently macrophages and endothelial cells. Other long pentraxins, such as rat neuronal pentraxin 1 and human neuronal pentraxin 2 (NPTX2), are expressed in the central nervous system (CNS). The present study was designed to investigate whether PTX3 is expressed in the brain and to define the structures and cells involved. I.c.v., but not i.v., injection of LPS induced high levels of PTX3 mRNA in the mouse brain. I.c.v. IL-1ß was also a potent inducer of PTX3 expression in the CNS, whereas TNFa was substantially less effective and IL-6 induced a barely detectable signal. Central administration of LPS and IL-1 induced PTX3 also in the periphery (heart) whereas the reverse did not occur. Expression of PTX3 was also observed in the brain of mice i.c.v. infected with *Candida albicans* or *Cryptococcus neoformans*. In situ hybridization revealed that i.c.v. injection of LPS induced a strong PTX3 expression in presumptive glial cells in the white matter (corpus callosum, fimbria) and meningeal pia mater as well as in dentate gyrus hilus and granule cells. No constitutive expression of PTX3 was detected. Central expression of PTX3 may amplify mechanisms of innate resistance and damage in the CNS. The possibility of a direct interaction of PTX3 with neuronal cells, as suggested for NPTX2, remains to be explored.

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Deoxycholic and Chenodeoxycholic Acids Inhibit Monocyte Function by Blocking fMLP Receptor

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Systemic infection and endotoxemia commonly occur in patients with severe cholestasis. Increased bile acid concentrations associated with cholestasis suppress cell-mediated immunity, although the mechanisms are not fully understood. The first line of defense against invading microorganisms is phagocytic leukocytes. Bacterially derived formylated peptides such as, fMLP, are potent chemoattractants for phagocytic leukocytes. We have investigated the ability of 7 bile acids to inhibit monocyte responses induced by chemoattractants. Co-incubation of fMLP with major components of human bile, deoxycholic acid (DCA) or chenodeoxycholic acid (CDCA), markedly inhibited chemotaxis by monocytes and RBL cells transfected to express only fMLP receptor (ETFR). Co-incubation of DCA or CDCA with 5 chemokines did not significantly inhibit chemotaxis. These bile acids also inhibited fMLP-induced calcium flux and ^{3}H-fMLP binding to monocytes and ETFR cells. DCA and CDCA inhibited fMLP responses at non-cytotoxic concentrations. Pretreatment of monocytes with DCA followed by washing did not increase the observed inhibition, suggesting that DCA interacts with fMLP. These results suggest that elevated levels of DCA and CDCA seen in cholestastic disease may impair cell-mediated immunity by inhibiting fMLP receptor function.

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T HELPER 2 CELLS SPECIFICALLY ENHANCE MMP-1 PRODUCTION ON HUMAN MONONUCLEAR PHAGOCYTES IN A CONTACT-DEPENDENT FASHION AND BY RELEASING IL-4. **CHIZZOLINI C, Rezzonico R, De Luca C, Burger D, Dayer J-M. Division of Immunology and Allergy, University Hospital, CH-1211 Geneva 14, Switzerland.**

Monocyte/macrophages (Mϕ) are directly involved in both tissue remodeling and destruction through the release of metalloproteinases (MMP). In the present study, we examined the effect of T-helper cell polarization on the production of MMP-1, MMP-9, and their inhibitor TIMP-1 by Mϕ activated by contact with T cells. Plasma cell membranes from antigen-activated Th1 and Th2 cell clones proved potent inducers of MMP-1 production by monocytic THP-1 cells, while TIMP-1 levels were not affected. By using neutralizing reagents cell membrane-associated TNF was found to be partially involved in MMP-1 induction by both Th1 and Th2 cells. In Th2 cells exclusively, membrane-associated IL-4 induced MMP-1 production by THP-1 cells. This membrane-associated IL-4 effect was additive to TNF and specifically observed on MMP-1 since MMP-9 production was not enhanced. Similarly, soluble IL-4 which induced THP-1 cells to produce MMP-1 and showed additive activity with soluble TNF, was blocked by IL-4 neutralization but unaffected by the presence of indomethacin or polymyxin-B. These effects were observed at protein and mRNA levels. When peripheral blood CD14+ monocytes were cultured for 7 d in the presence of GM-CSF, soluble IL-4 decreased spontaneous MMP-9 production and enhanced T cell-induced MMP-1 production both at protein and mRNA levels. In contrast with previously reported effects, Th2 and IL-4 specifically induce MMP-1 production by mononuclear phagocytes. This IL-4 activity may be relevant to some pathological conditions dominated by Th2 inflammatory responses.

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INFLAMMATORY CYTOKINE RESPONSES IN IRRADIATED GASTROINTESTINAL (GI) EPITHELIAL CELLS. B.M. Daly, C.J. Kovacs, and M.J. Evans. East Carolina University School of Medicine, Greenville NC 27858.

While the epithelial lining of the GI mucosal surface was traditionally viewed as a passive barrier, more recent evidence suggests that it functions as a sensitive indicator of oxidatative stress and, as such, is actively involved in the initiation of non-specific as well as specific host-defense responses. In order to better understand the mechanism(s) by which epithelial cells respond to oxidative stress, we established the expression of inflammatory cytokines and the activation of transcription factor NFκB in the GI epithelial cell line IEC-6 following ionizing (x) irradiation (xRT). Cells were exposed to doses of xRT ranging from 0 to 12 Gy and cytokine expression in the culture medium measured by ELISA. NFκB activation was measured in nuclear extracts by EMSA. The results indicated that IEC-6 cells constitutively produced of a number of inflammatory cytokines including IL-1 and IL-6. Moreover, the expression of both of these cytokines increased in a dose responsive manner. Similarly, the activation of NFκB in IEC-6 cells was observed to be related to xRT dose with a threshold of ≥ 1.5 Gy. Preliminary kinetic data suggests that there was an abbreviated delay between the activation of NFκB and cytokine exprtession. A comparison of the response of the IEC-6 cell line to xRT and the inflammatory cytokine TNFα is presented.

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HSP 27 SIGNALS MØ IL-10 VS IL-12 VIA DIFFERING PATHWAYS AND WITH DIFFERENT POTENCY FOR NORMALS VS IMMUNODEPRESSED TRAUMA PATIENTS. A.K.De, K.Kodys, C.Miller-Graziano, UMass Medical School, Worcester, MA 01655.

Large Hsps (60,70) induce proinflammatory cytokine production by human MØ. Paradoxically, increasing large Hsp levels is beneficial in endotoxin induced systemic inflammatory syndrome, suggesting that Hsps may induce different cytokine responses in unstimulated versus *in vivo* activated cells. Here, Hsp 27, an essential substrate for a protein kinase in the p38 mitogen activated protein kinase (MAPK) pathway leading to MØ cytokine production, was compared to SEB+MDP for its induction of IL-12, an immunostimulatory cytokine, and of IL-10, an anti-inflammatory cytokine, using both normal human MØ and MØ from immunodepressed or immunocompetent trauma patients (pt). Hsp 27 activation requirements for both the MØ Erk and p38 MAPKinase pathways were evaluated by Western blot for P-Erk and P-p38, by kinase assay of MAPKAPK-2, and with the specific MAPK inhibitors SB203580 (p38) or PB98059 (Erk). Hsp 27 stimulated normals' or immunocompetent trauma pts' MØ to 2.5-3.5 greater increases in IL-10 and IL-12 than SEB+MDP. Even trauma pt with depressed MØ IL-10 levels responded to Hsp 27 with a 2.5 fold increase in IL-10 production vs SEB+MDP induction. In striking contrast, pt MØ with highly depressed IL-12 production to SEB+MDP produced 8-10 fold more IL-12 in response to Hsp 27. Additionally, although Hsp 27-induced MØ IL-10 critically depends on p38 MAPK pathway activation, its induction of IL-12 depends neither on the p38 nor the Erk 1/2 pathways. This suggests Hsp 27 is not only a potent stimuli of both IL-10 and IL-12, but its potency for IL-12 induction differs depending on MØ activation status.

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ANTIGEN-INDUCED ARTHRITIS IN IL-6 KNOCK OUT MICE: DIFFERENT RELEVANCE OF HUMORAL AND CELLULAR IMMUNITY FOR DISEASE DEVELOPMENT

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Interleukin-6 (IL-6) is a multifunctional cytokine that is highly expressed during arthritis. Its exact function during arthritis is however not known. We studied the role of IL-6 in the murine antigen-induced arthritis model (AIA). Both wildtype (wt) and IL-6 knock out (IL-6-/-) mice were immunized and boostered with 100 µg methylated Bovine Serum Albumin (mBSA). Arthritis was induced by intra-articular (i.a.) injection of 60 µg mBSA. At day 1 of AIA, both IL-6-/- and wt mice developed joint inflammation. Joint swelling and influx of inflammatory cells (predominantly PMN's) were identical between the strains. The wt mice developed a severe and chronic arthritis. In the IL-6-/- mice however inflammation subsided after day 1. IL-6 has been shown to be important for immune development. We therefore looked at the role of the immune status for development of AIA. Prior to i.a. injection the IL 6-/- mice had developed lower antigen-specific antibody titers, mainly of IgG2a/2b. By immunizing wt mice with 10 instead of 100 µg mBSA they developed antigen-specific IgG2a titers comparable to IL-6 -/- mice. A reduced humoral immunity however did not prevent the development of arthritis in these wt mice. Cellular immunity was assessed by T cell proliferation. We found that lymph node cells (LN) from immunized animals were unable to respond to the antigen in the absence of IL-6. LN cells from wt mice immunized with 10 µg mBSA responded well after stimulation with mBSA while LN cells from IL-6 -/- mice could not respond. To conclude we found no differences at day 1 of AIA in the inflamed joints of wt and IL-6-/- mice. Antigen-specific antibody levels were not determining during onset of AIA. An impaired T cell response in the absence of IL-6 in contrast might contribute to the different disease development after day 1 in IL-6-/- mice.

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RADIATION DOSE AND TIME CONFIGURATIONS, LATE INTESTINAL EFFECTS, AND THE EXPRESSION OF INFLAMMATORY AND FIBROGENIC CYTOKINES IN FIBROSIS SENSITIVE MICE. M.J. Evans, C.J. Kovacs, and B.M. Daly. East Carolina University School of Medicine, Greenville, NC 27858.

The effects of radiation dose and time on the development of gastrointestinal (GI) late effects were studied in the C57Bl/6 fibrosis-sensitive mouse after either single, high dose (17.5 Gy), split-dose (13.75 Gy q10d x2) or conventional fractionated dose (2.0 Gy; q24hr x 5 → 74 Gy) irradiation (xRT). Intestinal adhesions first appeared at 16-20 weeks after each xRT schedule, increasing rapidly after the high-dose xRT, but more slowly after the split-dose and fractionated xRT. Fibrotic obstructions were apparent at the cecocolic junction following high-dose and split-dose xRT, but not fractionated xRT, during weeks 28-40. ELISA analysis demonstrated cyclic waves of enhanced plasma levels of IL-1α and TGFβ following each of the xRT schedules. In non-xRT control animals, the mean titer of IL-1α and TGFβ was 3.64±1.02 and 1.34±0.46 ng/ml, respectively. The cyclic waves of IL-1α, separated by the waves of TGFβ , were in excess of 200% of control and returned to control levels by 36 weeks. Conversely, plasma TGFβ remained elevated throughout week 48. During the first week of the acute colonic response, immunohistochemical analysis of tissue IL-1α and TGFβ were unremarkable between the three xRT schedules. However, a significant ($p<0.05$) dose response was observed in the activation of NFκB which regulates transcriptional expression of IL-1α. Conversely, the expression of tissue levels of TGFβ and collagen in the region of the cecocolic junction during week 20-28 following xRT was distinguished by the xRT schedule.

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LOCAL PRODUCTION OF IL-18, MIP-1α, and MIP-2 AT THE SITE OF TURPENTINE-INDUCED INFLAMMATION

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The subcutaneous administration of turpentine generates a sterile localized inflammation, which induces a systemic acute-phase response (APR). The necessary role for IL-1β in the development of turpentine-induced APR has been well characterized. However, the local cytokine production at the site of turpentine- induced inflammation remains unknown. Using turpentine-injected wild type (WT) and IL-1β-deficient (IL-1β KO) mice, we evaluated the kinetics and IL-1β-dependency of local and systemic IL-18, MIP-1α, MIP-2, IL-1α, TNFα, IFNγ, and IL-6 production. Local production of IL-18, as well as of the chemokines MIP-1α and MIP-2 in tissue homogenates peaked at 4 days following turpentine administration and declined thereafter, but was still significantly elevated 15 days after injection. Reduced IL-18, MIP-1α, and MIP-2 levels were observed in IL-1β KO mice, although substantial production of these cytokines was still present even in the absence of IL-1β, particularly at later time points. No systemic levels of IL-18, MIP-1α or MIP-2 were detectable at any time point. In fact, IL-6 was the only cytokine detectable in the systemic circulation and was reduced in IL-1β KO mice. On the other hand, in IL-1β KO mice local IL-6 levels were only reduced at 8 h following turpentine administration, but this was not seen at later time points. In addition, no increase in either local or systemic IL-1α, TNFα or IFNγ was observed at any of the time points evaluated.

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EFFECT ON CYTOKINES PRODUCTION AND LEUKOCYTE RECRUITMENT BY LOVASTATIN AND SQUALESTATIN. Paolo Fruscella, Diego Albani, Marcello Sottocorno,Marco Bianchi, Alessandra Bruno, Maria Romano, Mario Salmona and Luisa Diomede, Department of Biochemistry and Molecular Pharmacology, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milano, Italy (D.A., M.S., P.F., M.B., A.B., M.R., M.S., L.D.).

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the committed step for sterol and non-sterol mevalonate-derived intermediates, affecting not only cholesterol synthesis but also the production of other biologically active molecules. An anti-inflammatory action of HMG-CoA reductase inhibitors was suggested since *in vitro* these drugs down regulate cytokine and chemokine production. To clarify if another cholesterol-lowering drug, squalestatin, has any anti-inflammatory effect, we used an *in vivo* model of local inflammation. Lovastatin inhibited HMG-CoA reductase, down regulating the synthesis of sterol and non-sterol intermediates; squalestatin only affected the formation of sterol derivatives, since it reduced the activity of squalene synthase. Lovastatin, at doses which reduced the activity of HMG-CoA reductase without affecting blood cholesterol, significantly reduced lipopolysaccharide- or carrageenan-induced leukocyte recruitment. In the same animals local production of IL-6, MCP-1 and Rantes decreased. Lovastatin did not reduce leukocyte recruitment in IL-6 -/- mice, indicating that IL-6 is critical for the statin's anti-inflammatory effect. Squalestatin at doses that significantly lowered serum cholesterol and increased HMG-CoA reductase activity did not show any anti-inflammatory effect. In conclusion, our data indicate that the inhibition of sterol synthesis only does not block cytokine and chemokine production since their synthesis depends on non-sterol mevalonate metabolites.

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INTERLEUKIN-1 RECEPTOR ANTAGONIST IS PRODUCED AS AN ACUTE-PHASE PROTEIN IN VIVO. C. GABAY, G. Fantuzzi, J. Gigley, W.P. Arend, UCHSC, Denver, CO 80262.

To examine production of IL-1 receptor antagonist (IL-1Ra) by the liver in vivo, mice were injected intraperitoneally with LPS. Plasma levels of IL-1Ra increased in response to LPS. By RNase protection assay, high levels of sIL-1Ra, but not icIL-1Ra1, mRNA were detected in the liver in response to LPS. By Western blot analysis, both sIL-1Ra and icIL-1Ra3 proteins were present in liver tissue homogenates. By in situ hybridization, only sIL-1Ra mRNA was found in hepatocytes. Liver sIL-1Ra mRNA levels were also elevated following turpentine-induced inflammation. The kinetics of sIL-1Ra mRNA production were similar to serum amyloid A1 (SAA1) mRNA, a well-described acute-phase protein. Increased plasma levels of IL-6, but not IL-1β, were detected in mice treated with turpentine, suggesting that synthesis of both sIL-1Ra and SAA1 mRNA were stimulated by IL-6. To further examine the regulation of sIL-1Ra production in vivo following turpentine injection, we used IL-1β and IL-6 knockout (KO) mice. In IL-1β KO mice, circulating levels of IL-6 increased, but were significantly lower than in wild-type (WT) mice. Liver sIL-1Ra mRNA levels were also significantly lower in IL-1β KO mice than in WT mice. In IL-6 KO mice, both liver sIL-1Ra and SAA1 mRNA levels were significantly lower than in WT mice. In contrast to the liver, all the different IL-1Ra isoforms were detected at the site of inflammation and their production was not dependent on IL-6. In conclusion, our results show that sIL-1Ra and icIL-1Ra3 are produced by hepatocytes in response to local and systemic inflammation and that production of IL-1Ra is regulated differently in the liver than at the site of inflammation.

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DIFFERENTIAL TISSUE DISTRIBUTION OF IL-1 ALPHA AND BETA. M. Hacahm, S. Argov[1], E. Voronov, R. M. White, S. Segal and R. N. Apte. Ben-Gurion University, Beer-Sheva 84105, Israel.

Pro-inflammatory cytokines may play an important role in organ homeostasis, by participating in diverse functions, such as host-defense and proper metabolic functions, beyond the realm of immunology. Interleukin-1 (IL-1) is a pleiotropic cytokine that affects inflammatory and immune responses.It is possibly that within the producing cell, where the IL-1 molecules are expressed in different sub-cellular compartments, that their differential effects can be expressed. Thus, IL-1α is mainly active in its cytosolic precursor or as a membrane-associated form, while IL-1β is active only in its mature secreted form. We have assessed organ/tissue cytokine expression in young and old mice, by bioassays, ELISAs and immunohistochemistry. According to cytokine expression patterns, organs can be divided into main two categories; "lymphoreticular organs" (i.e. the liver, spleen, lungs and small intestine), that characterize the gates of entry of pathogens into the body, mount inflammatory responses based mainly on high IL-1 expression. This enables mounting potent inflammatory responses, even at the possible cost of tissue-damage. In contrast, homeostasis of highly vulnerable internal organs (i.e. the heart, muscle, kidney and brain), referred by us as "privileged organs" is characterized by inflammatory responses based on more docile cytokines, such as IL-6 and CSFs and not on IL-1-mediated responses. Hence, the privileged organs can develop milder and more tightly regulated inflammatory responses with less potential for tissue-damage. IL-1α and IL-1β were found to be differentially expressed in these two groups of organs. Thus, IL-1α is dominantly expressed in lymphoreticular organs, while IL-1β expression is more characteristic of the privileged organs. This patterns of expression are more accentuated at old age. The results may hint to the different physiological roles of IL-1α versus IL-1β in tissue homeostasis.

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STAT6 MEDIATES THE ANTI-INFLAMMATORY EFFECTS OF IL-4 AND IL-13 IN HEPATIC ISCHEMIA/ REPERFUSION INJURY. A.B. Lentsch, A. Kato, M.J. Edwards. University of Louisville School of Medicine, Louisville, KY 40292

Liver injury induced by hepatic ischemia and reperfusion (I/R) is characterized by activation of the transcription factor, NFκB, increased TNFα production, liver neutrophil accumulation and hepatocellular damage. The objective of the present study was to determine whether IL-4 and IL-13 could inhibit liver inflammatory injury induced by hepatic I/R and, if so, by what mechanism(s). Mice underwent 90 minutes of hepatic ischemia followed by 8 hours of reperfusion.

Group	NFκB (RI units)	STAT6 (RI units)	TNFα (pg/ml)	MPO (A655)	ALT (IU/ml)
sham	11 ± 1	10 ± .4	13 ± 5	0.0 ± 0	0.0 ± 0
I/R	20 ± 1	12 ± .5	86 ± 2	1.2 ± .2	1.1 ± .5
I/R+IL-4	21 ± 2	19 ± .8*	51 ± 13*	0.3 ± .1*	0.5 ± .2*
I/R+IL-13	20 ± 1	23 ± .7*	19 ± 12*	0.4 ± .2*	0.4 ± .1*

Data are expressed as mean ± SEM. *P<0.05 versus I/R group.

Hepatic I/R significantly increased (P<0.05) NFκB activation, serum levels of TNFα, liver accumulation of neutrophils (measured by myeloperoxidase [MPO] content) and hepatocellular damage (measured by serum alanine aminotransferase [ALT]) when compared to sham controls. Activation of STAT6 was not evident after I/R. Administration of 1 μg of IL-4 or IL-13 at reperfusion reduced serum TNFα, liver neutrophil accumulation (MPO) and hepatocellular injury (ALT). IL-4 and IL-13 had no effect on liver NFκB activation but significantly increased activation of STAT6. In STAT6 knockout mice, neither IL-4 nor IL-13 had any effect on TNFα, MPO or ALT values; the regulatory effects of these cytokines being completely abolished. These data suggest that activation of STAT6 may be essential for regulation of liver inflammatory injury.

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CONSTITUTIVE EXPRESSION OF PLD2 AND SELECTIVE INDUCTION OF PLD1 DURING PATHOGEN-MEDIATED MONOCYTE ACTIVATION

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Phospholipase D (PLD) activation is part of the complex signalling cascade induced during phagocyte activation. Two PLD isoforms have been cloned, but their role in phagocyte functions is still unknown. We report that resting monocytes selectively expressed PLD2, while PLD1 became detectable both at mRNA and protein level only following cell activation by pathogen-derived agonists (LPS, Candida albicans, Gram-positive bacteria and mycobacteria-derived arabinoside-terminated lipoarabidomannans) through a mechanism involving the pattern recognition receptor CD-14. Proinflammatory cytokines (IL-1β and TNFα) had only a weak effect, while immune cytokines (IL-6 and IFNγ), anti-inflammatory cytokines (IL-13) and chemoattractants (FMLP and MCP-1) were inactive. None of the agonists tested induced significant changes in PLD2 expression. Consistently with this, PLD enzymatic activity was increased in monocytes treated with pathogen-derived agonists. Thus, PLD2 is a constitutive enzyme in circulating monocytes that likely sustains the PLD activity rapidly observed after stimulation with chemoattractants and inflammatory agonists. Conversely, PLD1 is an inducible protein selectively expressed during cell activation and could play a role in immune responses against pathogens and in chronic inflammation.

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EXPRESSION OF IL-1β IN HUMAN FIBROBLASTS LEADS TO AUTOCRINE ACTIVATION. D. LOTTAZ, C. Solioz, K. Freiburghaus, Z. Beleznay, M. Bickel, University of Berne, 3010 Berne, Switzerland

Primary human dermal fibroblasts (HDF) treated with TNFα express IL-1β. To further investigate the function of IL-1β, we transiently transfected HDF and human embryonic kidney cells (HEK293) using expression plasmids containing the IL-1β coding sequence under the control of a SV40 promoter. Expression levels of IL-1β and of IL-8, as an indicator of inflammatory activation, were assessed using ELISA. In HDF, cell-associated IL-1β was highest three days after transfection, reaching similar levels as in untransfected TNFα-treated HDF. IL-1β was also released into the medium. IL-8 followed IL-1β with a delay of 24 h. The induction of IL-8 was inhibitable by a monoclonal anti-human IL-1β antibody (R&D MAB601), recombinant IL-1 receptor antagonist, or soluble IL-1 receptor type I. In contrast to HDF, transfected HEK293 kidney cells did not autonomously release IL-8. IL-1β in these cells was even 2 to 3 times higher than in HDF. In addition, exogenously added recombinant mature IL-1β induced an IL-8 response. We conclude that SV40-driven expression of IL-1β in HDF induces autocrine activation. Interestingly, this phenomenon is not observed in HEK293 cells, indicating that these cells may lack a component to generate biologically active IL-1β. We speculate that, in *vivo*, autocrine activation of fibroblasts induce and amplify a local inflammatory response.

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PERITONEAL ABSCESS FORMATION INDUCES IL-6, FIBRINOGEN AND ARDS-LIKE ABNORMALITIES IN SEPTIC RAT LUNGS. J.R. Lussier, N. Espina, and J.H. Siegel, UMDNJ-New Jersey Medical School & Graduate School of Biomedical Sciences, Newark, NJ 7103.

The development of a severe septic process is frequently associated with the subsequent evolution of the acute respiratory distress syndrome (ARDS). This study examines the relation of abscess development in a septic rat model, with the onset of ARDS-like changes. It employs the use of a rat model with peritoneal implantation of a fecal-agar pellet. Characterization of fibrin deposition and collagen synthesis was examined by H&E, and Gomori's Trichrome staining procedures. Interleukin-6 (IL-6) and fibrinogen mRNA were detected in lung tissues by *in situ* hybridization, with visualization by light microscopy. Lung tissue analysis demonstrated an increased IL-6 mRNA expression in the sterile inflammation and septic lungs as compared to controls, with a subsequently greater increase in the septic animals at days 2 and 3, as the abscess formed. Fibrinogen mRNA expression exhibited similar patterns, but at lower levels. The pattern of ARDS was evident in the lung with increased connective tissue deposition in the interstitium, associated with increased macrophage (mϕ) infiltration. The IL-6 mRNA expression corresponds to peak cytokine levels in circulation at day 3. We propose that the abscess plays a key role by acting as a functional cytokine producing organ, which results in a large lung infiltration of IL-6 producing mϕ's, during chronic septic inflammation with enhanced ARDS-like changes.

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IL-16 INDUCES THE PRODUCTION OF PRO-INFLAMMATORY CYTOKINES

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IL-16 acts as a chemoattractant for $CD4^+$ cells, as a modulator of T cell activation, and plays a key role in asthma. This study describes the cytokine inducing effects of IL-16 on total PBMCs and PBMC subpopulations. While $CD4^+$ T lymphocytes did not secrete cytokines in response to IL-16, $CD14^+CD4^+$ monocytes and maturing macrophages secreted IL-1β, IL-6, IL-15 and TNFα upon IL-16 stimulation. The mRNA species for these four cytokines were detected as early as 4h post stimulation, with protein being secreted by 24h. Secretion of IL-1β and IL-6 by total PBMCs was dose dependent, with maximal secretion being observed using 50 ng/ml IL-16. However, for IL-15 or TNFα maximal secretion by total PBMCs occurred with all concentrations between 5 ng/ml to 500 ng/ml IL-16. Purified monocytes/macrophages secreted maximal concentrations of all four cytokines in the presence of 500 ng/ml IL-16. The use of higher concentrations of IL-16 (1000 ng/ml) inhibited secretion of all four cytokines. While these IL-16 induced cytokines are likely to be involved in the immune system's response to antigen, the data suggest that IL-16 is likely to play a key role in initiating and/or sustaining an inflammatory response.

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SELECTIVE EXPRESSION AND REGULATION OF TOLL LIKE RECEPTORS (TLR) IN HUMAN LEUKOCYTES.

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Members of the Toll like receptor family (TLRs) probably play a fundamental role in pathogen recognition and activation of innate immunity. The present study analysed how different human leukocyte populations express the first 5 characterised TLR family members at the level of mRNA. TLR1 was expressed in all leukocytes examined, including monocytes, polymorphonuclear leukocytes, T and B cells and NK cells. In contrast TLR2, TLR4 and TLR5 were expressed only in myelomonocytic elements. Exposure to bacterial products, or to proinflammatory cytokines, increased TLR4 transcripts in monocytes and PMN, whereas IL-10 blocked this effect. TLR3 mRNA was only expressed in monocyte-derived dendritic cells (DC).

These results demonstrate that TLR1 through 5 are differentially expressed and regulated in human leukocytes. In particular, expression of TLR3 is restricted to DC that are the only elements which express the full TLR repertoire. These data suggest that TLRs can be classified based on expression pattern in Ubiquitous (TLR1), Restricted (TLR2, TLR4 and TLR5 in myelomonocytic cells), and Specific (TLR3 in DC) molecules.

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IRAK-M IS A MEMBER OF THE INTERLEUKIN-1 RECEPTOR-ASSOCIATED KINASE FAMILY.

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Interleukin-1 is a multifunctional cytokine of the innate immune system implicated in several pathophysiological conditions. IL-1 binding induces the recruitment of the kinases IRAK1 and IRAK2 to the receptor complex. IRAK-M has recently been identified as a third member of this family. Here we show that its overexpression activates NFκB in HEK293T or COS1 cells. This activation occurs in a dose-dependent manner, and can be inhibited by co-expression of dominant-negative TRAF6. Like the other two members of the family, IRAK-M contains an amino-terminal death domain (DD). Overexpression of IRAK-M death domain inhibits IL-1 and TNF-induced activation of NFκB in a dose-dependent manner. Furthermore, IRAK, IRAK2 and MyD88-induced activation of NFκB was also abrogated by overexpression of IRAK-M death domain. Taken together, these results suggest that IRAK-M may be involved in the IL-1 signalling pathways that lead to activation of NFκB.

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HEPARAN SULFATE-DERIVED DISACCHARIDES MODULATE CHEMOKINE-INDUCED T CELL ADHESION TO EXTRACELLULAR MATRIX. H. SCHOR, R. Hershkoviz, I. R. Cohen, L. Cahalon and O. Lider, Weizmann Institute of Science, Rehovot 76100, Israel.

In the course of migration towards an inflammatory site, leukocytes express a heparan sulfate-degrading endoglycosidase, designated heparanase. Heparanase yields specific degradation products from heparan sulfate in the form of disaccharides (DS). These DS have been shown to modulate TNFα secretion from immune cells *in vitro* and cell-mediated immune reactions *in vivo*. We now report that such DS can modulate the adhesion and migration of human T cells. We demonstrate that certain heparin- and heparan sulfate-derived DS induced, in a dose-dependent manner, the adhesion of human T cells to both ECM and immobilized fibronectin. This adhesion involves β_1 integrin recognition and activation and is associated with specific intracellular activation pathways. Although a first exposure of T cells to certain DS molecules induced cell adhesion, a subsequent exposure of T cells to pro-adhesive chemokines, such as MIP-1B or RANTES, but not to other pro-adhesive stimuli (for example, IL-2 or CD3 cross linking), inhibited T cell adhesion to and chemotactic migration through fibronectin. Hence, we propose that the breakdown products of tissues generated by inflammatory enzymes are part of an intrinsic functional program that provides the leukocytes with information about their environment to modulate their activities.

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IDENTIFICATION OF NEW GENES MODULATED DURING INFLAMMATORY RESPONSE. Aristóbolo M. Silva,[1,2] and Luiz F.L. Reis[1]. [1]Laboratory of Inflammation, Ludwig Institute for Cancer Research, São Paulo, SP, Brasil, and [2]Depto. de Microbiologia, ICB/UFMG, Belo Horizonte, MG.

The pro-inflammatory cytokine tumor necrosis factor (TNF) is one of the soluble mediators of inflammation being produced by leukocytes at the inflamed area. Once secreted, TNF binds to specific cellular receptors (TNFR1 and TNFR2), leading to the activation of several genes. Many actions of the pro-inflammatory cytokines, including IFNs, TNF and IL-1, are mediated by the transcription factor NF-κB. It has been proposed that anti-inflammatory salicylates (aspirin and sodium salicylate) act in part by inhibiting NF-κB activation, resulting in the blockage of NF-κB-regulated genes. By means of differential-display RT-PCR analysis, we have identified and characterized several genes whose expression is modulated on mouse embryonic fibroblasts (MEFs) upon stimulation by TNF and/or sodium salicylate, an non-steroidal anti-inflammatory drug. The identification of cDNA fragments corresponding to genes related to various aspects of cellular activation, as for example p34/cdc2 kinase, immunophilin FKBP51, biglycan, and a fragment homologous to the human eIF-3, could greatly contribute to the better understanding of the inflammatory response. We have also found that TNF-induced expression of N51/KC chemokine is not affected by sodium salicylate co-stimulation. Correlation of the expression of these genes and their promoter region has provided us with new insights on signaling within the cytokine network and on the mechanism of action of anti-inflammatory drugs.

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INTERLEUKIN-10 (IL-10) MEDIATED INHIBITION OF INFLAMMATORY CYTOKINE PRODUCTION BY HUMAN ALVEOLAR MACROPHAGES. M.J. Thomassen, B. Raychaudhuri, A. Malur, L. Buhrow, C. Farver, M.S. Kavuru and C.J. Fisher. Cleveland Clinic Foundation, Cleveland, OH 44195

Alveolar macrophages are an important source of inflammatory cytokines in the lung. Previous *in vitro* studies have shown that IL-10 inhibits inflammatory cytokine production by human alveolar macrophages (Clin Immunol Immunopath 80:321, 1996). The purpose of the present study was to determine the mechanism of IL-10 inhibition of cytokine production. Alveolar macrophages were obtained from healthy controls by fiberoptic bronchoscopy. Macrophages were incubated with LPS ± IL-10. Tumor necrosis factor-α (TNF) and interleukin-1-β (IL-1) mRNA levels were 2-6 fold decreased in the presence of IL-10. These results suggested that transcription might be affected by IL-10. Electrophoretic mobility shift assays were performed using end labelled nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) probe. NF-κB binding was decreased in extracts from macrophages incubated for 4h with LPS + IL-10 in comparison to those incubated with LPS alone. Supershift assays showed the presence of both p50 and p65 subunits of NF-κB. AP-1 was not affected by IL-10. IL-10 had no effect on the LPS-mediated degradation of the inhibitor protein IκB, but IL-10 prevented the nuclear translocation of the p65 subunit. These observations provide the first evidence that IL-10 inhibits cytokine production in human alveolar macrophages by preventing the nuclear translocation of NF-κB and thus down-regulates NF-κB dependent gene transcription.

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CONTINUOUS VENOVENOUS HEMOFILTRATION (CVVH) IN HORSES WITH LPS INDUCED SEPTIC SHOCK, J Veenman, C Dujardin, A. Hoek, W Klein, V Rutten, Utrecht Univ., Fac. Vet. Med., Utrecht, The Netherlands

Clinical efficacy of continuous venovenous hemofiltration (CVVH) and clearance of cytokines were investigated in an equine septic shock model. Ten male Shetland ponies received a slow infusion of LPS (2 µg/kg BW) under general anaesthesia. One group received treatment with CVVH (2 ml/kg BW/min) using a 75 kD polymethylmethacrylate (PMMA) filter. Hemodynamic and bloodvariable measurements were performed every 30' throughout the study. Blood samples were taken to determine bloodgases, blood chemicals, haematological parameters and cytokines (TNFα and IL-6). The cytokines were measured in serum samples before, and after the filter and in the ultrafiltrate. All ponies showed a typical reaction in MPAP, blood chemical and hematologic markers after LPS challenge. Only a slight increase in Cl and no decrease in MAP was seen. CVVH did not effect the course of hemodynamic, blood chemical and hematological parameters significantly between the groups. Clear cytokine responses were found in all ponies, although substantially different in magnitude between the individuals. Inspite of expectation only small of amounts of TNFα could be detected in the ultrafiltrate. No significant differences where observed in the cytokine levels before and after filtration. Although some decrease in IL-6, most likely due to adsorption to the filter, was repeatedly observed. In this study CVVH did not proof to have a significant benificial effect on the outcome of endotoxemia in horses.

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A ROLE FOR LEPTIN AND IL-18 IN T-CELL-MEDIATED ACUTE LIVER INJURY. R. Faggioni*, G. Fantuzzi**, C.A. Dinarello**, K.R. Feingold*, and C. Grunfeld*. *VAMC, University of California, San Francisco;**University of Colorado Health Sciences Center, Denver.

Leptin has been shown to have a direct effect on T-lymphocytes, enhancing T helper (Th) alloproliferative response, polarizing Th cells toward a Th1 phenotype. In the present study the role of leptin was investigate in a model of acute hepatitis induced by the administration of the T-cell mitogen ConA. In wild type (WT) mice, ConA administration (200 μg/mouse, iv) induced a marked increase in the serum levels of TNF-α, IL-18, IL-12, and IFN- γ as well as evidence of acute liver damage as manifested by increased serum alanine transferase (ALT). In contrast to these responses in WT mice, the induction of serum IL-18 and TNF-α was markedly reduced in leptin-deficient (*ob/ob*) mice (91% and 76% respectively) and ALT levels were also reduced in these mice following ConA administration (94%). Although *ob/ob* mice were protected against hepatic injury, these same mice produced levels of IL-12 and IFN-γ similar to those measured in WT mice. Because leptin deficiency protected mice from ConA-induced liver damage, we evaluated the role of IL-18 in mediating this T-cell-induced hepatic toxicity. Pretreatment of WT mice with a neutralizing anti-mouse IL-18 IgG significantly reduced the increase in ALT observed following ConA administration (50%). However, IL-18 neutralization did not inhibit ConA-induced production of IFN-γ. These results demonstrate that leptin deficiency leads to reduced production of IL-18 and TNF-α as well as to reduced T-cell-mediated hepatotoxicity. In addition, IL-18 appears to be an essential mediator of liver injury due to activated T-cells but the mechanism of IL-18 in mediating the acute hepatitis in this model is independent of IFN-γ.

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REDUCED LEPTIN LEVELS IN STARVATION INCREASE SUSCEPTIBILITY TO ENDOTOXIC SHOCK. R. Faggioni, K.R. Feingold and C. Grunfeld. Veterans Medical Center, Univ. California, San Francisco, CA, 94121.

Malnutrition compromises immune function, reducing resistance to infection. During starvation, the rapid fall in leptin levels is an important signal in neuroendocrine adaptation and has recently been proposed to play a role in the starvation associated immune dysfunction. Here we examine whether the decrease in leptin levels that occurs during fasting increases the susceptibility to LPS and TNF induced lethality. In mice, fasting for 48h enhances sensitivity to LPS. Decreasing the fasting-induced fall in leptin by administration of exogenous leptin markedly reversed the sensitivity to LPS. While basal serum leptin levels were decreased by fasting, LPS treatment increased leptin levels to the same extent as in fed animals. Fasting increased basal corticosterone levels; leptin treatment blunted this increase. LPS induced a marked increase in corticosterone levels in fed animals, which was blunted in fasted animals. Leptin treatment of fasted mice restored the corticosterone response. LPS induced a five fold greater increase in serum TNF levels in fasted mice; this increase was blunted by leptin replacement. Furthermore, fasting increased sensitivity to the lethal effect of TNF itself. This sensitization to TNF lethality was reversed by administration of leptin. These data suggest that leptin levels might be protective by both inhibiting TNF induction by LPS and by reducing TNF toxicity.

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EFFECT OF ETHANOL ON HEPATIC PRODUCTION OF INTERLEUKIN-6 (IL-6) FOLLOWING BURN INJURY IN MOUSE. A Colantoni, LA Duffner, N DeMaria, C Fontanilla, KAN Messingham, DH Van Thiel, MA Emanuele, EJ Kovacs. Loyola University Chicago, Maywood, IL, 60153

Elevated circulating levels of IL-6 are associated with a poor prognosis following thermal injury. The mortality rate is increased among burn patients in those with detectable blood alcohol levels (BAL). Aim of the study was to determine whether increasing levels of alcohol differentially enhance the hepatic production of IL-6 following thermal injury. To test this, mice were treated with saline or ethanol 30 min before scald (15% total body surface scald) or sham injury. Two doses of ethanol were administered to reach BALs of 100 mg/ml (E100) and 300 mg/ml (E300). The mice were sacrificed 24 h after injury and IL-6 levels measured by ELISA in serum and liver homogenates. Burn alone resulted in 15% mortality, as did sham and burn injury in the E100 group. Similarly, the mortality rate was 16% in sham E300 but, in contrast, 77% of the mice in the burn E300 group did not survive ($p=0.02$). In the absence of alcohol, IL-6 serum levels increased after burn injury to 45.3±8.8 pg/ml ($p=0.01$ vs sham). Accordingly, the hepatic IL-6 production was increased in burn as compared to sham mice ($p=0.03$). The serum and hepatic levels of IL-6 in sham mice were increased 2 fold in E100 and 5 fold in E300 as compared to saline group ($p=0.005$). IL-6 hepatic production following burn injury was higher in the E300 group (37.6±1.5 ng/mg protein) than in E100 (23.5±3.3 ng/mg) and saline (16.7±2.7 ng/mg, $p=0.001$). These results suggest that hepatic production of IL-6 is stimulated by both alcohol and burn injury. Since alcohol increases the early mortality rate following burn injury in a dose-dependent manner, this may be due to an increased hepatic production of IL-6 as a result of the combined effect of alcohol and burn. (Supported by NIH AA12034, GM55344, and AG16067)

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SOLUBLE GANGLIOSIDES INTERFERE WITH IFNγ ACTIVITY AND MODULATE THE DIFFERENTIATION OF CD4 T CELLS. R. Fernandez-Botran, B. Brewer and J. Vetvickova. Department of Pathology, School of Medicine, University of Louisville, Louisville, KY 40292.

Gangliosides are a group of acidic glycolipids ubiquitously present in cell membranes, but particularly enriched in the central nervous system (CNS). Gangliosides have been associated with immuno-suppressive activities, and are thought to contribute to the immuno-privileged status of the CNS and to the immune suppression associated with certain malignant diseases. In order to characterize the mechanisms responsible for their immunosuppressive activities, different types of gangliosides were tested for their effects on the activity of IFN-γ. Mono-, di- and trisialogangliosides (GM1, GD1a, and GT1b), but not asialoganglioside GM1, were found to inhibit the binding of iodinated IFNγ to membrane IFNγ receptors on COLO-205 cells. Consistently, the IFNγ-induced expression of MHC class II molecules on the same cells was also inhibited by the sialilated gangliosides. Moreover, the binding of IFNγ to heparin and to a monoclonal antibody specific for the C-terminal portion of IFNγ was also antagonized by the gangliosides. These inhibitory effects might have resulted from the ability of gangliosides to physically interact with IFNγ, as demonstrated by native gel electrophoresis. Addition of gangliosides (GM1, GD1a, and GT1b) significantly enhanced the production of IL-4 and reduced that of IFNγ in primary and secondary anti-CD3 stimulated splenic cell cultures, suggesting that they might promote the generation of Th2 cells. These results indicate that gangliosides might exert their immunosuppressive activity through the direct inhibition of IFNγ and through modulation of the natural balance of Th1/Th2 CD4 T cell subset differentiation.

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HIGHLY EFFICIENT EXPRESSION OF PORCINE IL-2 BY HYBRID BACULOVIRUS IN SILKWORM
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To produce cytokines efficiently, we are applying baculovirus - silkworm larvae gene expression system. Baculovirus (AcNPV and BmNPV) systems are popular as high efficiency and similarity of post translational modification as mammal one. Though the baculovirus - silkworm larvae system is known as most efficient among the baculovirus systems, there are only few reports about large scale production by this system. We, therefore, compared baculovirus systems with culture cells and silkworm larvae. Then studied about factors, possibly affect the efficacy when the larvae are used as a host. We used AcNPV and hybrid virus(HyNPV), which is able to infect both AcNPV host cells and silkworm, to construct porcine IL-2 recombinant viruses(AcPIL2 and HyPIL2). The SDS-PAGE showed that HyPIL2 and AcPIL2 infected cell culture fluid contained about 150 μg/ml of 18.6kDa recombinant products. In the hemolymph of HyPIL2 infected larvae, the product was accumulated 1 to 3mg/ml. This is about 7 to 20 times concentrated that AcPIL2 infected cell culture fluid. This data is comparable to the best data of *E. coli*, *B. brevis* and Yeast expression systems. Considering the similarity of post translational modification as mammal one, this expression system is one of the most suitable systems for cytokine mass production.

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THE PROMOTER POLYMORPHISM –174 OF THE IL-6 GENE REGULATES INDUCIBLE IL-6 PLASMA LEVELS
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In the promoter region of the IL-6 gene there is a single base exchange (G -> C) polymorphism at position –174. Recent findings suggest, that this polymorphism could affect the transcription rate of the IL-6 gene and IL-6 plasma levels. To analyse its biological significance we examined IL-6 plasma levels in relation to the expression of the IL-6G and IL-6C alleles.

Materials and Methods: Cord blood was collected from 50 healthy, full-term neonates after normal vaginal delivery. Adult samples were obtained from 63 Sjögren syndrome (SS) patients and from 400 healthy adult controls. The –174 polymorphism was analysed using the RFLP and PCR. Plasma levels were measured by an ELISA-method.

Results: IL-6 plasma levels of neonates were higher than those of adult controls ($p < 0,0001$); respective median values were 13,6 pg/ml (4,6 – 57,0) and 1,2 pg/ml (0,7 – 2,0). Non-carriers of the allele G secreted more IL-6 than carriers of the allele G ($p < 0,05$); respective median values were 33,2 pg/ml (11,1 – 145,8) and 8,7 pg/ml (4,1 – 50,4). Also IL-6 plasma levels of SS patients were elevated compared with those of adult controls ($p < 0,0001$); median value for SS patients was 3,0 pg/ml (2,1 – 5,9). Levels were increased in SS patients with the G/G genotype compared to patients with the G/C or the C/C genotypes; respective median values were 4,1 pg/ml (2,9 – 7,8), 2,8 pg/ml (2,1 – 6,2) and 2,5 pg/ml (1,9 – 5,0). No association was found between IL-6 plasma levels and different IL-6 genotypes in adult controls ($p = 0,55$).

Conclusions: We suggest, that the normal labour-related stress and the inflammation associated with the autoimmune disease could induce IL-6 secretion and that these inducible levels of IL-6 are regulated by the –174 polymorphism.

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N36, A SYNTHETIC N-TERMINAL DOMAIN OF HIV-1 ENVELOPE GP41, UTILIZES A SEVEN-TRANSMEMBRANE G-PROTEIN COUPLED RECEPTOR FPRL1 TO ACTIVATE HUMAN PHAGOCYTES.
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HIV-1 envelope protein gp 41 mediates viral fusion with human host cells. The peptide segment N36, located in the N-terminus of gp41, interacts with the C34 domain in the C-terminus of gp41 to establish a fusogenic conformation of the virus. Synthetic peptides containing approximately 40 residues from gp41 that overlap, or include all of, the residues in N36 have been shown to be effective inhibitors of HIV fusion and infection. In this study, we discovered that synthetic N36 peptide stimulates chemotaxis and calcium mobilization in human monocytes and neutrophils. The activity of N36 peptide on phagocytes was pertussis toxin sensitive, suggesting this peptide uses Gi-coupled seven-transmembrane receptor(s). Since high concentrations of bacterial chemotactic peptide fMLP partially desensitized the calcium mobilizing activity of N36 peptide in phagocytes, we postulated that N36 peptide might preferentially use a low affinity fMLP receptor. By using cells transfected to express cloned prototype chemotactic N-formyl peptide receptor FPR or its variant FPRL1, we established that N36 peptide uses FPRL1 to induce cell migration and calcium flux. Since the activation of FPRL1 in human phagocytic cells by its agonists can result in desensitization of cell response to other chemoattractants, we propose that the peptide domain of HIV-1 gp41 subsequent to the initial activation of FPRL1 expressing cells may interfere with the cell response to other proinflammatory stimuli. Thus immuno-modulatory agents could be designed based on the structure-function property of the N36 domain of gp41.

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THE ROLE OF GONADAL STEROIDS IN CONTROLLING IL-6 PRODUCTION AND IMMUNE FUNCTION AFTER THERMAL INJURY WITH PRIOR ETHANOL EXPOSURE.
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Previous studies from our laboratory have shown that gonadal steroids are involved in the suppression of immune function following thermal injury. We, and others, have reported that ethanol exposure prior to thermal injury exacerbates this immune dysfunction, both clinically and experimentally. Therefore, we examined the role of gonadal steroids in the immune dysfunction of male mice following a 15% body surface area scald (or sham) injury combined with ethanol (0.15 ml i.p. of a 20% ethanol solution) or vehicle given 30 minutes prior to burn treatment. At 48 hours post-injury, the delayed type hypersensitivity (DTH) response was suppressd in the mice receiving burn + ethanol by 45% when compared to sham + vehicle, but not in DTH in animals receiving either injury alone. This suppression was coincident with a near doubling in macrophage production of interleukin-6 (IL-6) in the burn + ethanol mice. Blocking testosterone in the burn + ethanol mice with flutamide did not restore the DTH response or reduce IL-6 production; however, treatment with an anti-estrogen resulted in increased production of IL-6 (from 462±100 to 596±210 pg/ml) with no effect on DTH. Furthermore, administration of 17β-estradiol to burn + ethanol mice completely restored the DTH response and reduced the macrophage production of IL-6 by 31%. These data suggest that estrogen, rather than testosterone, may be involved in the regulation of immune suppression in male mice subjected to thermal injury with prior ethanol exposure. (Supported by NIH GM55344, AA12034, and AG16067)

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MOLECULAR CLONING OF PORCINE INTERLEUKIN-18, INTERLEUKIN-1β CONVERTING ENZYME AND THEIR mRNA EXPRESSIONS IN PORCINE ALVEOLAR MACROPHAGES. Y. MUNETA, Y. MORI, Y. SHIMOJI, O. MIKAMI and Y. YOKOMIZO. Natl. Inst. Anim. Hlth, Tsukuba, Ibaraki, 305-0856, Japan

We have isolated cDNAs contain the coding sequence of porcine interleukin-18 (IL-18) and interleukin-1 β converting enzyme (ICE). Total-RNA prepared from porcine alveolar macrophages stimulated with 10 μg/ml lipopolysaccharide (LPS) was used to clone the cDNA of porcine IL-18 and ICE by reverse transcription polymerase chain reaction (RT-PCR). The open reading frame (ORF) of the porcine IL-18 cDNA is 579 base pairs (bp) in length and encodes 192 amino acids, which is 76.7%, 64.7% and 61.6% homologous to the predicted human, murine and rat IL-18 amino acid, respectively. The ORF of the porcine ICE cDNA is 1215 bp in length and encodes 404 amino acids, which is 72.5%, 62.6% and 64.1% homologous to the predicted human, murine and rat ICE amino acid, respectively. The recombinant IL-18 expressed by baculovirus and *E.coli* system induced IFN-γ production from porcine PBMC. We also established the anti-porcine IL-18 monoclonal antibodies, and applied it successfully to the purification, the development of sandwich-ELISA and the immunohistochemical staining of porcine IL-18. Moreover, the kinetics of mRNA expression of IL-18, ICE and interleukin-1β (IL-1β) in porcine alveolar macrophages after LPS stimulation revealed that ICE transcripts were weakly expressed in non-stimulated condition, but upregulated after LPS stimulation. IL-18 and IL-1β which need both cleavage by ICE are differently expressed after LPS stimulation, suggesting ICE regulates maturation of these pro inflammatory cytokines through the different pathway in pigs. These results described here will be useful for further investigation of these molecules in porcine inflammatory and immune reactions.

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L-GLUTAMATE AS IMMUNOMODULATOR. Nurieva R.[1], Navolotskaya E.[1], Kostanyan I[2], Lepikhova T[1], Malkova N[1], Lipkin V., [1] Branch of Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry Russian Academy of Sciences, Pushchino, Moscow Region, Russia. [2] Institute of Bioorganic Chemistry Russian Academy of Sciences, Moskow, Russia.

In addition to the important role that L-glutamate (L-Glu) and glutamate receptors (GluRs) play in the brain, recent data suggests a physiological role of glutamate in the endocrine and immune system. A specific interection of [^{3}H]L-Glu with T limphocytes from the blood of healthy donors (K_d = 0.236 μM) was revealed and described. It was found that unlabeled guisqualate, a structural analogue of L-glutamic acid, and unlabeled dipeptides Ala-Glu, Glu-Ala, and Glu-Glu competitively inhibit the specific binding of [^{3}H]L-Glu to T limphocytes (with K_i 0.19, 2.4, 3.4, and 1.2 μM, respectively). Binding experiments with conjugates of labeled and unlabeled glutamic acid with dextran showed that the receptors of [^{3}H]L-Glu are iocalized on the outer surface of the plasma membrane of T limphocytes. The experiments with murine peritoneal macrophages were shown that L-Glu (10^{-9}-10^{-7} M) inhibits the activity of these cells *in vitro* (the spreading, the adhesion to a glass surface). L-Glu (10^{-6} M) also inhibited the bacterial activity of macrophages: in its presence macrophages could not digest bacteria completely, even the nonvirulent stain, *Salmonella typhimurium* 416/71. Since we did not find glutamate receptors on macrophages, the logical proposal was made that L-Glu caused the inhibitory effects through its influence on reception of the cytokines, which are essential to macrophages functions (TNF-α, IL-1β, IL-2, IL-6, IFN-γ).

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EFFECT OF RECOMBINANT HUMAN IL-10 ON IN VITRO IL-2 AND IFN-r PRODUCTION OF PERIPHERAL BLOOD MONONUCLEAR CELLS. Q. Qian,Institute of Dermatol, Shenzhen,GD 518020, P.R. China.

In order to explore the regulation between Th1 and Th2 cytokines, the author studied the effect of recombinant human IL-10 on in vitro IL-2 and IFN-r production of PHA-activated human peripheral blood mononuclear cells(PBMC). The results showed 10ng/ml IL-10 inhibits IL-2 and IFN-r production by PBMC(P<0.02 and P<0.01, respectively). For concentration of 5ug/ml rabbit anti-human IL-10 antibody enhances IL-2 production(P<0.05) and in a dose-dependent fashion. The antibody also increases IFN-r production, but there was no significant difference. The data confirm that the production of IL-2 and IFN-r by PHA-activated PBMC was inhibited by IL-10 and enhanced by anti-human IL-10 antibody. The regulation between Th1 and Th2 cells mediated by IL-10, choosing the types of immune response to different antigens are of certain guided significance for the prevention and treatment of clinical diseases.

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β-CASOMORPHIN UPREGULATES THE LEVEL OF MHC-II EXPRESSION ON BOVINE PERIPHERAL BLOOD LYMPHOCYTES BUT SUPPRESSES THEIR PROLIFERATION FOLLOWING MITOGENIC STIMULATION

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The objective of this study was to examine the effect of β-casomorphin; a bioactive peptide derived from milk protein, on bovine peripheral blood lymphocyte (PBMC) and neutrophil function. Lymphocyte function was evaluated using lymphocyte proliferation and activation assays, whereas neutrophil phagocytosis assays were performed using FITC-conjugated Escherichia coli to determine the effect of β-casomorphin on neutrophil function. Our results demonstrated that β-casomorphin suppressed the proliferation of ConA stimulated PBMC by 3 to 13% relative to control samples. Naloxone, an opiate receptor antagonist when used in competitive binding assays with β-casomorphin was found to reverse the antiproliferative effect suggesting the β-casomorphin mediated its effects via the opiate receptor. β-casomorphin was found to increase the frequency of MHC-II+ PBMC and upregulate the level of MHC-II expression. β-casomorphin was found to induce a dose dependent increase in the phagocytic activity of neutrophils. Our findings suggest that β-casomorphin may have an immunomodulatory effect on bovine lymphocytes and neutrophils.

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PRIMARY SIV INFECTION LEADS TO AN INCREASED PREVALENCE OF IFNγ+CD8αβ+ T CELLS IN THE INTESTINAL EPITHELIUM OF RHESUS MACAQUES

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The objective of this study was to evaluate the alterations in the phenotype of CD8+ intraepithelial lymphocytes and their potential to produce IFNγ during primary SIV infection. The expression of CD8α and CD8β on intestinal IEL were determined using flow cytometry. To determine whether local proliferation was contributing to the increased prevalence of subsets of CD8+ T cells in the intestinal epithelium we determined the expression of Ki-67, a nuclear antigen expressed by proliferating T cells following SIV infection and compared them to uninfected control animals. The phenotype of IFNγ producing CD8+ T cell subsets was determined at a single cell level following short-term mitogenic stimulation followed by intracellular staining. Following SIV infection the frequency of CD8αβ+ intestinal IEL dramatically increased at 2 weeks p.i. In contrast the proportions of CD8αα+ T cells declined. A higher frequency of CD8αβ+ T cells were found to express the ki-67 antigen as early as 2 weeks p.i. suggesting that local proliferation of CD8αβ+ T cells may partially contribute to the increased prevalence of CD8αβ+ T cells in the intestinal epithelium. The CD8αβ+ T cells were the primary producers of IFNγ prior to and following SIV infection. The potential of CD8αβ+ IEL to produce IFNγ was found to be upregulated at 2 weeks p.i. and remained high at 8 weeks p.i. These findings suggest that primary SIV infection lead to an increased prevalence of CD8αβ+ T cells in the intestinal epithelium with a higher frequency of these CD8+ T cells capable of producing IFNγ. The IFNγ produced by these subsets of IEL may play a role in the immunopathogenesis of SIV/HIV infection.

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REDUCED IFN-G AND IL-2 EXPRESSION WITHIN LYMPHOID TISSUE AFTER ONE HIGHLY ACTIVE ANTIRETROVIRAL THERAPY.

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Objective: To evaluate the state of immune activation and reconstitution of T cell populations in lymphoid tissue after 1 year of highly active antiretroviral therapy (HAART) of HIV-1 infection.

Design and Methods: Cellular responses were measured in sequential tonsil biopsies obtained from HIV-1 infected volunteers prior to and after 4, 12 and 56 weeks of HAART. Computerized Image Analyses were used to study the expression of cytokines and surface antigens at the single cell level in immunohistochemically stained sections. The incidence of pro-viral HIV-1 DNA containing cells was determined by fluorescent in situ 5'nuclease assay in conjunction with plasma HIV-1 RNA levels and CD4: CD8 T cell counts in blood.

Results: Despite durable suppression of HIV-1 with viral load levels<400 HIV-RNA copies/ml for up to one year, a notable fraction of tonsil cells still harboured pro-viral DNA. A highly significant 4-8-fold increase of CD8+ T cells was demonstrated in the tonsillar tissue prior to HAART (22-33%). After 1 year of HAART, the frequency of CD8+ cells in tonsils was normalised and comparable with seronegative hypertrophied tonsils used as controls (3-5% of total cells). The frequency of recently immigrated dendritic CD1a+ cells was also highly reduced after HAART. The incidence of IL-2 and IFN-g expressing cells in HIV-1 infected tonsils before treatment was increased 10-15-fold compared with controls. The frequency of both IL-2 and IFN-g synthesising cells gradually returned to normal levels after 1 year of HAART, while IL-1a and IL-1b expression showed a slower decline correlating with persistence of pro-viral DNA content.

Conclusion: HAART induced immune changes are associated with a normalisation of immune activation in tonsils, despite the existence of a pro-viral reservoir in the tissue.

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HIV Nef, IL10 AND LPS ENHANCE CD14 EXPRESSION ON HUMAN MONOCYTES BY ACTIVATING DISTINCT MAP KINASE PATHWAYS.

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In this study, we investigated the membrane bound expression of CD14 (mCD14) on monocytes and soluble CD14 (sCD14) released into the culture supernatants of PBMC from HIV+ and HIV- individuals by flow cytometric analysis and ELISA respectively. Monocytes from HIV+ individuals demonstrated higher levels of mCD14 expression and sCD14 release compared to those of HIV- individuals. Studies to determine the role of the LPS, purified HIV Tat and Nef proteins, and the immunoregulatory cytokine IL10 revealed that stimulation of PBMC with either Nef, LPS or IL10 enhanced the expression of mCD14 on monocytes. In contrast, LPS and IL10, but not Nef, increased the levels of sCD14 in culture supernatants of both HIV+ and HIV- individuals. Increased expression of mCD14 and sCD14 in response to LPS was mediated by endogenously produced IL10, as addition of anti-IL10 antibodies blocked this effect. However, Nef-induced CD14 expression was not mediated by IL10 as addition of Nef did not result in IL10 production. To understand the regulation of mCD14 expression, the role of MAP kinases in LPS-, Nef-, and IL10-induced mCD14 expression by monocytes was determined using specific p38, p42/44 and JNK kinase inhibitory agents. The results show that p38 and JNK kinases were implicated in LPS- and Nef-induced expression of CD14 whereas p42/p44 MAP kinases were involved in IL10-induced expression of CD14 on monocytes. These results suggest that Nef, IL10 and LPS modulate the expression of mCD14 via mechanisms involving distinct MAP-kinases.

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REGULATORY DYSFUNCTION OF THE CYTOKINE NETWORK DURING HIV-INFECTION: ANALYSIS OF THE IL-2/IL-2RECEPTOR AND OF ITS THERAPEUTICAL APPLICATIONS.

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The dysregulation of T cell cytokines production appears very early during HIV-infection. The main and constantly reported sign of this regulatory dysfunction is the decreased production of IL-2 by CD4 T lymphocytes followed by their anergy and their increased susceptibility to apoptosis. *In vitro*, IL-2 protects CD4 T lymphocytes from HIV-infected patients against apoptosis by modulating Bcl-2. During HIV-infection, CD8 T lymphocytes are hyper-activated but their cytolytic activity is impaired. We have described that IL-2R loose their functionality after HIV-infection. CD8 T lymphocytes over-express IL-2R but remain insensitive to IL-2 growth signals. The Jak/STAT pathway may be involved in this defect. CD4 T lymphocytes of HIV-infected patients do not respond to IL-2 and we have shown that gp120 alone down-regulates IL-2R expression and reduces its functionality. Aggregation of CD4 molecules may change the intracellular distribution and localisation of some signal transduction molecules ($p56^{lck}$) and may explain the data. Under triple combination therapy (TCT), reduction of viral load is followed by a normalization of the function and of the expression of IL-2R. Our work provides the cellular and molecular basis to associate IL-2 and TCT in the treatment of HIV-infected patients.IL-2 has been shown to increase preferentially naïve CD45RA CD4 T lymphocytes and this may be beneficial for the restoration of the immune response against opportunistic infections and may help to maintain HIV in a latent stage.

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CD4-LOW RESPONDER HIV+ PATIENTS RECEIVING TRIPLE COMBINATION THERAPY. IMMUNOLOGICAL ANALYSIS BEFORE AND DURING IL-2 IMMUNO-THERAPY. D. David, J. THEZE, IGC, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France.

In the treatment of HIV-infected patients associating two reverse-transcriptase inhibitors and one protease inhibitor (Triple Combination Therapy, TCT), some failures have emerged. In some patients, the CD4 counts remain low (< 200 cells/mm^3) despite an efficient blockade of viral replication (plasma viral load < 50 copies/ml). Here, we report an analysis of the CD4 T lymphocytes (L) from a group of such patients and show that their poor immunological response can be reversed by addition of IL-2 to TCT. The data concerning both the hematopoietic cell lineage and the CD45RA/CD45RO ratio of the CD4 T L appear to exclude a profound defect of the primary lymphoid organs in these patients. On the contrary, peripheral CD4 T L display a lack of IL-2 reactivity and a poor expression of the anti-apoptotic molecule Bcl-2, both suggesting a defective maintenance of these cells at the periphery. The patients thereafter received 5-day cycles of subcutaneous IL-2 every six weeks. After three cycles, in addition to a significant increase in the CD4 counts (+ 70% at the median), IL-2 treatment leads to an increase in both IL-2 reactivity and Bcl-2 expression among the CD4 T L. We noticed that level of Bcl-2 expression at the baseline was predictive of the CD4 response after IL-2 therapy. After five IL-2 cycles, most of the patients reached CD4 count levels that are considered to be protective against opportunistic infections.

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ROLE OF SUPPRESOR OF CYTOKINE SIGNALING (SOCS) IN THE REGULATION OF β-CHEMOKINE EXPRESSION BY CYTOKINES IN ALVEOLAR MACROPHAGES (AM) FROM HIV-SEROPOSITIVE SUBJECTS.
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Objective: To determine the relationship of SOCS to MIP-1α expression in the presence of various cytokines in AM from HIV-seropositive subjects.
Methods: AM were isolated from 3 HIV-infected subjects by bronchoalveolar lavage. After incubation for 16h, the cultures were treated with lipopolysaccharide (LPS) and/or cytokines. Supernatants were collected after 24h and analyzed for the presence of MIP-1α protein by ELISA. mRNA was extracted and analyzed for β-chemokine and SOCS-1 expression by RT-PCR.
Results: LPS (100ng/ml) alone or in combination with TNFα or IFNγ, stimulated the highest levels of MIP-1α production by AM. TNFα, IL-11, IFNγ, IL-12, IL-4, IL-1β and IL-10 (all at 10 ng/ml, except IL-4 at 1 ng/ml) stimulated similar amounts of MIP-1α production. The expression of MIP-1α mRNA paralleled protein production under these conditions. The expression of SOCS-1 mRNA, known in systems involving IL-4 to be inversely related to cytokine effect, had no consistent relationship to the effects of LPS or the cytokines tested on MIP-1α production.
Conclusions: These results suggest that β-chemokine expression may be unrelated to the expression of SOCS-1 in AM from HIV-infected subjects.

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IL-1β IS AN EFFECTIVE ADJUVANT FOR NASAL VACCINES. H. F. Staats, N. Sparks, L. Casey, G. Nabors and R. S. Dondero, Duke University Medical Center, Durham, NC 27710, Pasteur Merrieux Connaught, Swiftwater, PA 18370 and CISTRON Biotechnology, Pine Brook, NJ 07058

Interleukin-1β was evaluated for its ability to act as an adjuvant for mucosal vaccines. BALB/c mice were intranasally immunized with 50 μg tetanus toxoid (TT) alone or in the presence of 0.2, 1, or 5 μg IL-1β on days 0, 21, and 42. IL-1β was administered with all three immunizations or with the first immunization only. IL-1β exhibited mucosal adjuvant activity as indicated by its ability to enhance the induction of anti-TT serum IgG, vaginal IgA and delayed-type hypersensitivity responses. Nasal immunization with TT only resulted in a serum anti-TT IgG end-point titer of 1:588. However, nasal immunization with IL-1β and TT induced serum anti-TT IgG end-point titers that ranged from 1:65,536 to 1:356,722, depending upon the dose of IL-1β and whether IL-1β was administered with the first or all three immunizations. Vaginal anti-TT IgA responses were induced in all groups receiving IL-1β. All mice that were intranasally immunized with TT and IL-1β survived when challenged with 10 LD_{50} of tetanus toxin while 100% (10 of 10) naïve mice and 90% (9 of 10) of animals nasally immunized with TT only became moribund. Additional studies have also been performed in rabbits. Therefore, IL-1β is an effective adjuvant for mucosal vaccines administered by the nasal route.

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Inhibition of apoptosis by the cytokine macrophage migration inhibitory factor (MIF) J. Bernhagen, E. Wagner, N. T. Mai, G. Tolle, H. Brunner; Laboratory of Biochemistry, Chair for Interfacial Engineering, University of Stuttgart, D-70569 Stuttgart, Germany

The classical T cell cytokine MIF has been redefined as pituitary hormone, macrophage and T cell cytokine, and enzyme with both thiol-protein oxidoreductase and tautomerase activities. However, no receptor has been cloned and the molecular mechanism of MIF action has remained elusive. MIF has been suggested to enhance the proliferation of T cells and tumor cell growth and has been proposed to play a role in the regulation of the cell cycle. Yet, the molecular pathways and targets of MIF action are poorly known. In an effort to further define the activities of MIF, we observed that MIF inhibited stress-induced apoptosis of various cell lines. These findings prompted us to investigate an anti-apoptotic function of MIF in a more detailed manner. Recombinant huMIF at a concentration of 0.5 to 2 μM was found to inhibit camptothecin-induced apoptosis of HL-60 cells and UV-induced apoptosis of Jurkatt T cells by > 40%. Using the tet-off system and the stably transfected HeLa cell line HtTA-MIF as well as the transiently transfected Kym-1 cell line, these data were confirmed. Recombinant mutant C60SMIF, which is devoid of any oxidoreductase activity, failed to inhibit apoptosis, suggesting that redox-based effects by MIF may be involved. The observed anti-apoptotic effect of endogously produced MIF, i.e. by transfection, suggests that inhibition of apoptosis by MIF may not be receptor-mediated. These studies should aid in identifying the molecular targets of MIF action.

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BCL-2 TRANSGENIC MICE ARE LESS SENSITIVE TO CCL4 INDUCED LIVER DAMAGE THAN WILDTYPE MICE

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Carbon Tetrachloride (CCl_4) is a widely used agent for the study of toxic liver damage. It is known that CCl_4 causes hepatic centrilobular necrosis followed by hepatic fibrosis. More recent results demonstrated that rat hepatocytes also undergo apoptosis after injection of CCl_4. Bcl-2 transgenic C57/Bl6-mice that show a high expression of human bcl-2 in the liver are resistant to FAS induced apoptosis.
We therefore used these mice to study the influence of Bcl-2 overexpression on toxic liver injury after CCl_4 administration. CCl_4 (2 µg/g body weight) was injected intraperitoneally into male bcl-2 mice (n=5 per point). Levels of aminotransaminases were measured 8 and 24 hours after injection. ALT levels were 913 ± 196 U/l after 8 and 15740 ± 2719 U/l after 24 hours in the wildtype mice compared to 693 ± 119 U/l and 5068 ± 932 U/l for bcl-.2 mice. Equivalent values for AST were 855 ± 168 U/l and 8539 ± 1095 U/l in controls and 584 ± 126 U/l and 5436 ± 134 U/l in bcl-2 mice. This suggests that toxic liver damage after CCl4 injection is inhibited by bcl-2 and that it may be caused by activation of apoptotic pathways.

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AN IL-10 ANALOGE IT9302 IS REGULATING PROLIFERATION AND APOPTOSIS IN HEPATO-CELLULAR CARCINOMA CELLS THROUGH A P53 DEPENDENT PATHWAY.

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IT9302, a nanopeptide homologues to the C terminal part of IL-10 posses a significant part of the effects of human IL-10 (B. Gesser et al Proc. Natl. Acad. Sci. 1997 94: 14620-25). IT9302 posses apoptotic effects on cutured normal cells and we wished to test if there is a possible relation between IL-10 and the tumor suppressor protein p53 in regulating cell proliferation. In a preliminery study on IT9302's effect on different hepato-carcinoma cell lines, (HEPG2 (p53 wilde type), HEP3B (p53 deleted) and PLC/PRF5 (p53 mutated)) we observed an exclusive dose-dependent anti-proliferative effect of IT9302 on HEPG2 cells cultured for 5 days. In a subsequent study, where IT9302 was added to the cells daily for seven days (0, 0.05, 0.5 and 5 µg/ml), we observed a significant inhibition of cell-proliferation ($p<0.01$) at 5 µg/ml of IT9302. The intracellular concentration of p53 was also determined and at day 2 we observed a significant induction of p53. By analyzing the DNA content it was found that the percentage of cells expressing DNA in G1, S and G2 phases at day 7 was unaltered when adding 0.05 µg of IT9302 while adding higher doses of IT9302, cells were expressing higher percentage of S phase DNA without entering the G2 phase. By adding Annexin V at day 7, in situ, it was shown that IT9302 induced significant apoptosis compared to the negative control. In conclusion: A nonapeptide identical to the C-terminal part of IL-10 regulates cell proliferation in the HEPG2 carcinoma cell line, a function which may be dependent on p53.

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THE ROLE OF SPHINGOLIPIDS IN THE ANTI-APOPTOTIC EFFECTS OF EGF IN PRIMARY PLACENTAL TROPHOBLAST CELLS.

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Sphingolipids have been shown to play a role in both cytokine-induced cell death and survival. Activation of sphingomyelinase, and the resulting increase in cellular ceramide, has been implicated in TNFα-induction of apoptosis while the activation of sphingosine kinase, and the increase in sphingosine-1-phosphate (S1P), has been shown to be anti-apoptotic. The combination of TNFα and IFNγ induces apoptosis in primary placental trophoblasts *in vitro* and is completely inhibited by cotreatment with epidermal growth factor (EGF). We therefore examined the role of sphingolipids in trophoblast apoptosis and how EGF may influence this. We find that both exogenous ceramide and acid SMase induce apoptosis in trophoblasts. As with TNFα/IFNγ-induced apoptosis, cotreatment with EGF completely abrogated ceramide and SMase-induced apoptosis. In addition, EGF lowered endogenous basal ceramide levels and reduced the level of acid SMase-induced ceramide. An acidic ceramidase inhibitor increased cellular ceramide levels and induced apoptosis that could not be blocked by cotreatment with EGF. Cotreatment with S1P also inhibits TNFα/IFNγ-induced apoptosis. Moreover, EGF activated sphingosine kinase and increased S1P levels. Inhibitors of sphingosine kinase induced apoptosis that could not be blocked by EGF. Together, these data suggest that the EGF abrogation of cytokine and ceramide-induced apoptosis in primary trophoblast cells is mediated, at least in part, by an EGF-induced reduction in cellular ceramide levels and an increase in S1P levels.

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DEVELOPMENT OF ANTI FAS, FASL OR TRAIL MABS AND ELISA KITS : NEW TOOLS FOR APOPTOSIS INVESTIGATION.

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Programmed cell death is the most common form of eukaryotic cell death. Proteins belonging to the TNF/TNFR superfamilies are potent mediators of apoptosis. We generated a panel of IgG isotype mAbs against the Fas (CD95), FasL or TRAIL antigens. By competitive flow cytometry assays, the epitope maps were determined. After stimulation of human peripheral mononuclear cells, the expression of Fas, FasL and TRAIL were compared. These mAbs were also tested on a panel of human cell lines. The ability of these mAbs to trigger apoptosis and/or to modulate this process were analysed. Both the Fas, FasL and TRAIL molecules occur in soluble form. ELISA kits detecting the recombinant and natural soluble Fas and FasL have been generated. The soluble Fas / FasL can be measured in human serum, plasma and cell culture supernatants.

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IDENTIFICATION AND GENE ORGANIZATION OF THREE NOVEL MEMBERS OF THE IL-1 FAMILY ON HUMAN CHROMOSOME 2.

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Members of the IL-1 family of cytokines are important in mediating inflammatory responses. The genes encoding IL-1α, IL-β and the IL-1Ra are clustered within 450kb on human chromosome 2q. By searching the EST databases and by sequencing through this region of chr. 2 we have identified three novel genes that show striking homology to the IL-1 family, that we have named IL-1δ, IL-1ε, IL-1ϕ. All three genes contain a signature motif common to the IL-1 family, and appear to be more closely related to IL-1Ra. Similar to the intracellular form of IL-1Ra, these genes also lack hydrophobic signal sequences. The expression of these genes appears to be highly restricted to various epithelial cell populations. Our results demonstrate the existence of additional genes within the previously defined IL-1 cluster and point to this region of chr. 2 as a evoloutionary hotspot for IL-1 gene duplication. These genes may prove to have an important role in inflammation.

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GENOMIC ORGANIZATION OF THE HUMAN INTERLEUKIN-12 RECEPTOR β2 GENE. J.G.J. van Rietschoten, H.H. Smits, R. Westland, C.L. Verweij, M.T. den Hartog, and E.A. Wierenga.
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Interleukin-12 (IL-12) is a potent skewer of T helper (Th) lymphocyte responses towards the Th1 phenotype by strongly enhancing IFNγ production. Therefore, IL-12 and responsiveness to this cytokine are of major importance for the outcome of specific immune responses. The IL-12 receptor (IL-12R) is composed of two subunits, referred to as ß1 and ß2. Both chains are necessary for high affinity IL-12 binding and signaling, although only the IL-12Rß2 chain contains the intracellular tyrosine residues responsible for STAT4 activation. We have dissected the intron-exon organization of the human IL-12Rß2 gene. PCR primers designed across the cDNA (U46198) were used to trace introns, by comparing PCR product sizes obtained using cDNA and genomic DNA as templates. PCR products spanning introns were sequenced to determine the exact splice sites and flanking regions. The coding region of the gene was found to consist of 15 exons and 14 introns. All intron-exon boundaries are consistent with the consensus sequence for splice junctions (5' GT/AG 3'). Comparison of the intron-exon organization with the human G-CSFR gene, indicated a remarkably well conserved genomic organization between these two class I cytokine receptors. Interestingly, we identified an alternatively spliced mRNA, encoding a putative, truncated protein, lacking all signaling potential.

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CYTOKINE SECRETION AND PLASMA LEPTIN IN HEALTHY WOMEN. Joseph G. Cannon, Esther Brooks-Asplund, Jane M. Daun, W. Larry Kenney. Penn State Univ., Univ. Park, PA 16802

Injecting exogenous IL-1 or TNF into rodents increases plasma leptin, as does injection of LPS into wild-type, but not IL-1β-deficient mice. Using samples obtained in a study of hormone replacement therapy and cytokine production after menopause, the present study tested whether plasma leptin was related to ex vivo cytokine secretion from mononuclear cells isolated from healthy women ranging in age from 25 to 71 years (n=36). The primary source of leptin is adipose tissue, thus body mass index (BMI) was a strong predictor of plasma leptin (R^2=0.598, P=0.0001). A calculated measure of IL-1 agonist and antagonist secretion (f[IL-1] = log IL-1β - log IL-1Ra - log IL-1sRII) also demonstrated a modest correlation (R^2=0.172, P=0.020), but not TNF or IL-6. However, adding IL-6 to a multivariate model including BMI and f[IL-1] yielded an overall R^2=0.673, p=0.0001). Plasma leptin was not significantly influenced by age or hormone replacement therapy. These data support the concept that inflammatory cytokine secretion may be a minor influence (7-17%) on circulating leptin concentrations in healthy women. Supported by NIH AG07004 and RR10732.

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INTERLEUKIN (IL)-6 IS AN IMPORTANT CIRCULATING MEDIATOR OF FEVER IN THE RAT.

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IL-6 has been proposed as an important circulatory mediator of the febrile response to infection and inflammation. Like IL-1, IL-6 produces fever when injected into the brain, however, unlike IL-1, IL-6 is not pyrogenic when given peripherally in rats. We have investigated the role of IL-6, both at the local site of inflammation and in the circulation, using the subcutaneous air pouch (ipo) system in the rat. Male, Sprague-Dawley rats (250-300g) were used for all experiments. Core temperature (Tb) was measured by remote telemetry. Injection of IL-6 (25μg/kg, ipo) alone, had no effect on Tb. Co-administration (ipo) of IL-6 (25ug/kg) with a non-pyrogenic dose of IL-1β (0.03ug/kg) induced a 0.9°C increase in Tb, and co-administration of IL-6 (25ug/kg) with a pyrogenic dose of IL-1β (0.3μg/kg), exacerbated the febrile response. In different experiments, administration (ipo) of LPS induced a 1.6°C rise in Tb (peak 3h) and significant increases in local (pouch; 20880 ± 4157 pg/ml) and circulating (411 ± 87 pg/ml) IL-6. The LPS-induced fever was abolished in the presence of IL-6 antiserum (1.8ml, intraperitoneal) injected 4h prior to LPS (ipo). These data support our earlier observations that IL-6 is an important mediator of fever which probably acts synergistically with other cytokines in the periphery, to activate brain mechanisms in disease.

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INVOLVEMENT OF STAT1 IN THE SYNERGISTIC EFFECT OF IFN-γ AND TNF.

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ME180 cells are extremely sensitive to the synergistic effects of IFN-γ and TNF-β. At low levels of both cytokines, which individually have no obvious effect on the cells, cell death occurs within 24 hours. We have isolated mutant cell lines resistant to the anti-proliferative and anti-viral effect of IFN-γ. These cells are lacking the enzyme indoleamine 2,3 dioxygenase,(IDO) a key enzyme in tryptophan catabolism. One of the cell lines, 3B6A is defective in STAT1 synthesis as shown by Western blot and EMSA assays. This cell line is resistant to the synergistic effect of IFN-γ and TNF-β. A second mutant cell line, 3B6B possibly defective in PKR and IRF-1 expression but retaining normal base line levels of STAT1 is sensitive to the synergistic effect of these cytokines. However this synergy (cell death) does not appear to work through the expression of IDO which is not made in 3B6B cells. We would conclude that STAT1 is an essential component of this synergistic effect and resulting cell death.

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INFLUENCE OF THE NATURAL COMPLEX CYTOKINE OBTAINED FROM AUTOLOGICAL MONONUCLEAR CELLS STIMULATED BY PHA ON THE REGENERATION OF CORNEA.

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We have evaluated the effect of the local application of the natural complex cytokine (NCC) on the regeneration of rabbit and human cornea. The NCC was obtained from the autological mononuclear cells stimulated by PHA. We have characterized the proteins of NCC. The biological test and ELISA revealed the presence of MIF, IL-6, TNF-α, TGF-β1 and IL-1β in the NCC. In experimental models of penetrating wounds of rabbit's cornea ore alkali burn of cornea the application of NCC reduced the inflammatory reaction and stimulated existentially the regeneration. It was shown that at the early stages of cornea regeneration NCC activated some functions of macrophages and neutrophils as migration towards the burn site, phagocitosis, production of reactive oxygen species and secretion of cytokines (IL-1 and TNF). Thus NCC assured resorbtion of debris and abrogation of inflammation. At the later stages of cornea regeneration NCC stimulated keratoblasts migration and proliferation. These results were the base for clinical approbation of autological NCC treating of some patients with ocular wounds. It was shown that the local application of NCC prevented the rough scar formation and deformation of cornea.

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INHIBITION OF INTERLEUKIN-12 PRODUCTION IN HUMAN MONOCYTE-DERIVED MACROPHAGES BY TUMOR NECROSIS FACTOR: IMPLICATIONS IN CYTOKINE CROSS-REGULATION DURING INFLAMMATION AND IMMUNE REACTIONS

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Interleukin-12 is a pivotal cytokine that links the innate and adaptive immune responses. Tumor necrosis factor-α (TNF-α) also plays a key role in orchestrating inflammation and immunity. The reciprocal influence of these two inflammatory mediators on each other may have significant impact on the cytokine balance that shapes the type and extent of immune responses. To investigate the relationship between TNF-α and IL-12 production, we analyzed the effects of exposure of human monocyte-derived macrophages to TNF-α on lipopolysaccharide (LPS)- or *Staphylococcus aureus*-induced IL-12 production in the presence or absence of IFN-γ. TNF-α is a potent inhibitor of IL-12 p40 and p70 secretion from human macrophages induced by LPS or *S. aureas*. IL-10 is not responsible for the TNF-α-mediated inhibition of IL-12. TNF-α selectively inhibits IL-12 p40 steady-state mRNA, but not those of IL-12 p35, IL-1α, IL-1β, or IL-6. Nuclear run-on analysis identified this specific inhibitory effect at the transcriptional level for IL-12 p40 without downregulation of the IL-12 p35 gene. The major transcriptional factors identified to be involved in the regulation of IL-12 p40 gene expression by LPS and IFN-γ, i.e. c-Rel, NFκB p50 and p65, IRF-1, and ets-2 were not affected by TNF-α when examined by nuclear translocation and DNA-binding. A novel nuclear activity named EBPX, was identified that binds to the ets site in the IL-12 p40 promoter and may play a functionally critical role in suppressing transcription in response to the combinatorial signaling of TNF and LPS.

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IFN-α AND IL-12 INDUCE IL-18 RECEPTOR GENE EXPRESSION IN HUMAN NK AND T CELLS.

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IL-18 is a proinflammatory cytokine that enhances innate and Th1-type immune responses. IL-18 exerts its effects in synergy with IFN-α and IL-12 to induce IFN-γ production in NK and T cells. Here we show that IFN-α and IL-12 strongly upregulated mRNA expression of the IL-18 receptor components, AcPL (accessory protein like) and IL-1Rrp1 (IL-1 receptor related protein 1) in human NK and T cells. In addition, IFN-α stimulation enhanced the expression of MyD88, an adaptor molecule involved in IL-18 signaling. Pretreatment of T cells with IFN-α or IL-12 enhanced IL-18-induced NF-κB activation and sensitized the cells to lower concentrations of IL-18. In T cells polarized with IL-12, AcPL and IL-1Rrp1 genes were strongly expressed whereas in IL-4-polarized T cells the respective genes were expressed at very low level. Therefore, it seems that AcPL and IL-1Rrp1 are preferentially expressed in Th1 cells. The results suggest that IFN-α and IL-12 enhance innate as well as Th1-type immune responses by inducing IL-18 receptor epression.

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INTERLEUKIN-4 INHIBITS LIPOPOLYSACCHARIDE INDUCED TNF-α mRNA PRODUCTION IN MOUSE MACROPHAGES. Scott W. Van Arsdell, Kevin P. Murphy, Csaba Pazmany, Diane Erickson, and Mark D. Moody
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Interleukin-4 has been reported to inhibit the LPS-induced production of TNF-α, and recently it has been suggested that the suppressive effect on TNF-α synthesis is mostly if not completely exerted at the translational level in J774 mouse macrophages (1). Here we report the the use of a novel mRNA quantification assay, the Xplore assay, to measure the steady-state levels of TNF-α mRNA found in the mouse macrophage cell line J774. Using this assay, we determined that the steady-state level of TNF-α mRNA increased rapidly to a peak level of 5900 molecules per cell at 60 minutes post-stimulation with 10 ng/ml of LPS. The steady-state level of TNF-α mRNA in cells treated with 5 ng/ml of IL-4 and 10 ng/ml of LPS showed similar kinetics of induction, peaking at 60 minutes. However, the level of TNF-α mRNA was reduced by approximately 30%, to 4100 copies per cell. Integration of time-course curves of mRNA levels over the 12 hours of the experiment showed that IL-4 reduced LPS induced TNF-α mRNA by 40%.
These results illustrate the value of accurate cytokine mRNA quantification when investigating the regulation of cytokine production.

(1) Mijatovic, T., Kruys, V., Caput, D., Defrance, P., and Huez, G. (1997) Interleukin-4 and –13 inhibit tumor necrosis factor-α mRNA translational activation in lipopolysaccharide-induced mouse macrophages. J. Biol. Chem. 272: 14394 – 14398.

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***IN VITRO* LPS INDUCED SPLENOCYTE PRODUCTION OF RAT INTERFERON GAMMA IS REGULATED BY IL-6 AS MEASURED WITH A SPECIFIC ELISA FOR RAT IFN γ.**

SF Orencole, LA Beausang, and CA Burns; Endogen, Woburn, MA

Interferon Gamma (IFNγ), in addition to its anti-viral activity, also acts as a prominent modulator of the immune system. Produced by T_{H1} and NK cells, IFNγ preferentially inhibits the proliferation of T_{H2} cells resulting in an expansion of the T_{H1} cell population and their secretory products (IL-2, IL-3, TNF-β, as well as IFNγ). Using a unique enzyme linked immunoassay (ELISA) developed to specifically detect rat IFNγ, we investigated the production and regulation of IFNγ in rat splenocytes by exogenous IL-6. The ELISA has a dynamic range of 8 to 500 pg/ml and a lower limit of detection of <2 pg/ml. Normal rat sera or plasma IFNγ levels were detectable at the 2 pg/ml level while recovery of either recombinant or natural rat IFNγ spiked sera and plasmas ranged 90-105%. Cross reactivity of the NIH/NIBSC Murine IFNγ reference(Gg02-901-533) read 4% of expected value while other rat, mouse or human cytokines do nct cross react or interfere with the assay. *In vitro* LPS stimulated splenocyte cell culture supernatants generated in the presence or absence of increasing amounts of rat IL-6 were assessed for IFNγ production using the above mentioned ELISA. LPS stimulated supernatants exhibited peak production at T=3Hr. Supernatants from cultures in the presence of IL-6 showed decreasing IFNγ production inversely proportional with IL-6 concentration. All rat IFNγ levels reported were completely neutralized when incubated with a rabbit polyclonal anti-murine IFNγ IgG. These results demonstrate the Endogen Rat IFNγ ELISA as a specific, and accurate tool useful for the quantitative analysis of IFNγ in experimental rat samples.

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IL-15 EXPRESSION IS INDUCED BY IFNα IN A SMALL CELL LUNG CANCER CELL LINE.

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IL-15 is a cytokine with IL-2-like functions. Although the IL-15 transcript is widely expressed in several normal tissues and tumors, there is a strong discordance between IL-15 mRNA expression and detection of synthesis and secretion. It has been suggested that IL-15 mRNA may exist in translationally inactive pools and cells might respond to intracellular infections or other stimuli transforming IL-15 message into protein.
IFN α is a pleiotropic cytokine produced by many cell types in response to viral infection. We studied the effect of IFN α on IL-15 expression in the human small cell lung cancer cell line (SCLC) NCI-H82. Treatment of NCI-H82 cells with IFNα induced a 2-3 fold increase in the expression of IL-15 mRNA at 6 hrs. The intracellular IL-15 protein level increased at 12 hrs upon IFNα treatment as detected by FACS analysis. According to these results, IFNα could be included as an inducer of the IL-15 expression. To our knowledge, this is the first report on activation of IL-15 by IFNα. Consequences of this induction are discussed.

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INTERLEUKIN 16 ENHANCES THE IL-12 AND IL-18 DRIVEN PRODUCTION OF INTERFERON-γ

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IL-12 is a cytokine that induces the production of IFNγ by T and NK cells. Since the enhancing effect of IL-16 on IL-2 driven IFNγ production has already been shown, the possible co-stimulatory effect of IL-16 and IL-12 together was studied. PBMCs stimulated with IL-16 plus IL-12 produce more IFNγ than those stimulated with either cytokine alone. Using FACS analysis, the IFNγ producing cells were identified as $CD16^+$ NK cells, $CD8^{low+}$ T lymphocytes and $CD14^+$ monocytes. Interestingly, $CD4^+$ T lymphocytes were not positive for IFNγ. The possible involvement of IL-18, an IFNγ-co-inducing factor, was therefore tested. IL-16 and IL-16/IL-12 stimulated PBMCs were found to produce IL-18, both at the protein and at the mRNA level. Furthermore, blocking of IL-18 activity by anti-IL-18 mAb decreased the level of IL-16/IL-12 induced IFNγ production.

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THE CD14 LIGANDS LIPOARABINOMANNAN AND LPS DIFFER IN THEIR REQUIREMENT FOR TOLL-LIKE RECEPTORS. T. K. Means[1], E. Lien[2], At. Yoshimura[2], D. T. Golenbock[2], and M. J. Fenton[1]. [1]The Pulmonary Center and [2]Infectious Disease Section, Department of Medicine, Boston University School of Medicine, Boston MA 02118.

Mammalian Toll-like receptor (TLR) proteins mediate cellular activation by bacterial products, such as LPS, via a signaling pathway that is largely shared by the type I IL-1 receptor. We showed that Chinese hamster ovary fibroblasts engineered to express CD14 (CHO/CD14) were responsive to LPS, but not to a distinct CD14 ligand, mycobacterial lipoarabinomannan (LAM). Chinese hamsters, and thus CHO cells, possess a frame-shift mutation within the TLR2 gene which results in their inability to express functional TLR2 protein. Thus, we hypothesized that TLR2 was required for LAM signaling. We found that CHO/CD14 cells that over-express functional TLR2 protein acquired the ability to be activated by LAM. Similarly, over-expression of TLR2 in murine macrophages conferred enhanced LAM-responsiveness. Conversely, peritoneal macrophages from TLR2-deficient Chinese hamsters could be activated by LPS, but not by LAM. Together, our data demonstrate that the distinct CD14 ligands LAM and LPS utilize different TLR proteins to initiate intracellular signals. These findings suggest a novel receptor signaling paradigm in which the binding of distinct ligands is mediated by a common receptor chain, but cellular activation is initiated via distinct signal transducing chains that confer ligand specificity.

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HUMAN TOLL-LIKE RECEPTORS MEDIATE CELLULAR ACTIVATION BY *M. TUBERCULOSIS*. T. K. Means[1], E. Lien[2], A. Yoshimura[2], D. T. Golenbock[2], and M. J. Fenton[1]. [1]The Pulmonary Center and [2]Infectious Disease Section, Dept. of Medicine, Boston Univ. Sch. of Med., Boston MA 02118.

Recent studies have implicated a family of mammalian Toll-like receptors (TLR) in the activation of macrophages by bacterial products such as Gram-negative bacterial lipopolysaccharide (LPS) and the mycobacterial glycolipid lipoarabinomannan (LAM). Here we show that viable *Mycobacterium tuberculosis* bacilli activated both CHO cells and murine macrophages that over-expressed either TLR2 or TLR4. This contrasted with *M. avium* that activated cells via TLR2, but not TLR4. Neither membrane-bound nor soluble CD14 were required for bacilli to activate cells in a TLR-dependent manner. TLR-dependent cellular activation required functional TRAF6. We also found that TLR2, but not TLR4, could confer responsiveness to LAM isolated from rapidly-growing *Mycobacteria*. In contrast, LAM isolated from *M. tuberculosis* failed to induce TLR-dependent activation. Lastly, both soluble and cell wall-associated mycobacterial factors were capable of mediating activation via distinct TLR proteins. A soluble heat-stable and protease-resistant factor was found to mediate TLR2-dependent activation, whereas a heat-sensitive cell-associated mycobacterial factor mediated TLR4-dependent activation. Together, our data demonstrate that Toll-like receptors can mediate cellular activation by *M. tuberculosis* via CD14-independent ligands that are distinct from the mycobacterial cell wall glycolipid LAM.

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DIFFERENTIAL *EX VIVO* RESPONSE TO IFN-β AND IL-12 IN CUTANEOUS LEISHMANIASIS PATIENTS

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The murine model of experimental leishmaniasis has been instrumental for the development of the actual Th1/Th2 paradigm, with Th1 standing for protection and Th2 for progression of the disease. In human leishmaniasis, however, the situation is less clear, but polarized Th1 or Th2 responses have been described in both cutaneous and visceral leishmaniasis patients. We have previously shown that IFN-β is able to antagonize the protective effect of IFN-γ in *Leishmania*-infected human macrophages, thus behaving as Th2-like. To further characterize the role of IFN-β in Th1/Th2 development, we studied its possible interaction with IL-12, the key Th1 cytokine. We found that IFN-β by itself induced modest amounts of IFN-γ, but was able to strongly synergize with IL-12 for IFN-γ induction in healthy controls, both *in vitro* (purified PBMC) and *ex vivo* (whole-blood assay). In cutaneous leishmaniasis patients, however, neither IFN-β, nor IL-12 were able to induce *ex vivo* IFN-γ production ($p<0.01$ for patients vs. controls), but simultaneous addition of both cytokines resulted in low, but significant IFN-γ induction, confirming their synergistic action. Infection with *Leishmania in vitro* significantly ($p<0.01$) inhibited IL-12-induced IFN-γ secretion in healthy controls, but drastically increased basal and IL-12-induced, but not IFN-β-induced IFN-γ levels in patients. In conclusion, IFN-β synergizes with IL-12 for IFN-γ synthesis, which is inhibited by *Leishmania* infection *in vitro* as well as *in vivo*, implying different molecular mechanisms for IL-12- or parasite-induced IFN-γ induction.

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CONSTITUTIVE EXPRESSION OF GP-130-BINDING CYTOKINES IN BONE MARROW: IMPLICATIONS FOR MYELOMA PATHOLOGY. B.E. BARTON and T. Murphy, UMDNJ-New Jersey Medical School, Newark, NJ 07103.

Myeloma is a neoplasm of plasma cells accompanied by osteolytic bone lesions due to factors induced or secreted by myeloma cells which accumulate within the marrow. Previously it was assumed that myeloma cells "home" to bone marrow to proliferate. However, there is little experimental evidence to show that myeloma cells migrate to bone marrow from other organs; moreover, proliferating myeloma cells have been recovered from other tissue. While investigating the pattern of gp-130-binding cytokine expression important for myeloma growth, we analyzed spleen and bone marrow cells from SCID mice transplanted with human myeloma cells. We also examined cells from tumor-free SCID, C57BL/6J, and Swiss Webster mice. Utilizing intracellular staining techniques and flow cytometry, we found that IL-6 and IL-11 were constitutively expressed by bone marrow but not spleen cells of all 3 types of mice. Some oncostatin M (OSM) expression was seen in the spleen cells of C57BL/6J, but not SCID or Swiss Webster mice. Moreover, tumor-bearing SCID mice expressed IL-6, IL-11, and oncostatin M in splenocytes at the same time human myeloma-derived paraprotein was detected in their sera. IL-6 and oncostatin M were shown to be growth factors for the human myeloma line in vitro by the prevention of dexamethasone-induced apoptosis. Low serum levels of IL-6 and OSM detected in tumor-bearing mice when significant human paraprotein was present may indicate that these cytokines drive myeloma proliferation in vivo. Currently we are phenotyping the cell(s) synthesizing the cytokines. We conclude that myeloma cells do not migrate to bone marrow, but rather the constitutive expression of myeloma growth factors in bone marrow induces more proliferation there than at other sites in the body. The dysregulation of marrow function may contribute to the development of bone lesions characteristic of the disease.

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EVIDENCE FOR A KEY ROLE OF IL-6 IN ANTIBODY-MEDIATED EXPERIMENTAL MYASTHENIA GRAVIS PATHOGENESIS. P. CHRISTADOSS, C. Deng, and E. Goluszko, Univ. of Texas Medical Branch, Galveston, TX 77555.

Myasthenia gravis (MG) and its experimental model experimental autoimmune myasthenia gravis (EAMG) are antibody-mediated autoimmune diseases. Elimination of the functions of IFN-γ or IL-12 only partially affected the autoimmunity to acetylcholine receptor (AChR). Therefore, it is crucial to identify the cytokine(s), which could act on the efferent limb of an autoimmune response to AChR in driving B cells to produce pathogenic anti-AChR antibodies. Besides other functions, a pro-inflammatory cytokine, IL-6, promotes B cell maturation, activation, differentiation, and IgG_1 antibody isotype switching. Also, MG patients' thymic epithelial cells over produce IL-6, suggesting the involvement of this cytokine in thymic hyperplasia and germinal center formation. To precisely study the role of IL-6 in EAMG, we immunized and boosted IL-5 gene knockout (KO) mice in the C57BL6 (B6) background and wild type B6 mice with AChR in CFA. The IL-6 KO mice developed normally, and the total number of lymph node cells after AChR immunization were similar to that of B6 mice. A dramatic reduction in the clinical EAMG incidence (16.7%) and suppressed anti-AChR antibody response were noted in IL-6 KO mice compared to B6 mice (clinical disease in 83.3%). Both the early and late AChR and dominant α146-162 peptide-specific lymphocyte responses and IFN-γ and IL-10 production were suppressed in IL-6 KO mice compared to B6 control. This is the first evidence for a key role of IL-6 in the autoimmune response to AChR and in the development of EAMG. Therefore, IL-6 antagonists could be used in the treatment of EAMG/MG.

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ARTERITIS IN MICE LACKING IL-1RA

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In the high pressure arterial system, branch points and flexures have long been recognized as sites of unusually high turbulence and consequent stress, and in humans are foci for atherosclerotic lesions. We show that mice that are homozygous for a null mutation in the gene encoding an interleukin-1 receptor antagonist (IL-1ra), developed lethal arterial inflammation involving branch points and flexures of the aorta and its primary and secondary branches. The disease is focal. We have observed aneurysm formation, with massive transmural infiltration of neutrophils, macrophages and $CD4^+$ T-cells, destruction of elastic laminae and invasion of the vessel intima by smooth muscle cells. Animals have usually died as a result of vessel wall collapse, causing organ infarction, or less frequently as a result of haemorrhage from ruptured aneurysms. Few heterozygotes have died within a year of birth, but small lesions have been found in prospectively killed individuals, which suggests that a reduced level of interleukin-1 receptor antagonist is insufficient to control inflammation fully in arteries. Our results demonstrate a surprisingly specific role for interleukin-1 receptor antagonist in the control of spontaneous inflammation in constitutively stressed artery walls, suggesting that expression of interleukin-1 is likely to have a significant role in signalling artery wall damage. Apart from occasional sialoadenitis, inflammation was not found at other sites. However, work from other laboratories suggests that IL-1ra deficiency on different genetic backgrounds causes specific inflammatory lesions at other sites.

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ANTI-INTERLEUKIN-6 ANTIBODY TREATMENT RESTORES SOME BUT NOT ALL IMMUNE FUNCTIONS AFTER THERMAL INJURY. EJ Kovacs, LA Duffner, and MS Gregory. Loyola University Chicago, Maywood IL, 60153

We recently reported that cell mediated immune responses (delayed type hypersensitivity (DTH) and splenocyte proliferation) were suppressed after being subjected to a 15% total body surface area scald injury and that this suppression occurred at times when circulating levels of interleukin-6 (IL-6) were significantly elevated. To further examine the role of IL-6 in the control of cell mediated immunity after scald injury, animals were given i.p. injections of anti-IL-6 antibody (or control IgG) on days 4, 6, and 8 post injury and examined for DTH and splenocyte proliferative responses and cytokine production on day 10. There was an 84% decrease in the DTH response in IgG treated mice at 10 days following thermal injury relative to IgG treated sham controls which did not differ from untreated mice. Anti-IL-6 treatment resulted in a significant 39% recovery in the DTH response in scalded mice when compared to IgG treated thermally injured animals ($p<0.05$). In contrast to the partial recovery in the DTH response, anti-IL-6 antibody treatment fully restored the Con A mediated splenocyte proliferative. The proliferative response of splenocytes isolated from IgG treated thermally injured mice was decreased by 43% when compared to sham animals given IgG ($p<0.05$), while the proliferative response of splenocytes isolated from anti-IL-6 treated scalded animals was indistinguishable from sham levels. Thus, splenocyte proliferation appears to be more sensitive to IL-6 treatment than the DTH response. Future studies will further explore this differential sensitivity to determine whether there is potential for use of anti-IL-6 therapy to improve immune status of the critically burned patient. (Supported by NIH GM55344, AG16067, and AA12034)

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ROLES OF INTERFERON CONSENSUS SEQUENCE BINDING PROTEIN AND PU.1 IN REGULATING IL-18 GENE EXPRESSION IN MOUSE MACROPHAGES.

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IL-18, previously known as IFN-γ-inducing factor, is expressed in a variety of cell types and functions as a cytokine playing various regulatory roles in the immune system. Its gene expression is regulated by two promoters located upstream of exon 1 (5'-flanking region) and upstream of exon 2 (intron 1). We have cloned both promoter regions into reporter plasmid to yield p1-2686 for the 5'-flanking promoter and p2-2.3 for the intron 1 promoter. Both promoters showed basal constitutive activity and LPS-inducibility when transfected into RAW 264.7 macrophages. To learn the regulatory elements mediating constitutive and LPS-inducible promoter activity, 5'-serial deletion and site-directed mutants of both promoters were constructed. For the activity of p1-2686 promoter, interferon consensus sequence binding protein (ICSBP) binding site between –39 to –22 was critical. EMSA using an oligonucleotide probe encompassing the ICSBP-binding site showed that LPS treatment increased the formation of DNA binding complex. In addition, when supershift assays were performed, the retardation of the protein-DNA complex was seen by the addition of anti-ICSBP antibody. For the activity of p2-2.3 promoter, PU.1 binding site between –31 to –13 was important. EMSA with a PU.1-specific oligonucleotide demonstrated that PU.1 binding is constitutive and increased by LPS treatment. The addition of PU.1-specific antibody to LPS-treated nuclear extracts resulted in the formation of a supershifted complex. Furthermore, co-transfection of ICSBP or PU.1 expression vector increased p1 promoter or IL-18 expression, respectively. Taken together, these results indicate that ICSBP and PU.1 are critical elements for IL-18 gene expression.

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Expression of the Novel Cytokine IL-B30 in Transgenic Mice Induces a Multi-Organ Inflammatory Disease

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A novel cytokine (IL-B30) was cloned from ESTs in the public database. Sequence analysis grouped it into the IL-6/G-CSF family of long-chained cytokines. Transgenic expression of IL-B30 from the β-actin promoter induced a striking phenotype characterized by runting, systemic inflammation, infertility and death. Microsopically, founder animals had infiltration of inflammatory cells into lung, liver and skin and inflammation of the digestive track. This inflammatory response was associated with increased number of platelets, increased immunoglobulin synthesis, and constitutive expression of acute phase proteins in the liver. While IFN-γ, IL-1 and TNF-α were expressed at high levels in IL-B30 transgenic animals, no expression of IL-6 could be detected. This suggests that IL-B30 is a novel cytokine with functions similar to IL-6. However in contrast to IL-6 expression of functional IL-B30 appears to be restricted to hemaotpoietic cells, since liver-specific transgene expression of IL-B30 did not result in a phenotype. In contrast, bone marrow transferred from β-actin – IL-B30 transgenic animals into lethally irradiated recipients induced a phenotype similar to the one detected in donor transgenic animals. We are now expressing IL-B30 conditionally in transgenic animals to further analyze its biology in vivo.

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HMG-1 INDUCES TNF SYNTHESIS IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS.

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Lethal endotoxemia stimulates the release of macrophage-derived cytokines (e.g. TNF and IL-1β) that mediate tissue injury and shock. Death, however, frequently occurs several days later when serum TNF and IL-1β levels have returned to basal levels, implicating other "late" mediators in endotoxin lethality. Recently, we reported that HMG-1 is released from macrophage and human peripheral blood mononuclear cell (HuPBMC) cultures following stimulation with LPS, TNF, or IL-1β. HMG-1 levels are increased significantly 8 hours after the onset of endotoxemia (Science 285: 248-251, 1999). Passive immunization of mice with anti-HMG-1 antibodies protected against the LPS lethality, and administration of purified HMG-1 protein is itself toxic. To elucidate mechanisms underlying HMG-1-mediated toxicity, we examined effects of HMG-1 on the synthesis of TNF in HuPBMCs. Administration of HMG-1 to HuPBMC cultures (0 - 1000 ng/ml) induced TNF protein synthesis in HuPBMCs in a time- and dose-dependent manner, with maximal effect at 8 hours after stimulation. The up-regulation of TNF protein synthesis was accompanied by a delayed increase of TNF mRNA levels (peaking at 8-10 hours, vs. 2-4 hours after LPS stimulation). Thus, amplification of the inflammatory cytokine cascade by HMG-1 might contribute to the endotoxin lethality.

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DISTINCT AND OVERLAPPING ROLES OF LYMPHOTOXIN AND TNF IN DEVELOPMENT OF LYMPHOID TISSUES: CORRELATIONS WITH GENE EXPRESSION PATTERN.

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Previous studies have established the role of two signaling pathways, LT/LTβR and TNF/TNFRp55, in the development of lymphoid tissues and in the maintenance of their functional microarchitecture. We have compared occurrence and structure of lymphoid tissues in LTα, LTβ, TNF, TNFRp55, double LTβ/TNF and triple TNF/LTα/LTβ KO mice. Unlike recently reported LTβ/TNFRp55 double KO mice, the double LTβ/TNF KO mice develop mesenteric lymph nodes, indicating distinct role for LTα signaling via TNFRp55. Although LT/LTβR pathway appears to play the major role in development of spleen and lymph nodes, our data suggest non-redundant role of TNF signaling in positioning of stromal elements which control compartmentalization of lymphoid cell populations. In order to identify genes whose expression may be associated with phenotypic alterations, we subtractively cloned several dozen known and novel genes from spleens of mice with combined TNF/LT deficiency. The gene products include receptor of marginal zone macrophages, components of neutrophil granules, chemokine of lymphoid organs, novel phospholipase A2 (SPLASH) and other. Decreased expression of most of these genes is associated with disrupted LT/LTβR, but not with TNF/TNFR signaling (supported in part by NIH Contract NO1-CO-56000).

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HUMAN β-DEFENSINS USE CCR6 AS A RECEPTOR TO CHEMOATTRACT DENDRITIC AND T CELLS.

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Defensins have antimicrobial activity and disrupt the cytoplasmic membrane of microorganisms. We observed that human β-defensins (HBDs) were also chemotactic for immature dendritic cells (DCs) and memory T cells, but not for mature DC and naïve T cells. The chemotactic effect of HBDs on DCs and T cells was inhibitable by pertussis toxin, suggesting that HBDs might use a G-protein coupled seven transmembrane domain receptor. Investigation of cells stably transfected with chemokine receptors known to be expressed by both immature DCs and memory T cells revealed that HBDs selectively chemoattracted CCR6-transfected cells in dose-dependent, pertussis toxin-sensitive manner. HBD-induced DC migration was inhibited by anti-CCR6 antibodies. Furthermore, the binding of iodinated LARC, the chemokine ligand for CCR6, to CCR6-transfected cells was competitively displaced by HBDs. Thus, HBDs utilize CCR6 as a receptor and may be considered as "microchemokines" that promote adaptive immune responses by recruiting DC and T cells through interaction with CCR6 to the site of microbial invasion.

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IDENTIFICATION OF A NOVEL FOUR-TRANSMEMBRANE DOMAIN PROTEIN ON TH1 LYMPHOCYTES.

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Differentiation of helper T (Th) cells induces programmed expression of specific sets of genes in Th1 and Th2 lymphocytes. Using a differential substraction approach, we identified a novel four-transmembrane protein which is specifically expressed by Th1 but not Th2 cells. Expression of the corresponding mRNA is seen in Th1 cells from Stat4 deficient mice, and in IL-12 and anti-IL-4 treated T cells from IFN gamma-deficient mice. The mRNA was also detected in T cells from Stat6 deficient mice following activation through the antigen receptor. However, Th1 polarization conditions were required to induce expression of this protein in T cells from Stat4- or IFN gamma-deficient mice. These observations indicate that the expression of this transmembrane protein in Th1 cells does not require signal transduction events mediated via the two transcription factors, Stat1 or Stat4. However, expression of this protein in Th2 populations can be prevented by Stat6-dependent signaling events. Overexpression of this four-transmembrane protein results in a potent inhibition of IL-4-mediated gene transcription, while it modestly augments IFN gamma-induced gene transcription. These observations suggest that expression of this novel four-transmembrane protein can potentially downregulate inappropriate IL-4-mediated signal transduction in Th1 lymphocytes.

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CLONING AND CHARACTERIZATION OF MOUSE AND HUMAN TIF, A NEW IL-10-RELATED CYTOKINE.

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TIF (T cell-derived Inducible Factor) is a new cytokine recently identified as a gene induced by IL-9 in murine T lymphocytes. The cDNA of its human homologue was cloned based on homology with the mouse sequence. Both human and mouse TIF consist of a 179 amino acid long protein, including 4 cysteins, and showing 22% amino acid identity with IL-10. The human TIF gene has been mapped on chromosome 12, at 12q15, in a region where a genetic linkage with asthma has been reported. Both human and mouse genes consist of 6 exons spreading over approximately 6 kb. This gene was found to be duplicated in some mouse strains such as FVB and 129. The second copy of the gene, called TIFß, shows 95% nucleotide identity with TIFα,. The deduced amino acid sequence showed only one non conservative difference, suggesting that the TIFß and TIFα proteins share similar functional properties. However, the TIFß gene is characterized by a deletion of the region containing the non-coding exon 1 and the first part of the promoter including the TATA box, suggesting a distinct transcriptional regulation.

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BLYS, A NOVEL MEMBER OF THE TUMOR NECROSIS FACTOR FAMILY AND POTENT B LYMPHOCYTE STIMULATOR. D. M. Hilbert, P. A. Moore, O. Belvedere, Y. Li, V. Roschke, J. Fikes, B. Nardelli and J. Giri, Human Genome Sciences, Inc., 9410 Key West Avenue, Rockville, MD 20850

The TNF superfamily of cytokines includes both soluble and membrane bound proteins with critical roles in the regulation of immune and inflammatory responses. BLyS (B Lymphocyte Stimulator) is a novel member of the human TNF-ligand family that specifically induces B cell proliferation, differentiation and Ig secretion. BLyS is expressed on normal human monocytes and can be upregulated following activation with IFN-γ. A naturally occurring soluble form of BLyS which lacks the first 133 NH_2-terminal residues functions as a potent B cell growth and differentiation factor in co-stimulatory assays. Administration of soluble rBLyS to mice resulted in a disruption of splenic B and T cell zones accompanied by the appearance of a mature $(CD45R(B220)^{dull}$, $ThB(Ly6D)^{bright})$ B cell population. BLyS administration also resulted in a significant increase in serum levels of IgM, IgA, and IgG. The B cell tropism displayed by BLyS is consistent with its receptor expression on B-lineage cells obtained from tonsil, peripheral blood, and B cell tumors. The biological profile of BLyS suggests that it is a critical component of monocyte/macrophage driven B cell activation.

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ANALYSIS AND FUNCTIONALITY OF ISOFORMS OF NATURALLY OCCURRING IL-18 BINDING PROTEIN (IL-18BP)

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Four isoforms in human and two isoforms in murine IL-18BPs are present in five different cDNA libraries. Each isoform has an identical mature N-terminus. The major difference in each isoform is at the C-terminus as a consequence of mRNA splicing. There are four putative glycosylation sites in human IL-18BP isoforms "a" and "c" and in murine isoforms "c" and "d". However, human IL-18BP isoforms "b" and "d" are deleted at their C-termini. Of importance, human IL-18BP isoform "b" does not possess glycosylation sites whereas IL-18BP isoform "d" has one of the four sites.

Recombinant IL-18BPs were expressed transiently in COS-1 cells, and examined for blocking IL-18 induced IFN-γ in human PBMC, the NK92 cells and primary murine splenocytes. Human IL-18BP "a" and "c" completely blocked human IL-18. In addition, IL-18BPs completely blocked murine IL-18 in splenocytes. In contrast, human IL-18BP "b" and "d" did not affect the biological activity of human or murine IL-18 even of 3-4 molar excesses. Interestingly, the full-length murine IL-18BP isoforms "c" and "d" inhibited murine IL-18 but only the murine IL-18BP "d" was active against human IL-18. These studies demonstrate that the binding of IL-18 to different naturally occurring isoforms of human and murine IL-18BPs may affect the ability of IL-18BP to suppress the Th1 response in vivo by acting as either inhibitors or carriers of IL-18.

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INTERFERON-GAMMA INDUCED DESENSITIZATION (IGID) FOR HOUSE DUST MITES : MODULATION OF IMMUNE STATUS FROM TH2 TO TH1 USING INTERFERON-GAMMA AS A NEW THERAPEUTIC CONCEPT FOR ATOPIC DERMATITIS. G.W. NOH and K.Y. Lee, Sungkyunkwan Univ. Sch. Med., Seoul, Korea 100-380 and Yonsei Univ. Col Med, Seoul, Korea 120-749.

Interferon-gamma is a Th1 cytokine which maintains Th1/Th2 balance in the immune response. Atopic dermatitis is one of allergic diseases in which immune status is deviated to Th2 with decreased interferon-gamma and increased interleukin-4. IgE antibody response in Th2-deviated status lead patient to allergic status. A total of 170 patients who showed apparent allergy mainly to house dust mites were selected. Reverse of Th1/Th2 imbalance and desensitization in this reversed Th1/Th2 status for house dust mites were tried in 100 patients. Simple interferon-gamma therapy was conducted on 30 patients and simple desensitization was conducted on another 10 patients. The remaining 30 patients received none of the above therapies. Interferon-gamma induced desensitization (IGID) for house dust mites was successfully achieved in 84% of subjects. Total IgE, specific IgE for house dust mites, and skin reactivity to house dust mites allergens were all dramatically decreased. The ratio of interferon-gamma-containing cells to interleukin-4-containing cells was also effectively decreased. Conclusively, IGID was successful and it has been found that house dust mites may play a pathogenic role in a subgroup of atopic dermatitis. Desensitization for house dust mites was effectively achieved by an appropriate immune modulation. Th1/Th2 balance may be clearly present in human immune regulation especially in those concerning the development of allergy.

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XANTHINE OXIDASE AS A MEDIATOR OF CYTOKINE ACTION IN ARTHRITIS AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS. P. GHEZZI, P. Villa, M. Fratelli, P. Van der Meide, D. Agnello. Lab Neuroimmunology, Istituto "Mario Negri", 20157 Milan, Italy; and Dept. Immunology, Biomedical Primate Research Centre, Rijswijk, The Netherlands.

Oxidative stress has been implicated in inflammatory diseases and in demyelinating diseases. However, the source of the reactive oxygen species (ROS) in these diseases is not clear. We have investigated the effects of cytokines on the ROS-generating enzyme, xanthine oxidase (XO), and found that administration of IFN and IL-12 to mice induce its activity in various tissues. We found an induction of XO in the joint of rats with adjuvant arthritis, but not in the circulation. This was associated with depletion of glutathione (GSH), indicating oxidative stress. XO was also elevated in the spinal cord, not in circulation, of rats with experimental autoimmune encephalomyelitis (EAE). This was associated with GSH depletion in the spinal cord. XO induction was inhibited by anti-IFNg antibodies, suggesting it might mediate part of the central actions of IFNg. Treatment of EAE rats with rat IFNb, while ameliorating the disease, did not decrease the levels of XO in the spinal cord. However, IFNb induced a marked elevation of XO in the circulation. We hypothesize that induction of peripheral XO by IFNb might result in ROS-mediated immunosuppression and contribute to the pharmacological action of IFNb. Thus, XO might be a mediator of the action of IFN with different effects depending on the compartment where it is induced.

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TRANSFORMATION BY TNF-α IN BALB/3T3 CELLS AND OVEREXPRESSION OF IL-1 GENE IN THE TRANSFORMANTS. M. SUGANUMA, S. Okabe, E. Sueoka, and H. Fujiki. Saitama Cancer Center Res. Inst. Saitama 362-0806, Japan

Using two-stage carcinogenesis experiments in TNF-α-deficient mice, we demonstrated that TNF-α is an essential cytokine for tumor promotion. Next, we examined whether TNF-α itself has carcinogenic activity: Treatment with either human TNF-α or mouse TNF-α (10 ng/ml) significantly induced transformed foci of BALB/3T3 cells, 2.08 foci/dish and 0.92 foci/dish compared with 0.13 foci/dish for non-treated cells, indicating that TNF-α does indeed have carcinogenicity. To investigate genetic changes in trans-formants induced by TNF-α, we established 18 transformed clones and named them TNF-α-transformed BALB/3T3 in Saitama (TABS) clones. All TABS clones showed tumori-genicity in nude mice and colony forming activity in soft agar, of various potencies. We chose the 3 clones (TABS-2, -37, and -56) showing the most malignant phenotypes for further examination. Study on chimeric cells of TABS clones (-2, -37, and -56) and BALB/3T3 cells indicated that TABS-2 and -56 had dominant malignant phenotypes, and TABS-37, recessive phenotypes. Mutations of oncogenes in TABS-2 and -56, and of suppressor genes in TABS-37, are now expected. Furthermore, we found that all TABS clones commonly overexpressed IL-1 gene. These results suggest-ed that TNF-α is the instigator for transformation of cells and that induced IL-1 plays a key role in carcinogenesis.

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IMMUNOTHERAPY OF RENAL CARCINOMA SUPPRESSES NF-κB RESPONSE AND INCREASES SUSCEPTIBILITY TO CYTOTOXIC EFFECTORS. U. Junker, B. Henzgen, T. Steiner, K. Nuske, S.K. Durum. Inst. Clin. Immunol., Univ. Jena, D-07740 Jena, Germany

In immunochemotherapy (ICT) for advanced renal cell carcinoma (RCC), the used cytokines are considered to activate the immune response. However interferons also modulate the resistance of tumor cells to proapoptotic effectors. Here we show that NF-κB signaling is suppressed by IFNα and IFNγ in several permanent cell lines and in primary cultures of RCC. Simultaneously we observe increased cell killing by TNFα in some cell lines only. This is not caused by diminished NF-κB protein levels, but by lower transactivating capacity. In an in vitro model of ICT (IL-2, IFNα, 5-fluorouracil, doxorubicin, Caelyx®) pretreatment of RCC with IL-2 and IFNα leads to a diminished NF-κB response to TNFα and a diminished baseline NF-κB activity. In certain tumors, this correlates with increased susceptibility to TNFα, doxorubicin or Caelyx® but not to 5-FU. We find that a dominant negative IκB construct leads to the same effect as the IL-2 / IFNα pretreatment as shown by predominant elimination of the transfected cells from the overall population, while constitutively active p65 leads to a partial rescue from the effect of IL-2 and IFNα, but not from baseline killing. We conclude that besides of immunomodulating effects, treatment of RCC with IL-2 / IFNα leads to a proapoptotic state in certain tumors. In an ongoing study we are investigating whether these are the patients who can be expected to respond to ICT.

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CCR8-DEPENDENT ACTIVATION OF THE RAS/MAP-KINASE PATHWAY MEDIATES ANTI-APOPTOTIC ACTIVITY OF I-309 AND vMIP1.

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We have previously shown that the CC-chemokine I-309 protects mouse thymic lymphomas against corticoid-induced apoptosis. Here, we focused on the signal transduction pathways involved in this activity on BW5147 lymphoma. Inhibition of the I-309 activity by Pertussis toxin suggested the involvement of a G protein-coupled receptor. In addition to CCR8, previously shown to bind I-309, BW5147 cells expressed CXCR4, CCR4, CCR5 but chemokines such as SDF-1, MCP1, MIP1α and Rantes failed to protect cells against apoptosis suggesting that CCR8 chemokine receptors activate a specific signal transduction pathway. By contrast, vMIP1, another CCR8-ligand identified from the genome of a T-cell transforming Herpes virus, shared the anti-apoptotic activity. Further analysis demonstrated that I-309 activates the MAP-kinase pathway in CCR8-transfected CHO cells. The role of this pathway was demonstrated by the fact that PD98059, an inhibitor of MAP-kinases, as well as dominant negative forms of the Ras protein specifically blocked the anti-apoptotic activity of I-309 but not that of IL-9. Finally, protection against apoptosis was also conferred to BW5147 cells by expression of an activated form of Ras, showing that activation of this pathway mimics I-309 activity in these cells.

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THE MECHANISM OF MGSA/GROα MEDIATED TRANSFORMATION IN MOUSE MELANOCYTES. D. Z. WANG, J. Du, J. Luan, P. Liang, K. Matsumoto, K. Tsubakimoto, T. Endo, A. Richmond, Vanderbilt Univ. Sch. Med., Nashville, TN 37232, VA Medical Center, Nashville, TN 37212, and Chiba Univ., Japan.

MGSA/GRO is characterized as an autocrine growth factor for melanoma cells. We have previously demonstrated that immortalized mouse melan-a clones continuously expressing the human MGSA/GROα, β, or γ transgenes exhibit enhanced ability to form large colonies in soft agar and tumors in SCID and nude mice. To determine the effects of constitutive expression of MGSA/GRO on the expression of other genes involved in melanocyte tumor progression, differential display was performed. M-ras was identified as over-expressed in MGSA/GRO-expressing melan-a clones. In the *in vitro* transformation assay, over-expression of M-ras induced cellular transformation, while over-expression of dominant negative M-ras blocked MGSA/GRO induced transformation. To further characterize the downstream targets of the MGSA/GRO enhanced ras expression, we examined the potential effects of ras on AP-1 and NF-κB . Transfection of either M-ras or the dominant active form of M-ras into non-MGSA/GRO expressing melan-a cells increased basal AP-1 and NF-κB transactivation, while transfection of the dominant-negative M-ras into MGSA/GRO expressing cells inhibited endogenous AP-1 and NF-κB transactivation. Moreover, MGSA/GRO induced melanocyte transformation requires the activation of NF-κB, based on the observation that over-expression of IκBα blocked the MGSA/GRO induced transformation and induced apoptosis in MGSA/GRO expressing melan-a cells. In conclusion, MGSA/GRO enhanced ras expression is essential for MGSA/GRO induced melanocyte transformation, while activation of NF-κB protects transforming cells from apoptosis.

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DIFFERENTIAL ACTIVITY OF IL-1 ALPHA AND IL-1 BETA IN TUMOR DEVELOPMENT. E.Voronov*, T. Dvorkin*, E. Fima*, X. Song*, R. White*, E. Cagnano*, D. Benharroch*, O Bjorkdahl**, A. Gjorloff Wingren**, M. Dohlsten**, R. Apte*. Department of Immunology and Microbiology Faculty of Health Sciences Ben-Gurion University of the Negev*, Israel, University of Lund, Sweden**.

IL-1 consists of a family of two proteins, namely IL-1a and IL-1b, which overlap in their biological activities and bind to the same receptors. IL-1a previously was shown, to be a good candidate for the treatment of different metastatic and non-metastatic murine tumor models. In this study, we evaluated the mechanism of IL-1a anti-tumor activity and the role of IL-1b in tumor immunology. As a model we used IL-1a and IL-1b-transduced fibrosarcoma cells, injected into NFS mice. IL-1a-transduced fibrosarcoma cells lose their tumorigenicity and either do not grow in mice or start to grow and subsequently regress. In contrast, IL-1b-transduced cells show even more tumorigenicity than wild-type fibrosarcoma cells. In immunohistochemical studies, a dense infiltrate of mononuclear cells, composed mainly of CD8+ T cells and NK cells was shown to invade IL-1a positive fibrosarcomas, starting from the first week after inoculation of the malignant cells, and ultimately replacing the tumor cells by 40-45 days after tumor inoculation. In contrast, in wild-type and IL-1b transfectants, we see an infiltrate composed mainly of neutrophils predominantly located at the periphery of the tumor, the first week after tumor injection with progressively increased tumor mass. Regression of IL-1a-positive fibrosarcomas is mainly mediated by T cells, predominantly CD8+ lymphocytes. In vivo depletion of CD8+ cells abrogates regression of the tumor, in contrast to depletion of CD4+ lymphocytes, which does not. In addition, we have found that IL-1a-transduced fibrosarcoma cells can secrete stimulatory cytokines, such as IL-6 and thus stimulate the function of tissue-resident APCs. IL-1a-positive cells were found to express the co-stimulatory molecule B7, which increases activation of anti-tumor T cell-mediated immune responses. Thus, we concluded that membrane-associated IL-1a may serve as an adhesion molecule, promoting efficient cell-to-cell interaction between malignant tumor cells and immune effector cells and as a focused cytokine with a strong adjuvant activity. The progressive growth of IL-1b-transduced fibrosarcoma cells shows the different effect of these cytokines on the tumor development. Probably, IL-1b can induce tumor growth by affecting interaction between tumor cells and endothelial cells and by inducing angiogenesis.

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INTERACTION OF THE SKI ONCOPROTEIN WITH SMAD3 REGULATES TGF-ß SIGNALING.

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TGF-ß treatment of cells induces a variety of physiologic responses including growth inhibition, differentiation and induction of apoptosis. Some TGF-ß-initiated signals are conveyed through Smad3; TGF-ß binding to its receptors induces phosphorylation of Smad3 which then migrates to the nucleus where it functions as a transcription factor. We describe here the association of Smad3 with the nuclear proto-oncogene protein Ski in response to the activation of TGF-ß signaling. Association with Ski represses transcriptional activation by Smad3, and overexpression of Ski renders cells resistant to the growth-inhibitory effects of TGF-ß. The transcriptional repression as well as the growth resistance to TGF-ß by overexpression of Ski can be overcome by overexpression of Smad3. These results demonstrate that Ski is a novel component of the TGF-ß signaling pathway and shed light on the mechanism of action of the Ski oncoprotein.

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OVEREXPRESSION OF THE BCL-2 FAMILY MEMBER A1 IN CD4+ T CELLS FROM IL-2 DEFICIENT MICE. S.Wagner, Burkhard Kneitz, A. Schimpl, Univ. Würzburg, 97078 Würzburg, Germany

IL-2 deficient mice characteristically develop lymphoadenopathy and multiorgan infiltration as a consequence of uncontrolled activation of $CD4^+$ T cells, a high proportion of which exhibit a memory phenotype ($CD62L^{lo}$, $CD44^{hi}$, $CD45RB^{lo}$). One of the reasons for accumulation of peripheral T cells may be the inability of T-cells stimulated in the absence of IL-2 to undergo CD95 mediated cell death, although these cells express the same levels of CD95 and molecules forming the "death inducing signaling complex" as do wildtype T cells. The same or higher levels of caspases were investigated. Using RNase protection assays, expression levels of Bcl-2 family members were investigated. A1 was found to be highly overexpressed in IL-2 deficient T cells, that were activated in vitro, whereas Bcl-2 was barely detectable. A1 has been previously shown to rescue cells of the hematopoetic lineage from apoptosis. Experiments are currently in progress to assess wether the overexpression of A1 is causative for abnormal cell survival in IL-2 deficient mice or a consequence of the accumulation of CD95 resistent cells.

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MULTIPLE MECHANISMS OF TUMOR LYSIS BY ACTIVATED T CELLS. T.J. Sayers, A.D. Brooks, N. Seki, A.M. Malyguine, M.J. Smyth and B.R. Blazar, IRSP, SAIC-Frederick, NCI-FCRDC, Frederick, MD 21702-1201.

Cytotoxic T cells (CTL) and natural killer (NK) cells lyse virally-infected or tumor target cells using 2 established mechanisms (1) directed release of lytic granules containing perforin and granzymes, (2) expression of Fas ligand (FasL) or TNF by effector cells, which on engagement of appropriate receptors on the target cell triggers apoptosis. We have examined mechanisms of target cell lysis using activated T cells triggered to lyse tumor target cells by cross-linking of CD3 on their surface. Furthermore, we have utilized T cells from both gld mice and mice gene-targeted for perforin (pfpko). Activated T cells from pfpko mice were very effective in lysing the murine renal cancer Renca in long-term (16-18hr) cytotoxicity assays. Part of the lysis by T cells from pfpko mice was due to Fas-mediated killing. However, in contrast to FasL-mediated killing, this lysis was not completely blocked by the caspase inhibitor zVAD-FMK. To further investigate this lytic mechanism, we generated activated T cells from gld x pfpko mice. Activated T cells from these mice could lyse a variety of tumor targets including Renca, YAC, and P815, in long-term cytotoxicity assays, yet no lysis was seen in short-term (4-6hr) assays. This lysis by gld x pfpko T cells was not efficiently inhibited by zVAD-FMK which often blocks "death-receptor" mediated killing. Furthermore these tumor cells were insensitive to TNF or recombinant human TRAIL. This data suggests that T cells can use an alternative mechanism to lyse certain tumors, which is perforin, TNF, FasL, and TRAIL independent, and does not seem to involve caspase signaling in target cells.

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HUMAN AND MURINE CELLULAR MODELS FOR CASPASE INDEPENDENT DEATH INDUCED BY TNF OR dsRNA. M. Kalai, G. Van Loo, G. Denecker, D. Vercammen, P. Vandenabeele. Laboratory of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Ghent, B-9000 Ghent, Belgium

Cellular death induced by double stranded RNA with or without pre-treatment with interferon is usually described as apoptosis and was often shown to be inhibited by caspase-inhibitors We have recently reported that murine L929 fibrosarcoma cells treated with TNF die in a necrotic way. Furthermore, stable over-expression of the viral serpin-like caspase inhibitor CrmA in these cells renders them much more sensitive to TNF-mediated death. Treatment of L929 cells with poly(I;C) or type I or II interferon alone is hardly cytotoxic. However, pre-treatment with interferon followed by poly(I;C) is highly cytotoxic. Although the dying cells have necrotic morphology, their death is independent of endogenously produced TNF since their survival is not affected by antagonistic antibodies directed against murine TNF. Furthermore, L929R1 mutant cells that lack TNF receptor and are resistant to TNF cytotoxicity still die when pre-treated with interferon and challenged with poly(I;C). Interestingly, L929 cells over-expressing CrmA are more responsive to the cytotoxic effect of the combination of interferon and poly(I;C) than the parental cells. We obtained similar results with human leukemic T lymphoma Jurkat cells. These cells die by anti-Fas or poly(I;C) treatment. Their response to the latter is enhanced by pre-treatment with type I or II interferon. However, they are resistant to TNF cytotoxicity. Interestingly, a variant clone derived from these cells which over-expresses Bcl-2 and is caspase-8 deficient is much more sensitive to TNF and to poly(I;C) mediated cell death than the parental cells, demonstrating again a caspase independent cellular death mode.

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MOLECULAR MECHANISMS INVOLVED IN THE LITHIUM-INDUCED SENSITIZATION OF TUMOR CELLS TO TNF-INDUCED APOPTOSIS. Rudi Beyaert and Peter Schotte, Dept. Mol. Biol., University of Gent - VIB, Belgium.

Lithium salts are widely used in psychiatry for the treatment of manic depressive disease states. We previously demonstrated that lithium could sensitize tumor cells to the cytotoxic effect of TNF in vitro and in mouse tumor models. The molecular mechanism which is responsible for the TNF-sensitizing effect of lithium is still unclear. We now demonstrate that LiCl considerably increases the TNF-induced activation of caspases in KYM cells. This was associated with a 200-fold increase in TNF-induced apoptosis, whereas LiCl as such had no effect. Pretreatment of cells with the broad spectrum caspase inhibitor zVAD.fmk or the caspase-3 like inhibitor zDEVD.fmk completely abolished TNF+LiCl induced apoptosis, whereas the caspase-1 like inhibitor Ac-YVAD.cmk was only partially protective. Similar results were obtained in several other cell lines although the relative protection by the different caspase inhibitors was cell line dependent. Mitochondria are believed to contribute to the induction of caspase activation and apoptosis by releasing cytochrome c. LiCl was found to increase also the TNF-induced release of cytochrome c, before any detectable activation of apoptosis. This was associated with an increased proteolytic activation of caspase-3 and caspase-8. In contrast to TNF-induced apoptosis, TNF-induced activation of NF-κB was not modulated by lithium. Moreover, cell death induced by etoposide, staurosporine or anti-Fas treatment was also not affected by lithium. The above observations suggest that lithium increases the apoptotic effect of TNF by acting at an early point during the TNF-initiated signalling pathway.

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INTERLEUKIN(IL)-7 INDUCES RAPID ACTIVATION OF PYK2 WHICH IS BOUND TO JAK 1 AND IL-7Rα

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IL-7 receptor signaling begins with activation of the tyrosine kinases Jak1 and Jak3, which are associated with the receptor complex. To identify potential targets of these kinases, we examined Pyk2 (a member of the focal adhesion kinase family) using an IL-7-dependent murine thymocyte line, D1. We demonstrate that stimulation of D1 cells by IL-7 rapidly increased tyrosine phosphorylation and enzymatic activity of Pyk2, with kinetics slightly lagging that of Jak1 and Jak3 phosphorylation. Conversely, IL-7 withdrawal resulted in a marked decrease of Pyk2 phosphorylation. Pyk2 was found to be physically associated with Jak1 prior to IL-7 stimulation, and to increase its association with IL-7Rα chain following IL-7 stimulation. Pyk2 appeared to be involved in cell survival, since antisense Pyk2 accelerated the cell death process. Activation of Pyk2 via the muscarinic and nicotinic receptors using carbachol, or via intracellular Ca^{++} rise using ionomycin/phorbol myristate acetate, promoted survival in the absence of IL-7. These data support a role for Pyk2 in coupling Jak signaling to the trophic response.

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TRANSIENT INFILTRATION OF NEUTROPHILS INTO THE THYMUS IN ASSOCIATION WITH APOPTOSIS INDUCED BY WHOLE BODY X-IRRADIATION.

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Generally, the process of apoptosis does not cause leakage of noxious cytosolic contents and is therefore non-inflammatory. However, as previously shown, macrophages ingesting apoptotic CTLL-2 cells produced pro-inflammatory cytokines, in particular, IL-8 and MIP-2, a murine IL-8 homologue (BBRC 239, 799, 1997; JI 161, 6245, 1998). This predicted that rapid and massive apoptosis may induce neutrophil accumulation in vivo. In this study, we tested this prediction by inducing apoptosis by whole body X-irradiation in mice. After exposure to 4 Gy X ray irradiation, mice exhibited considerable apoptosis of thymic cells, which was associated with transient infiltration of neutrophils as well as MIP-2 mRNA expression. In contrast, in p53-deficient mice, in which irradiation-induced apoptosis was suppressed as has been reported, infiltration of neutrophils into the thymus was also suppressed to a similar extent. Taken together, these results suggest that massive and rapid apoptosis can result in infiltration of neutrophils.

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THE NEUTROPHIL CHEMOATTRACTANT GCP-2 SELECTIVELY ANTAGONIZES THE INTERACTION OF IP10 WITH CXCR3

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IP10, MIG and I-TAC are three interferon-inducible CXC chemokines thought to be responsible for the recruitment of activated T and NK cells to sites of inflammation by virtue of their common ability to bind to and activate CXCR3, a G-protein coupled receptor (GPCR) expressed on these cell types. Additional biological properties have also been ascribed to IP10, including the ability to block the signal transducing properties of other GPCRs to which CXC chemokines bind with high affinity. For instance, IP10 potently inhibits the *in vitro* and *in vivo* angiogenic activity mediated by CXCR1/2 ligands IL-8 and GCP-2 on endothelial cells. IP10 also inhibits the constitutive signaling of Kaposi's Sarcoma-associated Herpesvirus GPCR, a property it shares with CXC chemokine SDF-1 $\cdot$, but interestingly not with MIG. We asked if this was a reciprocal phenomenon , ie if CXC chemokines other than MIG or I-TAC could block the interaction of IP10 with CXCR3. We found that the neutrophil chemoattractant GCP-2, but not IL-8, a closely related chemokine with similar biological properties, potently competes for the binding of radiolabeled IP10 to CXCR3 receptors on transfected RBL (rat basophil leukemia) cells and activated human T-cells. Furthermore, GCP-2 does not induce the activation of CXCR3 expressing cells, but blocks IP10-induced chemotaxis and calcium mobilization. Surprisingly, the antagonism of GCP-2 is selective for IP10 as GCP-2 does not inhibit the ability of MIG or I-TAC to bind to or activate CXCR3 expressing cells. Using GCP-2 as a radioligand, we explored the mechanism of GCP-2 inhibition of IP10 binding and function, and determined that GCP-2 binds with high potency not to CXCR3, but to a cell surface receptor on transfected and untransfected RBL cells which is pharmacologically distinct from the GCP-2 receptors characterized to date. Thus, our data suggest that the interaction of GCP-2 with a novel cell surface receptor leads to cooperative and selective displacement of IP10 from CXCR3. These findings demonstrate one way in which the apparent promiscuity of the chemokine/ chemokine receptor system may be biologically manipulated in order to confer non-redundant roles on individual chemokines.

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LYMPHOID TISSUE HOMING CHEMOKINES ARE EXPRESSED IN CHRONIC INFLAMMATION.

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Secondary lymphoid tissue chemokine (SLC) and B lymphocyte chemoattractant (BLC) are homing chemokines that have been implicated in the trafficking of lymphocytes and dendritic cells in lymphoid organs. Lymphotoxin-α (LTα), a cytokine crucial for development of lymphoid organs, is important for expression of SLC and BLC in secondary lymphoid organs during development. Here we report that transgenic expression of LTα induces ectopic expression of SLC and BLC in the adult animal. LTβ was not necessary for induction of BLC and SLC, whereas, in contrast, TNFR1 was found to be important for the LTα mediated induction of these chemokines. The expression of LTα is associated with a chronic inflammation that closely resembles organized lymphoid tissue and this lymphoid neogenesis can also be seen in several chronic inflammatory diseases, including in the pancreas of the prediabetic nonobese diabetic (NOD) mouse. Expression of SLC protein was also observed in the pancreas of prediabetic NOD mice. This study implicates BLC and SLC in chronic inflammation and presents further evidence that LTα orchestrates lymphoid organogenesis both during development and in inflammatory processes.

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IFN-γ attenuates IL-2 induced CXCR3 function in primary human lymphocytes: Implications for Th1 responses. Venugopal Gangur*, Ji-Ming Wang*, Wanghua Gong‡, Howard A. Young§ and Joost J. Oppenheim* *Laboratory of Molecular Immunoregulation, §Laboratory of Experimental Immunology, National Cancer Institute, ‡Intramural Research Support Program, SAIC, Frederick, Maryland 21702-1201.

Since IFN-γ is a potent inducer of IP-10, I-TAC and MIG, we examined the impact of this prototypic type-1 cytokine on CXCR3, the receptor for these CXC chemokines. Coculture of IL-2 stimulated human primary lymphocytes with IFN-γ unexpectedly abrogated lymphocyte chemotaxis to these agonists in a dose and time dependent manner. This inhibitory effect of IFN-γ was not due to the loss of intrinsic migration ability of lymphocytes *per se*. Ligand binding experiments using [^{125}I]IP-10 indicated that IFN-γ almost completely abolished the IL-2 induced specific binding sites for IP-10 on lymphocytes. Flow cytometry analyses using an anti-CXCR3 mAb indicated depletion of CXCR3. In contrast, analysis by ribonuclease protection assay indicated no significant effect of IFN-γ on CXCR3 mRNA level. The receptor was not merely down regulated by ligands since neutralizing antibodies to CXCR3 agonists (IP-10 and MIG) did not reverse the effect of IFN-γ, whereas I-TAC is not known to be produced by lymphocytes. Based on these data, we propose that responsiveness to IFN-γ may play a key role in retention of lymphocytes in Th1-dominated inflammatory sites by modulating CXCR3 function.

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INTERACTION OF RECEPTOR BINDING EPITOPS OF IL-6 TYPE CYTOKINES: THE SPECIFITY OF SITE III DEPENDS ON SITE I

KALLEN, K.-J., Aasland, D., Grötzinger, J., Galle, P.R.G., Gascan, H., Rose-John, S.

I. Medizinische Klinik, Universtität Mainz; Institut für Biochemie, RWTH Aachen, Germany; INSERM Unit CHU Angers, France

Four helical bundle cytokines of the IL-6 family have a modular architecture. With site I they may bind to specific α-receptor, whereas sites II and III are responsible for signal induction by formation of either a gp130 homodimer (IL-6, IL-11) or a gp130/ LIF receptor (LIFR) heterodimer (LIF, OSM, CNTF, CT-1). OSM, but none of the other family members, can additionally signal via a heterodimer of gp130 and the specific OSMRß.

On CNTF the site III LIFR binding epitope consists of the C-terminal A-helix/ N-terminal AB-loop, the BC-loop and the N-terminal CD-loop/ C-terminal D-helix. We have recently created a new designer cytokine, by exchanging the site III LIF receptor binding epitope of CNTF with the corresponding gp130 binding epitope of IL-6. The resulting IL-6/ CNTF hybrid cytokine, termed IC7, binds to the specific IL-6 α-receptor , but like CNTF induces a signalling gp130/ LIFR heterodimer via its CNTF module (site III) which is responsible for LIFR binding. It is inactive on cells only expressing the IL-6 α-receptor and gp130.

Surprisingly, however, IC7 can illicit a signalling heterodimer of gp130 and OSMRß upon binding to the IL-6 receptor. In contrast, CNTF bound to theCNTFRα does not induce signalling via gp130 and OSMRß, although at the same concentrations of CNTF and CNTFRα optimal cellular responses via gp130 and LIFR are achieved. This suggests that an identical receptor binding epitope (here site III) can change its specificity depending on the receptor bound to site I. Thus, the α-receptor binding to the cytokine site I is not just a docking site, but may strongly influence the specificty of IL-6 type cytokines.

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CYTOMEGALOVIRUS HARBORS ITS OWN UNIQUE IL-10 HOMOLOG. S.V. KOTENKO, L.S. Izotova, S. Saccani, O.V. Mirochnitchenko and S. Pestka. Dept. of Molecular Genetics & Microbiology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854-5635, USA.

IL-10 is a powerful immunosuppressor and anti-inflammatory agent. Many hematopoietic and solid tumors express IL-10. The presence of IL-10 at the host-tumor interface helps tumors to survive immune surveillance by inhibiting cell-mediated immunity. It is no surprise that viruses utilize the same strategy to protect virus-infected cells from immune defenses and thus enhance survival of virus and virus-infected cells in the host. Two herpes viruses capable of infecting humans harbor homologs of IL-10. Epstein Barr virus (EBV) encoded IL-10 was the first viral IL-10 (vIL-10 or ebvIL-10) cloned. ebvIL-10 shares many but not all of the biological activities of cellular IL-10 (cIL-10) and may play an important role in the host-virus interaction. In addition to EBV, another virus, the Orf poxvirus (OV), has its own IL-10 homolog (ovIL-10). vIL-10s demonstrate more than 85% identity to human IL-10. We have discovered that another herpes virus, cytomegalovirus (CMV), encodes its own IL-10 homolog, designated cmvIL-10. We demonstrated that cmvIL-10 binds to the human IL-10 receptor complex and competes with human IL-10 for receptor binding sites despite the fact that these two proteins have minimal homology (27% identity). We also demonstrated that cmvIL-10 requires both subunits of the IL-10 receptor complex to induce signal transduction events and biological activities. Moreover, cmvIL-10 is able to induce signal transduction in peripheral blood mononuclear cells. Although the sequence of the entire CMV genome has been known since 1990, because of the minimal homology between cmvIL-10 and IL-10, this homolog of IL-10 was not previously recognized as such. Furthermore, the structure of the cmvIL-10 gene is unique. The gene retained two of four introns of the IL-10 gene, but the length of the introns was reduced. In conclusion, we have identified a unique homolog of IL-10 encoded by the CMV genome.

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CELLULAR DISTRIBUTION OF A BINDING PARTNER FOR CLF-1.

Greg C.A. Elson, Amelie Benoit de Coignac, Jean-Yves Bonnefoy and Jean-François Gauchat.
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The recently identified soluble protein cytokine-like factor 1 (CLF-1) shares significant homology with the type-I cytokine receptor family. This has lead to speculation that CLF-1 is either a soluble ligand binding chain of a cytokine receptor complex or a component of a heterodimeric cytokine. CLF-1 is extremely highly conserved, suggesting that the protein has an important physiological function. This is supported by the observation that CLF-1 knockout mice failed to survive after birth. Three forms of CLF-1 are known to exist in the mouse, containing C-terminal variations generated by alternative splicing. By database screening and RT-PCR, we have identified 4 forms of hCLF-1, also varying at the C-terminus and being generated by alternative splicing. In an attempt to further characterize this novel protein, we first studied its mRNA tissue distribution and saw highest expression in adult aorta, placenta, brain, heart and skeletal muscle. In the developing foetus, strongest expression was seen in the lung. Extending this study to cell lines, we saw expression of CLF-1 in a number of tumour derived lineages. Using a CLF-1-Fc homodimeric recombinant fusion protein a CLF-1 binding partner was detected on a number of different cell lines and could be upregulated on certain primary immunocompetent cells. It is therefore tempting to speculate that a CLF-1 homodimer is capable of binding specifically to the appropriate receptor, similar to IL-12 p40$_2$ binding to IL-12R.

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CYTOKINE DISREGULATION IN THE RHEUMATOID SYNOVIUM: MECHANISMS OF TNFα REGULATION AND DISEASE CHRONICITY

M. Feldmann, F.M. Brennan, J. Bondeson and B.M.J. Foxwell. Kennedy Institute of Rheumatology, London, U.K.

In a local inflammatory disease, the tissue provides an admirable tool for studying disease pathogenesis. We have used it to define TNFα as an effective target for therapy in rheumatoid arthritis, so subsequently we turned our attention to the mechanism of TNF over production in RA. This was studied at the molecular level, using adenoviruses. An adenovirus over expressing IκBα was found to inhibit TNFα production by 70%, as well as many other proinflammatory cytokines metalloproteinases, but not the anti-inflammatory cytokines or TIMP, suggesting that local blockade of NFκB might be very useful in RA. At the cellular level we have studied the activation of synovial macrophages, the cells producing most of the TNFα. Spontaneous synovial TNFα production was not blocked by neutralizing antibodies to many cytokines, it was by T cell removal. RA T cells co-cultured with fresh monocytes were able to induce TNFα, verifying their role in TNFα induction. Comparing the RA T cell phenotype with that of T cells activated via the TCR, or by a cocktail of cytokines, revealed that RA T cells resemble cytokine activated T cells, by a number of criteria. This suggests that a vicious cycle of 'bystander' activated T cell-macrophage interactions is at the heart of the disregulated cytokine network in RA, and could be a new target for therapy

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MOLECULAR CLONING OF A NOVEL MACROPHAGE-DERIVED CYTOKINE (SMAF-1) AND ITS IMMUNO-MODULATING CAPACITIES.

L. Fransen, P. De Baetselier[1], L. Nelles[2], W. Deleersnijder, L. Brijs[1], K. Devos, F. Bosman, M.A. Buyse, D. Huylebroeck[2], E. Sablon, A. Van De Voorde and H. Van Heuverswijn.

Innogenetics, Industriepark 7 box 4, B-9052 Ghent, Belgium. [1]Dienst Cellulaire Immunologie, IMOL II, VUB, Brussels, Belgium. [2]Dept. cell Growth, Differentiation and Develop, Flanders Interuniversity Institute for Biotechnology, Leuven Belgium.

A new macrophage-derived cytokine (SMAF-1) with the ability to down-regulate Th1 (and possibly also Th3)-like responses was isolated by plus-minus hybridization of an LPS-induced PU5.1.8. mouse monocytic cDNA library. The full-size human and mouse cDNAs of this suppressive macrophage associated factor (SMAF-1) were cloned from the mRNA of *in vitro*-activated human (THP-1) or mouse (PU5.1.8.) macrophage cell lines and code for a protein of 311 amino acids sharing 77.4% homology. SMAF-1 gives rise to a secreted mature protein of 30 kDa. No homology exists between known database sequences and the primary structure. However, we were also able to identify a related family member (SMAF-2). SMAF-1 is mainly produced by macrophages and can be up-regulated by Th2 cytokines (IL-4, IL-10) and down-modulated by Th1 cytokines (IFN-γ, TNF-α).

C57/BL6 SMAF-1-deficient mice demonstrated a clear bias towards a Th1 (and possibly also a Th3)-like response when challenged with different antigens as reflected in the higher IgG2a (and IgA) levels in serum and IFN-γ production. Moreover, in wild-type mice, the antigen-induced IFN-γ response was reversed upon co-injection with immunoaffinity-purified recombinant mouse protein. Collectively, the data point to a new and important immunoregulatory role of SMAF-1 in the down-regulation of Th1 type responses.

Sponsored by IWT grants 940054 and 970228.

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IL-7 WITHDRAWAL INDUCES A RAPID RISE IN INTRA-CELLULAR pH CAUSING: A) BAX TRANSLOCATION TO MITOCHONDRIA AND B) TRANSIENT MITOCHONDRIAL HYPER-POLARIZATION. A. KHALED, K. Kim, K. Muegge and S.K. Durum, National Cancer Institute, Frederick, MD, 21702

During T lymphocyte development, IL-7 functions as a trophic factor by an unknown mechanism. Here we report a novel process by which IL-7 withdrawal activates the pro-death protein Bax and produces a transient hyperpolarization of the mitochondrial inner membrane. Following IL-7 withdrawal from a dependent cell line, intracellular pH rose steadily within the first hours of cytokine withdrawal, peaking over pH 7.8 before returning to neutrality (a similar alkalinization was observed following IL-3 withdrawal from a dependent cell line). Intracellular acidification occurred later, 30-40 hours post-cytokine withdrawal. The transient alkalinization triggered a conformational change in the death protein Bax via positively and negatively charged residues at the N– and C-termini, exposing hydrophobic domains that targeted it to mitochondrial membranes, initiating the apoptotic process. Site-directed mutagenesis, substituting neutral residues for the charged residues at the termini, confirmed the pH dependence of Bax. A second consequence of alkalinization was also observed: transient hyperpolarization of the mitochondrial inner membrane, an effect blocked by oligomycin. Therefore, we have shown that cytokine withdrawal, perhaps by deregulating membrane ion exchangers, prompts a rise in intracellular pH that activates Bax, by changing its conformation, as well as inducing a transient hyperpolarization of the mitochondrial membrane, leading to apoptotic death.

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AN INTERFERON-INDUCED PROTEIN OF THE BCL-2 FAMILY HAS TWO INDEPENDENT ACTIVITIES: IT PROMOTES APOPTOSIS AND SYNTHESIZES 2-5 (A). G.C.Sen, A.Ghosh and S.N. Sarkar, Dept. of Mol. Biology, Lerner Res. Inst., Cleveland Clinic Foundation, Cleveland, OH 44195

Interferon induces the synthesis of many proteins through which it mediates its various cellular functions. Among them are the enzymes, 2-5 (A) synthetases, which, in combination with RNase L, cause RNA degradation in virus infected cells. Here, we report that 9-2, a specific isozyme of 2-5 (A) synthetase cloned in our laboratory, has a direct cell-killing activity. Expression of 9-2 in human and mouse cells caused apoptosis as monitored by TUNEL assay, DNA laddering, FACScanning for hypodiploidy, Caspase activation and PARP cleavage. Surprisingly, an enzymatically inactive mutant of 9-2 was equally proapoptotic. Among the many isozymes of 2-5 (A) synthetases, only 9-2 had the cell-killing activity. Even 3-9 and E-18, two other isozymes, which, along with 9-2 are produced from same primary transcript by alternative splicing, lacked the proapoptotic effect. This was due to the unique C-terminal of 9-2 that was shown to contain a Bcl-2 Homology Domain 3 (BH3). Cellular apoptosis is known to be regulated by interactions among the pro and anti-apoptotic members of the Bcl-2 family of proteins. Like other proteins which contain the BH3 domain, 9-2, but not 3-9, interacted with Bcl-2 and Bcl-X_L, the cellular anti-apoptotic proteins. Point mutations in the BH3 domain of 9-2, which disrupted this interaction, also abolished its cell-killing activity without affecting its enzyme activity. Thus, we have identified 9-2 as a unique dual-function protein: it has 2-5 (A) synthetase activity in its N-terminal domain and apoptotic activity in its C-terminal domain. It is also the first member of the Bcl-2 family of proteins that is known to be interferon induced.

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MOUSE γ c MUTANTS WITH IMPAIRED BINDING OF IL-2, IL-4, AND IL-7. F. Olosz and T. R. Malek, University of Miami School of Medicine, Miami, FL 33101.

The common γ chain (γc) is a shared subunit of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors that plays a critical role in cytokine signaling, and also acts as a modulator of cytokine binding. The aim of our study was to identify regions of the γc ectodomain that are required for the upregulation of ligand binding in the heterodimeric IL-2, IL-4, and IL-7 receptors. For this, we carried out a detailed alanine scanning mutagenesis study of putative ligand binding regions in the mouse γc extracellular domain, based on predictions of recently published theoretical models of γc-containing receptors. Mutant γc variants were transiently coexpressed with other subunits in COS7 cells, and studied for expression, mAb binding, and the ability to enhance the binding of radiolabeled cytokines. We found that at least three loops of the γc ectodomain contain sites that are important for IL-2, IL-4, and IL-7 binding, and that the cytokines likely interact with overlapping regions of γc. The epitope of the antagonistic TUGm2 mAb has been mapped to the same area. This putative regulatory region contains a tyrosine that is homologous to an important ligand binding site in the growth hormone receptor, and two cysteines that appear to be important for both γc stability and cytokine binding. We propose that a hypothetical disulfide bond between these cysteines may be required for maintaining the functional conformation of γc.

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EFFICIENT INTERNALIZATION OF IL-2 DEPENDS UPON THE DISTAL PORTION OF THE CYTOPLASMIC TAIL OF THE IL-2R γ c SUBUNIT AND A LYMPHOID CELL ENVIRONMENT. A.-X. Yu and T. R. Malek. University of Miami School of Medicine, Miami, FL 33101.

An early consequence of IL-2/IL-2R interaction is rapid receptor-mediated endocytosis of the receptor-ligand complex. In the present study, we establish that this rapid endocytosis of IL-2 in a T cell tumor line is dependent upon the cytoplasmic tail of γ c. Deletion mutants of the cytoplasmic tail mapped this activity to the carboxyl 40 amino acids of γc which contain an essential Jak3 binding subdomain. In contrast, ligand-independent constitutive endocytosis of γc occurred more slowly and was dependent upon a more membrane proximal region of the cytoplasmic tail of γc. Thus, this receptor subunit may use distinct sorting signals for its constitutive regulation and ligand endocytosis. Rapid endocytosis of IL-2 was inhibited by the tyrosine kinase inhibitor geneistein, implicating a role for a signal transduction pathway in IL-2 internalization. Inefficeint endocytosis of IL-2 was noted after transfection of the COS7 epithelial cell line with the IL-2R and further reconstitution of these cells with Jak/STAT proteins did not enhance this internalization. Collectively, these latter findings indicate that rapid endocytosis of IL-2 is dependent upon cellular signaling in lymphoid cell environment that is not solely a consequence of the presence of the Jak/STAT pathway.

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INTERLEUKIN-1 GENES AND ARTERY DISEASE. Gordon Duff, Martin Nicklin, Julian Sorrel, Franco diGiovine, Univ of Sheffield; Peter Berger, Mayo Clinic; Katherine Stephenson, Ken Komman, Interleukin Genetics Inc; James Pankow, James Beck, Steve Offenbacher (ARIC), Univ of North Carolina.

Immuno-inflammatory responses play a significant role in coronary artery disease (CAD) with inflammatory cells and mediators characterising every stage of the development and progression of atherosclerosis. Interleukin-1 (IL-1) is one of the main cytokines involved in the regulation of immuno-inflammatory responses and we tested the hypothesis that genetic polymorphism of the IL-1 cluster contributes risk of CAD. We have now completed multiple gene frequency studies involving over 3,000 patients. The results indicate that specific IL-1 gene polymorphisms are significantly associated with cardiovascular disease. Genotypes of the IL-1 cluster were associated with increased risk for coronary artery stenosis, as measured angiographically. In addition these studies revealed a significant interaction of IL-1 cluster genotype with cholesterol, producing an increased risk for CAD. The polymorphisms in the IL-1 gene cluster have been further shown to be associated with increased carotid arterial intimal-medial wall thickness which reflects the development and progression of atherosclerosis and is a predictor of CAD. Disruption of the IL-1 receptor antagonist gene in mice demonstrated a patho-physiological role for IL-1 in the major arteries. These knockouts displayed excess mortality compared with wild-type and had striking mononuclear cell inflammatory infiltrates in mid- and large-sized arteries. Many of the homozygous knockouts died of aneurysms, indicating extensive destruction of extracellular matrix in the arterial wall. The clinical findings in patient populations along with the animal data support a substantial role of the IL-1 gene cluster in arterial disease, including CAD, and IL-1 genotypes may be useful predictors of disease risk in humans.

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NORMAL HOMEOSTASIS, LACK OF AUTOIMMUNITY, BUT SEVERELY IMPAIRED IL-2R FUNCTION BY MATURE T CELLS AFTER THYMIC EXPRESSION OF THE IL-2R β CHAIN IN IL-2R β-DEFICIENT MICE. T. R. Malek, B. O. Porter, E. K. Codias, P. Scibelli, and A.Yu. University of Miami School of Medicine, Miami, FL 33101.

The importance of IL-2R β function for immune regulation is highlighted by the severe impairment in lymphoid cell function in IL-2R β-deficient mice. It has been speculated that failed IL-2/IL-2R signaling in peripheral T cells causes the associated autoimmunity, imbalanced peripheral lymphoid homeostasis, and defective T cell function. This study explored the function of IL-2R β in thymocytes by thymic-specific IL-2R β transgene expression in IL-2R β-deficient mice. We show that enforced thymic expression of IL-2R β prevents lethal autoimmunity, restores normal production of B lymphocytes, and results in a peripheral T cell compartment that is responsive to triggering through the TCR, but not the IL-2R. The dysfunction of the IL-2R is illustrated by the near complete failure of mature T cells to proliferate to IL-2, to differentiate into CTL, and to upregulate IL-2R α expression. These data indicate that lymphoid homeostasis is maintained despite a non-functional IL-2R in mature T lymphocytes and suggest that IL-2R β provides an essential signal during thymic development to regulate self-reactivity.

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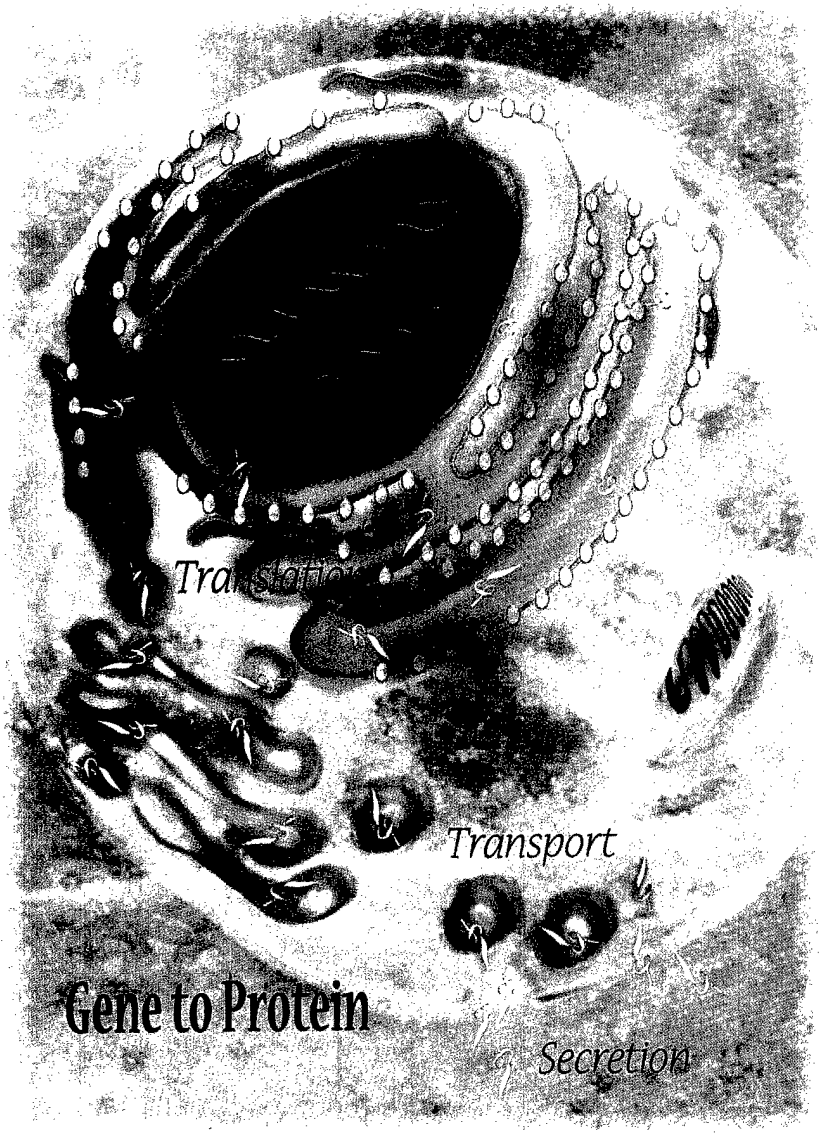

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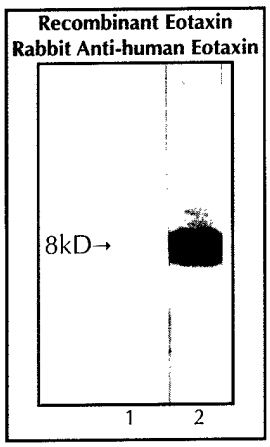

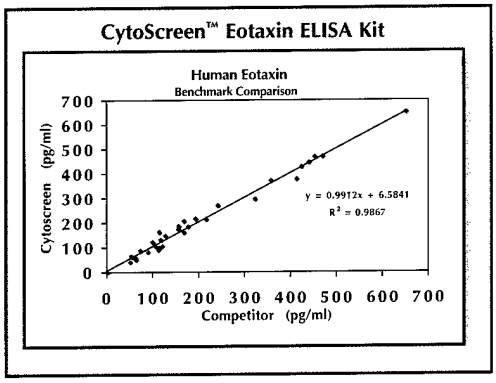

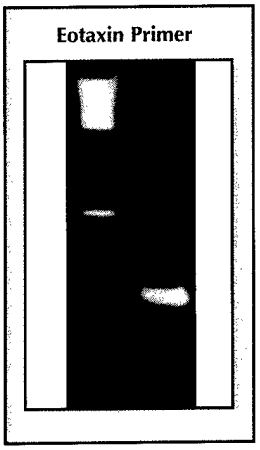

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