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ABSTRACT

"Human Pregnancy-Specific Glycoproteins Function as Immunomodulators In Vitro by Inducing Secretion of IL-10 and IL-6 in Human Monocytes"

Sara Kathleen Snyder, Doctor of Philosophy, 2000

Thesis directed by Gabriela Dveksler, Associate Professor, Molecular and Cellular Biology Program

The lack of rejection of the semiallogeneic fetus by the maternal immune system is brought about in part by the maintenance of an anti-inflammatory immune environment at the maternal-fetal interface. The fetoplacental unit produces an array of cytokines and other regulatory molecules that assist in the implantation, survival and development of the fetus. Pregnancy specific glycoproteins (PSGs) are a family of highly conserved, secreted proteins abundantly produced by the placenta in various species including human, mouse and rat. PSGs are composed of repeated immunoglobulin (Ig) related domains, and are part of the Ig superfamily. Abnormally low levels of PSGs in maternal serum have been correlated with complications of pregnancy including spontaneous abortion. A peptide derived from the N-terminal domain of human PSG11 has been shown to bind cells of the promonocyte lineage, suggesting a role for PSGs in modulation of macrophage function during pregnancy.

We investigated the ability of three recombinant human PSGs (PSG1, PSG6 and PSG11), produced using a baculovirus expression system, to regulate the *in vitro* production of cytokines by human monocytes. Cytokine secretion by monocytes at 24 hours after treatment was measured by quantitative sandwich ELISA. All three PSGs induced dose-dependent secretion of IL-10 and IL-6, but not secretion of TNF-α, IL-1β or IL-12. In order to examine the role of the N-terminal Ig-variable-like domain in PSG function, we produced a fusion protein consisting of only the N-terminal domain of PSG6. The PSG6 N-terminal domain was shown to be sufficient for induction of monocyte secretion of IL-10 and IL-6, demonstrating that this domain mediates the interaction with a putative PSG receptor on monocytes. As shown by RT-PCR, increased IL-10 and IL-6 secretion was accompanied by an increase in mRNA after PSG6 treatment. PSG6 induction of IL-10 and IL-6 secretion was inhibited by the tyrosine kinase inhibitor Herbimycin A, the protein kinase C inhibitor Calphostin C, and the specific protein kinase A inhibitor (Rp)cAMPS, suggesting a possible role for these intracellular signalling molecules in PSG signal transduction in monocytes. Also, the specific phosphodiesterase type IV inhibitor and cAMP elevating agent, rolipram, increased monocyte secretion of IL-10 and IL-6 after treatment with PSG6, indicating that increased production of these cytokines in response to PSGs may be mediated by an increase in cAMP. We also showed that PSGs exhibit cross-species activity in cytokine induction using human PSG treatment of a mouse macrophage cell line, RAW 264.7, and mouse PSG18 N-domain protein treatment of human monocytes, indicating that PSG function may be highly conserved between species. Our results are consistent with a role for PSGs in modulation of macrophage inflammatory responses at the maternal-fetal interface where PSGs are in high concentration.

Human Pregnancy-Specific Glycoproteins Function as Immunomodulators In Vitro by Inducing Secretion of IL-10 and IL-6 in Human Monocytes

By

Sara Kathleen Snyder

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This dissertation is dedicated to my husband, Denny Snyder, for his unending support, encouragement and cheerfulness throughout the years I have been working on this doctoral degree.

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I. Introduction

Pregnancy-specific Glycoproteins are Members of the CEA Gene Family

The pregnancy-specific glycoproteins (PSGs) are a group of highly conserved, secreted proteins produced by the placenta. The PSGs form a large family of closely related proteins, which comprises one subgroup of the carcinoembryonic antigen (CEA) family (Wanatabe and Chou, 1988). There are 29 genes in the human CEA gene family, which are found within a 1 Mb region on the long arm of chromosome 19 (Thompson et. al., 1990, Teglund et. al., 1994). Differences in primary sequence divide the CEA gene family into two major subgroups, the CEA subgroup including CEA and its classical crossreacting antigens, and the PSG subgroup. PSGs and CEAs probably evolved through duplication of the same primordial gene (Wanatabe and Chou, 1988). Members of the CEA subgroup are 65-75 % homologous to PSGs at the nucleotide level, and are estimated to have separated from the PSG subgroup 72 million years ago (Streydio et. al., 1990). A third subgroup of the CEA family exists containing six genes (CGM13-18), none of which are known to be active (Teglund et. al., 1994).

Strong similarities between members of the CEA subgroup initially made it difficult to distinguish between these proteins immunologically as members showed high crossreactivity with available antibodies. Molecular cloning and the generation of specific monoclonal antibodies, as well as the use of techniques to identify expression of distinct mRNA species, have allowed identification of individual genes within the subgroup. CEA family members are highly glycosylated, containing up to 40-60% carbohydrate. Proteins of the CEA subgroup are membrane-bound via a C-terminal transmembrane region as integral membrane proteins, or by a glycosylphosphatidyl-inositol anchor (Hefta et. al., 1988), while the PSGs are secreted proteins (Plouzek et. al., 1991, Chen et. al., 1993). CEA is a cell surface glycoprotein that was first identified by Gold and Freedman (1965) as a tumor marker for adenocarcinoma of the colon, but has since been shown to be expressed in much lower amounts in normal adult colon. The presence of CEA in serum has been used diagnostically to monitor progression of patients with colon cancer (Shively and Beatty, 1985). CEA transcripts have not been found in placenta (Thompson et. al., 1990). CEA family members that crossreact with antisera to CEA include nonspecific crossreacting antigen (NCA) found largely in granulocytes and macrophage/monocytes (Burtin et. al., 1975, Bordes et. al., 1975), the biliary glycoproteins (BGPs) (Svenburg, 1976), and CGM1, 2 (Thompson et. al., 1989), CGM6 (Arakawa et. al.,

1990), and CGM7 (Thompson et. al., 1991). CEA is presumed to have evolved by gene duplication from the simpler NCA gene. Since NCA is found in lower mammals and CEA is not, it is hypothesized that the duplication event occurred relatively recently (Neumaier et. al., 1988).

The CEA Gene Family is within the Ig Superfamily

The CEA family is part of the immunoglobulin (Ig) gene superfamily. Human CEA family members are composed of one Ig variable-like domain (IgV) and a varying number of constant-like domains (IgC). Amino acid analysis of CEA family members shows that they have domains containing cysteine residues and other invariant amino acids (9 of 13) of Ig superfamily members (Williams, 1987, Paxton et. al., 1987). A disulfide bond within the IgC-like domain connects two predicted anti-parallel β sheets, and N-linked carbohydrate is located on the surface of the molecule, as in immunoglobulins. The finding of the presence of β strands of an Ig folding pattern held together by disulfide bonds explained the fact that most epitopes recognized by antibodies to CEA are conformationally dependent and lost upon reduction of cystine. The Ig sandwich domains of CEA family members are bounded by proline-rich sequences, which disrupt ordered folding structures, and therefore delineate boundaries between Ig-like domains. The N-terminal domains of CEA family members have been shown to have close structural similarity to the variable domains of Igs (Williams, 1987) including the extra β strands, C' and C'', which form an additional loop in one β sheet, a hypervariable region within the C'/C'' connecting loop, and arginine and aspartic acid residues which form a salt bridge. Although the N-terminal domains do not contain cysteine residues like the IgV domain, the putative salt bridge is thought to stabilize the Ig fold of the N-terminal domain of CEA family members (Thompson et. al., 1989).

PSGs in Pregnancy

PSGs, also known as Schwangerschafts protein (SP-1), and pregnancy-specific β1-glycoprotein (PSβG), are a group of major proteins produced by the human placenta (Bohn and Sedlacek, 1975). At term PSGs are the most abundant placental protein in maternal serum, at twenty times the amount of human chorionic gonadotropin (Sorensen, 1984). PSGs are detectable in maternal blood at the time of blastocyst implantation (Gordon et. al., 1977, Bischof 1984, Rosen, 1986), are increasingly produced during gestation (Wurz et. al., 1981, Towler et. al., 1976), and can reach serum concentrations of 200-400 μg/ml at term (Lin et. al., 1974, Wurz et. al., 1981, MacDonald et. al., 1983, Silver et. al., 1993). PSGs are also detected

in the urine of pregnant women (Engvall et. al., 1982). Experiments involving explants of placental tissue showed that PSGs make up 3-5 % of total protein produced by placenta (Actis et. al., 1981). PSGs are also produced at lower levels by the human amnion and chorion (Plouzek et. al., 1993), and are present in amniotic fluid (Koh and Cauchi, 1981, Bischof 1984). After birth, PSGs disappear from the circulation with a relatively long half-life of 17-45 hours (Towler et. al., 1976, Bischof, 1984). The large amounts of PSGs produced by the placenta suggest an important role for these proteins in pregnancy, but no definitive PSG function has yet been shown.

Low serum levels of PSGs are associated with pregnancy complications, such as spontaneous abortion (Masson et. al., 1983, Hertz and Schultz-Larsen, 1983), intrauterine growth retardation (Gordon et. al., 1977, Tamsen et. al., 1983), pre-eclampsia (Grudzinskas et. al., 1983), and fetal hypoxia (MacDonald et. al., 1983). In addition levels of PSGs in serum are significantly correlated with birth weight (Gordon et. al., 1977, Ismail and Fahmy, 1992). In further support for the idea that PSGs are important in maintaining pregnancy, it has been shown that administration of antibodies against PSGs in monkeys (Bohn and Weiman, 1974) and mice (Hau et. al., 1985) induces abortion, and immunization of primates with PSG protein reduces fertility (Bohn and Weinmann, 1976). One hundred percent fetal resorption occurred when pregnant outbred mice were injected with rabbit anti-mouse PSG antiserum in one uterine horn one day after onset of implantation, compared to a maximum of 46 % resorption when mice were injected with nonspecific or pre-absorbed antisera (Hau et. al., 1985). (Fetuses in the right uterine horn were unaffected by the treatment.) Elevated levels of PSGs are found in most patients with choriocarcinoma (Tatarinov et. al., 1976, Tatarinov, 1978), hydatidiform mole (Bocco et. al., 1989), and invasive mole (Leslie et. al., 1990). Hydatidiform molar tissues preferentially express PSG6 transcripts (Leslie et. al., 1990). PSG levels have been used as a marker for diagnosis and post-treatment follow-up of these gestational tumors (Sakuragi, 1982). Elevated levels of serum PSGs have also been associated with non-trophoblastic tumors like ovarian cancer (Searle et. al., 1978), tumors of the breast (Horne et. al., 1976b), lung, liver, and uterus, and multiple myeloma (Sorenson, 1984).

Despite their name, PSGs are found in very low concentration (< 0.5 μg/l) in the blood of normal, nonpregnant individuals (Ho et. al., 1988, Wurz, 1979). PSG mRNAs are synthesized in normal extraplacental tissues such as endometrium (Arnold et. al., 1999), fetal liver (Khan et. al., 1989,

Zimmermann et. al., 1989), salivary gland (Zoubir et. al., 1990), testes (Borjigin et. al., 1990), intestine (Shupert and Chan, 1993), myeloid cell lines (Oikawa et. al., 1989a), PMNs, monocytes (Heikinheimo et. al., 1987), B cells and T cells (Wu et. al., 1993). PSG protein synthesis has been demonstrated for testes (Borjigin et. al., 1990), intestine (Shupert and Chan, 1993), and human fibroblast cell lines in vitro (Rosen et. al., 1979, Engvall et. al., 1982).

PSG Protein and Gene Structure

PSGs are classified as a subfamily of the Ig superfamily based on amino acid sequence analysis (Zheng et. al., 1990). PSG proteins consist of an Ig variable-like domain at the N-terminus, followed by several constant-like domains. PSG sequences predict a characteristic domain structure like that of the Igs, including the conserved disulfide bridges (in IgC-like domains) and β-sandwich conformation within Ig domains. Except for the very short C-terminal domain, PSGs display greater than 90 % sequence similarity at the nucleotide level (Streydio et. al., 1990). Amino acid sequences of human PSGs are 85-95% identical (except in the non-Ig-like C-terminal domain), and are believed to be the result of recent gene duplication events involving unequal crossing over (Thompson et. al., 1991). According to sequence analyses, the PSG gene family arose concomitantly with the expansion of placental mammals (Streydio et. al., 1990). The high similarity between PSGs makes it difficult to determine expression patterns immunologically, since crossreactivity between members is high. Therefore detection of mRNA species with specific RNA or DNA oligonucleotide probes, or RT-PCR using gene-specific primers is generally used.

There are 11 PSG genes in humans (Khan et. al., 1992). These genes have been cloned and mapped to a tandem array on the 19q13.2 region of chromosome 19 within the CEA gene family region that spans 1.2 Mb (Thompson et. al., 1990, Thompson et. al., 1991), interspersed with members of the CEA subgroup genes (Teglund et. al., 1994). The human PSG genes are designated PSG1-8 and 11-13 (Teglund et. al., 1994). PSG9 and PSG10 cDNAs have been shown to be allelic variants of PSG4 and PSG6, respectively (Teglund et. al., 1994). Allelic variants, some of which are pseudogenes, have been identified for all human PSGs except PSG2 (Teglund et. al., 1994).

PSG proteins are hydrophilic and are predicted to have a 34 amino acid leader sequence (which is cleaved upon secretion), a 109-110 amino acid N-terminal Ig variable-like domain, 2-3 Ig constant-like domains of type A (92 or 93 amino acids) or type B (85 amino acids) containing two cysteine residues at

invariable positions, and a variable length, relatively hydrophilic C-terminal domain of 3-21 amino acids (Figure 1). All PSG genes contain L, L/N, A1, B1, A2 and B2 exons. However, the B1 exon is not found in mRNAs, probably due to an aberrant intron 3' splice site (splice acceptor signal) (Oikawa et. al., 1988), or the presence of stop codons within some (PSG4 and PSG5) B1 exons (Thompson et. al., 1990, Teglund et. al., 1994). The first exon (L) encodes the 5' untranslated region (5'UTR) and the first 21 amino acids of the signal peptide. The second exon, L/N, encodes the rest of the leader sequence and the N-domain. "A" and "B" Ig constant-like domains are encoded by individual exons, a characteristic gene structure of Ig superfamily members. Amino acid sequence similarity for the IgC-like domains of PSG genes is 90-95 % for corresponding domains between genes (e.g. between A1 domains) but only 43-46 % between domains within a gene (e.g. A1 and B2). This suggests that an exon encoding an Ig-like domain was duplicated to produce a multi-Ig domain gene prior to duplication of the whole gene unit (Thompson et. al., 1990).

Alternative splicing between Ig-like domain-encoding exons results in several different types of domain structures for PSG proteins. The domain structure of most PSG cDNAs can be described by three types: L-N-A1-A2-B2-C (type I), L-N-A1-B2-C (type IIa), and L-N-A2-B2-C (type IIb). However, fetal liver has been shown to contain PSG transcripts of the rare domain structures, L-N-B2-C (type III) and L-A1-B2-C (type IV), (Teglund et. al., 1995). It is notable that although PSGs may lack one or more IgC-like domains, all PSGs identified in adult tissues contain an N-terminal IgV-like domain that is thought to be critical to PSG function. Alternative splicing near the 3' end of the PSG genes generates multiple transcripts containing C-terminal domains of varying length. Most C domains are short and contain 3-21 amino acids, but the product of one alternatively spliced transcript, PSG11w, has a unique 81 amino acid hydrophobic C-domain (Zheng et. al., 1990). Due to the C-terminal sequence, this protein is retained within the endoplasmic reticulum and degraded (Chen et. al., 1993). The C-terminal domain of most other PSGs is not hydrophobic, and they are known to be secreted proteins (Plouzek et. al., 1991, Chen et. al., 1993), which is consistent with the finding of PSG protein at high levels in maternal serum. PSG genes can be divided into three subclasses based on the organization of the C-terminal exons (Thompson et. al., 1991). Subclass 1 genes (PSG1, 4, 7, 8) contain four alternative C-terminal domains, Ca, Cb, Cc and Cd, encoded by separate exons which include the 3' UTR (Lei et. al., 1992). Subclass 2 genes (PSG2, 3, and 5) express a Cm/n domain and partial 3' UTR encoded by the Cm/n exon, and contain an additional exon

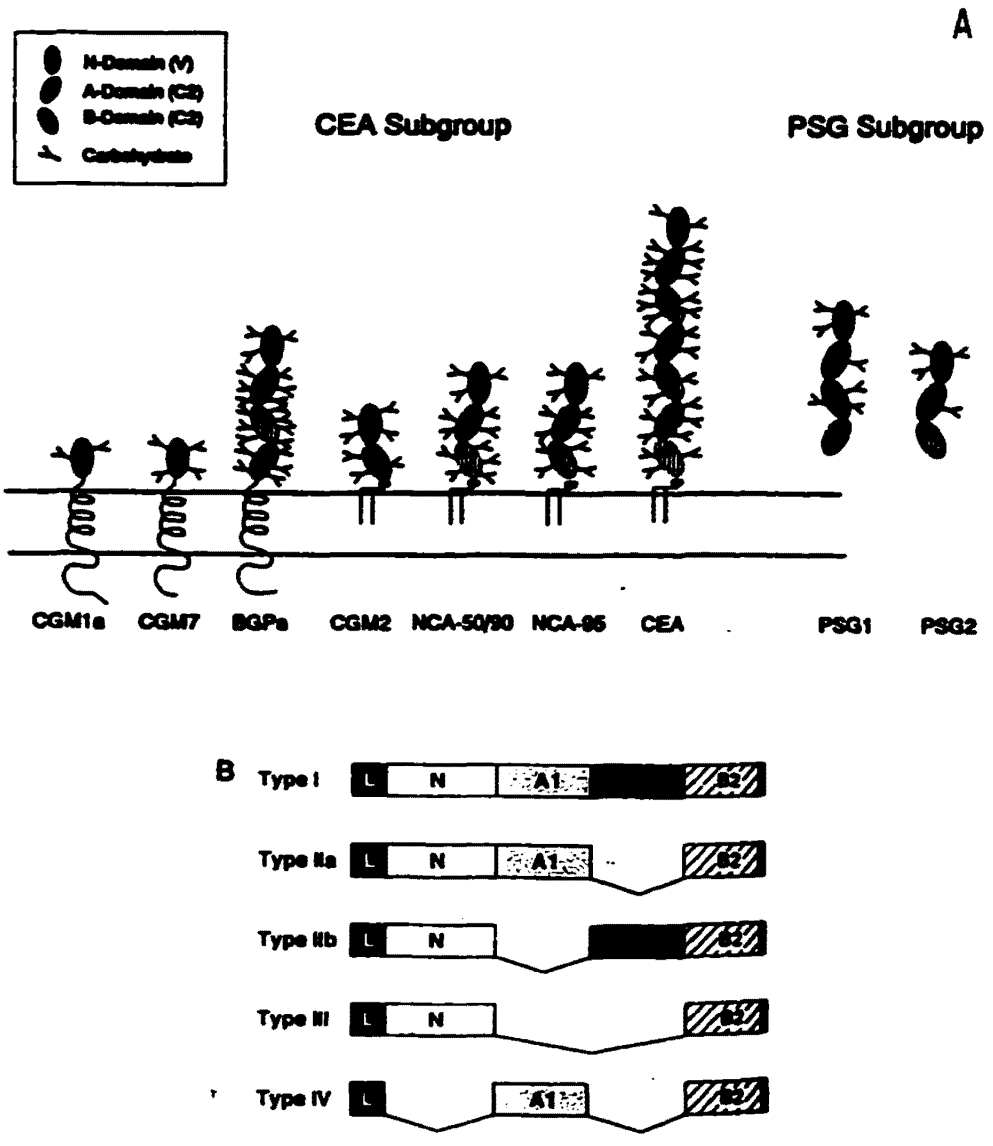

Figure 1. Diagrammatic representation of the CEA family proteins and domain arrangements of PSG cDNAs. **(A)** CEA family protein structure. Immunoglobulin domains are shown as ovals. The N-terminal Ig variable-like domains are black and the Ig constant-like domains A and B are striped. CEA subgroup members are membrane-bound, and PSG subgroup members are secreted proteins. (Taken from Thompson, 1995, p12.) **(B)** PSG cDNA domain structure. L indicates the PSG leader sequence, N is the N-terminal Ig variable-like domain, A_1, A_2, and B_2 indicate the Ig constant-like domains. (From Teglund et. al., 1995, p 662.)

encoding the remainder of the 3' UTR (Thompson et. al., 1990). Subclass 3 genes (PSG6, 11, and 12) can express three different C-terminal domains encoded by Cw, Cr and Cs exons which include the 3'UTR (Zimmermann et. al., 1989, Joe et. al., 1994). Seven human PSGs (PSG2, 3, 5, 6, 7, 11, and 13) contain an Arg-Gly-Asp (RGD) sequence motif located at a conserved position within the N-terminal IgV domain. This motif in several extracellular matrix proteins has been shown to bind to integrin cell surface receptors.

PSGs produced by the placenta vary in size. Major species of 110, 68-94, and 42 kD have been identified by gel filtration, SDS-PAGE, and ultracentrifugation (Engvall, 1980, Osborne et. al., 1982). Higher molecular weight species have been reported and may represent PSG oligomers. PSGs are thought to be single polypeptide chains because the relative molecular weight is unaffected by mercaptoethanol (Engvall, 1980); however, aggregates have been found in the absence of denaturing agents (Osborne et. al., 1982), suggesting a possible non-covalent association between PSGs. Small PSG proteins of 35 and 32 kD have also been identified. Most known human PSG cDNAs encode proteins with the domain structure L-N-A1-A2-B2-C, which has a predicted unglycosylated molecular mass of approximately 45 kD. In fact the majority of PSGs range in size between 324 and 435 amino acids with estimated unglycosylated molecular weights of 37-49 kD (Chan, 1991). The potential for alternative splicing between domain-encoding exons, resulting in PSG species lacking in one or more of the IgC-like domains, could account for the lower molecular weight species. In addition, variation in extent of glycosylation could generate variability in estimated PSG size. Glycosylated proteins are known to behave anomalously in gel electrophoresis, generating unpredictable relative molecular weights. PSGs, like the CEA subgroup proteins, are highly glycosylated with a carbohydrate content of about 30% (Bohn et. al., 1972, Osborne et. al., 1982), due to the presence of 4-8 potential N-linked glycosylation sites of the type Asn-X-Thr/Ser at conserved positions. That the carbohydrate of PSGs is probably all N-linked is shown by the fact that only N-acetylglucosamine and no galactosamine has been found in these proteins (Engvall, 1980, Osborne et. al., 1982). Wanatabe and Chou (1988) identified a major placental PSG band at 72 kD by SDS-PAGE, and minor bands at 64 kD and 54 kD. Using in vitro translation of poly(A) RNA from human placenta, these researchers showed that three major unglycosylated PSG proteins of 50, 48, and 36 kD were synthesized, confirming the heterogeneity of PSG polypeptides.

The isoelectric point of PSGs ranges from 2.5 to 4.5 (Lin et. al., 1974), indicating that PSGs have a net negative charge at physiological pH.

Placental Expression of PSGs

PSGs were first identified as pregnancy-specific serum proteins with a β electrophoretic mobility (Tatarinov and Masyukevich, 1970), and were found in human placenta by Bohn (Bohn, 1971). Human PSGs are synthesized by the syncytiotrophoblast of the placenta, as shown by in situ hybridization (Lei et. al., 1992) and monoclonal antibody analysis (Zhou et. al., 1997), and are found on its surface (Horne et. al., 1976a, Tatarinov et. al., 1976, Lin and Halpert, 1976). Highly specific monoclonal antibodies have demonstrated the presence of PSG protein in the three main cellular compartments involved in biosynthesis of secreted proteins, i.e. rough endoplasmic reticulum, Golgi complex, and secretory vesicles (Zhou et. al., 1999). Due to high crossreactivity between human PSG proteins, it has been difficult to determine the placental expression patterns of different PSG protein species during pregnancy. However mRNA analyses have shown that human PSGs are co-expressed in the placenta (Streydio and Vassart, 1990, Thompson et. al., 1990, Wu et. al., 1993), although at different levels (Wu et. al., 1993). PSG1-6, gene products predominate in human placenta (Streydio and Vassart, 1990, Pan et. al., 1994), and PSG6 is also preferentially expressed by hydatiform mole (Leslie et. al., 1990). The differential expression of PSGs may be attributable to observed differences in promoter sequences (Chamberlain et. al., 1994). Very little information regarding promoter structure and regulation of PSG transcription and translation is currently available. PSG genes fall into two classes based on the amount of minimal promoter sequence (Chamberlain et. al., 1994). The class I genes (PSG1, PSG3 and PSG12) contain an imperfect binding site for Sp1, and class II genes (PSG5, PSG6 and PSG11) have a smaller minimal promoter and contain a perfect Sp1 recognition site which is necessary for promoter activity (Chamberlain et. al., 1994). The transcription factors responsible for human PSG expression by trophoblast have not been specifically identified. Proteins of 78 kD and 53 kD from human term placental extracts were shown to bind to a critical 27 base pair motif in the promoter of PSG5 near the 5' cap site, and mutation of this sequence abolished PSG5 expression in transient transfection experiments using HeLa and JEG-3 cells (Koritschoner et. al., 1996). Secretion of PSG protein has been reported to be increased in placental extracts by angiotensin II (Kalenga et. al., 1994). Two transcriptional activation elements designated PISI and PISII

have been identified within the promoter of a rat PSG gene, rnCGM3, (Chen et. al., 1994), and the transcription factor C/EBPβ, which is plentiful in placental extracts, has been shown to transactivate rnCGM3 in vitro through binding to the PISII site (Chen et. al., 1995a).

Function of PSGs

PSGs were discovered in the early 1970s, but to date little is known about their function. Some early experiments utilizing complex PSG mixtures obtained from serum suggested a role in immuno-regulation. Preincubation with PSGs inhibits phytohemagglutinin-stimulated proliferation of human lymphocytes (Majumdar et. al., 1982), and PSGs suppress stimulated lymphocytes in mixed lymphocyte culture (Johannson et. al., 1976, Harris et. al., 1984). The time and location of a gene's expression can provide clues to its biological role. Human granulocytes and adherent (differentiated) monocytes show cell surface and cytoplasmic immunofluorescent staining for PSGs, as does the promyelocytic cell line HL-60 after induced differentiation, while lymphoblastoid and erythroleukemic cell lines do not (Heikinheimo et. al., 1987). The high levels of PSG transcripts in fetal liver, the site of hematopoiesis, and their presence in freshly isolated blood cells (Wu et. al., 1993), suggests an effect on hematopoietic cells. Recently it was reported that treatment with human PSGs enhanced platelet and white blood cell recovery after bone marrow transplant into gamma-irradiated mice (Blomberg et. al., 1998). A peptide sequence of PSG11, which includes a Arg-Gly-Asp (RGD) motif, has been shown to mediate binding to cells of the promonocytic lineage, but not to T or B cell lines (Rutherford, et. al., 1995). Moreover, it was reported recently that although mRNA for PSG1-6 and PSG11 is normally expressed in human endometrium, the expression of PSG11 was significantly lower in women who suffered from recurrent spontaneous abortion (RSA) (Arnold et. al., 1999). Immune system dysfunction is believed to be a major cause of RSA in humans and mice. In the same study it was demonstrated that PSG11, but not PSG1, enhanced the secretion of the anti-inflammatory cytokine IL-10 by LPS-activated monocytes and induced IL-10 protein in two monocytic cell lines. These investigators did not see any effect of purified recombinant PSGs on the mixed lymphocyte reaction or mitogen-stimulated T cell proliferation, in contrast to results from earlier studies. It was concluded that PSGs may act to downregulate an inflammatory immune response via induction of IL-10, and that low production of PSG11 in particular may increase susceptibility to a harmful inflammatory immune response to the fetus resulting in abortion. Taken together these findings imply a

possible immune system function for PSGs. The presence of PSGs in intestine, fetal liver and blood, in conjunction with the large number of PSG genes and alternatively spliced forms of mRNA, has led to the speculation that at least some PSGs have cellular growth promoting activity, while others may act as immunosuppressants in testes and placenta (Wu et. al., 1993, Shupert and Chan, 1993).

The location of the tripeptide RGD sequence in a conserved and surface-exposed position in the N-terminus (amino acids 93-95) of most human PSGs (PSGs 2, 3, 5, 6, 7, 11 and 13) has also suggested a possible role for PSGs in integrin binding. An RGD motif is part of the cell-binding domain of many extracellular matrix proteins (Ruoslahti and Pierschbacher, 1987) and snake venom disintegrins (Blobel and White, 1992). This motif is present in fibronectin, collagen and vitronectin, and other adhesive proteins, and it is recognized by several integrins ($\alpha_5\beta_1$, $\alpha_3\beta_1$, $\alpha_{IIb}\beta_3$, and $\alpha_v\beta_3$) that are the main receptors by which cells attach to the extracellular matrix. It has been proposed that interaction of PSGs with integrin receptors may disrupt cell-cell matrix interactions within the basal lamina of the uterine decidua and thus contribute to trophoblast invasion within the uterus (Rebstock et. al., 1993). Alternatively PSGs could promote trophoblast invasion by blocking adherence of large granular lymphocytes (LGL) via integrin receptor binding in the uterus, thus preventing LGL inhibition of trophoblast invasion (Rebstock et. al., 1993). The high production of PSGs in choriocarcinoma and invasive mole is consistent with a role for PSGs in invasion of the uterine wall.

The N terminal IgV-like domain is implicated in PSG function. Amino acid sequence variation between PSG family members has been reported to be clustered in the N-domain, especially in or near regions analogous to the hypervariable complementarity determining regions (CDRs) of the Igs (Khan et. al., 1982, McLenachen et. al., 1996). The nonrandom accumulation of nonconservative amino acid changes at these sites in the N-domain argues for a role in PSG biological activity, and may indicate that PSGs bind different receptors or have different effects on the same receptor. In addition, the RGD motif of PSGs lies on a solvent-exposed loop within the N-domain variable region, according to a structural model of PSGs based on the tertiary structure of other Ig family members (McLenachan et. al., 1996). Moreover, all adult PSG proteins contain an N-terminal IgV-like domain, although the other domains may be absent due to alternative splicing. These observations suggest that the N-domain (and perhaps the RGD motif) is important in PSG protein function. The involvement of the N-domain RGD motif of PSG11 in binding to a

putative PSG receptor on monocyte cell lines, reported by Rutherford, also supports this notion. Recently it was shown that a truncated form of the mouse PSG18 protein, consisting of only the N-terminal domain, induced expression of IL-10 and IL-6 in mouse macrophages and the RAW 264.7 macrophage cell line (Wessells et. al., submitted).

In general, members of the Ig superfamily function in intercellular recognition involving homophilic or heterophilic binding at the cell surface. The Ig domain provides for presentation of unique sequence determinants at the bends of loops or on the faces of the β sheets (Williams, 1987). In addition the protease resistance of the Ig domain is important in the extracellular environment. For example, the cell adhesion molecule N-CAM (neural cell adhesion molecule) contains an RGD motif, is a member of the Ig superfamily, and acts in cell surface recognition. In vitro studies involving cells transfected to express CEA proteins indicate that CEA (Benchimol et. al., 1989), NCA (Oikawa et. al., 1989b, Oikawa et. al., 1991), and BGPs (Rojas et. al., 1990) can mediate homophilic or heterophilic cell-cell binding interactions involving the N-terminal domain (Oikawa et. al., 1991). (CEA and NCA, which are highly glycosylated, also bind lectins on some strains of bacteria [Leusch et. al., 1990, 1991]). Since PSGs are secreted proteins, a cell-cell adhesion function is not postulated, but a cell binding function seems probable.

Murine PSGs

Expression of PSGs has been observed in species other than humans, including various primates (Bischof, 1984), rat (Tatarinov et. al., 1986, Chan et. al., 1988) and mouse (Rudert et. al., 1992). As in humans, mouse PSGs form a subgroup of the CEA gene family, and high (55-60 %) sequence similarity is seen between the CEA-like and the PSG subgroups (Thompson et. al., 1991). Genes encoding the murine PSG family are located on the centromere-proximal region of chromosome 7 (Rudert et. al., 1992, Zimmermann et. al., unpublished), which is syntenic to the region of human chromosome 19 containing the human CEA gene family (Stubbs et. al., 1996). CEA gene families in human and mouse apparently underwent a parallel but independent evolution. In contrast to the human genes, which contain only one Ig variable-like domain, mouse (Rudert et. al., 1992) and rat (Rebstock et. al., 1990) PSGs contain 2-3 variable-like domains, designated N1, N2 and N3, and only one constant-like domain. Murine PSGs have no counterpart to the short but highly variable C-terminal domain of human PSGs. Rudert and coworkers first showed that mouse PSGs demonstrate similar exon organization to human PSGs, wherein individual

exons encode separate Ig-like domains (Rudert et. al., 1992). In the same study they also identified a semi-conserved sequence motif in the N1 domain, consisting of a positively charged amino acid (Arg or His), followed by a glycine, and a positively or negatively charged amino acid (Lys or Glu). This motif corresponds in location to the RGD or RGD-like motifs found in the N-terminal domains of human PSGs, and its presence is indicative of a common function for this domain for both murine and human PSGs.

The murine PSG family consists of at least 15 genes, PSGs 16-29 and CEA6 (Chen et. al., 1995, Zimmermann et. al., unpublished). Like human PSGs, rodent PSGs are expressed by the placenta, and genes exhibit differential splicing (Kromer et. al., 1996). The concentration of mouse PSG protein in pregnant sera has not been reported. Mouse PSG transcripts have been localized in secondary trophoblast giant cells and in the spongiotrophoblast of the placenta by in situ hybridization (Kromer et. al., 1996). The trophoblast giant cell layer corresponds to human cytotrophoblast, and this layer forms the interface between maternal and fetal tissue in rodents. PSG transcripts have been detected in mouse placenta as early as day 6.5 of gestation, or two days after implantation (Wessells et. al, submitted). Kromer and colleagues (1996) reported that PSG17-19 are coordinately expressed from day 12.5 to day 18.5. Zimmermann has analyzed expression of PSG16-29 by RT-PCR and found increasing expression by placenta from gestational day 15.5 to 17.5 for all PSGs examined except PSG28 (unpublished data). (PSG28 may have been inefficiently amplified by the degenerate oligonucleotide primers used for PCR in this study.) Although coordinately expressed, murine PSG mRNAs were shown to be produced at varying levels by Zimmermann. In contrast to human PSGs, mouse PSG transcripts (except for PSG16) have not been detected in extraplacental tissues. Murine PSGs 17-19 are not expressed in colon, uterus, ovary, liver, kidney (Rudert et. al., 1992), brain, thymus, heart, spleen, lung, or testicle (Kromer et. al., 1996).

Until recently only five murine PSG genes had been identified, including PSG16/bCEA (Chen et. al., 1995), PSG 17-19 and CEA6 (Rudert et. al., 1992, Kromer et. al., 1996). CEA6 has a single base pair deletion within the leader peptide sequence in five mouse strains examined and is considered to be a pseudogene (Rudert et. al., 1992, Zimmermann et. al., unpublished). PSG16/bCEA is expressed predominantly in the brain, but also weakly in placenta, and in brain acts as a receptor for mouse hepatitis virus (Chen et. al., 1995b). There is very little information concerning murine PSG family proteins in comparison to human PSGs. At present cDNA sequences for only CEA6 (Rudert et. al., 1992), PSG16

(Chen et. al., 1995), PSG17, PSG19 (Rudert et. al., 1992, Kromer et. al., 1996), and PSG18 (Wessells et. al., unpublished) are available. For the remaining murine PSGs only partial genomic sequences have been identified. Exons encoding the L and N1 domain of PSG20 and PSG21, and exons encoding part of the L and the complete N1 domains for PSG22-29 have been sequenced (Zimmermann et. al., unpublished). The N1 domains contain between 104 and 108 amino acids and share between 54 % and 94 % amino acid sequence homology. The cloning and characterization of cDNAs for the remaining mouse PSG genes is needed to develop a mouse model of PSG function that will allow in vivo experimentation with this important gene family.

Structure and Function of the Placenta

The survival and proper development of the human embryo and fetus depends upon the function of the placenta. The placenta is an endocrine organ that produces hormones and other molecules essential for maintenance of pregnancy, and provides for transfer of nutrients (water, oxygen, carbohydrates, amino acids and lipids) and wastes (carbon dioxide, urea, uric acid and bilirubin) between the maternal and fetal blood. In addition to PSGs, the placenta secretes human chorionic gonadotropin (hCG), placental lactogen (PL), human chorionic corticotropin, progesterone, estrogen, placental alkaline phosphatase (PLAP), insulin-like growth factors, prolactin, relaxin, endothelin, prostaglandins, and cytokines. Hormone secretion by the placenta modifies the maternal metabolism and uterine environment to benefit fetal survival and growth. The early trophoblast secretes hCG which supports the activity of the corpus luteum (the remains of a ruptured follicle), that would otherwise spontaneously degenerate 14 days after ovulation. Thus hCG continues progesterone production by the ovary until the placenta secretes enough progesterone to maintain the endometrium. The main function of PL is as an insulin antagonist, causing mobilization of maternal fatty acids that aid in energy production and maintenance of high glucose levels in maternal blood for fetal consumption. Another function of placenta is to protect the fetus from infection. Placental cells called trophoblasts have many properties and functions in common with macrophages, including formation of syncytia, production of cytokines, and phagocytosis, which aid in fetal immune defense.

The human placenta is a disk or circular plate 15-20 cm in diameter and 2-3 cm thick that weighs 500-600 g at term. The placenta is a unique organ in that it is comprised of cells from two different individuals. The maternal portion is the decidual basalis, and the fetal portion is the chorionic plate,

chorionic villi and the cytotrophoblast shell of the basal plate (Figure 2). The implantation of the blastocyst into the uterine wall results in a change in the adjacent endometrial stroma called the "decidual reaction". The stromal cells differentiate into secretory cells that accumulate lipid and glycogen. Some decidual cells rupture and thus provide nutrients for the early stage embryo, which obtains nutrients and eliminates wastes by diffusion until the uteroplacental circulation is in place. The human placenta is "hemochorial" in that maternal blood directly bathes the outermost trophoblast layer. In hemochorial placentation the barrier between maternal and fetal blood consists only of fetal trophoblast, connective tissue, and endothelium.

Mouse and rat placentation is similar to human placentation in that the placenta is discoid and hemochorial. The mouse placenta can be divided into four zones. The first zone, which is farthest from maternal tissue, is the chorionic plate. The second zone is the "labyrinth", containing the chorionic villi and maternal blood sinuses, and lined by labyrinthine trophoblast. A third zone consists of "spongiotrophoblast" in a compact cellular tissue called the spongy zone or the junctional zone. The fourth zone opposes the maternal decidua and consists of trophoblast cells called giant cells, which are multinucleated and secrete steroid hormones. This mouse trophoblast layer corresponds to the human cytotrophoblast of the basal plate. Mouse placental invasion into the uterus is shallower than human placental invasion.

Implantation and Development of Hemochorial Placentation

The trophoblast is formed at the 8-16 cell stage by differentiation of embryonic cells to form an outer cell mass (trophoblast) and an inner cell mass (embryoblast). Implantation of the blastocyst into the endometrial lining of the uterine wall begins at day 6 or 7 after fertilization and is complete by day 9. The trophoblast penetrates through the basement membrane of the uterine epithelia, invades the endometrial stroma, and multiplies and differentiates into two layers, the outer multinucleated syncytiotrophoblast and the inner, mitotically active mononuclear cytotrophoblast. The external mitotically inactive syncytiotrophoblast layer arises from the fusion of cytotrophoblast cells. The syncytiotrophoblast secretes hydrolytic enzymes that degrade the endometrial ECM, allowing cellular processes from syncytiotrophoblast to penetrate between endometrial cells. Eventually the lytic activity of the trophoblast results in rupture of maternal arterial blood vessels. Blood from the maternal vessels (spiral arteries) fills spaces that have formed in the syncytiotrophoblast layer called trophoblastic lacunae. On days 11-13

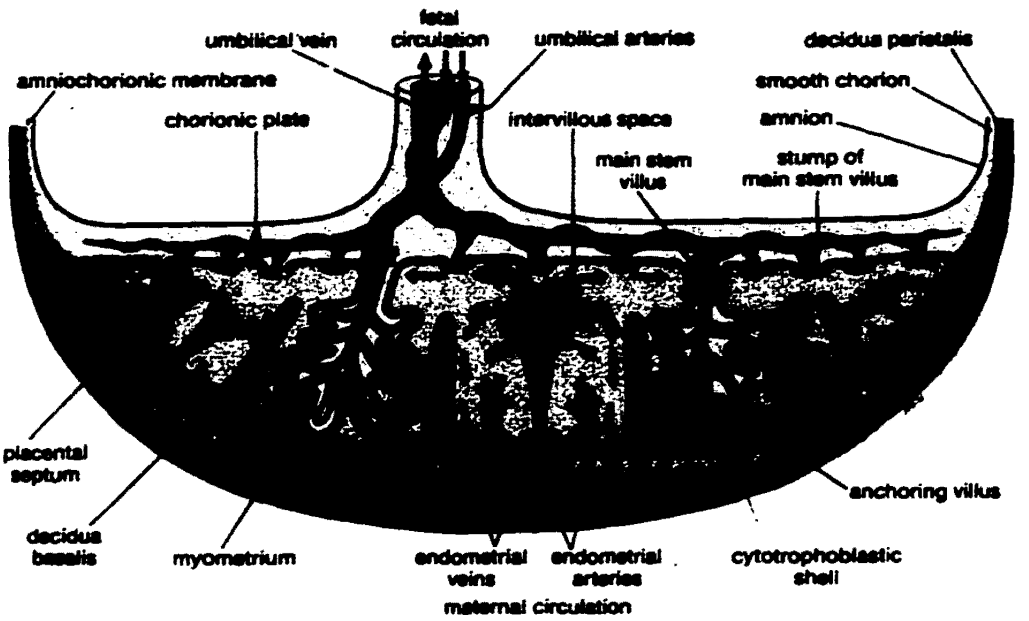

Figure 2. Schematic diagram of the human placenta. The decidua basalis and the cytotrophoblast shell form the basal plate of the placenta. The fetal portion of the placenta consists of the chorionic plate, chorionic villi and the cytotrophoblast of the basal plate. The maternal portion of the placenta consists of the decidua basalis (decidualized uterine endometrium). The chorionic villi containing fetal blood vessels extend from the chorionic plate into the intervillous space, which is filled with maternal blood. The syncytiotrophoblast layer covering the chorionic villi and the cytotrophoblast shell of the basal plate, and in direct contact with maternal blood in the intervillous space, is shown as a continuous black line. Fetal arteries are shown in blue and fetal veins are red. (Taken from Moore, 1989, p92.)

The cytotrophoblast forms extensions that grow into and push the overlying syncytiotrophoblast deeper into the lacunae. These structures, which penetrate into the blood-filled lacunae, are called primary stem villi. Lacunae will later coalesce to form the intervillus space. Secondary stem villi are formed when fetal extraembryonic mesoderm enters the core of the primary stem villus. The villus mesoderm eventually forms fetal blood vessels, thus producing the tertiary stem villi. The villus blood vessels connect with embryonic vessels in the chorionic plate to create the uteroplacental circulation. Deficiency in the uteroplacental circulation results in fetal hypoxia and growth retardation.

Syncytiotrophoblast cells are responsible for the initial invasion of the endometrium, while deeper invasion is accomplished by the cytotrophoblast. Cytotrophoblast cells proliferate and extend through the syncytiotrophoblast layer of villi in some locations to form a complete shell of cytotrophoblast that attaches the placenta to the uterine decidua. This cytotrophoblast layer forms part of the basal plate and is in direct contact with maternal uterine tissue. Cytotrophoblast cells also colonize the decidua and myometrium and are called interstitial extravillous cytrophoblast. Cytotrophoblast enters the lumen of maternal spiral arteries, replaces the vessel endothelium, and destroys the medial elastic and muscular tissue. The result is that the thick walled vessels are converted into flaccid, low resistance, sinusoidal vessels that can accommodate the increasing blood flow needed to support the growing fetus. Shallow cytotrophoblast invasion and defective cytotrophoblast modification of maternal arteries is associated with pregnancy pathologies such as preeclampsia and intrauterine growth retardation.

Syncytiotrophoblast

There are no plasma membrane cell boundaries within the syncytiotrophoblast, such that it forms an uninterrupted cytoplasmic layer that covers the entire surface of the villus tree, and lines the intervillus space. Thus the syncytiotrophoblast is in direct contact with maternal blood. The single plasma membrane and the presence of microvilli on its surface facilitate the transfer of substances across the syncytium, whose primary function is nutrient, waste and gas exchange between the maternal and fetal circulations. The syncytiotrophoblast contains a large amount of dilated rough endoplasmic reticulum (rer), prominent Golgi apparatus, and many pinocytotic vesicles, granules and lipid droplets. In contrast, the cytotrophoblast contains significantly fewer organelles, rer, and secretory and pinocytotic vesicles. In tissue culture,

cytotrophoblast differentiates into syncytiotrophoblast in the presence of fetal bovine serum (Kao et. al., 1988). HCG, PLAP and PSGs are produced primarily by the syncytiotrophoblast.

Pathologies of Pregnancy

In complete hydatidiform molar pregnancy the trophoblast develops into the placental membranes, but the embryoblast either does not develop, or it degenerates quickly, and the fetus is entirely missing. Complete moles have a diploid karyotype but all chromosomes are paternally derived. This results from the fertilization of an oocyte lacking a nucleus. In partial hydatidiform mole some embryonic development occurs, and usually a triploid karyotype is observed. It is thought that the paternal chromosomes are responsible for early development of the placenta, and maternal chromosomes for early development of the embryo, thus explaining the absence of embryonic tissue in complete moles. In invasive hydatidiform mole, trophoblast penetrates deep into the myometrium. Invasive mole becomes manifest clinically when hemorrhaging occurs after removal of the mole from the uterus. Invasive mole can penetrate the uterine wall, but this is not necessarily a neoplastic sign since normal trophoblast can invade through the uterine wall on rare occasions. Choriocarcinoma often follows molar pregnancy and consists of malignant trophoblast, one of the most metastatic tumors known. It is clear that regulation of trophoblast invasion is a critical issue. Trophoblast produces the serine protease urokinase-type plasminogen activator, and matrix metalloproteinases (MMP-1 or interstitial collagenase, MMP-2, and MMP-9 or 92 kD type IV collagenase/gelatinase), which degrade the endometrial ECM (Librach et. al., 1991). Invasiveness of trophoblast decreases during gestation, in part through downregulation of proteinase production (Librach et. al., 1991). In human pregnancy decidual and trophoblast cells secrete transforming growth factor-β (TGF-β) (Graham et. al., 1992), and cytotrophoblast secretes IL-10 (Roth et. al., 1996) into the extracellular matrix. TGF-β functions to control invasion by promoting differentiation of the cytotrophoblast and subsequent upregulation of metalloproteinase inhibitors, while IL-10 downregulates MMP-9 production and trophoblast invasiveness in vitro (Roth and Fisher, 1999). Maternal decidual production of proteinase inhibitors may also control trophoblast invasion. Pregnancy complications such as preeclampsia (Zhou et. al., 1993) and intrauterine growth retardation (Gerretsen et. al., 1981) are associated with shallow trophoblast invasion, which may be partly a result of MMP-9 dysregulation. (Disregulation of

cytotrophoblast adhesion molecule expression affecting trophoblast invasiveness also occurs in preeclampsia.)

The Fetus as an Allograft

The fetus is semi-allogeneic, expressing both maternal and paternal antigens, and as such, is considered an allograft which should be rejected by the mother, as first proposed by Medawar in 1953 (Medawar, 1953). Rejection of allografts is mediated by T cell recognition of non-self peptide-major histocompatibility complexes (MHC) on grafted tissue, and is a cell mediated immune response (CMI). The effector cells of CMI are activated macrophages, inflammatory and cytotoxic T cells and natural killer cells (NKs). Recipient T cells can directly recognize allograft antigens presented on the surface of donor antigen presenting cells (APCs), as well as alloantigens presented on self APCs. Alloreactive T cells include MHC class II-restricted CD4+ helper T cells and MHC class I-restricted CD8+ cytotoxic T cells. T cell activities, including effector cell mechanisms like delayed type hypersensitivity and cytotoxic T cell activity are essential in allograft rejection. Killing by NKs occurs through the lack of expression of host class I MHC molecules by cellular targets. The NK cell expresses inhibitory receptors which recognize host class I MHC antigens (HLA-A, B, and C), and when these receptors are bound they prevent cell killing by activated NK cells. Thus, virally-infected and malignant cells, that have downregulated MHC class I molecule expression in order to escape killing by cytotoxic T cells, are lysed by NK cells. Allogeneic tissues, that express different (non-self) MHC class I molecules, can therefore be targeted by NK cells. NK cells are activated to become lytic/cytotoxic by IFN-α, IFN-β, IL-2 and IL-12.

Explanations for the lack of maternal rejection have included that the fetus is not immunogenic, the maternal immune system is suppressed during pregnancy, the uterus is an immunologically privileged site, and that the placenta forms an anatomical/ immunological barrier between mother and fetus. In fact, the maternal immune system can respond to paternal fetal antigens, indicating that fetal antigens are immunogenic. Pregnancy-induced antibodies against paternally-encoded human leucocyte antigens are found in the sera of multiparous women, and are commonly used as reagents in MHC class I serological tissue typing. (These antibodies are largely generated during feto-maternal hemorrhages at delivery.) Also if the fetus alone is transplanted to another anatomical location in the mother, it is rejected (Woodruff, 1958). That the immune system in pregnant females is not generally suppressed is shown by the fact that

pregnant H-2^k mice transgenic for a TCR recognizing H-2^b reject subcutaneous H-2^b grafts unless pregnant with an H-2^b conceptus (Tafuri et. al., 1995). (This experiment does suggest that maternal T cells may acquire a transient state of tolerance directed toward paternal antigens during pregnancy.) Additionally, pregnant women can mount an immune response to infection during pregnancy. This response is not identical to the immune response in nonpregnant women. A bias toward an humoral immune response (production of antibody) and a shift away from a strong cell-mediated response has been observed by many investigators in both pregnant humans and mice. In fact, a central theory explaining lack of immune rejection of the fetus postulates that cell mediated immunity, and therefore allograft rejection, is downregulated during pregnancy, while humoral immunity is upregulated. There is a growing amount of experimental evidence to support this theory, which is discussed in greater detail below. Paternal or fetal tissue transplants at intrauterine sites in non-pregnant experimental animals are rejected, showing that the uterus is not generally a site of immune privilege (Vince and Johnson, 1996). However, the placenta may act to create immune privilege during pregnancy. Fas ligand is expressed by trophoblast and its expression may result in deletion of active maternal T cells (Runic et. al., 1996), but Fas-negative females have successful allogeneic pregnancies suggesting that this mechanism is not essential for fetal survival (Chaouat and Clark, 1988). The TNF-related apoptosis-inducing ligand TRAIL has also been found in syncytiotrophoblast, but its role in pregnancy is unknown (Phillips et. al., 1999).

The placenta does form a type of immunological barrier between mother and fetus. The stromal mesenchyme of placental villi express conventional class I HLA antigens, and class II HLA is expressed by Hofbauer cells (fetal macrophages) within villi. These antigens are not directly exposed to the mother due to the covering of syncytiotrophoblast on placental villi. Human villus syncytiotrophoblast itself does not express classical class I (Sunderland et. al., 1981) or class II HLA antigens (Hunt et. al., 1988). Extravillous cytotrophoblast, which intermingles with maternal cells at various sites in the uterus and maternal vessels, expresses a nonclassical class I HLA antigen, HLA-G, associated with β-2 microglobulin (Redman et. al., 1984, Ellis et. al., 1989, Kovats et. al., 1990, McMaster et. al., 1995), and HLA-C only during the first trimester (King et. al., 1996). Mouse trophoblast giant cells do not express class I antigens and low expression is seen in labyrinthine trophoblast (Hunt and Orr, 1992). It is unknown whether mice express a class I HLA-G counterpart. The downregulation of classical MHC antigens by trophoblast

prevents presentation of fetal antigens to maternal T cells, thus avoiding allorecognition and cytotoxic T cell-mediated immune rejection. The expression of HLA-G prevents killing of trophoblast by NK cells (but not by lymphokine activated killer cells), which recognize the absence of MHC class I molecules (Pazmany et. al., 1996). However, the trophoblast is not a complete barrier to tissue exchange between fetus and mother since maternal lymphocytes pass into the fetus (Pitrowski and Croy, 1996), and both fetal cells (Herzenberg et. al., 1979, Bonney and Matzinger, 1997) and fetal DNA (Lo et. al., 1997) have been found in the maternal circulation.

That multiple mechanisms exist for control of the immune system during pregnancy, to induce tolerance of the semi-allogeneic fetus, is an accepted principle in the biology of reproduction. It is also acknowledged that in some instances these mechanisms must be faulty and result in immune abortion. Measurements of human chorionic gonadotropin in urine have been used to assess early embryo loss. HCG values above a nontrophoblastic HCG threshold value, (determined via analysis of samples from sterilized women), indicated the presence of early embryos in women, who were subsequently monitored for development of a clinically recognized pregnancy (Edmonds et. al., 1982). Such studies have revealed that an estimated 30%- 60% of human fetuses may be spontaneously aborted, with the majority of these occurring during the first month of pregnancy (Edmonds et. al., 1982, Wilcox et. al., 1988). Because a substantial number of spontaneous abortions occur before a women knows she is pregnant, the cause of pregnancy loss is frequently undetermined or undeterminable. In cases of recurrent spontaneous abortion, defined as three or more sequential pregnancy losses, a large fraction of these abortions cannot be attributed to known causes, such as chromosomal defects, anatomical abnormalites of the uterus, endocrinological insufficiency, or infection (Harger et. al., 1983, Stray-Pederson and Stray-Pederson, 1984, Scott and Branch, 1995). In the 50% of recurrent miscarriages of no recognized cause, an immune-mediated etiology is suspected. Studies indicate that an inflammatory uterine environment increases the risk of spontaneous abortion. The discovery that the placenta and uterine tissues, and not simply leukocytes within these tissues, were an abundant source of bioactive cytokines initiated investigation into their function in pregnancy. It is now believed that placental trophoblast and maternal uterine leukocytes, decidua and epithelia all utilize multiple cytokines to communicate with each other in the effort to prevent harmful immune responses, and to promote and control growth and invasion by the placenta. Cytokines are

especially suited for such a role since they are potent at low concentration, act locally, are pleiotrophic and affect a wide range of cell types. In fact, exogenously administered cytokines have been shown to be capable of both preventing and inducing murine spontaneous abortion. Although an intact adaptive immune response is not necessary for successful pregnancy (mice lacking both T cells and NK cells can reproduce [Croy and Chapeau, 1990]), it is clear that the immune system can both benefit and harm the outcome of pregnancy. The sharing of many intracellular signaling molecules and growth factors and their receptors between the immune and reproductive systems guarantees such interactions.

Th1 and Th2 Helper T Cell Subtypes

Intimate contact between the developing fetus and maternal tissue is necessary for exchange of nutrients and wastes during human fetal development. Although the placenta is in direct contact with maternal tissue and blood, the semi-allogeneic fetus is not rejected by the mother. Studies suggest that an immune response dominated by humoral immunity is beneficial during pregnancy, and that inflammatory or cell-mediated immunity is damaging to the fetus (Wegmann et. al., 1993). These two types of immune response are mediated by different T helper (CD4+) cell subsets. Helper T lymphocytes organize an immune response by promoting intracellular killing by macrophages via release of IFN-γ, clonal expansion of cytotoxic (killer) T lymphocytes via synthesis of IL-2, and antibody production by B cells through IL-4 secretion (Figure 3). Human and mouse helper T lymphocytes can be divided into two major phenotypic subsets based on their lymphokine secretion patterns (Mosmann et. al., 1986, Mosmann et. al, 1997). Th1 "inflammatory" T cells secrete IL-2, INF-γ, and TNF-β (lymphotoxin), and mediate CMI, including delayed-type hypersensitivity and inflammation. This response provides protection against intracellular parasites, protozoa, fungi and bacteria. Th2 "helper" T cells regulate humoral immunity including immediate hypersensitivity (allergy) and B cell activation and antibody production, enhance eosinophil proliferation and function, protect against extracellular pathogens such as bacteria and multicellular parasites, and soluble toxins, and provide B cell help for IgE isotype switching. Th2 helper T cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13.

Development of the appropriate Th subset is important during infection because certain pathogens are most effectively controlled by either predominantly cellular or humoral immune responses. The wrong type of response can result in chronic disease. A Th2 response to the intracellular parasite *Leishmania*

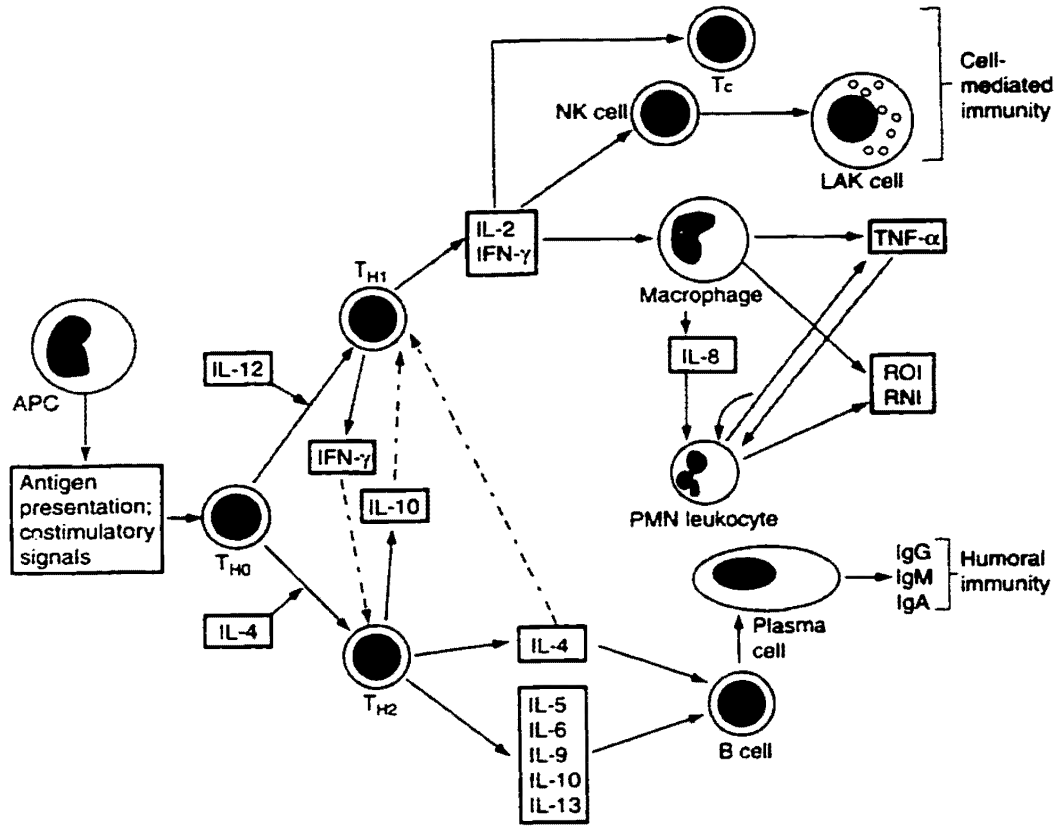

Figure 3. Schematic representation of the roles of cytokines in cell-mediated and humoral immune responses. During T cell activation Th1 cells develop from Th0 precursors in the presence of IL-12, and Th2 cells develop in the presence of IL-4. Th1 cells release IL-2 and IFN-γ to activate effector functions of Tc (cytotoxic T cells), NK (natural killer) cells, and macrophages. Macrophages and polymorphonuclear (PMN) leukocytes are activated to release TNF-α and reactive oxygen (ROI) and nitrogen (RNI) intermediates, which are involved in killing tumor cells and microorganisms. Through release of IL-4, IL-5, IL-6 and IL-10 Th2 type T cells promote differentiation of B cells to antibody-secreting plasma cells. Th1 and Th2 cells suppress each other's function through production of IFN-γ and IL-4/IL-10, respectively. Solid lines indicate activation and broken lines indicate suppression. (From Pugh-Humphreys and Thomson, 1998, p 907.)

major (*L. major*) in the Balb/c mouse strain results in fatal disseminated disease, while the normal Th1 response mounted by other mouse strains controls infection (Scott et. al., 1988). Immune responses polarized toward Th1 or Th2 are mutually exclusive due to cross-regulation by individual Th subset cytokines (Sher and Coffman, 1992, Seder and Paul, 1994). Some Th subset cytokines act as autocrine growth factors and promote differentiation to naïve T cells of the same subset, while inhibiting development and activity of cells of the other Th subset. Therefore, once one helper subset becomes strongly activated, it tends to perpetuate its own existence and dominate the response.

Development of Helper T Subsets

The early cytokine environment during an immune response directs Th subset development (Seder and Paul, 1994). Th1 type T cells develop in the presence of IL-12 and IFN-γ, in the absence of IL-4 (Manetti et. al., 1993). The source of IL-12 early in a response to a pathogen is thought to be cells of the innate immune system, such as activated macrophages and dendritic cells. Cells of the innate immune system interact with pathogens before the development of specific immunity. Microbial components signal the presence of infection by binding receptors on the surface of cells of the innate immune system. These cells have receptors which recognize conserved molecular patterns expressed by microbial pathogens and not by mammalian cells. Molecules, called pattern recognition receptors (PRRs), recognize the lipopolysaccharide (LPS) of Gram-negative bacteria, glycolipids of mycobacteria, lipoteichoic acids of Gram-positive bacteria, mannans of yeasts, and double-stranded RNA of viruses. For example, LPS receptor binding at the surface of macrophage cells induces mediators such as IL-1β, IL-6, IL-12, TNF-α, and costimulatory molecule expression, and results in the induction of the acute phase response, enhancement of microbicidal activity of macrophages, promotion of growth of helper T cells, and activation of T cells via costimulation. Recognition of LPS by immune system cells and subsequent release of TNF and other cytokines by macrophages allows clearance of gram-negative organisms before disseminated infection occurs. Importantly, LPS and microbial lipoproteins stimulate macrophages to secrete IL-12 (Brightbill et. al., 1999, D'Andrea et. al., 1992), which activates both T cells and natural killer (NK) cells to release IFN-γ. The main source of IFN-γ for Th1 cell differentiation is IL-12-activated NK cells (D'Andrea et. al., 1992). Together these two cytokines direct the development of Th1 type T cells. IFN-γ inhibits Th2 subtype development and enhances macrophage antigen presentation, killing of

pathogens, and inflammatory cytokine secretion. It also induces immunoglobulin class switch to anti-bacterial, complement binding isotypes, which promote phagocyte-mediated defense.

Th2 cells develop when IL-4 is present during activation of naïve T cells (Le Gros et. al., 1990). The main source of IL-4 for Th2 subset differentiation is unclear, since classical APCs don't produce IL-4. T cells themselves produce IL-4 initially upon activation (Abbas et. al. 1996). Therefore accumulation of IL-4 in response to TCR engagement, in the absence of significant quantities of IL-12, may result in a Th2-like response. Eosinophils, basophils, NK1.1+ CD4+ T cells, and γδ T cells have also been implicated in early IL-4 production. Recently it was shown that in mouse cells exogenous IL-6 alone can direct the differentiation of CD4+ cells to Th2, and impair IL-12-induced development of Th1 cells (Rincon et. al., 1997). This effect was dependent on the endogenous production of IL-4 by T cells in response to IL-6 produced by APCs. However, experiments using IL-6 knockout mice show that IL-6 is not required for an in vivo Th2 response (La Flamme and Pearce, 1999). Type 2 responses are typically induced by large doses of antigen, and probably have a regulatory function in inhibiting acute and chronic inflammation, and tissue injury brought about by toxic substances produced by activated macrophages. IL-4 prevents IL-12 signaling in Th cells and commitment to the Th1 pathway by inhibition of expression of the β2 chain of the IL-12 receptor (Szabo et. al., 1997). IL-4 inhibits macrophage activation, provides B cell help for non-complement fixing antibodies, and promotes growth of eosinophils and mast cells. The Th2 cytokine IL-10 inhibits cytokine synthesis by Th1 cells (Fiorentino et. al., 1989), and inhibits IL-12 production by monocytes (D'Andrea et. al., 1993), thus helping to polarize the immune response toward Th2.

Interleukin-10

The anti-inflammatory Th2 cytokine IL-10 is implicated in protection from spontaneous abortion. IL-10, a noncovalent homodimer of 18 kD polypeptide chains, is produced by CD4+ T cells (Moore et. al. 1993), B cells (Matthes et. al., 1993), monocytes (de Waal Malefyt, 1991b), and UV-irradiated keratinocytes (Rivas and Ullrich, 1992). IL-10 is also expressed by human placenta in substantial quantities (Cadet et. al., 1995), and bioactive IL-10 has been shown to be produced by cytotrophoblast cells (Roth et. al., 1996). The biological activity of IL-10 is mediated through high affinity binding (K_D = 50-200 pM) to a cell surface receptor (Tan et. al., 1993, Liu et. al., 1994a) that is structurally related to the

interferon receptors (Ho et. al., 1993). IL-10 receptor mRNA is expressed by human monocytes, B cells, T cells and NK/LGL cells, and placenta (Liu et. al., 1994a).

IL-10 was identified as a factor that inhibits cytokine synthesis by Th1 clones, in particular IFN-γ production (Fiorentino et. al., 1989). IL-10 inhibits antigen-specific proliferative responses of both Th1 and Th2 cell subsets (de Waal Malefyt et. al., 1993), including proliferation of alloreactive T cells in mixed lymphocyte reactions when allogeneic cells are used as stimulators (Bejarano et. al., 1992). Since such T cells play a major role in allograft rejection, the production of IL-10 by cytotrophoblast should aid in prevention of immune rejection of the fetus. Certain T cell populations express the IL-10 receptor constitutively (Weber-Nordt et. al., 1994), and IL-10 can directly inhibit IL-2 synthesis and proliferation by T cells (Hisatsune et. al., 1992, de Waal Malefyt et. al., 1993, Taga et. al., 1993).

However, IL-10's T cell inhibitory function is mediated primarily indirectly, via its effects on antigen presenting cells (APCs). IL-10 downregulates many APC cell surface molecules that are required for antigen presentation and T cell activation. In macrophages IL-10 inhibits induction of the costimulatory molecule B7 (Ding et. al., 1993), the adhesion molecule ICAM-1 (Willems et. al., 1994), and MHC class II expression (de Waal Malefyt et. al., 1991a). IL-10 also inhibits human macrophage production of the proinflammatory cytokines IL-1α, IL-1β, IL-6, TNF-α and IL-8 at the transcriptional level, as shown in nuclear run-on experiments (de Waal Malefyt, 1991b, Wang et. al., 1994), and inhibits IL-1, IL-6 and TNF expression in mouse (Fiorentino et. al., 1991a). The production of toxic reactive nitrogen and oxygen species by macrophages is also downregulated by IL-10 (Bogdon et. al., 1991, Cunha et. al., 1992, Gazzinelli et. al., 1992). TGF-β mRNA expression by activated human macrophages is not affected by IL-10 (de Waal Malefyt, et. al., 1991b). IL-10 upregulates monocytic IL-1 receptor antagonist (IL-1RA) production (Howard and O'Garra, 1992). IL-1RA competes for binding of the IL-1 receptor and so is a competitive inhibitor of IL-1α/β. Due to the inhibition of TNF-α synthesis, IL-10 reduces endotoxin-mediated lethality in mice (Gerard et. al., 1993). In addition IL-10 prevents LPS-induced transcription of the IL-12 p40 chain (p35 chain is constitutively expressed) in human monocytes (D'Andrea et. al., 1993). Since IL-12 is the main inducer of IFN-γ synthesis by T and NK cells, IL-10's effect on IL-12 production, and its inhibition of T cell activation via macrophage antigen presentation, provide a mechanism for its suppression of IFN-γ synthesis and the Th1 response. IL-10 is produced maximally at 20-48 h after LPS

activation of macrophages, whereas inflammatory cytokines are secreted earlier at 4-8 h (de Waal Malefyt et. al., 1991b). The late release of IL-10 supports the notion that it functions in vivo as a dampener of the inflammatory immune response. Human IL-10 inhibits its own production by LPS-activated macrophages, indicating the presence of a negative feedback mechanism in IL-10 regulation (de Waal Malefyt et. al., 1991b).

In contrast to its effect on macrophages, IL-10 does not inhibit the ability of B cells to induce IFN-γ secretion by T cells (Fiorentino et. al., 1991b). IL-10 also does not inhibit dendritic cell ability to stimulate proliferation of T cells in response to antigen, but does inhibit dendritic cell-induced IFN-γ production in MLR, which would prevent T cell-induced macrophage activation and Th1 development (Macatonia et. al., 1993). IL-10 stimulates the growth of B cells (Go et. al., 1990) and mast cells (Thompson-Snipes et. al., 1991). Human NK cells express IL-10 receptor mRNA (Liu et. al., 1994), and IL-10 indirectly inhibits IL-2-induced synthesis of IFN-γ and TNF-α by activated human NK cells (Hsu et. al., 1992).

IL-10 inhibits activated monocyte/macrophage production of MMP-1 (interstitial collagenase) and MMP-9 (type IV collagenase/gelatinase) via inhibition of PGE2 production, and therefore may be a regulator of connective tissue destruction during inflammation (Mertz et. al., 1994). IL-10 is also an autocrine inhibitor of trophoblast PGE2 synthesis (Goodwin et. al., 1998), MMP-9 production, and trophoblast invasion (Roth and Fisher, 1999). Thus, this cytokine may help regulate two important processes during pregnancy, suppression of harmful maternal immune responses to the embryo, and control of placental invasion into the uterus.

Not all the effects of IL-10 on immune system cells are inhibitory. IL-10 stimulates the growth of B cells (Go et. al., 1990) and mast cells (Thompson-Snipes et. al., 1991), and enhances M-CSF-mediated growth and differentiation of human macrophages in culture (Hashimoto et. al., 1997).

IL-10 knockout mice develop chronic enterocolitis, and postnatal growth retardation and anemia due to uncontrolled immune responses to enteric antigens (Kuhn et. al., 1993). The lack of IL-10 during gestation in these mice does not result in fetal rejection and loss, but as the fetus is syngeneic to the mother, allo-rejection would not be expected. It was reported that IL-10 knockout mice had an average birth weight of two-thirds that of litter mate controls (Kuhn et. al., 1992). Wegmann et. al. have postulated that an

increase in placental IFN-γ, in the absence of IL-10, leading to a decrease in placental growth factors (GM-CSF), is the cause of the observed runting of IL-10 deficient mice (Wegmann et. al., 1992). Some pregnancy complications brought about by the presence of trophoblast and fetal-specific antigens may be prevented by activities of other cytokines (IL-4, TGF-β) and immune mediators (PGE2) in IL-10 deficient mice. Mice deficient in other cytokines that are thought to be important during pregnancy, such as TGF-β1, are fertile (Shull et. al., 1992). Cytokines are remarkably functionally redundant, accounting for the lack of severe phenotype often seen in knockout mice.

Interleukin-6

Interleukin-6 (IL-6) is a pleiotropic cytokine that is important in inflammatory immune responses, acute phase reactions, hematopoiesis, and induction of B cell differentiation and immunoglobulin secretion (Kishimoto, 1989, Hirano et. al., 1990). IL-6, originally called interferon-β2, 26 kD protein, and B-cell stimulatory factor is produced by a variety of cells, and is induced in response to tissue injury, and bacterial or viral infection. IL-6 is produced by fibroblasts, T and B lymphocytes, endothelial cells, monocytes/ macrophages and many other cell types (Le and Vilcek, 1989, Papanicolaou et. al., 1998). Cytokines such as IL-1, TNF, IL-2 and IFN-β induce IL-6 production by various cell types. Macrophage secretion of IL-6 is induced by LPS, TNF and IL-1, and the activities of IL-6 overlap with those of TNF and IL-1 (May et. al., 1988). IL-6 inhibits the secretion of TNF and IL-1 in a negative feedback loop (Aderka et. al., 1989, Schlinder et. al., 1990b). Endotoxin (1 ng/ml) elicits strong IL-6 production and secretion by monocytes in culture, as does IL-1β, although in lower amounts (Bauer et. al., 1988). In T cells, IL-6 is induced by mitogens or antigenic stimulation. Viruses induce IL-6 production by fibroblasts (Sehgal et. al., 1988).

Human IL-6 consists of a 184 amino acid mature protein with a mass of 21-28 kD, depending on variable N- and O-linked glycosylation. The IL-6 receptor is composed of an 80 kD α chain that binds IL-6, and a homodimer of 130 kD β chains, which acts as the signal transducing component of the receptor. The gp130 chain is shared by other cytokine receptors including CNTF (ciliary neutotrophic factor), LIF (leukemia inhibitory factor), oncostatin M and IL-11. For this reason these cytokines share some functional properties with IL-6. The receptor α chain also exists in a soluble form, which can interact with membrane gp130 when the α chain is bound to IL-6 (Kishimoto et. al., 1994). The IL-6 receptor is expressed in many

cells including T cells, activated B cells and macrophages. In human monocytes high levels of IL-6 reduce the expression of the IL-6 receptor (Bauer et. al., 1989).

IL-6 is involved in T cell activation, growth and differentiation (reviewed in Houssiau and Van Snick, 1992). It can function as a second signal for IL-2 production by CD4+ T cells, and promotes growth of human T cells stimulated by PHA. Recombinant mouse IL-6 inhibits apoptosis of T cells in vitro and may be an important survival factor for T cells in vivo (Teague et. al., 1997). IL-6 also synergistically induces IL-2 receptor α chain expression by T cells (Houssiau et. al., 1989). IL-6 synergizes with other cytokines to induce generation and differentiation of cytotoxic T cells (Takai et. al., 1988).

IL-6 induces proliferation of hematopoietic stem cells by recruiting quiescent progenitor cells into the cell cycle, and acts synergistically with GM-CSF and M-CSF to support granulocyte differentiation and macrophage colony formation, respectively, from hematopoietic progenitors (Caracciolo et. al., 1989, Bot et. al., 1989). Ruppert and Peters (1991) demonstrated that IL-6 induces increased accessory cell activity (defined as ability of macrophages to increase lymphocyte proliferation in response to mitogens) of human macrophages in culture. IL-6 also induces growth inhibition and macrophage differentiation of human myeloid cell lines, suggesting a role for IL-6 in macrophage maturation (Onozaki et. al., 1989).

IL-6 may have an important role in differentiation of T cells to the Th2 phenotype. Th2 type T cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13. These T cells mediate B cell activation, antibody production and control of extracellular pathogens. Th2 responses develop in the presence of IL-4 (Le Gros et. al., 1990), but the cellular source of IL-4 during T cell activation is unclear. Rincon and colleagues (1997) showed that IL-6, a cytokine produced by cells of the innate immune system in initial responses to pathogens, can induce production of IL-4 by naïve Con A-stimulated CD4+ murine T cells, and in an autocrine fashion the resulting IL-4 drives differentiation to the Th2 phenotype in vitro. IL-6 also inhibited IFN-γ production by Th1 cells (Rincon et. al., 1997). Subsequently, however, it was demonstrated that IL-6 is not required for in vivo development of a Th2 response to *Shistosoma mansoni* eggs in mice, although CD4+ T cells from IL-6 deficient mice produced less IL-4 than wild type (La Flamme and Pearce, 1999).

The acute phase response consists of physiologic changes that occur rapidly after infection or trauma, and is characterized by fever, and shifts in vascular permeability and biosynthesis by various tissues, especially the liver. The acute phase response is initiated by cytokines such as IL-1, IL-6, TNF-α

and glucocorticoids that are produced in response to infection or tissue damage. This response enhances host survival by promoting neutralization of the inflammatory agent and stimulation of repair processes. IL-6 induces production of acute phase proteins such as fibrinogen, α1-antichymotrypsin, serum amyloid, C-reactive protein and α1-antitrypsin by human hepatocytes (Castell et. al., 1988).

IL-6 has been described as both a proinflammatory and antiinflammatory cytokine. It is often induced in conjunction with TNF and IL-1 secretion, and enhances immune responses to intracellular infection, but some evidence points to an antiinflammatory role for IL-6. When acute inflammatory responses are induced in mice by exposure to endotoxin, significantly increased extent of inflammation and levels of TNF-α are seen in IL-6 deficient mice (Xing et. al., 1998). The ability of IL-6 to downregulate expression of IL-1, TNF and IFN-γ, to increase IL-1RA and soluble TNF-α receptor (Tilg et. al., 1994), and perhaps to drive Th2 cell development is likely to be a major cause of the observed antiinflammatory properties.

Deregulated expression of the IL-6 gene has been implicated in a variety of diseases including chronic inflammation, autoimmune disease and malignancies such as myeloma (Le and Vilcek, 1989). Transgenic mice that overexpress IL-6 show massive plasmacytosis, hypergammaglobulinemia and thrombocytosis, and resemble human patients with Castleman syndrome (Suematsu et. al., 1989). IL-6 knockout mice are impaired in acute phase protein response to tissue damage and infection, and have reduced microbial resistance (Kopf et. al., 1994). These mice also have reduced numbers of thymocytes and peripheral T cells, specific IgG responses, mucosal IgA and activity of CTLs.

IL-6 may have important functions during pregnancy. The trophoblast is known to constitutively produce IL-6 (Kameda et. al., 1990), and IL-6 receptors are present on the trophoblast layer of the placenta (Nishino et. al., 1990). One function of trophoblast-derived IL-6 is in stimulation of the release of hCG (Nishino et. al., 1990) and placental lactogen (Stephanou and Handwerger, 1994) by trophoblast. Since hCG supports cytotrophoblast growth, IL-6 may be acting as a growth factor for trophoblast (Nishino et. al., 1990). IL-1 and TNF-α synergistically stimulate trophoblast to release IL-6, which results in IL-6-mediated hCG release (Masuhiro et. al., 1991, Li et al., 1992). As in macrophages, IL-6 inhibits cytotrophoblast synthesis of IL-1α/β mRNA, (Stephanou et. al., 1995).

Human uterine endometrial stromal cells secrete IL-6 in culture, which is upregulated in response to IL-1 and TNF (Tabibzadeh et. al., 1989). Mouse uterine epithelium also constitutively secretes IL-6 (Robertson et. al., 1992). IL-6 is believed to function in angiogenesis occurring in maternal decidua in response to embryonic implantation (Motro et. al., 1990). Uterine stromal cell production of IL-6 may function in control of monthly renewal of endometrial blood vessels, as well as angiogenesis during pregnancy. Pretreatment with recombinant IL-6 has been shown to inhibit LPS-stimulated induction of TNF-α by human monocytes, and to inhibit monocyte cytotoxicity toward tumor cells (Aderka et. al., 1989). Synthesis of IL-6 by uterine and placental cells may function to protect the fetus from the demonstrated harmful effects of overproduction of TNF-α. IL-6 increases prostaglandin production by human amnion and decidua, and thus may function in suppression of inflammation, as well as initiation of term and preterm labor, since PGE2 is also a major myometrial contractile agent (Mitchell et. al., 1991). IL-6 has also been suggested to function in differentiation of uterine macrophages (Robertson et. al., 1994). Despite a number of potential functions, IL-6 is apparently not essential to pregnancy since interbreeding of IL-6 deficient mice results in a normal size litter of pups (Kopf et. al., 1994). However, cytokine redundancy makes it difficult to form conclusions regarding the impact of a particular cytokine in any biological process based solely on evidence from gene knockout mice.

A Th1-type Immune Response is Harmful and a Th2-type is Beneficial during Pregnancy

It is believed that the lack of immune rejection of the semiallogeneic fetus by the maternal immune system is brought about in large part by the maintenance of an anti-inflammatory Th2-like environment at the maternal-fetal interface (Wegmann et. al., 1993). Both maternal and fetal cells participate in creating the local immune environment during pregnancy by secretion of cytokines and other immune mediators. Several studies have shown that an inflammatory Th1 response can induce fetal resorption in a mouse model of spontaneous abortion. Specifically, the inflammatory cytokines IL-2, TNF-α and IFN-γ are implicated in immune-mediated complications of pregnancy. The immune system is responsible for fetal resorption in the abortion-prone mating combination CBA/J x DBA/2 (Baines et. al., 1994). Resorption of up to 50 % of a litter (compared to 7% in matings with males bearing the same H-2 haplotype as DBA/2), occurs when CBA/J females ($H\text{-}2^k$) are mated with DBA/2 males ($H\text{-}2^d$) (Tangri and Raghupathy, 1993), preceded by leukocyte infiltration consisting predominantly of NK-like cells

(DeFougerolles and Baines, 1987, Gendron and Baines, 1988). Mice of this mating combination have increased placental expression of the Th1 cytokines IL-2, TNF-α and IFN-γ compared to non-resorption prone mice (Tangri and Raghupathy, 1993). In addition, maternal CBA/J lymphocytes secrete significantly higher levels of these cytokines in response to CBA/J x DBA/2 placental antigens in mixed lymphocyte-placental reactions (MLPR) than in response to CBA/J x Balb/c placental cells (Tangri et. al., 1994). Evidence suggests that the responder cells are CD8+, and that CBA/J mice are previously primed for the response by environmental antigens. (In fact normal resorption rates are seen in this mating combination when the mice are maintained in sterile conditions). In humans, non-uterine parasitic infection, which results in a strong Th1 response, imperils pregnancy (Brabin and Brabin, 1992). Spontaneous abortion can be induced in normal mice by infection with the Th1-inducing protozoan organism *L. major*, and is accompanied by a decrease in production of IL-4 and IL-10, and an increase in TNF and IFN-γ (Krishnan et. al., 1996). Spontaneous abortion in normal mice is also induced by LPS (Chaouat, 1994), or by treatment with the Th1 cytokines IL-2 (Tezabwala et. al., 1989, Chaouat et. al., 1990), TNF-α, or IFN-γ (Chaouat et. al., 1990). IFN-γ and TNF-α cause developmental arrest and embryo deterioration when added to mouse embryos in culture (Hill et. al., 1987). IFN-γ inhibits trophoblast growth in vitro, and together with TNF-α, inhibits fetal development (Haimovici et. al., 1991). Recently it was shown that TNF-α and IFN-γ coadministration can induce abortion in CBA/J xDBA/2 matings in the absence of NK cells and macrophages via cytokine-triggered ischemia caused by thrombosis and inflammation (Clark et. al., 1998). In one study IFN-γ levels in maternal serum were increased by 50% in human spontaneous abortion compared to normal first trimester pregnancy (Vassiliadis et. al., 1998).

Although large amounts of TNF-α may be harmful during pregnancy, smaller quantities at certain times in pregnancy may be important in regulating cellular processes such as differentiation and cell proliferation (Hunt et. al., 1996), and promoting vascularization and blood flow through the placenta. TNF-α is produced by mouse (Hunt et. al., 1993), and human placenta (Chen et. al., 1991). Both inhibition of proliferation of rat trophoblast (Hunt et. al., 1989), and induction of apoptosis in human villous cytotrophoblast (Yui et. al., 1994), have been demonstrated activities of TNF-α in vitro. Thus, TNF-α may help control placental proliferation and invasion of the uterus. TNF-α also induces release of hCG by

placenta via induction of IL-6 (Li et. al., 1992). Normal preimplantation embryonic development is promoted by early TNF-α injection in models of mouse pregnancy failure (Tartakovsky and Ben-Yair, 1991). IFN-γ is also produced by normal mouse placental tissue in the first trimester of pregnancy, but its potential function is unknown (Lin et. al., 1993).

Intrauterine growth retardation (IUGR) is a major cause of neonatal morbidity and mortality. Placental insufficiency associated with maternal vascular disease and compromised uterine blood flow is one recognized cause of IUGR. Another proposed mechanism resulting in IUGR is the release of inflammatory and cytotoxic mediators, which affect placental and fetal growth. Administration of low dose LPS to pregnant rats for 7 days results in significant fetal growth retardation, and concomitant administration of IL-10 reverses this effect (Rivera et. al., 1998). It was postulated that the beneficial effect of IL-10 in this model was due to its inhibition of TNF- and NO-mediated apoptosis (Rivera et. al., 1998).

Interestingly, stress-triggered abortion in mice is accompanied by an increase in the TNF-α/TGF-β2 concentration ratio in supernatants from uterine decidua, and is prevented by anti-sialoGM1 antibody, which depletes NK cells (Arck et. al., 1995). This suggests that stress-induced abortion can be immunologically mediated and has similarity to the mouse model of recurrent spontaneous abortion. Stress is known to have an effect on the immune system and can increase incidence or severity of autoimmune disease, allergy and infection.

There is evidence that abortion can be prevented by a Th2 response. Placenta from CBA/J x DBA/2 matings produces less IL-4 and IL-10 than placenta from non-resorption-prone matings (Chaouat et. al., 1995). Both IL-4 and IL-10 inhibit the development and function of Th1 cells and inflammatory macrophages, and promote the development of a Th2-like response. Third party allo-immunization of abortion prone pregnant mice with spleen cells carrying the paternal MHC antigen haplotype H-2^d increases IL-4 and IL-10 production by placental tissue and decreases abortion (Chaouat et. al., 1983, Kiger et. al., 1985). In humans, alloimmunization with paternal cellular antigen also decreases spontaneous abortion in women prone to this condition (Mowbray et. al., 1985). Significantly, injection of recombinant IL-10 alone prevents increased fetal resorption in CBA/J x DBA/2 matings, and blocking of IFN-γ or TNF reduces resorption (Chaouat et. al., 1995). Also, in abortion prone matings injection of anti-IL-10 antibody increases incidence of abortion (Chaouat et. al., 1995). The majority of abortions in women with recurrent

spontaneous abortion (RSA, diagnosed as three or more sequential pregnancy losses) cannot be attributed to chromosomal defects, anatomical anomalies, endocrine disorders or infections (Harger et. al., 1983, Stray-Pederson and Stray-Pederson, 1984). It has long been suspected that immunological factors are responsible for many unexplained cases of RSA and other cases of early pregnancy loss. Peripheral blood mononuclear cells (PBMC) from women with RSA commonly react to trophoblast antigens by release of IFN-γ and TNF-β, and low levels of IL-10, whereas those from non-RSA-prone women produce only IL-10 (Hill et. al., 1995). Together with the alloimmunization studies, this suggests that in a normal pregnancy maternal immune system cells do recognize fetal trophoblast antigens, but the recognition causes a protective Th2 response. A shift from production of the type 1 cytokines IL-2 and IFN-γ by in vitro mitogen-stimulated PBMC to the type 2 cytokines IL-4 and IL-10 can be observed during the third trimester in normal pregnancies (Marzi et. al., 1996). In the same study, pathologic pregnancies, such as spontaneous abortion, were associated with an increase in IL-2 and IFN-γ, and reduced IL-10 production in (MLR) mixed lymphocyte reaction compared to normal pregnancies (Marzi et. al., 1996). A recent study showed a defect in leukemia inhibitory factor (LIF), IL-4 and IL-10 synthesis by in vitro activated decidual T lymphocytes from women who had undergone unexplained recurrent spontaneous first trimester abortion (Piccinni et. al., 1998). This defect was not observed in the peripheral T cells from the same women, indicating that immune regulation of the local environment during pregnancy may not necessarily affect the systemic immune system. These studies suggest that successful human pregnancy is associated with a Th2-like immune environment.

It is evident that spontaneous abortion can be influenced, both positively and negatively, by immune system mediators, but this does not necessarily mean that immunity is the causal factor in unexplained abortion. Because the immune and reproductive systems utilize common cytokines and other immune mediators, the etiology of spontaneous abortion is difficult to prove. However, the preponderance of evidence points to inflammatory cytokines as causal factors in many instances of pathology of pregnancy.

Mechanism of Th1 Cytokine Harmful Effects

The deleterious effect on pregnancy of Th1 cytokines is largely mediated by induction of macrophage, NK and lymphokine activated killer (LAK) activity. A main function of the cytokines IFN-γ,

TNF-α and IL-2, which are known to be detrimental to pregnancy, is to activate macrophage and NK-like cell activity. There are relatively few T cells in mouse uterus (Gambel et. al., 1985), and T cells compose only 8% of all decidual cells in human uterus (Vince et. al., 1990). Human and rodent pregnancy is accompanied by an increase in NK cells in the uterus (Croy and Kiso, 1993), with human large granular lymphocytes (LGLs), which are related to NK cells, constituting 75% of bone marrow derived decidual cells in the first trimester (Vince et. al., 1990). In mice, NK-like cells, called granulated metrial gland cells (GMGs) or uterine NKs, are the counterpart to human LGLs, and localize to a small region of the implantation site in the uterine musculature called the mesometrial triangle to form a part of a structure called the metrial gland (Peel, 1989).

High levels of NK cells in pregnant women with recurrent spontaneous abortion compared to non-abortion prone women have been demonstrated (Kwak et. al., 1995). In mice enhanced fetal loss correlates with local NK activity at the implantation site (Gendron and Baines, 1988). When NK cells are exposed to cytokines such as IL-2, IL-12 and interferons, they become "activated NK cells", which lyse a different set of target cells. Factors which stimulate NK activity, such as IL-2 (O'Garra, 1989), double-stranded RNA, and TNF, are abortifacient (deFougerolles and Baines, 1987, Tezabwala et. al., 1989, Chaouat et. al., 1990, King et. al., 1990). LAK generation from the NK cell lineage requires IL-2, IL-12, or IL-2 and TNF-α (Trinchieri et. al., 1995), and both mouse (Drake and Head, 1989) and human trophoblast are susceptible to killing by these cells (Parhar et. al. 1988, King and Loke, 1990). Human decidual LGLs are abundant in first trimester decidua (Starkey et. al., 1988) and also kill trophoblast when stimulated with IL-2 (Ferry et. al., 1991, King and Loke, 1993). Treatment with anti-asialoGM antibody, against the mouse NK cell marker ASGM1, depletes NK cells and inhibits fetal resorption in CBA/J x DBA/2 matings (de Fougerolles and Baines, 1987). IL-10, which inhibits spontaneous abortion in the mouse model of RSA, inhibits the IL-2 induced synthesis of cytokines by activated NK cells (Hsu et. al., 1992).

Trophoblast is not normally susceptible to killing by NK cells (King et. al., 1989) or cytotoxic T cells (Zuckermann and Head, 1987). NK-like cells cause fetal loss only after conversion to LAKs in the presence of activating cytokines. NK killing is also inhibited at the maternal fetal interface by PGE2 produced by uterine decidual macrophages (Lala et. al., 1990). In normal pregnancy NK cells are thought to protect against infection, and prevent uncontrolled invasion of the uterus by trophoblast (Lala, 1990).

Macrophages are believed to play an important role in spontaneous abortion. In pregnancy the number of macrophages in the uterus increases, and they are found abundantly in human decidua (Bulmer and Johnson, 1984), and in mouse myometrium, endometrial stroma and metrial glands (Hunt, 1989). Macrophages make up 20% of decidual cells in human uterus (Vince et. al., 1990). One source of the LAK-generating cytokines IL-12 and TNF-α, is activated uterine macrophages, which are significantly increased in number in resorbing embryos (Duclos et. al., 1994). Activated decidual macrophages are capable of directly causing embryo cell death through the production of nitric oxide (NO) (Haddad et. al. 1995). Both IFN-γ and TNF-α are implicated as critical cytokines in immune-mediated abortion and these two cytokines are macrophage activating factors. It has been suggested that the essential role of NK cells in spontaneous abortion may be the production of IFN-γ and subsequent activation of decidual macrophages (Haddad et. al., 1997.) There are increased numbers of decidual macrophages at implantation sites in the uteri of abortion-prone mice before signs of fetal damage are visible (Duclos et. al., 1994). Also, treatments that decrease the number and activity of decidual macrophages increase embryo survival in the mouse model of recurrent abortion (Duclos et. al., 1994). Macrophages are involved in activation and suppression of lymphocyte proliferation and effector function, production of the inflammatory response, and its downregulation, and they produce factors that can directly promote growth of (GM-CSF) (Athanassakis et. al., 1987) or harm (NO) fetal tissues.

Monocytes develop from bone marrow progenitor cells, which are induced to differentiate to monoblasts by IL-3, CSF-1, GM-CSF and IL-6 (review in Rutherford et. al., 1993). Monocytes are released from the bone marrow into the blood where they have a half-life of 8-9 hours. Circulating monocytes migrate to tissues where they differentiate irreversibly into long-lived resident macrophages. Tissue macrophages further differentiate into region-specific types as a result of local environmental signals, including hormones, growth factors and cytokines such as CSF-1, GM-CSF, TNF and IL-6 (Rutherford et. al., 1993). Macrophages from different tissues express different surface antigens and receptors. For example, peritoneal macrophages express high amounts of CD14 (the LPS receptor), but alveolar macrophages do not (Andreeson et. al., 1990). The use of tissue macrophages of different phenotypes can result in conflicting experimental results when these cells are used in functional studies. The number of tissue macrophages, including liver Kupffer cells, peritoneal macrophages, alveolar lung

macrophages, brain microglia and bone osteoclasts, are 500-1000 times the number of bone marrow and blood monocytes (Cohn, 1983).

Blood monocytes and tissue macrophages are morphologically and biochemically distinct. Macrophages are larger than freshly isolated blood monocytes and show more extensions on their surface. Circulating monocytes express higher levels of proteases than resident macrophages, presumably in order to penetrate blood vessels and tissues. Monocytes can be separated from other mononuclear leukocytes by by their ability to adhere to a substratum. Since adherence is a first step in extravasation and entry into tissues, adherence may prime monocytes for maturation via intracellular signals generated by surface receptor binding to ECM and cellular ligands (Eierman et. al., 1989). Adherent monocytes display increased transient mRNA expression for TNF and CSF-1, but secretion depends on a secondary stimulus such as LPS (Haskill et. al., 1988). Monocyte maturation to macrophages is accompanied by reduced capacity to produce reactive oxygen species and reduced anti-microbial activity, that can be reactivated in response to cytokines and/or microbial products (Nakagawara et. al., 1991). Human blood monocytes in culture with human serum differentiate into mature macrophages and multinucleated giant cells, which can remain viable for weeks. Without either serum, LPS, M-CSF, G-CSF or CSF-1, human monocytes in suspension culture die in 3-5 days (Becker et. al., 1987, Young et. al., 1990). Adherent monocytes in serum-free media survive (but don't differentiatiate to macrophages) due to autocrine production of CSF-1 and other factors (Andreesen et. al., 1990). Differentiation of monocytes in vitro takes 5-7 days and results in surface antigens and morphology similar to tissue macrophages (Musson, 1983).

Macrophage activation is acquisition of enhanced competence to carry out various macrophage functions, such as tumor cell killing or inflammatory cytokine secretion. Resident tissue macrophages, in the absence of stimulation, express low levels of MHC class II molecules and do not secrete significant amounts of cytokines. Macrophage activation is a step-wise process that is incompletely understood. Initial signals, such as IFN-γ, induce differentiation to an intermediate non-cytotoxic state with enhanced MHC class II molecule expression and antigen presentation. Secondary signals from microbial products then generate fully activated macrophages in usually less than 24 hours. Activated macrophages have enhanced ability for chemotaxis, phagocytosis, adhesion, MHC class II molecule expression and antigen presentation, antibody dependent cellular cytotoxicity (ADCC), secretion of TNF, NO and cytotoxic

factors, and are unable to proliferate. Primary and secondary signals synergize in macrophage activation. For example, LPS-stimulated macrophages secrete TNF-α, but if first primed with IFN-γ or GM-CSF they secrete several-fold more TNF-α in response to LPS (Heidenrich et. al., 1989). In addition, macrophages primed for one function often have decreased ability to perform other functions. Macrophages that have strong tumoricidal activity are poor antigen presenters.

After implantation there is an influx of macrophages into the uterus, believed to be a result of estrogen-mediated uterine epithelial cell production of macrophage chemotactic factors, such as CSF-1, GM-CSF and TNF (Hunt and Robertson, 1996). Once in the endometrium, macrophage differentiation is subject to influences by steroid hormones and endometrial and placental cytokines, which result in a phenotype appropriate to pregnancy. McCay et. al. (1991) have reported that components of murine uterine decidual cell ECM inhibit macrophage tumor cell lysis, and this inhibition occurs only in regions of the decidualized endometrium where both maternal and fetal cells are present, suggesting that discrete areas within the uterus contain variable macrophage phenotypes during pregnancy (Redline and Yu, 1988). Very little is known about the precise in vivo concentrations and effects of uterine and placental factors on macrophage differentiation. Estrogen seems to stimulate phagocytosis of human macrophages (Butterworth and Loke, 1995, Miller and Hunt, 1996). Physiological concentrations of progesterone increase monocyte PGE2 synthesis and suppress macrophage cytotoxicity (Yagel et. al., 1987, Feinberg et. al., 1992). Although progesterone receptors have not been found in monocytes, progesterone can bind to glucocorticoid receptors, which are present in macrophages. In fact, human decidual macrophages from early pregnancy have been reported to be of a suppressive phenotype (Lala et. al., 1986). Macrophages comprise 40% of fetal placental villous stromal cells (Goldstein et. al., 1988). Fetal macrophages in placenta are phagocytic (Wilson et. al., 1983), microbicidal (Wilson and Westall, 1985), express MHC class II antigens (Goldstein et. al., 1988), produce IL-1 (Flynn et. al., 1985) and secrete PGE2 in response to progesterone in culture (Yagel et. al., 1987).

Control of monocyte/macrophage activity is critical during pregnancy. The deleterious effects of Th1 cytokines on the fetus is mainly a function of their induction of cytotoxic macrophage, NK cell and LAK cell activity. Factors that stimulate NK cell and macrophage activity, such as TNF-α and IL-2, are abortifacient in normal mouse matings (Chaouat et. al., 1990), and administration of IL-10, which

downregulates inflammatory cytokine production, and NK cell and macrophage activity, prevents spontaneous abortion in murine abortion-prone matings (Chaouat et. al., 1995). Human maternal macrophages are found adjacent to fetal membranes throughout pregnancy (Lessin et. al., 1988). Activated macrophages can directly kill embryo cells, and products of monocytes, like IL-12 and TNF-α, generate LAK cells, which have been shown to kill trophoblast (Parhar et. al., 1988, King and Loke, 1990). Because of their ability to significantly influence pregnancy in both positive and negative ways, and their central role in cytokine networks, we chose to evaluate the effects of human PSGs on cytokine synthesis by human monocytes.

Immune abortions appear to be due to nonspecific effector cells (NK-like cells and macrophages, rather that antigen-specific cytotoxic T cells), but the cytokine activators of these cells (IL-2, TNF, and IL-12) can be produced by cells of both adaptive immunity and nonspecific immunity, as well as fetal trophoblast, and uterine decidua (see below). Communication between maternal and fetal tissues results in a complex cytokine network that acts to promote the maintenance of pregnancy. Dysregulation of the production of intercellular signaling molecules (e.g. TNF-α) by or in response to the fetus, or the presence of microorganisms or small amounts of LPS, may be enough in certain females to trigger the production of inflammatory mediators which activate NK-like cells and macrophages and result in abortion. In normal pregnancy cytokines such as GM-CSF, TGF-β1, PGE2 and IL-10, produced by both uterine macrophages and uterine epithelia, act to inhibit detrimental actions of macrophages, NK cells and T lymphocytes.

Pregnancy Induces an Anti-Inflammatory Immune Environment

In 1993 Wegmann proposed that pregnancy induces a Th2 dominant immune environment to protect the fetus (Wegmann et. al., 1993). It is well established that pregnancy modifies the immune status of the mother. Pregnancy potentiates the humoral immune response in mice (Dresser, 1991), and humoral autoimmune diseases in humans, such as myasthenia gravis (Fennel and Ringell, 1987), systemic lupus erythematosus (SLE), autoimmune thrombocytopenic purpura and Graves' Disease (Kendall-Taylor, 1993), are exacerbated during pregnancy (Varner, 1991). In addition CMI is attenuated in pregnant mice (Holland et. al., 1984), and resistance to intracellular pathogens is decreased (Luft and Remington, 1984). In women some infectious diseases caused by intracellular pathogens are worsened during pregnancy, including leprosy, tuberculosis, malaria, HIV and toxoplasmosis, suggesting a decrease in Th1 immunity (Weinberg,

1984, Wegmann et. al., 1993). In humans inflammatory macrophage function is inhibited specifically in the decidua as shown in a model of intrauterine infection by *Listeria monocytogenes* (Redline and Lu, 1988). During pregnancy malignancies are more frequent (Gleicher et. al., 1979, Janerich, 1980), and skin homograft survival is increased (Andreson and Monroe, 1962), indicating a decrease in CMI. Also, Th1-driven autoimmune diseases such as rheumatoid arthritis and multiple sclerosis often improve during pregnancy, presumably due to the presence of an antiinflammatory environment. These observations indicate that changes in cytokine production associated with pregnancy can have systemic effects.

Pregnancy is accompanied by an influx of leucocytes into the uterus, including macrophages, NK cells and T lymphocytes. Decidual tissue contains 45% bone marrow derived cells (Vince et. al., 1990). These cells, the feto-placental unit, and uterine decidua and epithelia produce immune cytokines which contribute to the local immune environment, and may affect the systemic immune system. The feto-placental unit produces immunomodulatory cytokines that result in a Th2-like environment. Mouse placental tissue secretes IL-3 and the Th2 cytokines IL-4, IL-5, and IL-10 (Lin et. al., 1993). Levels of the Th2-inducing cytokine IL-4 in mouse placenta rise sharply and remain elevated throughout pregnancy (Delassus et. al., 1994). In abortion-prone mice the secretion of Th2 cytokines is reduced, and an increase in Th1 cytokine production by placenta is seen (Chaouat et. al., 1995). The Th2 bias during pregnancy is apparently overcome in response to serious infectious assault, e.g. *L. major* infection in normal mice, which allows the mother to fight off the infection and then re-establish pregnancy.

Trophoblast is also a key regulator of the immune environment at the human feto-placental interface, preventing immune attack of the fetal semi-allograft. In addition to hormones, human trophoblast is known to produce cytokines including IL-1β (Paulesu et. al., 1991), TNF-α (Jaattela et. al., 1988), IL-6 (Stephanou et. al., 1995), IL-4 (DeMoraes-Pinto et. al., 1997), IL-10 (Cadet et. al., 1995, Roth et. al., 1996), and TGF-β (Lysiak et. al., 1995). The proinflammatory cytokines IL-1β and TNF-α are thought to help regulate trophoblast invasion by stimulating MMP-9 production (Yui et. al., 1994, Shimonovitz et. al., 1996, Librach et. al., 1994), controlling trophoblast differentiation, and inducing prostaglandin E synthesis (Shimonovitz et. al., 1985). Trophoblast-derived IL-6, TNF-α and IL-1 stimulate release of placental hCG (Nishino et. al., 1990, Li et. al., 1992, Masuhiro et. al., 1991). Cytotrophoblast cells from human placenta synthesize and secrete biologically active IL-10, which probably constitutes one of the mechanisms by

which immune rejection is prevented (Roth et. al., 1996). IL-10 may also have a role in controlling trophoblast invasion through its inhibition of trophoblast MMP-9 production. Trophoblast cells have been compared to macrophages because these cell types share functional and morphologic characteristics (Guilbert et. al., 1995). Both form syncytia, exhibit invasiveness, and express CD14, FcR, non-specific esterase, GM-CSF, CSF-1, IL-1, IL-6, TNF-α, TGF-β, and receptors for these cytokines (Guilbert et. al., 1995). CSF-1 is a growth factor for both macrophages and trophoblast (Athanassakis et. al., 1987). Trophoblast is the first line of defense for the fetus against infectious agents, is phagocytic and produces reactive oxygen species. Like macrophages, trophoblast can respond to LPS by increased production of IL-10 (Roth et. al., 1996). As macrophage giant cells encircle pathogens, so the trophoblast surrounds and protects the fetus.

The production of anti-inflammatory factors at the maternal-fetal interface suppresses macrophage, NK cell and cytotoxic T cell activity. GM-CSF, IL-3, IL-4, TGF-β, and IL-10 are implicated in inhibition of NK-type cell activity (Reiter and Rappaport, 1993, Hsu et. al. 1992). Maternal uterine decidual macrophages produce high levels of prostaglandin E2 (PGE2) (Parhar et. al., 1988), which inhibits generation of both cytotoxic T cells and LAKs (Tawfik et. al., 1986). PGE2 may also help to prevent a T cell-mediated response to the fetus by inhibiting T cell activation and IL-2 production. Mouse uterine epithelia and decidua produce TGF-β1, which inhibits the generation and cytolytic activity of cytotoxic T cells, NK cells and LAKs, and suppressed lymphokine-activated killing by LGLs (Roberts and Sporn, 1990, Tamada et. al., 1990). Human decidua also expresses TGF-β throughout most of pregnancy (Graham et. al., 1992). In addition, human (Dungy et. al., 1991) and mouse (Lysiak et. al., 1995) trophoblast also produce TGF-β (Lysiak et. al., 1995). TGF-β has widespread anti-inflammatory effects including inhibition of tissue macrophage activation, deactivation of macrophages by inhibition of the respiratory burst (Tsunawaki et. a l., 1988), and inhibition of inflammatory monokine production (Letterio and Roberts, 1997). TGF-β also acts as a positive regulator of differentiation of Th2 cells and an inhibitor of Th1 cell differentiation (Schmitt et. al., 1994, Strober et. al., 1994). Basal PGE2 production by cultured human term placenta is increased by TGF-β (Goodwin et. al., 1998). TGF-β, in addition to its anti-inflammatory properties, has been reported to inhibit human placental growth, differentiation to syncytiotrophoblast, and invasion (Graham et. al., 1992, Morrish et. al., 1991, Caniggia et. al., 1997).

Decidual murine non-T lymphocytes also produce a soluble suppressor of allospecific cytotoxic T cells and LAKs, identified as TGF-β2 (Lea et. al., 1992).

Steroidal hormones produced by the placenta may contribute to producing an anti-inflammatory environment. Both estrogen and progesterone have anti-inflammatory properties (Szekeres-Bartho, 1982). During pregnancy levels of estrogen, progesterone and the corticosteroid hormone cortisol are increased, and T helper subset cytokine production may be regulated in part by these hormones. Glucocorticoids suppress production of the Th1-associated cytokines IL-12, IFN-γ, TNF-α and IL-2, and promote IL-4 production (Daynes and Araneo, 1989). Progesterone, which is in high concentration at the maternal-fetal interface, has been shown to favor the development of Th2 cytokine-secreting clones, enhance IL-4 production by human Th1-like clones (Piccinni et. al., 1995), and to block lymphocyte function in vitro (Stites and Siiteri, 1983). Progesterone induces PIBF (progesterone-induced blocking factor), a factor that inhibits NK cell and macrophage cytotoxicity and is anti-abortive in mice (Szekeres-Bartho et. al., 1997). High levels of estrogen, similar to physiological circulating levels during pregnancy, inhibit TNF and potentiate antigen-stimulated IL-10 and IFN-γ production (Gilmore et. al., 1997). Progesterone, estrogen, hPL and prolactin inhibit lymphocyte reactivity in vitro (Siiteri and Stites, 1982).

There is ample evidence that in normal pregnancy the combined actions of progesterone and estrogen, and immune factors produced by the placenta and uterus, bring about an anti-inflammatory environment, which protects the fetus from damage by NK-like cells and macrophages, and results in amelioration of clinical symptoms of several inflammatory autoimmune diseases. Uterine and placental hormones, cytokines, prostaglandins and growth hormones act in concert to inhibit inflammation and CMI, but the presence of maternal antibody to paternal antigens suggests that Th2 responses do occur during pregnancy. Thus, rather than a general immunosuppression, a bias toward a Th2-like response in pregnancy is believed to be the solution to the immune problem of fetal antigenicity and uterine invasion.

The Immunotrophic Hypothesis

In addition to protection against toxic factors, certain cytokines, produced by maternal cells in response to fetal antigens, may actively promote growth and survival of the feto-placental unit. The immunotrophic hypothesis, first proposed by Wegmann (Wegmann, 1984), states that T cell-derived cytokines, such as IL-3 and GM-CSF, from maternal alloreactive cells, promote fetal survival by

stimulating placental growth, while the presence of suppressors of the detrimental activities of alloreactive T cells prevents harm (Mogil and Wegmann, 1988). According to this theory, the failure to respond to paternal or embryonal antigens with appropriate cytokine production would lead to poor placentation, fetal growth retardation and even fetal death. This hypothesis provides a mechanism to explain the protective effects of alloimmunization with paternal antigens on human and mouse pregnancy outcome. (Alloimmunization also induces the generation of non-complement binding serum antibodies directed against paternal antigens, which become adsorbed to the placenta and thus may mask fetal antigens from recognition and maternal immune attack [Chaouat et. al., 1985].)

IL-3 normally functions as a multi-colony stimulating factor for hematopoietic cells, while GM-CSF promotes growth of granulocytes and macrophages, and regulates macrophage maturation and activity. Both GM-CSF and IL-3 have been shown to enhance growth of mouse and human trophoblast in vitro (Athanassakis et. al., 1987, Armstrong and Chaouat, 1989, Loke et. al., 1992), while IFN-γ inhibits the production of GM-CSF and trophoblast proliferation (Berkowitz et. al., 1988). The cytokines IL-1, IL-6 and TNF-α also affect trophoblast function, specifically placental hormone secretion. Both IL-1 and IL-6 are produced by differentiating human cytotrophoblast and act in an autocrine fashion to induce the release of hCG (Yagel et. al., 1989, Nishino et. al., 1990), and hPL (Stephanou and Handwerger, 1994) by trophoblast. GM-CSF and IL-3, and alloimmunization decrease LPS-induced and NK activation-induced fetal resorption (Chaouat, 1994), and prevent resorption in CBA/J x DBA/2 pregnancy (Chaouat et. al., 1990). GM-CSF and IL-3 downregulate NK-like cell cytotoxicity (Reiter and Rappaport, 1993).

In mixed lymphocyte-placenta reactions (MLPR) stimulation of maternal lymphocytes by placental antigens was much higher for lymphocytes from mice of non-abortion-prone matings than from CBA/J x DBA/2 matings (Tangri and Raghupathy, 1994). (In addition, TNF-α, IFN-γ, and IL-2 were significantly higher in supernatants from MLPR of the abortion-prone mating combination.) This suggests that appropriate T cell alloreactivity toward fetal antigens may benefit pregnancy. Because genetically T cell-deficient mice are reproductively competent (Croy and Chapeau, 1990), and there are relatively few T cells in the uterus during pregnancy, it is now believed that immunotrophic cytokines are produced mainly by decidual and placental cells. In mice and humans uterine macrophages (Tawfik et. al., 1986), uterine epithelial cells (Robertson et. al., 1992), and placental cells (Burgess et. al., 1977, Kanzaki et. al., 1991)

secrete GM-CSF. IL-3 is produced by mouse placenta (Lin et. al., 1993). Although an intact adaptive immune system is not necessary for maintenance of pregnancy, T cell removal in vivo can cause decreased placental size and even fetal death in some mouse strains (Chaouat et. al., 1989).

The role of the immune system in maternal and fetal health during pregnancy is becoming a major topic of interest for reproductive biologists, particularly since cells of the innate immune system (macrophages and NK-like cells) appear to be some of the primary targets of feto-placental regulatory mechanisms. The interplay of hormones, cytokines, bioactive lipids and growth factors, produced by different cells during pregnancy is complex and incompletely understood. That the immune environment is critical to a successful pregnancy, and that it is altered during pregnancy has been clearly documented over the years since Medawar's first proposal of consideration of the fetal unit as an allograft. Small increases in the cytokines TNF-α and IFN-γ are generally harmful to pregnancy; single doses can induce fetal resorption in murine abortion-prone matings. Yet these cytokines are normally found at the maternal-fetal interface, highlighting the fact that a finely balanced cytokine network exists during pregnancy. GM-CSF, IL-10, TGF-β and PGE2 induce "immunoinhibitory" macrophages that inhibit T cell and NK cell activation (Robertson et. al., 1994). GM-CSF and CSF-1 enhance trophoblast growth. Of these cytokines, GM-CSF and IL-10 have been shown to protect against fetal resorption in mouse recurrent abortion. Cytokine dysregulation is also implicated in intrauterine growth retardation. Since immune-mediated abortion appears to be due to non-specific effectors (NK-like cells and macrophages), but the activators of these effectors are produced both in specific immunity (Th1-induced inflammation) and nonspecific inflammatory defense, it is reasonable that a bias toward a Th2-like anti-inflammatory environment would be beneficial. The production of anti-inflammatory mediators primarily functions to prevent activation of NK-like cells and macrophages locally, and secondarily perturbs the systemic immune response, with the observed effects on maternal immunity and autoimmunity during pregnancy.

It is likely that the feto-placental unit itself orchestrates the creation of the proper immune environment necessary for fetal survival, and experimental evidence supports this contention. Placental trophoblast is a key regulator of cytokine production during pregnancy (Robertson et. al., 1994). The placenta is known to synthesize factors that modify the maternal metabolism and uterine environment to benefit the fetus. Pregnancy-specific glycoproteins are products of the placenta produced in large

quantities throughout pregnancy. PSGs are hypothesized to function, at least in part, in moderating the immune environment during pregnancy. Support for this idea includes their production by immune system cells at sites of hematopoiesis in adult and fetus, the binding of one member of the family to cells of the monocytic lineage, their membership in the immunoglobulin gene family, the fact that low levels of these proteins in maternal serum and endometrium are correlated with spontaneous abortion, and that PSG11 increases IL-10 secretion by activated macrophages. Interestingly, increased levels of PSG protein in maternal serum have also been correlated with improvement of rheumatoid arthritis, a Th1-mediated inflammatory disease, during pregnancy (Fialova et. al., 1991). We postulate that the PSGs, a family of glycoproteins produced abundantly by the placenta, may mediate expression of one or more immunoregulatory molecules, as part of the feto-placental control of the local immune environment. We investigated the ability of three human PSGs to regulate the production of immune mediators such as IL-10, TNF-α, and IL-6 by macrophages. The capability to synthesize a large number of regulatory cytokines in response to microbial and immune signals places the macrophage at a central position in uteroplacental cytokine networks. Knowledge of such regulatory mechanisms may be of major importance in development of clinical therapies for treatment of female infertility, maintenance and termination of pregnancy, prevention of intrauterine growth retardation, and possibly in autoimmune diseases arising during or exacerbated by pregnancy.

II. Materials and Methods

Cloning of Murine PSG23 and PSG28 cDNAs

Total RNA (1-4 μg) from placentae isolated from 18-day pregnant Swiss Webster mice (Pel-Freez Biologicals, AR) was reverse transcribed using Ready-to-Go You-Prime First Strand beads (Pharmacia Biotech, NJ) and 1 μM of an oligo$(dT)_{18}$-Not I oligonucleotide 3' anchor primer (Pharmacia Biotech, NJ) at 37° C for 60 min. followed by incubation at 90° C for 5 min. The resulting cDNA were amplified by PCR using 5' oligonucleotides derived from murine PSG20 and PSG21 5'UTR exon 1 sequences and an oligo(dT) oligonucleotide. All oligonucleotides were synthesized with an Applied Biosystems Inc. (CA) DNA Synthesizer at the USUHS Biomedical Instrumentation Center, and had at least 95% purity.

PSG28 cDNA was amplified using 30 pmol of the 5' primer 5'-GGTCTTCTCCTAGAGAAAG-3' from the PSG20 5' UTR and 1 μM of an oligo(dT) primer in a 100 μl reaction with 2.5 U of Amplitaq DNA Polymerase (Perkin Elmer Cetus, CA), 1 mM each of dATP, dCTP, dTTP and dGTP and 1X PCR buffer (10X buffer contains 500mM KCl, 100 mM Tris-HCl [pH 8.3], and 15 mM $MgCl_2$) for 30 cycles (denaturation at 94° C for 1 min., annealing at 50° C for 1 min., extension at 72° C for 1.5 min.) in a DNA Thermal Cycler 480 (Perkin Elmer, CA). PSG23 cDNA was amplified using the 5' primer 5'-TGAAGAG-ATATGGAGTGA-3' from the PSG21 5' UTR and coding region containing the start codon (underlined), and an oligo(dT) in a reaction similar to that for PSG28 except that the annealing temperature was 49° C.

The PCR products were separated by electrophoresis on a 0.8% agarose gel, stained with ethidium bromide, and bands of the appropriate size (1.7 kb) were cut out of the gel. DNA was eluted from the agarose using GenElute Agarose Spin Columns (Supelco, PA). The eluted DNA was precipitated with one-tenth volume of 3 M Sodium Acetate in ethanol, washed with 70% ethanol, and subcloned into pCR2.1 (TA cloning kit, Invitrogen Corp., CA) using Ready-to-Go T4 DNA Ligase (Pharmacia Biotech, NJ) by the TA cloning method. The linearized pCR2.1 vector has 3' single deoxythymidine residues for efficient ligation of Taq PCR products. Recombinant pCR2.1 was propagated by transformation into *E. coli* INVαF' competent cells using the Invitrogen "One Shot" system (Invitrogen Corporation, CA) according to the manufacturer's instructions. Bacterial colonies were grown on Luria Bertani (LB) agar plates (Difco Laboratories, MI) with 50 μg/ml ampicillin (Sigma, MO), and plasmids were isolated after growth in liquid

LB media (Sigma, MO) from single colonies by boiling lysis mini-prep protocol (Holmes and Quigley, 1981).

PCR2.1 digested with *EcoRI* to release the inserts, was then electrophoresed on 1.5 % agarose gels, transferred to Nytran membranes (Schleicher and Schuell, NH) overnight in 20X SSPE, and analyzed by Southern blot. (1X SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, 1 mM EDTA, pH 7.7.) DNA on the blots was crosslinked with UV light for 2 min. and baked onto the Nytran membrane at 90° C for 25 min. in a vacuum oven. Blots were probed with a mixture of two ^{32}P-labeled mouse PSG-specific universal oligonucleotide probes (M5' UNI, 5'-TCTTCTCCAGGTCCATAA'3 and M3' UNI, 5'ATTG(GA)-TACTCTCCAGCAT-3'). 5' nucleotide end-labeled oligonucleotide probes were prepared using $\gamma[^{32}P]$ATP (ICN Pharmaceuticals, Inc., CA) and T4 polynucleotide kinase (New England Biolabs, Inc., MA). Blots were prehybridized in 3 ml of Rapid-hyb (Amersham Pharmacie Biotech, NJ) at 42° C for 30 min., incubated with 1 x 10^6 cpm/ml of labeled probe for 1 hour at 42° C, and then washed three times in 2X SSPE, 0.1% sodium dodecyl sulfate (SDS) for 5 min. at 25° C, followed by two 20 min. washes at 51° C (high temperature T_m - 5°C) in 5X SSPE, 0.1% SDS. Blots were exposed to Kodak XAR film (Eastman Kodak Company, NY) overnight. Positive clones were selected and plasmid DNA was prepared for sequencing by Qiagen maxiprep plasmid protocol (Qiagen Inc., CA).

Two independent PSG28 cDNA clones and one PSG23 clone were further analyzed. These clones were sequenced on both strands by the cycle sequencing method, utilizing incorporation of fluorescently labeled dideoxynucleotides from a Taq Dyedeoxy Terminator Cycle Sequence kit (Applied Biosystems, CA) by a thermostable polymerase in a thermal cycler. Fluorescently labeled products were run on 4% polyacrylamide, 8 M Urea gels, and nucleotide sequence was determined using an Applied Biosystems Model 377 Fluorescent Sequencer. Sequencing was begun at both ends of the cDNA using universal oligonucleotide primers (T7S and M13 reverse) that flank the cloning site in the vector. Newly determined sequence was used to design sequential internal primers to sequence both strands of the entire cDNA. Consensus sequences were compiled and analyzed using the AssemblyLIGN Sequence Assembly Software version 1.0 and MacVector software programs (Oxford Molecular Ltd, Oxford, UK).

Sites of PSG23 and PSG28 mRNA Expression

Tissue expression of PSG23 and PSG28 was analyzed by RT-PCR. Total RNA (2μg) from adult Swiss Webster mouse bone marrow, liver, brain, thymus, heart, kidney, lung, testes, ovary (Ambion, Inc., TX), and 18 day placenta (Pel-Freez Biologicals, AR) was converted to cDNA using random hexamer primers and Ready-to-Go You-Prime First Strand Beads, as described above. PSG28-specific sense (5'-CCCACGAATGACAAATTTGAG-3') and antisense (5'-CTTGTTAAATACAATCAGCCC-3') primers from the N1 and N2 domains were used to amplify PSG28 with 2.5 U of AmpliTaq DNA Polymerase, dNTPs and 1X PCR buffer as described above for 30 cycles (94° C for 45 s, 53° C for 30 s, 72° C for 45 s) in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer, CA). PSG23 was amplified using a universal PSG primer set derived from the N3 and A domains (M5' UNI, and M3' UNI) that hybridizes to most murine PSG cDNAs, for 30 cycles (94° C for 45 s, 51° C for 30 s, 72° C for 45 s). A fragment of the housekeeping gene, hypoxanthine phosphoribosyl transferase (HPRT), was amplified for each RNA sample as a control for RNA degradation. HPRT was amplified using sense (5'-GTTGGATACAGGCCAGACT-TTGTTG-3') and antisense (5'-GATTCAACTTGCGCTCATCTTAGG-3') primers for 22 cycles (94° C for 1 min., 53° C for 30 s, 72° C for 1 min.). All primer sets spanned more than one exon to identify amplification of genomic DNA. Controls without template were included to detect DNA contamination. Ten μl of each reaction were electrophoresed on a 1.5% agarose gel, transferred to a Nytran membrane, and Southern blotted. After prehybridization, blots were probed with a ^{32}P-labeled PSG28-specific probe (5'-TGTACTATAGTTTAACAGCG-3'), a PSG23-specific probe (5'-TAACTATAGTGTCCGAGAC-3') or an HPRT-specific probe (5'-GTTGTTGGATATGCCCTTGAC-3') for 1 hour at 42° C. The PSG28-specific probe spanned a 12 nucleotide gap in the N1 domain that is unique to PSG28. Blots were washed two times for 5 min. each in 2X SSPE, 0.1% SDS at 25° C, followed by two 20 min. washes in 5X SSPE, 0.1 % SDS, at temperatures of 52° C, 50° C, and 55° C for PSG28, PSG23 and HPRT probes, respectively, and the blots were exposed to Kodak XAR film (Eastman Kodak Company, NY).

Generation of Recombinant Baculovirus Transfer Vectors

Complementary DNAs encoding PSG1(d) (426 amino acids, predicted Mr 47.9 kD; minus leader 44.1 kD), and PSG6(r) (435 amino acids, predicted Mr 48.8 kD; minus leader 45.1 kD), originally isolated from fetal liver (Zimmermann et. al., 1989) and PSG11(s) (426 amino acids, predicted Mr 48 kD; minus

leader 44.4 kD), isolated from human placenta (Brophy et. al., 1992) were cloned into the pAcSecG2T baculovirus transfer vector (Pharmingen, CA). The pAcSecG2T vector provides a strong baculovirus promoter (polyhedrin), a baculovirus leader sequence for secretion (gp67) and an N-terminal glutathione S-transferase (GST) tag, plus an *E. coli* origin of replication and an ampicillin resistance gene for plasmid propagation in *E. coli*. PSG cDNAs were introduced into the baculovirus genome of *Autographa californica* nuclear polyhedrosis virus (AcNPV) by homologous recombination via cotransfection of the transfer vector and AcNPV baculovirus DNA into *Spodoptera frugiperda* (Sf9) insect cells. The pAcSecG2T plasmid contains a promoter and multiple cloning site flanked by baculovirus DNA. A modified version of AcNPV virus ("BaculoGold DNA", Pharmingen, CA), containing a lethal deletion (near the target site for insertion of the foreign gene) that is rescued by recombination with the transfer vector, allows positive survival selection of recombinant virus. GST fusion proteins produced from pAcSecG2T contain a thrombin cleavage site (6 amino acids) between the GST tag and the protein of interest to facilitate release of untagged protein if desired. A control protein XylE, a product of *Pseudomonas putrida*, was also produced as a fusion with GST in insect cells.

PSG1(d), PSG6(r), and PSG11(s) cDNAs were kindly provided by Dr. Wolfgang Zimmermann (University of Freiburg, Germany) as full-length cDNA clones in Bluescript (Stratagene, CA). These cDNA sequences were amplified by PCR using AmpliTaq DNA polymerase (Perkin Elmer Cetus, CA), dNTPs and 1X PCR buffer as described above, and specific 5' oligonucleotides containing a *Bgl* II restriction site at the 5' end. The 5' primers consisted of sequence from the beginning of the N1 domain of the PSG sequences. A leader sequence from the baculovirus transfer vector was used in place of the PSG signal peptide to effect secretion of the recombinant proteins. The 3' oligonucleotides included an *Eco*R1 site at the 5' end, and PSG sequence from the C-terminal domain ending with the stop codon. The PSG1 sense and antisense primers were 5'-GCAGATCTCAAGTCACGATTGAAGCC-3' and 5'-TGGAATTC-*CTA*AAACCCTATTGCCAAC-3', respectively, where the restriction sites are underlined and the stop codon is italicized. The PSG6 sense and antisense primers were 5'-GCAGATCTCAAGTAATAATT-GAAGCC-3' and 5'-TGGAATTC*TCA*CAGCAGAAGGATTTC-3', respectively. The PSG11 sense and antisense primers were 5'-GCAGATCTGAAGTCACGATTGAAGCC-3' and 5'-GCGAATTC*TCA*-TGACTGAGACTCTGT-3', respectively. PSG1, PSG6 and PSG11 cDNAs were amplified using 30 pmol

of the 5' and 3' primers for 30 cycles (94° C for 1 min., 55° C for 1 min. annealing for PSG1, PSG6, and PSG11, and 72° C for 1.5 min.). The PSG PCR products were agarose gel-purified, digested with *Bgl* II and *Eco*R1, and ligated into the baculovirus transfer vector pAcSecG2T (previously digested with *Bam* H1 and *Eco*RI). The presence of PSG cDNAs within recombinant pAcSecG2T was confirmed by Southern blot hybridization with ^{32}P-labeled internal specific oligonucleotide probes for each PSG sequence. The PSG1-, PSG6- and PSG11-specific oligonucleotide probe sequences from the N-terminal domains are 5'-ATCATATGTAGTAGACGGTGAAATAA-3', 5'-CAAATTATATATGGGCCTGCCTA-3', and 5'-CATTATATCGTATATAGTTGATGGTAA-3', respectively. Blots were washed first in 2X SSPE, 0.1% SDS as described above, followed by two washes in 5X SSPE, 0.1% SDS at temperatures of 57° C, 59° C, or 59° C, for PSG1, PSG6 and PSG11, respectively.

To produce a GST fusion protein containing only the N1 domain of PSG6, the linearized pAcSecG2T-PSG6 construct was used as template for a PCR reaction using sense primer 5'-GGGAATTCATGCTACTAGTAAATCAGTC-3', containing an *Eco*RI site (underlined), and the antisense primer 5'-AAGCTTGTATAAGGTGACAGTGAA -3', with a *Hind*III site. PSG6N was amplified using 2.5 U of AmpliTaq DNA polymerase for 2 cycles of 94° C for 30 s, 46° C for 30 s, and 72° C for 50 s, followed by 23 cycles of 94° C for 30 s, 55° C for 30s, and 72° C for 50 s in a thermal cycler. The product, GST-PSG6N, contained the gp67 leader, the N-terminal GST tag sequence and the N1 domain PSG6 sequence. The PCR product was subcloned into the *Eco*RI-*Hind*III sites of pcDNA3.1(-)/Myc-His (Invitrogen Corp., CA) to obtain a 3' Myc-6XHis tag and a stop codon. The GST-PSG6N insert containing the Myc-His sequence (21 amino acids) was subsequently excised from pcDNA3.1(-)/Myc-His using *Pme* I (a blunt cutter) and subcloned into the pFastBac1 donor plasmid (Life Technologies, MD), which had been digested with the blunt cutter *Stu* I. The presence of the N1 domain was confirmed by Southern blot using the same PSG6-specific internal oligonucleotide probe used to detect the PSG6 sequence in pAcSecG2T. The orientation of the blunt-ended insert in pFastBac1 was determined by restriction digestion.

Production of Recombinant Baculovirus Expressing PSG cDNAs

Recombinant virus expressing GST-PSG1, GST-PSG6 and GST-PSG11 were obtained by cotransfection of the recombinant transfer plasmid pAcSecG2T-PSG1, -PSG6, or -PSG11, respectively,

with BaculoGold DNA into Spodoptera frugiperda (Sf9) insect cells (Invitrogen Corporation, CA). Insect cells were grown in Sf900 II media (Life Technologies, MD) in the presence of 5 % fetal bovine serum (Hyclone, VT) at 27°C. After five days the cell supernatant was collected and progeny virus was plaque-purified following the manufacturer's instructions (PharMingen, CA). The recombinant virus was identified by an altered plaque morphology (occlusion negative), characterized by the absence of occluded virus in the nucleus of the infected Sf9 cells. Virus was isolated from the chosen plaques and propagated in Sf9 cells for four or five cycles of infection and virus harvest to generate high titer virus stocks for the production of GST-PSG1, GST-PSG6 and GST-PSG11 proteins. Viral titers were determined by plaque assay according to the manufacturer's instructions (PharMingen, CA).

Recombinant virus expressing the control fusion protein GST-XylE was obtained by cotransfection of BaculoGold DNA and the baculovirus transfer vector pAcGHLT-XylE (PharMingen, CA) into Sf9 cells as indicated above for PSG-containing virus. The recombinant PSG glycoproteins were secreted into the supernatant of infected Sf9 cells, while the GST-XylE protein was produced cytoplasmically and was isolated from Sf9 cell lysates as a non-glycosylated protein.

Recombinant baculovirus expressing GST-PSG6N was produced using the Bac-to-Bac Baculovirus Expression System (Life Technologies, MD). This system is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector or "bacmid" propagated in *E. coli* (Figure 4). The bacmid contains a kanamycin resistance marker and a segment of DNA encoding the lacZα polypeptide. The bacmid is propagated in DH10Bac cells (Life Technologies, MD), where it complements a lacZ chromosomal deletion to form blue colonies in the presence of Bluo-gal or X-gal substrate and the inducer IPTG. DH10Bac *E. coli* also contains a helper plasmid that provides elements necessary for Tn7 transposition. Within the lacZα sequence of the bacmid is an attachment site (mini-attTn7) for the bacterial transposon Tn7. Recombinant bacmids are constructed by transposing a mini-Tn7 element from a pFastBac donor plasmid to the mini-attTn7 attachment site in the bacmid. The transposition event disrupts the lacZα sequence leading to the loss of complementation of the lacZ gene, and colonies containing recombinant bacmid are white in a blue background. Within the mini-attTn7 element of the pFastBac1 plasmid is a multiple cloning site downstream of the strong polyhedrin promoter, and an SV40 polyadenylation signal. After transposition and selection of (white) *E. coli* clones, the

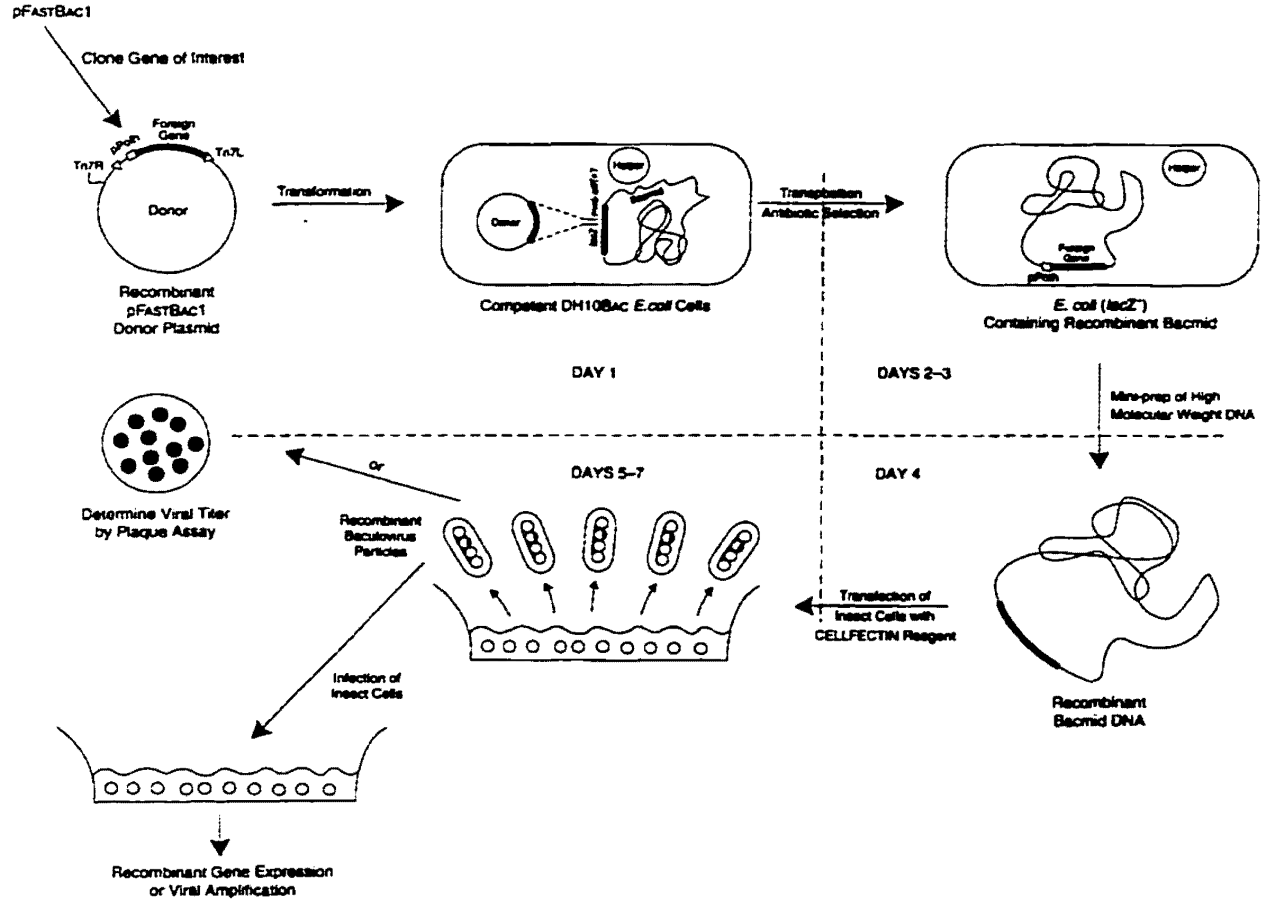

Figure 4. Generation of recombinant baculoviruses with the Bac-to-Bac expression system. From "Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques", (Life Technologies, Gaithersburg, MD, p 4.)

recombinant bacmid DNA is isolated by high molecular weight mini-prep, and transfected into insect cells to produce virus.

DH10Bac competent *E. coli* was transformed with Qiagen maxiprep-purified pFastBac1-GST-PSG6N plasmid DNA, and high molecular weight DNA was isolated from selected white colonies (grown on LB agar plates containing kanamycin [Sigma, MO], Bluo-gal and IPTG [Life Technologies, MD]), by miniprep according to the manufacturer's instructions. The high molecular weight bacmid DNA was transfected into Sf9 cells with CellFECTIN reagent (Life Technologies, MD). Passage-1 (P1) virus was collected 3 days later and was propagated as described above to generate high titer P4 virus stock.

Production of Recombinant Human PSG Proteins

To produce recombinant PSG1, 6, 11, PSG6N, and XylE protein, Sf9 cells were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 5. Infected cells were grown in Sf900 II SFM media (Life Technologies) supplemented with 5% fetal bovine serum (FBS, low endotoxin, Hyclone, VT), 50 units/ml penicillin and 50 μg/ml streptomycin (Quality Biologicals, MD) for 24 to 72 h at 27° C in ambient air. The recombinant PSG proteins were secreted into the supernatant fluid as N-terminal GST fusion proteins from baculovirus-infected Sf9 insect cells. Virus-infected insect cell supernatant was collected and stored at -20° C. To obtain recombinant GST-XylE protein, infected insect cells were lysed with RIPA buffer (0.1 M NaCl, 0.001 M EDTA [pH 7.4], 0.1% Nonidet P-40, 0.1% deoxycholate, 1% phenylmethylsulfonyl fluoride [PMSF] and 1% aprotinin) for 45 min. on ice. Cellular debris was removed from the lysate by centrifugation at 1000 x g for 5 min. The protease inhibitors PMSF (1%) and leupeptin (1%) (Sigma, ML) were added to harvested insect cell supernatant or cell lysate, which was stored at -20° C or 4 °C.

Time Course of Recombinant Human PSG Protein Production

In order to determine the optimal protein harvest time, a time course assay for protein production was performed. Baculovirus-infected insect cell supernatant or lysate was harvested at 24, 48, 72 and 96 hours after infection, clarified by centrifugation at 1000 x g for 5 min., and GST-fusion protein was detected by immunoblot using a monoclonal antibody to the GST tag (PharMingen, CA). For immunoblot, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4-12% NuPAGE Bis-Tris HCl-buffered polyacrylamide gels (Novex, CA), transferred to nitrocellulose

membranes (Schleicher and Schuell, NH) and blocked overnight in TBST (25 mM Tris [pH 7.6], 150 mM NaCl, 0.1 % Tween 20) supplemented with 5% powdered milk at 4°C. The membranes were incubated with 1 μg/ml anti-GST antibody in TBST-milk for 1 h at 25°C. After three 5 minute washes in TBST, the membranes were incubated for 1 hour with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibody (Biorad Laboratories, CA). After three 20 minute washes in TBST, signal was detected using the Super Signal West Pico Chemiluminescent Substrate (Pierce, IL) by autoradiography using Kodak XAR film. The 72 h time point resulted in maximal protein production for all recombinant proteins, so this time point was used to harvest GST-XylE and GST-PSG protein in all experiments.

Purification and Analysis of Recombinant Human PSG Proteins

GST-tagged proteins were purified by three sequential rounds of affinity chromatography using glutathione-sepharose beads or concanavalin A-sepharose 4B beads (Sigma, MO) (Figure 5). All procedures were carried out aseptically to prevent bacterial LPS contamination of recombinant protein. Clarified cell supernatant or lysate was incubated with glutathione-linked or concanavalin A-sepharose (con A) beads from 2 hours to overnight at 4° C. The beads were washed with cold phosphate buffered saline (PBS is 0.25 M NaCl, 1 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 7.4, Quality Biological Inc., MD), and fusion protein was eluted from the beads by incubation with 6 mM reduced glutathione (Sigma, MO) in 50 mM Tris pH 8, or 500 mM methyl-α-D-mannopyranoside (Sigma, MO) in 20 mM Tris pH 7.4, 0.5 M NaCl, for glutathione- and con A-linked beads respectively, for 30 min at 4°C. Elutions were repeated three times to collect maximum recombinant protein from the beads. The insect cell supernatant collected after one round of binding to beads was found to contain significant amounts of recombinant protein, so supernatant was re-incubated with sepharose beads for a total of three incubations, and protein was eluted as described above. Elutions were pooled, concentrated 10-15 fold using a Centriprep 10 ultrafiltration concentrator with a molecular weight cut-off of 10 kD (Millipore Corporation, MA), and dialyzed by addition of 10 volumes of PBS, followed by reconcentration by Centriprep 10. The control GST-XylE fusion protein was purified from the insect cell lysate by the same procedures used for the PSG fusion proteins, except that since XylE is not glycosylated, con A-linked beads were not used. The control supernatant, termed "XylE sup", was prepared by collecting supernatant from GST-XylE baculovirus infected cells, incubating it with glutathione-linked beads, eluting with 6 mM reduced glutathione, and

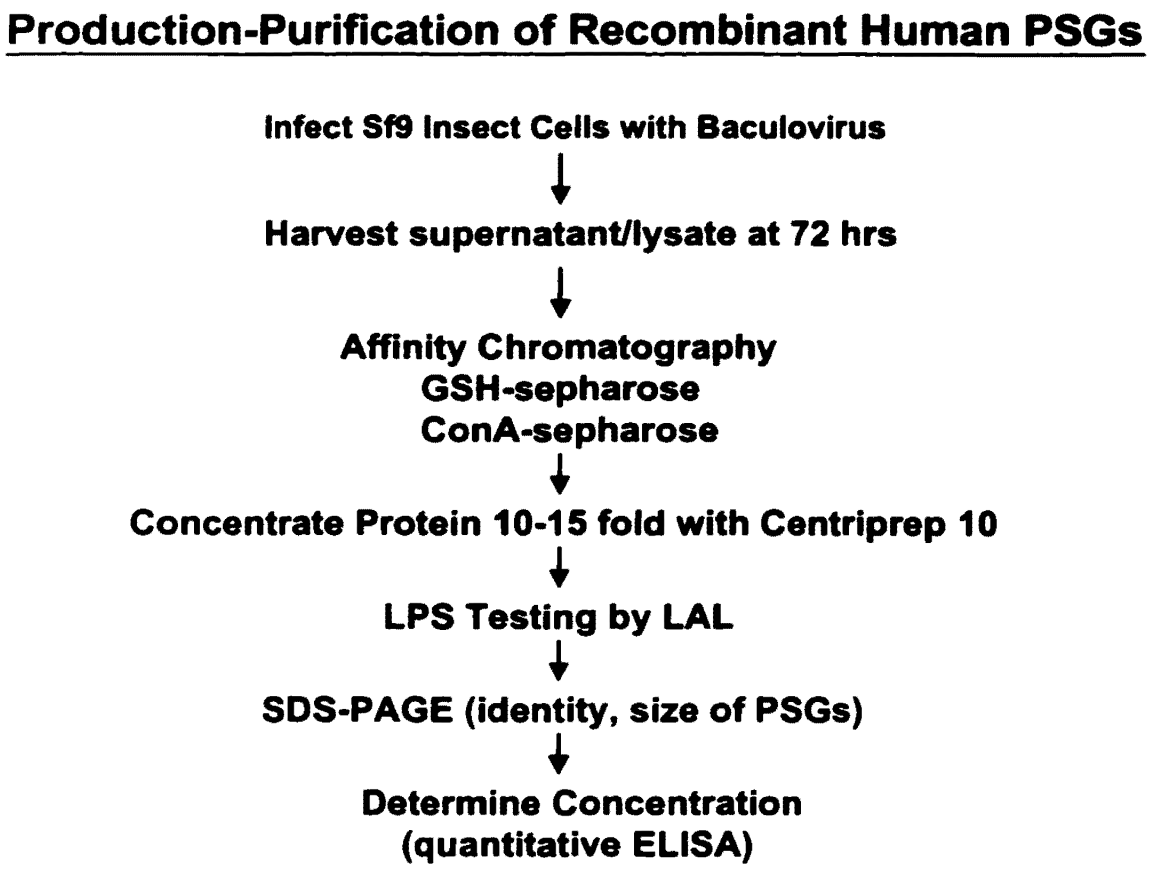

Figure 5. Production and purification of recombinant PSG proteins

concentrating with Centriprep 10 as described for PSG protein-containing supernatant.

Recombinant PSG protein purity, molecular weight, and concentration were evaluated. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted as described above. Recombinant protein was detected by Western blot using a monoclonal antibody to PSGs (kindly supplied by Dr. W. Zimmermann, University of Freiburg), monoclonal antibody to the GST tag (PharMingen, CA), or rabbit polyclonal anti-PSG antisera (Boehringer Mannheim Corp., IN; Dako Corporation, CA). Primary antibody binding was detected using HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Biorad Laboratories, CA) and Super Signal Enhanced Chemiluminescence (Pierce, IL). Protein purity was assessed by Coomassie staining after SDS-PAGE using Gel Code Blue (Pierce, IL). PSG and XylE protein concentration was measured by comparison to bovine serum albumin (BSA) concentration standards (10 – 100 ng/μl) on Coomassie stained SDS-PAGE gels using the Eagle Eye II Still Video System and Eagle Sight version 3.1 software, (Stratagene, CA). Concentration of GST-tagged recombinant proteins was also measured using a GST detection enzyme-linked immunosorbant assay (ELISA) (Pharmacia Biotech, NJ), which has a detection range of 0.1 pg/μl – 1 ng/μl. To show glycosylation of recombinant PSG protein produced in insect cells, recombinant PSG proteins were precipitated with concanavalin A-sepharose (Sigma, MO) as described above. Evidence for glycosylation of PSGs was also suggested by apparent relative molecular weight in SDS-PAGE compared to expected unglycosylated size (70 kD with GST tag). LPS contamination of recombinant protein preparations was measured by the QCL-1000 Limulus Amoebocyte Lysate (LAL) assay (BioWhittaker Inc., MD), which has a detection range of 0.1 - 1.0 Endotoxin Units/ml. Molecular weight of recombinant proteins was calculated by comparison to Benchmark Molecular Weight Standards (Life Technologies, MD) on autoradiographs from Western blots using the Eagle Sight Version 3.1 software and the Eagle Eye II Still Video System (Stratagene, CA).

Measurement of Recombinant Human PSG Induction of Monocyte Cytokine Secretion

We measured induction of IL-10, IL-6, TNF-α, IL-1β and IL-12 protein secretion by human monocytes incubated with GST-PSG1, -6, and -11 fusion proteins at 24 h post-treatment. All treatments were done in triplicate in at least three independent experiments, and results were compared to cytokine induction by control GST-XylE protein or XylE sup. LPS treatment (100 ng/ml) was used as a positive

control in all experiments. Protein-free *E. coli* K235 LPS was obtained by hot phenol-water extraction and was kindly provided by Dr. Stefanie Vogel (USUHS, MD). Elutriated human monocytes (96 % pure) from healthy adult donors were obtained from Dr. Larry Wahl (NIDR, NIH, Bethesda, MD). Human peripheral blood cells were obtained by leukapheresis of normal volunteers at the Department of Transfusion Medicine at the National Institutes of Health. These cells were diluted in endotoxin-free PBS with out Ca^{2+} and Mg^{2+} (BioWhittaker, MD), and layered over 20 ml of endotoxin-free lymphocyte sedimentaton medium (Organon Teknika, NC) in 50 ml tubes. After density sedimentation at 400 x g for 30 minutes, the monocytes in the mononuclear cell layer were purified by counterflow centrifugal elutriation using a Beckman (Beckman, CA) elutriation system (Wahl et. al., 1984). Counterflow centrifugal elutriation is a centrifugal separation method based on cell size and density that does not lead to monocyte activation. Freshly isolated human monocytes in Macrophage-Serum Free Medium (M-SFM, Life Technologies, MD) were added to 96-well tissue culture plates (Corning, NY) at 0.4 x 10^6 cells/well.

Monocytes were allowed to adhere to the dish for 1 hour at 37° C in a CO_2 (5%) incubator. (Monocytes are strongly adherent at temperatures above 20° C.) Media was removed from the wells, and 100 µl of M-SFM (untreated control), LPS (100 ng/ml) in M-SFM, or various concentrations of affinity-purified GST-PSG or GST-XylE (negative control) in M-SFM were added to the adherent cells in triplicate wells. After 3 hours of incubation at 37°C, 100 µl of M-SFM was added to each well. At 24 h after the initial treatment, supernatant fluid was harvested from each well and frozen at –20°C. Cytokine protein in the supernatant was quantitated using enzyme immunoassay sandwich ELISA kits by comparison to recombinant cytokine standard curves (Immunotech, Marseille, France). The lower limits of detection were 5 pg/ml for IL-10, IL-12, and IL-1β, and 10 pg/ml for IL-6 and TNF-α. Data was analyzed for significance using one-way analysis of variance (ANOVA) and pairwise comparisons were done by the Student-Newman-Keuls method with $p< 0.05$, using the SigmaStat statistical program (Jandel Scientific Software, CA).

PSG Dose Response Curves for IL-6 and IL-10 Induction

Dose response curves for IL-6 and IL-10 secretion by monocytes using all three PSG fusion proteins were performed by varying the PSG dose over a 100-fold range. Monocytes from two different donors were treated in 96-well tissue culture dishes with equal concentrations of each recombinant GST-

PSG protein or GST-XylE, and cytokine secretion at 24 hours after treatment was measured by quantitative sandwich ELISA as described above. Monocytes were treated with PSG1 at 0.1, 1, 2, 3, 5, 8 or 11 μg/ml, PSG6 at 0.08, 0.8, 1.5, 2.3, 3.8, 6.1 or 8.5 μg/ml, or PSG11 at 0.17, 1.7, 3, 5, 8, 13 or 18 μg/ml. LPS dose response curves for IL-10 and IL-6 induction by monocytes were generated to compare to PSG induction of these cytokines. Monocytes from three different donors were treated with LPS concentrations of 0.01, 0.1, 1, 10, 100, and 1000 ng/ml. Data was analyzed using two-way ANOVA and pairwise multiple comparisons were done using Student-Newman-Keuls method with $p < 0.05$.

Immunodepletion of PSG Protein

To show the specificity of cytokine induction by the GST-PSG fusion proteins, PSG-containing insect cell supernatants were depleted of PSG protein by immunoprecipitation. Concentrated GST-PSG6 and GST-PSG11 (2 ml, 20μg/ml) were incubated with 10 μl polyclonal rabbit antiserum to PSGs or normal rabbit serum (NRS) (Boehringer Mannheim Corp., IN) overnight at 4°C. Protein A-sepharose beads (100 μl of a 50% slurry) were added to the solution and incubated for 1 hour at 4°C to precipitate the immune complexes. The beads were removed by centrifugation, and the immunoprecipitation was repeated for a total of three times in order to remove all PSG protein from the protein solution. After testing for LPS by limulus amoebocyte lysate assay, samples of the starting material, control (NRS) and the PSG-depleted supernatants were tested for presence of GST-PSG by Western Blot with antibody to the GST tag. Monocyte cells were treated in triplicate in 96-well tissue culture dishes in three independent experiments with equal volumes of the control (NRS) immunodepleted protein (approximately 8 μg/ml PSG) or PSG-depleted protein as described above, and IL-10 and IL-6 induction at 24 hours was measured by ELISA. Data was analyzed for significance using one-way ANOVA and Student-Newman-Keuls test.

Time Course of IL-10 and IL-6 Protein Secretion after Treatment of Monocytes with PSGs

We determined the kinetics of IL-10 and IL-6 secretion by monocytes after induction by recombinant PSGs. Monocytes were treated in triplicate wells of 96-well dishes in two independent experiments with 12 ug/ml GST-PSG1, GST-PSG6, GST-PSG11, GST-XylE or 100 ng/ml LPS in 96-well plates as described above. Cell supernatant was collected at 2, 6, 10, 13 and 22 hours after treatment, and IL-10 and IL-6 protein was measured by sandwich ELISA (Immunotech, Marseille, France). Data was analyzed for significance using two-way ANOVA and Student-Newman-Keuls test with $p < 0.05$.

Semi-quantitative RT-PCR Analysis of IL-10 and IL-6 mRNA in PSG6-treated Monocytes

Monocytes (3.5 x 10^6/well) seeded into 12-well tissue culture dishes (Corning, NY) were treated in triplicate in 3 independent experiments with 10μg/ml GST-PSG6 or GST-XylE in 500μl total volume of M-SFM media, with or without 5 μg/ml cycloheximide (Sigma, MO). Monocytes were also treated with cycloheximide alone as a control. Two hours after treatment, 300 μl M-SFM was added to each well. Five hours after treatment, total RNA was harvested from each well using 900 μl TRIzol Reagent (Life Technologies, MD), extracted with 180 μl chloroform, and precipitated overnight with 450 μl isopropanol according to the manufacturer's instructions (Life Technologies, MD). After precipitation, RNA was resuspended in 30 μl sterile DEPC (diethyl pyrocarbonate) H_20 (Quality Biological Inc., MD), and quantified by UV spectrophotometry. Three μg total RNA from each treated well were reverse transcribed using 50 μM random hexamer primers and Ready-to-Go You-Prime First Strand Beads (Pharmacia Biotech, NJ) in a 33 μl reaction volume as described above. Three μl of the cDNA reaction were used for each PCR reaction with 1.25 U of Taq DNA polymerase (Promega Corp., WI), dNTPs and 1X PCR buffer. IL-6 was amplified using the sense 5'-GATTCCAAAGATGTAGC-3' and antisense 5'-GATTTTCACCAGGCAAGTCTCC-3' primers for 22 cycles (94° C for 30 s, 44° C for 40 s, 72° C for 40s) to generate a 243 bp product. IL-10 was amplified using sense 5'-CGGGAAGACAATAACTG-3' and antisense 5'-CATTTCCGATAAGGCTTGG-3' primers for 25 cycles (94° C for 30 s, 60° C for 40 s, 72° C for 40 s) to generate a 427 bp product. Glyceraldehyde-phosphate dehydrogenase (GAPDH) was amplified using sense 5'-CCATGGAGAAGGCTGGGG -3' and antisense 5'-CAAAGTTGTCATGG-ATGACC -3' primers for 18 cycles (94° C for 30 s, 50° C for 40 s, 72° C for 40 s) to generate a product of 195 bp. 15 μl of each PCR reaction were electrophoresed on a 1.8% agarose gel, and transferred to a Nytran membrane overnight in 20X SSPE. The amount of PCR product was quantified by Southern blotting with specific ^{32}P-labeled oligonucleotide probes (5'-GGATGCTTCCAATCTGGA-3' for IL-6, 5'-GGACTGCCTTCAGCCAGGTGAAGACTTT-3' for IL-10, and 5'-CTAAGCATGTGGTGGTGCA -3' for GAPDH). Blots were washed at 47° C, 57° C, or 53° C in 5X SSPE, 0.1% SDS for IL-6, IL-10 and GAPDH, respectively, followed by densitometric analysis using a Storm 860 PhosphorImager and the ImageQuaNT program (Molecular Dynamics, CA). The densitometric values for IL-10 and IL-6 mRNA

were normalized by dividing by the amount of GAPDH PCR product amplified from each well, and were expressed as fold induction over the XylE control value. Data was analyzed by one-way ANOVA as described above.

Measurement of IL-6 and IL-10 Secretion by Monocytes After Treatment with PSG6N

Monocytes were seeded into 96-well tissue culture dishes and treated in triplicate in three independent experiments with 8μg/ml GST-XylE, GST-PSG6 or the truncated fusion protein GST-PSG6N as described above. Cell supernatant was harvested after 24 hours, and induction of IL-6 and IL-10 was analyzed by quantitative sandwich ELISA. Dose response curves for PSG6N induction of IL-6 and IL-10 were performed using 1, 2.5 and 10 μg/ml of GST-PSG6N or GST-XylE. Statistical significance was determined using two-way ANOVA and Student-Newman-Keuls test.

Analysis of PSG6 Intracellular Signal Transduction Pathways

To investigate the intracellular signals involved in PSG-stimulated IL-6 and IL-10 secretion by monocytes, we used a strategy of treatment with PSG6 and specific inhibitors or activators for signal transduction pathways together with measurement of cytokine secretion. At the tested concentrations, the inhibitors had no effect on cell viability compared to untreated cells as assessed by trypan blue exclusion assay of the recovered cells, which were approximately 80% viable at 24 hours after treatment. Monocytes in 96-well tissue culture dishes were treated in triplicate in three independent experiments with 12 μg/ml GST-PSG6 or GST-XylE, with or without either 500 nM Herbimycin A (Calbiochem, CA) in DMSO, 1 μM Calphostin C (Calbiochem, CA) in DMSO, 1 μM rolipram (Calbiochem, CA) in H_20, or 300 μM (Rp)cAMPS (Sigma, MO) in H_20. The IC_{50} (50% inhibitory concentration) of Herbimycin A is 900 nM, Calphostin C is 50 nM, Rolipram is 800 nM and (Rp)cAMPS is 11 μM. Monocytes were also treated with each inhibitor alone and with DMSO alone to assess the effects of these chemicals on cytokine secretion. In addition, monocytes were treated with GST-PSG6 plus DMSO to determine whether DMSO inhibited PSG6 induction of cytokines. After 3 hours 100 μl of M-SFM media was added to each well. Supernatant fluid was harvested at 24 hours after treatment and assayed for IL-6 and IL-10 cytokine secretion by quantitative sandwich ELISA. Data was analyzed for significance using one-way ANOVA and Student-Newman-Keuls test. None of the inhibitors induced IL-6 or IL-10 secretion by monocytes, and DMSO did not decrease PSG6 induction of IL-6 or IL-10.

Cross-Species Induction of IL-6 and IL-10 by PSGs

To investigate whether PSGs had any cross-species activity, we treated a mouse macrophage cell line RAW 264.7 (American Type Culture Collection, VA) with recombinant human GST-PSG1, GST-PSG6, GST-PSG11 and GST-XylE, and measured IL-6 and IL-10 secretion. RAW 264.7 cells were seeded into 24-well tissue culture dishes in DMEM with high glucose (Irvine Scientific, CA) supplemented with 10% FBS, 0.25 μg/ml amphotericin B, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM glutamine (Quality Biologicals Inc., MD). Adherent RAW 264.7 cells were treated in triplicate in three independent experiments with 8 μg/ml GST-PSG1, GST-PSG6, GST-PSG11 or GST-XylE in 300 μl total volume DMEM, and were incubated at 37° C in a 5% CO_2 incubator. 24 hours after treatment cell supernatant was collected and stored at -20° C. Mouse IL-10 secretion was measured using a murine IL-10 sandwich ELISA (Endogen Inc., MA) and mouse IL-6 was measured by Dr. Florence Rollwagon (USUHS, MD) using an IL-6 ELISA assay (Biosource, CA). The lower limits of detection of these assays were 12 pg/ml and 10 pg/ml, respectively.

To examine if mouse PSGs could induce cytokine secretion in human monocytes, cells were treated with recombinant protein consisting of the N-terminal Ig variable-like domain of PSG18 (PSG18N). This protein was synthesized using the baculovirus insect cell expression system and purified by SDS-PAGE (J. Wessells, 1999 doctoral thesis, USUHS). Human monocytes were seeded into 96-well dishes as described above, and treated in triplicate in three independent experiments with 30 μg/ml PSG18N. Twenty-five μg/ml PSG18N induces IL-10 secretion in RAW 264.7 cells. Cell supernatant was harvested 24 hours after treatment and analyzed for IL-10 and IL-6 secretion by quantitative sandwich ELISA. Data was analyzed for statistical significance using one-way ANOVA and Student-Newman-Keuls test.

III. Results

Cloning Murine PSG23 and PSG28 cDNAs

As a basis for future in vivo functional studies, a murine model for the PSG family of proteins is being developed in our laboratory. At the present time, although 15 PSG genes have been identified, only five murine PSG cDNAs (CEA6, Psg16/bCEA, Psg17, Psg18 and Psg19) have been cloned. Cloning and sequencing new murine PSG family members will allow future studies of the functions of individual PSGs and the identification of their specific receptors. A complete mouse model for PSG function requires the delineation of the primary structures of all mouse PSG proteins, and determination of the site and temporal pattern of their expression. To further characterize the murine PSG family, we obtained novel full-length murine PSG cDNA clones from placental mRNA and determined whether extraplacental sites of expression existed.

Partial genomic sequences, consisting of the first two exons or just the second exon, for 10 newly discovered murine PSGs have been identified (Zimmermann et. al., submitted). As measured by RT-PCR, PSG23 has been shown to be one of the most highly expressed mouse PSGs (Zimmermann et. al., submitted). PSG28 has a QGE sequence motif in the N1 domain, while PSG23 has the more RGD-like HGE sequence. We used a PCR-based cloning strategy to clone cDNAs encoding these two members of the mouse PSG family. The cloning method was designed to produce full-length cDNA clones from placental RNA using specific 5'UTR sequences and oligo(dT) 3' end primers. Using the partial genomic sequences, we designed specific oligonucleotide primers from the 5' end of PSG20 and PSG21 (the PSGs for which the 5' UTR is available), consisting of sequences upstream of, or including the start codon, where there are known differences between these PSGs and other family members. PCR products were sub-cloned into the vector pCR2.1 (Invitrogen Corp., CA), and selected by hybridization to mouse PSG-specific universal oligonucleotide probes. The plasmid inserts of two PSG28 clones and one PSG23 clone were sequenced. The sequences of the two PSG28 cDNA clones were found to be identical. Both the PSG23 and PSG28 cDNAs code for a 34 amino acid leader peptide (L), three IgV-like domains (N1-N3) and an IgC-like "A" domain. The N2 and N3 domains were preceded by hydrophobic partial leader-like sequences (L') of 9-12 amino acids, as is characteristic of mouse PSGs. The putative PSG23 and PSG28 preproteins contain 471 and 472 amino acids, respectively, and the mature glycosylated proteins are

estimated to have a molecular mass of 77 kD, including N-linked carbohydrate (3-4 kD per site) at seven potential sites within the IgV-like domains. PSG23 cDNA (Genbank accession number AF113599) contains an open reading frame of 1413 bp, encoding an N1 domain of 107 amino acids, N2 and N3 domains of 108 amino acids each, and an A domain of 93 amino acids (Figure 6). The N1 domain has a one amino acid gap at codon 41, and a three amino acid gap at the beginning of the L'3 partial leader sequence compared to PSG17-19, CEA6 and PSG28. This PSG cDNA has an RGD-related motif, HGE, in a conserved position within the N-terminal domain. (Nucleotides 1-9 and 13-19 of PSG23 cDNA are uncertain because they are part of the primer designed from the PSG21 sequence.) PSG28 (Genbank accession number AF113598) contains an open reading frame of 1416 bp, encoding an N1 of 105 amino acids, N2 and N3 domains of 108 amino acids, and an A domain of 93 amino acids (Figure 7). The N1 domain shows a three amino acid gap after codon 81 in comparison to the other known mouse PSG cDNAs. An RGD-like motif, QGE, is present in the N1 domain. The IgC-like A domains of both clones contain the conserved cysteine residues of an Ig domain. The 3' untranslated region of the PSG28 cDNA has a degenerate polyadenylation signal (AUUAAA) located 331 nucleotides after the stop codon. Both the polyadenylation signal and a poly(A) tail are missing from the PSG23 clone. The N1 domains of the mouse PSG genes, including PSG23 and PSG28, share between 54% and 94% amino acid similarity (Zimmermann et. al., submitted).

Tissues Sites of Mouse PSG23 and PSG28 mRNA Expression

Expression of mRNA encoding human PSGs has been reported for various tissues, and protein expression in testes, intestine and fibroblast cell lines has been observed (Borjigin et. al., 1990, Shupert and Chan, 1993, Engvall et. al., 1982). Therefore we examined whether expression of murine PSG23 and PSG28 could be detected in placental and nonplacental tissues (Figure 8). RT-PCR produced an amplification product of 487 bp for PSG23, a product of 375 bp for PSG28, and a 164 bp product for HPRT. PSG23 and PSG28 were detected only in placental tissue, and not in bone marrow, liver, brain, thymus, heart, kidney, lung, testes or ovary. The amounts of the HPRT PCR product were similar in all tissues examined. These results are similar to those obtained for mouse PSG17, 18 and 19 (J. Wessells, USUHS doctoral thesis), which were found to be expressed in placenta and whole embryo only. Thus far it

Psg23 cDNA

Figure 6. Nucleotide and derived amino acid sequence of PSG23. Nucleotide numbers are shown in the left margin. The start sites of the leader (L), N1, N2 and N3 Ig variable-like domains and their corresponding leader-like sequences (L'), and the Ig constant-like A domain are indicated by arrows above the sequence. Potential N-linked glycosylation sites are thinly underlined, and the RGD-related motif in the N-terminal domain is thickly underlined. The stop codon is represented by three asterisks. The conserved cysteine residues of the Ig constant-like domain are highlighted.

Psg28 cDNA

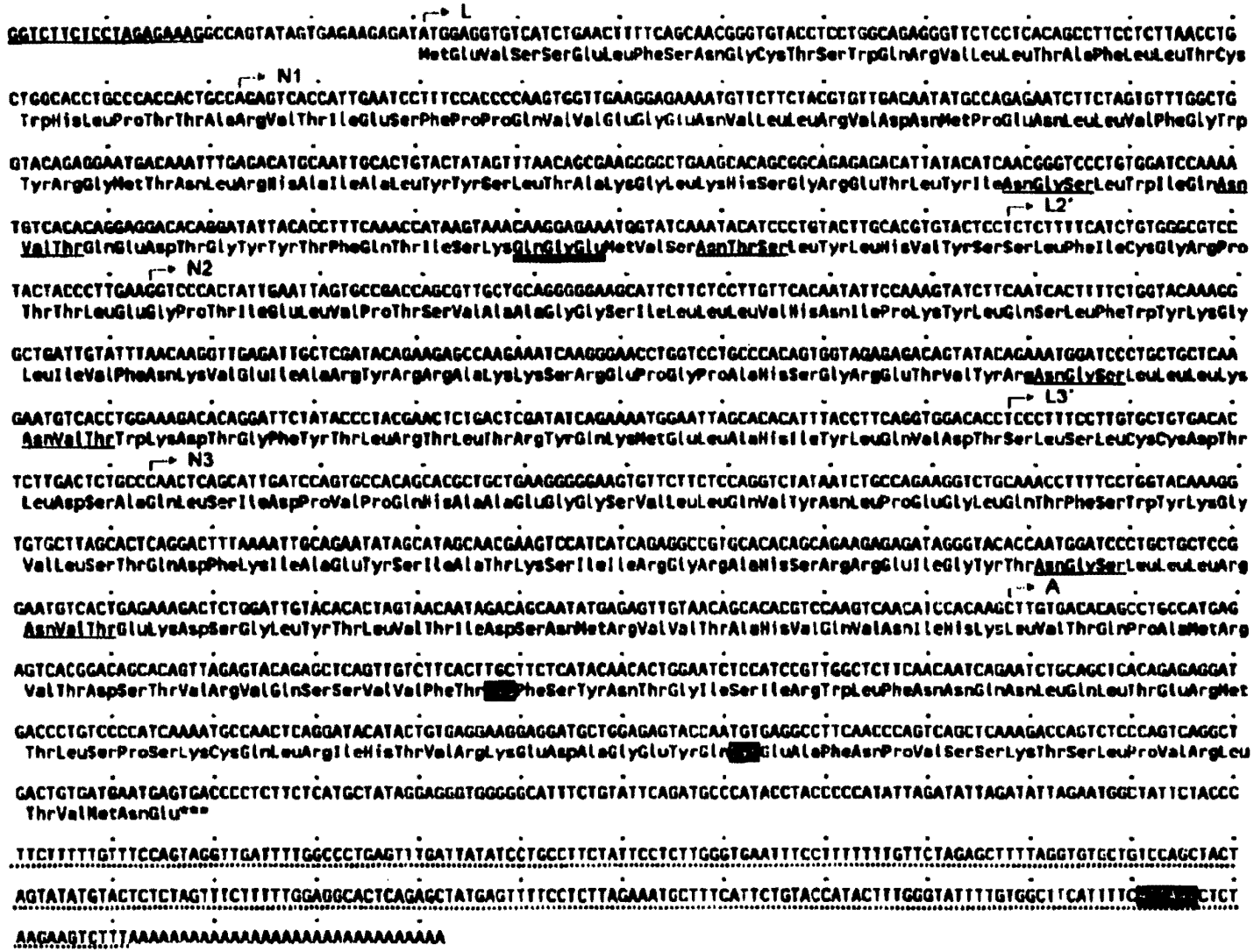

Figure 7. Nucleotide and derived amino acid sequence of PSG28. Nucleotide numbers are shown in the left margin. The start sites of the leader (L), N1, N2 and N3 Ig variable-like domains and their corresponding leader-like sequences (L'), and the Ig constant-like A domain are indicated by arrows above the sequence. Potential N-linked glycosylation sites are thinly underlined, and the RGD-related motif in the N-terminal domain is thickly underlined. The stop codon is represented by three asterisks. The 3' UTR is indicated by broken lines, and the conserved cysteine residues of the Ig constant-like domain and the polyadenylation signal are highlighted.

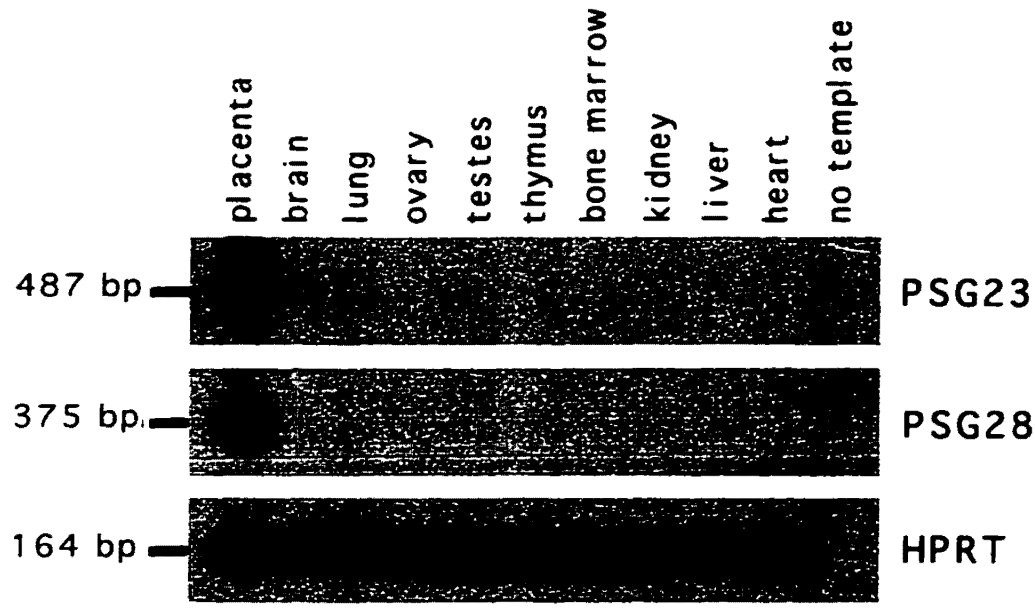

Figure 8. Sites of PSG23 and PSG28 tissue expression. Tissue expression of murine PSG23 and PSG28 was examined by RT-PCR in mouse placenta, brain, lung, ovary, testes, thymus, bone marrow, kidney, liver, and heart. A fragment of the housekeeping gene, HPRT, was amplified for each RNA sample as a control for RNA degradation. All primer sets spanned more than one exon to identify amplification of genomic DNA. Controls without template were included to detect DNA contamination. PSG23 and PSG28 mRNA were detected only in placental tissue.

appears that mouse PSGs have a different expression pattern from that of human PSGs. The mouse PSG gene family evolved independently, and resulted in a PSG domain structure consisting of several IgV-like domains with partial leader sequences and one constant-like domain, rather than the single IgV-like domain and several IgC-like domains of human PSGs. The independent evolution may have also resulted in different biological functions for the mouse and human proteins, although some functions of these two families of proteins are similar. Members from both mouse (J. Wessells, USUHS doctoral thesis) and human families (see below) can increase IL-10 and IL-6 production by monocytes/macrophages. Development of activity in tissues other than placenta may have been precluded in the mouse due to structural differences between mouse and human PSG genes.

Production of Recombinant Human PSG Proteins

Pregnancy specific glycoproteins (PSGs) are a family of highly conserved, secreted proteins produced by the placenta in various species including human (Bohn, 1971), mouse (Rudert et. al., 1992) and rat (Tatarinov et. al., 1986). Abnormally low levels of PSGs in maternal serum have been correlated with important complications of pregnancy including spontaneous abortion, intrauterine growth retardation, and fetal hypoxia. Administration of antibodies to PSGs is abortifacient in mice and monkeys (Hau et. al., 1985, Bohn and Weiman, 1976). These observations, and the fact that PSGs are the major proteins produced by the human placenta, indicate an important role for PSGs in maintaining pregnancy, but no definitive PSG function has yet been shown. Some experiments have indicated a role for PSGs in immunoregulation. It has been proposed that the lack of rejection of the semiallogeneic fetus by the maternal immune system is brought about by the maintenance of an anti-inflammatory immune environment at the maternal-fetal interface (Wegmann et. al., 1993). The immune environment is produced by cytokines and other immune mediators synthesized by cells of the placenta, the uterine decidua and maternal immune system. Our goal was to begin to define the role and mechanism of action of PSGs in normal pregnancy by evaluating the ability of human PSGs to regulate the production of cytokines by macrophages.

We synthesized recombinant human PSG1, PSG6 and PSG11 using a baculovirus insect cell expression system. PSG1 and PSG6 gene products are highly expressed by placenta (Streydio and Vassart, 1990, Pan et. al., 1994). PSG11 is expressed by placenta and has been shown to enhance LPS-mediated

induction of IL-10 by human monocytes (Arnold et. al., 1999). PSG6 and PSG11 contain an RGD sequence motif within the N-terminal domain, and PSG1 (KGD) does not. The RGD motif has been thought to be important in PSG function due to its presence at a conserved position in the N-terminal domain of 7 of 11 human PSGs, and because a peptide from PSG11 including the RGD motif was shown to mediate binding to monocytic cells (Rutherford et. al., 1995). Functional experiments utilizing both RGD-containing PSGs and a PSG lacking a canonical RGD motif may shed light on the relevence of this sequence motif in PSG function.

We used the baculovirus insect cell expression system to synthesize recombinant human PSG proteins as fusions with glutathione S-transferase (GST). GST is a 26 kD protein of *Schistosoma japonicum*. Its use as a recombinant fusion partner allows protein purification in a single nondenaturing affinity step using glutathione-coupled beads. The baculovirus eucaryotic expression system is capable of providing large amounts of soluble, functionally active protein. Many post-translational modifications of vertebrate cells such as N- and O-linked glycosylation, phosphorylation, isoprenylation, and signal peptide cleavage occur in insect cells, which provide an environment usually resulting in proper folding, disulfide bond formation and oligomerization of recombinant protein. Baculoviruses are large (130 kb) double-stranded DNA viruses that infect many species of insects, but no known vertebrate host.

Time Course of Recombinant Human PSG Production

Sf9 insect cells were infected with recombinant baculovirus at an MOI of 5, and recombinant PSG proteins were secreted into the supernatant fluid as N-terminal GST fusion proteins. To obtain GST-XylE protein from insect cell cytoplasm, infected insect cells were lysed with RIPA buffer. In order to determine the optimal protein harvest time, a time course for protein production was performed. Baculovirus-infected insect cell supernatant or lysate was harvested at 24, 48, 72 and 96 hours after infection, and was analyzed for the presence of recombinant protein using immunoblot with an antibody to the GST tag. The 72 hour time point resulted in maximal protein production for all recombinant proteins (Figure 9), so this time point was used to harvest GST-XylE and GST-PSGs for all experiments

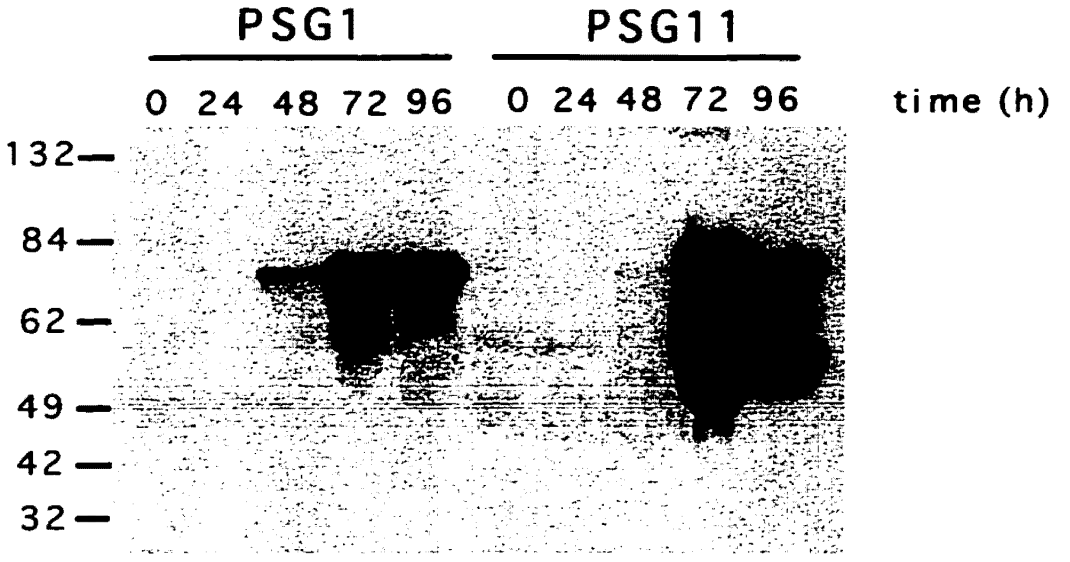

Figure 9. Time course of recombinant PSG protein production by insect cells. GST-PSG1, GST-PSG6, GST-PSG11 and GST-XylE were produced using the baculovirus insect cell expression system. To determine the optimal protein harvest time, baculovirus-infected insect cell supernatant was harvested at 24, 48, 72 and 96 hours after infection, clarified by centrifugation, and GST fusion protein was detected by immunoblot using an monoclonal antibody to the GST tag. Sizes of molecular weight markers are shown in the left margin. The 72 hour timepoint resulted in maximal protein production for all recombinant proteins (shown here for PSG1 and PSG11).

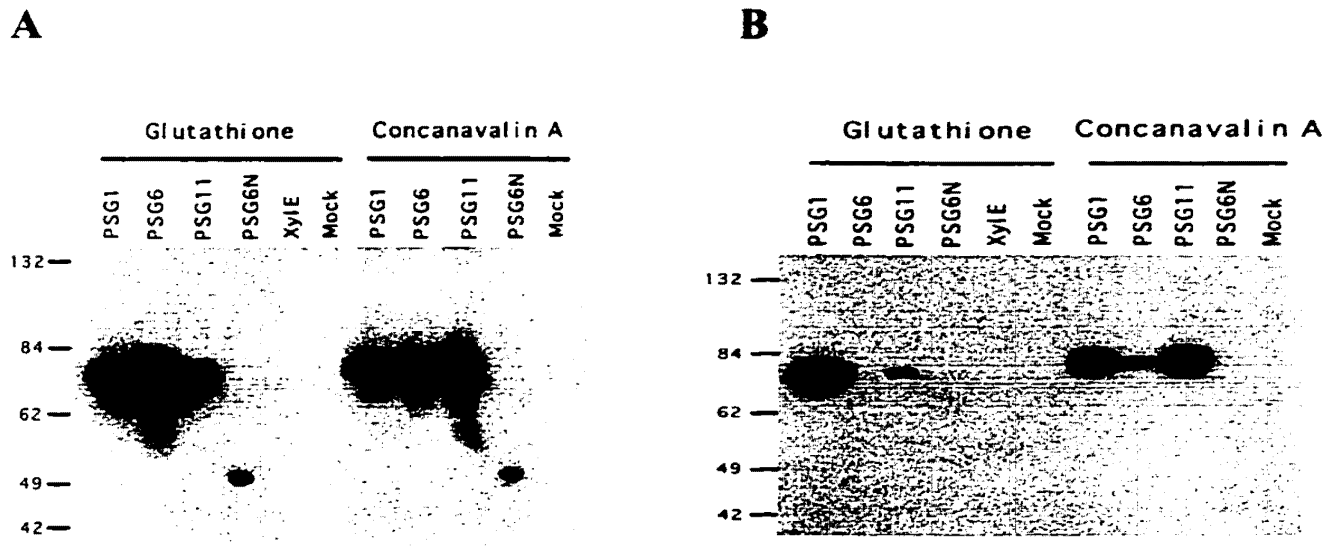

Figure 10. Recombinant human PSG and XylE GST fusion proteins produced in insect cells. **(A)** GST-PSG1, GST-PSG6, GST-PSG11, GST-PSG6N and GST-XylE purified using either glutathione-linked or concanavalin A-linked beads were analyzed by Western blot using a specific rabbit polyclonal antiserum to PSGs. All recombinant PSG proteins were detectable using this antiserum, but GST-XylE and protein from mock-infected insect cell supernatant were not. **(B)** Purified GST-PSG1, GST-PSG6, GST-PSG11, GST-PSG6N and GST-XylE were analyzed by Western blot using the specific mouse monoclonal antibody for PSGs, BAP-1. The full-length GST-PSG1, GST-PSG6 and GST-PSG11 were detected using this antibody, but the truncated PSG6N fusion protein was not detectable. Sizes of molecular weight markers are shown on the left.

Purification and Analysis of Human Recombinant PSG Protein

Recombinant GST-PSG1, 6, 11, GST-PSG6N and GST-XylE were purified with glutathione or concanavalin A beads, and analyzed by immunoblot using a rabbit polyclonal antiserum specific for PSGs (Figure 10). This antiserum detected PSG1, PSG6, PSG11 and the truncated N-domain protein PSG6N, but did not bind to GST-XylE or proteins from mock-infected insect cell supernatant. A monoclonal antibody to human PSGs, BAP-1, also detected GST-PSG1, GST-PSG6 and GST-PSG11, but not the truncated GST-PSG6N, presumably because this monoclonal antibody recognizes an epitope that is not located in the N-terminal domain (Figure 10). An antibody specific for GST detected all PSG recombinant proteins and GST-XylE in immunoblot (Figure 11). Size of the recombinant proteins was calculated by relative mobility in SDS-PAGE compared to molecular weight standard proteins. GST-PSG1 is 75 kD, GST-PSG6 is 77 kD, GST-PSG11 is 74 kD, and GST-PSG6N is 47 kD. Since the predicted relative molecular weight of all the full-length GST-PSG fusion proteins is 70 kD and that of the GST-PSG6N is 40 kD, the increased apparent molecular weights probably indicate glycosylation of PSGs produced in insect cells, because these proteins were also efficiently precipitated by the lectin concanavalin A (Figures 10 and 11). Glutathione affinity chromatography purification did not result in single bands for the recombinant proteins, as determined by Coomassie blue staining of protein after SDS-PAGE. Since other components of the Sf9 cell supernatant were apparently binding to the beads used for purification, supernatant from GST-XylE baculovirus-infected cells (designated "XylE sup") was treated identically to that of PSG-infected supernatant (incubated with beads, eluted and concentrated), and used as a control. Concentration of recombinant proteins used in these experiments ranged from 20-50 μg/ml as measured by GST detection ELISA, and in comparison to BSA protein standards on Coomassie-stained gels. LPS contamination of protein samples was less than 1×10^{-6} ng/ ng protein, as measured by limulus amoebocyte lysate assay (LAL).

PSG Induction of Cytokine Secretion in Human Monocytes

Knowledge of the effect of PSGs on mononuclear phagocyte production of several immune mediators will begin to define their role as immunomodulators in pregnancy. The deleterious effect on pregnancy of Th1 cytokines is largely mediated by induction of macrophage, NK cell and LAK activity. Macrophages are involved in the activation and suppression of NK and LAK cytotoxicity, production of an

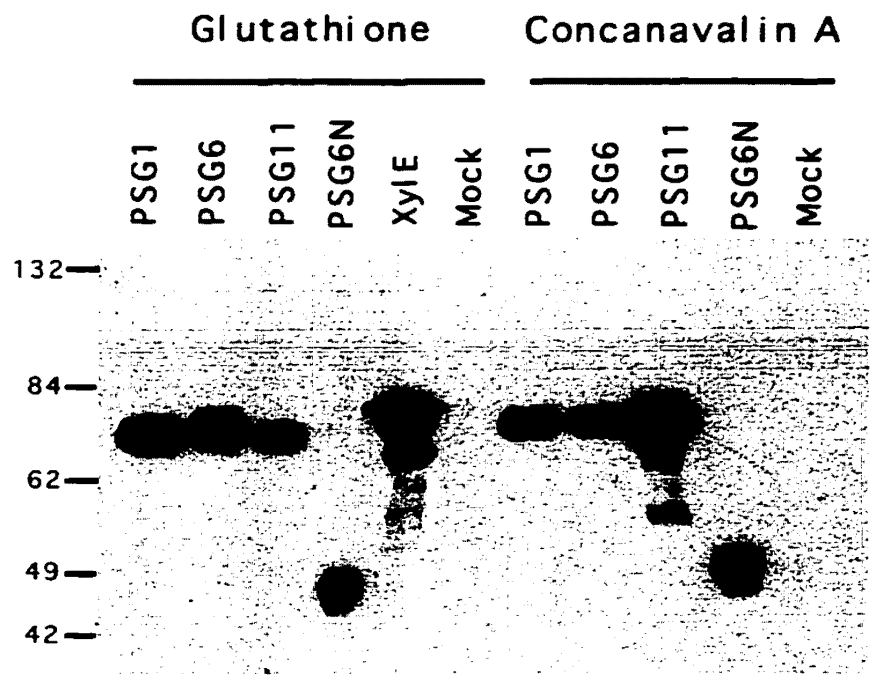

Figure 11. Recombinant human PSG and XylE GST fusion proteins detected by an antibody to the GST tag. GST-PSG1, GST-PSG6, GST-PSG11, GST-PSG6N and GST-XylE purified using either glutathione-linked or concanavalin A-linked beads were analyzed by Western blot using a specific monoclonal antibody to the GST tag. All recombinant fusion proteins were detectable using this antibody. Protein from mock-infected insect cell supernatant was not detected by antibody to GST. Sizes of molecular weight markers are shown at left.

inflammatory response, and can directly kill embryonic cells in a mouse model of recurrent abortion (Haddad et. al., 1995). Activated macrophages are a crucial part of the first line of defense against infection and play a central role in the regulation of both the innate and adaptive immune responses. Macrophages are widely distributed in tissues, including the pregnant uterus, as resident mononuclear phagocytes. Tissue macrophages are morphologically, antigenically, and functionally heterogeneous due to maturation pathways induced by the specific tissue microenvironment encountered after migration from the circulation. The microenvironment includes chemokines, cytokines, hormones, ECM components, and microbes. In the pregnant uterus high concentrations of PSGs, among other factors, may serve to bring about development of functional macrophage phenotypes appropriate for fertility and pregnancy. We used freshly isolated, undifferentiated blood monocytes in experiments examining PSG effects on cytokine production.

IL-10 seems to play a critical role during pregnancy. An aberrant T cell response to trophoblast antigens, defined by abnormally low levels of IL-10, and increased levels of IFN-γ and TNFβ, produced by lymphocytes from women prone to recurrent spontaneous abortion, may be an important factor in unexplained pregnancy loss in these patients (Hill et. al., 1995). Treatment with IL-10 abrogates increased incidence of abortion in the mouse abortion-prone mating combination (Chaouat et. al., 1995). IL-10 can deactivate inflammatory macrophages (Bogdan et. al., 1991) and inhibit NK cell cytotoxicity (Hsu et. al., 1992). Pregnancy-specific glycoproteins are hypothesized to modify the immune environment during pregnancy to prevent fetal damage or loss. Abnormally low levels of PSGs in maternal serum (Masson et. al., 1983, Hertz and Schultz-Larson, 1983) and endometrium (Arnold et. al., 1999) have been correlated with spontaneous abortion. PSG 11 has been shown to enhance secretion of IL-10 by monocytic cell lines (Arnold et. al., 1999). Therefore, we first investigated whether treatment with PSGs could induce IL-10 production by human monocytes. The effect of PSGs on monocyte production of other immune mediators that have established roles in pregnancy was also measured. Freshly isolated human monocytes were seeded into 96-well tissue culture dishes, allowed to adhere, and then were treated with various concentrations of recombinant PSGs, XylE control, or LPS as a positive control. We measured induction of IL-10, IL-6, TNF-α, IL-1β and IL-12 at 24 hours after treatment using quantitative sandwich ELISA.

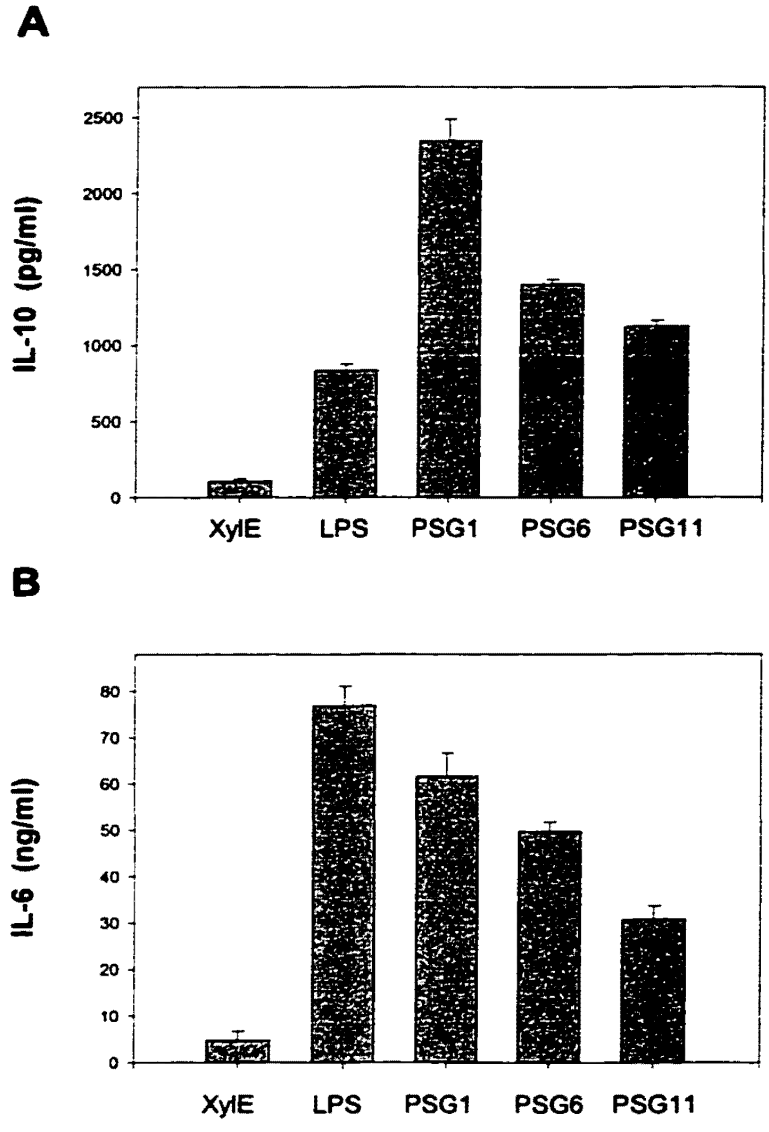

Figure 12. PSG induction of IL-10 and IL-6 secretion by monocytes. Monocytes (0.4 x 10^6/well) in 96-well plates were treated with 12 μg/ml GST-PSG1, GST-PSG6, GST-PSG11, GST-XylE or 100 ng/ml LPS, and secretion of **(A)** IL-10 and **(B)** IL-16 into the supernatant fluid was measured at 24 hours by quantitative ELISA. All three recombinant PSG proteins induced significant amounts of IL-10 and IL-6 secretion compared to the control XylE sup.

Human recombinant GST-PSG1, GST-PSG6 and GST-PSG11 induced significant amounts of IL-10 and IL-6 protein secretion by elutriated human monocytes compared to the control protein GST-XylE (Figure 12). A control, consisting of GST-XylE baculovirus-infected insect cell supernatant or "XylE sup", was tested for activity in these cytokine assays because the preparations of PSG recombinant protein were not completely pure. Since other components of infected-cell supernatant might induce cytokine secretion by monocytes, and because the GST-XylE protein was purified from insect cell lysate rather that supernatant, the "XylE sup" control was used to treat monocytes. (The volume of XylE sup control added to monocytes was equal to the highest volume of purified PSG protein in all experiments.) In fact a slight but significant induction in IL-10 and in IL-6 secretion was seen in some experiments using the XylE sup control. Thus, a small proportion (1 – 15 %) of the IL-10 and IL-6 protein induction by GST-PSGs may have been due to other components from insect cell supernatant that were retained in the purification procedure. PSG1 consistently induced significantly more IL-10 and IL-6 secretion by monocytes than PSG6 or PSG11 when concentrations of recombinant proteins were adjusted so that equal volumes of each were added to the cells. This indicates that PSG1 may be a stronger inducer of monocyte activity than the other two PSGs, although PSG1 does not contain the RGD motif. All GST-PSG proteins purified via affinity chromatography using either glutathione-linked or concanavalin A-linked beads (data not shown) induced significant amounts of IL-10 and IL-6 protein secretion by human monocytes.

Examination of Inflammatory Cytokine Secretion by Monocytes Treated with PSGs

LPS activation of human monocytes results in a well-characterized burst of secretion of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6, followed by later secretion of the anti-inflammatory mediator, IL-10. Monokines such as IL-1, IL-6, and TNF are produced maximally at 4-8 h after LPS treatment and high levels (>50 ng/ml) of IL-10 at 20–48 h after LPS treatment (de Waal Malefyt et. al., 1991). TNF-α acts as part of a negative feedback loop that controls the acute inflammatory response in macrophages (Wanidworanun and Strober, 1993, Platzer et. al., 1995). Elevation of TNF and cAMP, which are both downstream of LPS activation, can together induce IL-10 protein production by monocytes (Meisel et. al., 1996). PSGs may downregulate the expression of inflammatory cytokines by macrophages directly or via the action of IL-10. It is also possible that PSGs induce an early inflammatory response by macrophages, followed by a delayed induction of IL-10, as is the case for LPS. Therefore we measured

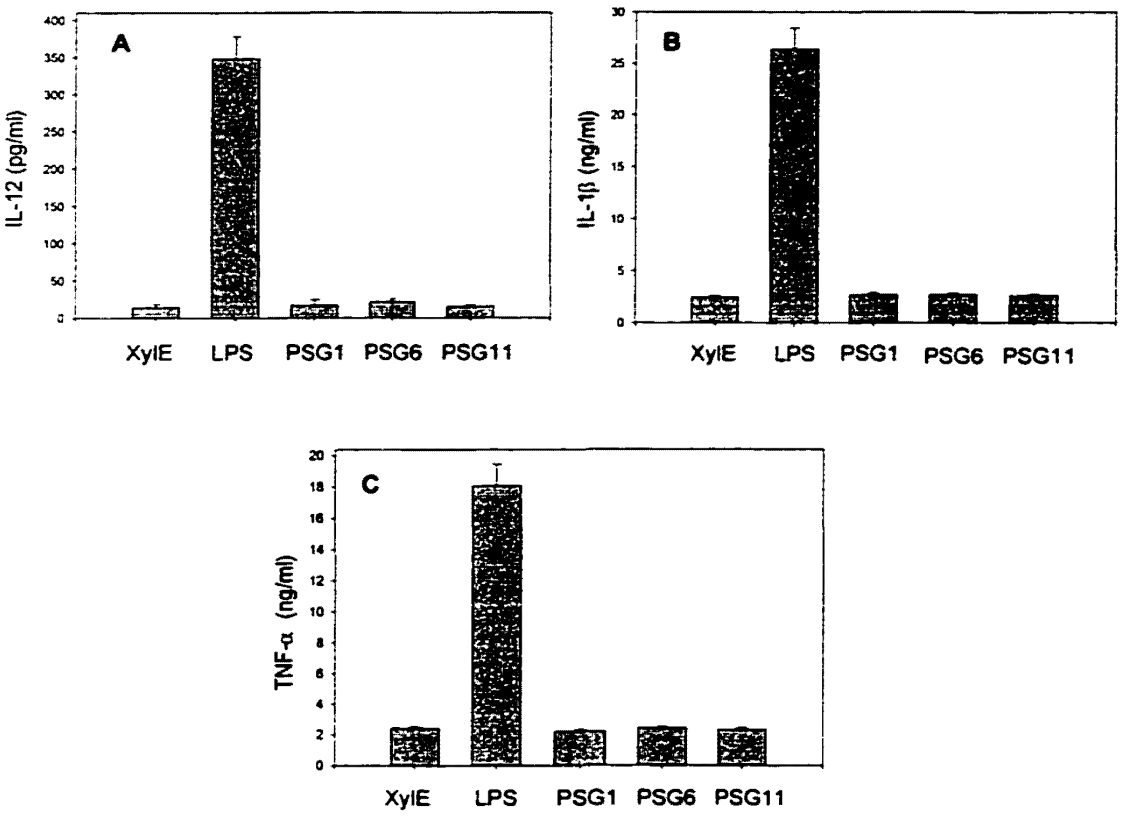

Figure 13. Recombinant PSGs do not induce IL-12, IL-1β or TNF-α secretion by monocytes. Monocytes (0.4×10^6/well) in 96-well plates were treated with 12 μg/ml GST-PSG1, GST-PSG6, GST-PSG11, GST-XylE or 100 ng/ml LPS, and secretion of IL-12 **(A)** IL-1β **(B)** and TNF-α **(C)** into the supernatant fluid was measured at 24 hours by ELISA. None of the recombinant PSG proteins induced significant secretion of IL-12, IL-1β or TNF-α compared to the XylE sup control.

PSG inducton of TNF-α, IL-1β and IL-12 protein secretion in monocyte cell supernatant by sandwich ELISA (Figure 13). Monocytes were treated in triplicate with a single dose (12 μg/ml) of GST-PSG1, 6, 11, and GST-XylE or 100 ng/ml LPS as described above, and cell supernatant was harvested 24 hours after treatment.

GST-PSG1, 6, and 11 did not induce statistically significant amounts of TNF-α, IL-1β or IL-12 by monocytes compared to GST-XylE or XylE sup. However, recombinant PSGs and the XylE sup control did induce up to 2.6 ng/ml of TNF-α and IL-1β. (The IL-12 secretion by PSGs, XylE and XylE sup were the same as background levels of untreated monocytes.) Monocyte secretion of TNF-α and IL-1β after treatment with PSGs is assumed to be due to other components of the insect cell supernatant, because of the similar induction by XylE sup, but not XylE protein purified from insect cell lysates. IL-10 inhibits the production of TNF-α, IL-1β, IL-12 and IL-6 by monocytes if it is present early (before 4 hours) in a response to LPS (D'Andrea et. al., 1993, Wang et. al., 1994). It was possible that an early induction of IL-10 by PSGs inhibited TNF-α production in these experiments. It will be shown below that significant amounts of IL-10 are not induced by PSGs until 13 - 22 hours after treatment. The results of these experiments show that PSGs do not induce an early release of inflammatory cytokines before synthesis of IL-10 and IL-6, and suggest that PSGs probably do not act as inflammatory mediators in vitro.

Induction of IL-6 and IL-10 Secretion by Monocytes is a Specific Effect of PSG Protein

In order to show that PSG induction of IL-10 and IL-6 was a specific effect of PSG protein in the purified insect cell supernatant, we removed PSG protein from the concentrated recombinant protein samples by immunodepletion. Concentrated PSG protein was incubated with a specific rabbit polyclonal antiserum to PSGs or with normal rabbit serum (NRS) as a control. Immune complexes were precipitated with protein A-sepharose. No PSG protein could be detected in the PSG-immunodepleted samples by Western blot, and the NRS-treated PSG samples had amounts of PSG protein similar to the starting material (shown for PSG6 and PSG11 in Figure 14). Monocytes were treated with equal volumes of the control NRS-treated or the immunodepleted PSG6 and PSG11, and IL-10 and IL-6 induction at 24 hours was measured by ELISA (Figure 15). Cytokine induction was a specific effect of the PSG proteins as shown by the fact that PSG-immunodepleted samples showed a reduction in IL-10 secretion to 14% (PSG11) or 11% (PSG6) of that induced by the control normal rabbit serum immunoprecipitated samples.

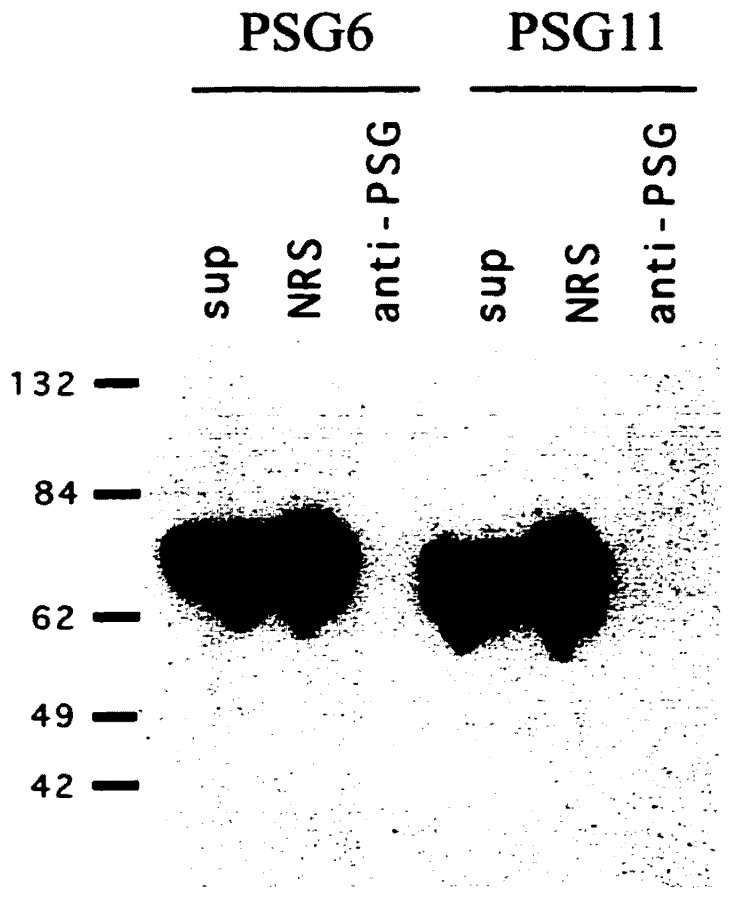

Figure 14. Immunodepletion removes PSG protein from concentrated recombinant PSG protein samples. Affinity chromatography purified and concentrated samples of PSG6 and PSG11 were immunoprecipitated with a rabbit polyclonal antiserum specific for PSGs or with normal rabbit serum. After precipitation the samples were analyzed for the presence of recombinant PSG protein by Western blot using an antibody to the GST tag. The concentrated PSG protein starting material is designated "sup" in the figure. "NRS" indicates PSG protein incubated with normal rabbit serum and "anti-PSG" indicates PSG protein incubated with rabbit anti-PSG antiserum. Specific immunoprecipitation removed all detectable PSG protein from the concentrated protein solution, while the control immunoprecipitated samples had the same amount of PSG protein as the starting material. Sizes of molecular weight markers are shown on the left.

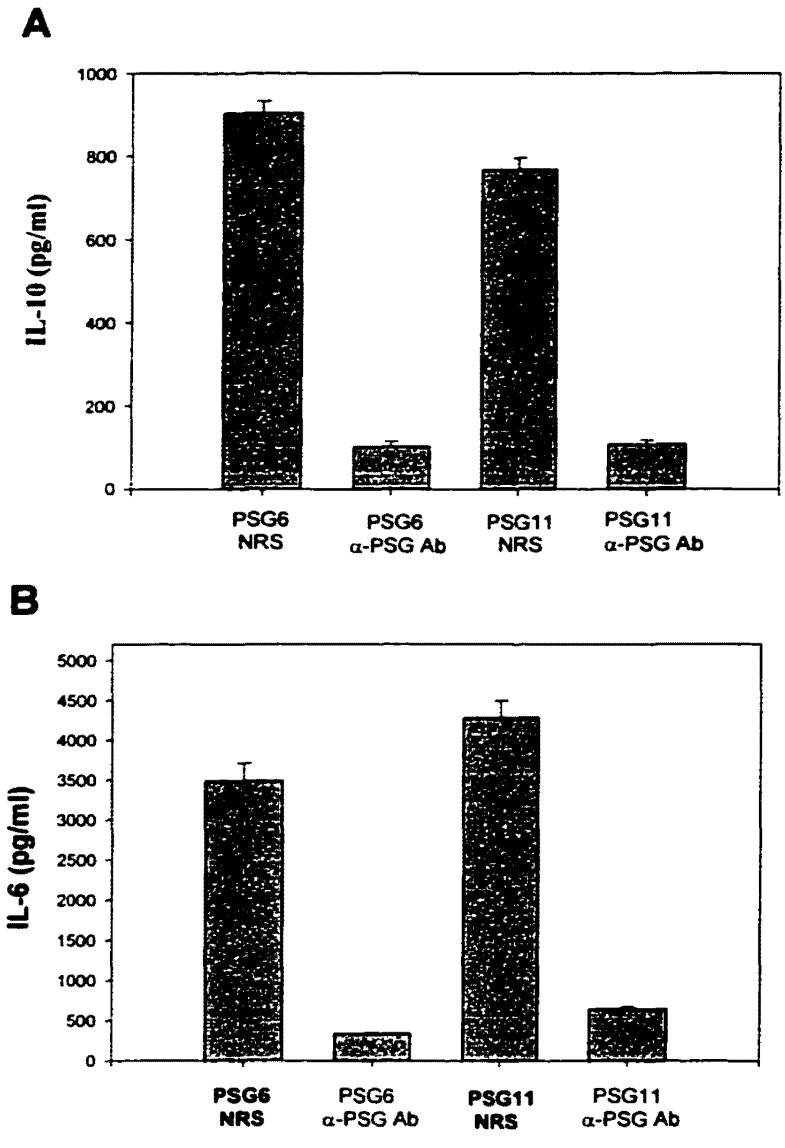

Figure 15. Induction of IL-10 and IL-6 secretion by monocytes is a specific effect of PSG protein. Monocytes (0.4 x 10^6/well) were treated in 96-well tissue culture dishes with equal volumes of normal rabbit serum (NRS) immunoprecipitated (8 μg/ml) or anti-PSG antibody immunoprecipitated PSG6 or PSG11 protein. **(A)** IL-10 and **(B)** IL-6 secretion was measured in the supernatant fluid at 24 hours after treatment. Specific removal of PSG protein resulted in reduction of IL-10 and IL-6 secretion to 10 – 15% of the control NRS-immunoprecipitated samples, indicating that induction of IL-10 and IL-6 secretion is a specific effect of PSG protein.

IL-6 secretion induced by PSG-depleted samples was reduced to 10-15% of the NRS control value. The 10 -15% activity seen in the PSG-immunodepleted samples is probably due to the presence of insect cell supernatant components or FBS in the purified recombinant protein samples. Also low levels of PSGs in the immunodepleted protein solution (PSG that has lost the GST tag and is therefore not detectable by anti-GST Western blot) could have induced IL-10 and IL-6 secretion. In such a situation the presence of antibody to PSGs in the immunoprecipitated solution might increase the efficiency of receptor clustering and potentiate cytokine induction by low levels of PSG protein.

PSG Dose Response Curves for IL-10 and IL-6 Secretion

Since all three GST-PSGs induced specific IL-10 and IL-6 protein secretion by monocytes when tested at one concentration, we performed IL-10 and IL-6 dose response curves for the three PSGs and the control protein GST-XylE by varying the concentration of recombinant protein over a 100-fold range. Monocytes from two different blood donors were used for each experiment and triplicate wells of cells were treated at each concentration. LPS dose response curves for IL-10 and IL-6 protein secretion were also generated using monocytes from three blood cell donors. Cell supernatant was harvested at 24 hours after treatment and analyzed for cytokine secretion by ELISA.

There is large variation in IL-10 production by blood monocytes from different individuals in response to LPS. In one study of 132 adults, IL-10 secretion induced by 1 μg/ml LPS ranged almost 10-fold from 600-5000 pg/ml (Eskdale et. al., 1998), and was partially attributed to differences in IL-10 promoter haplotypes based on microsatellite analysis. Our GST-PSG and LPS dose response curves showed distinct variations in induction of cytokines between cells from different blood donors. One μg/ml LPS induced between 1451 - 3300 pg/ml IL-10 and 34 - 50 ng/ml IL-6, and the curves were beginning to plateau at this concentration of LPS (Figure 16). LPS induction of IL-10 and IL-6 by monocytes was similar to that reported previously (Eskdale et. al., 1998, Wang et. al., 1994).

PSG1, PSG6 and PSG11 induced a dose dependent secretion of IL-10 and IL-6 compared to the XylE control protein (Figures 17-19). The PSG1 dose response curves reached a plateau at 8 μg/ml (approximately 107 pmol/ml PSG1) at IL-10 values between 1500-2000 pg/ml, and IL-6 values of 13-27 ng/ml (Figure 17). Significant IL-10 and IL-6 induction was observed when monocytes were treated with between 5-8 μg/ml of PSG1. The PSG6 and PSG11 dose response curves did not plateau at the maximum

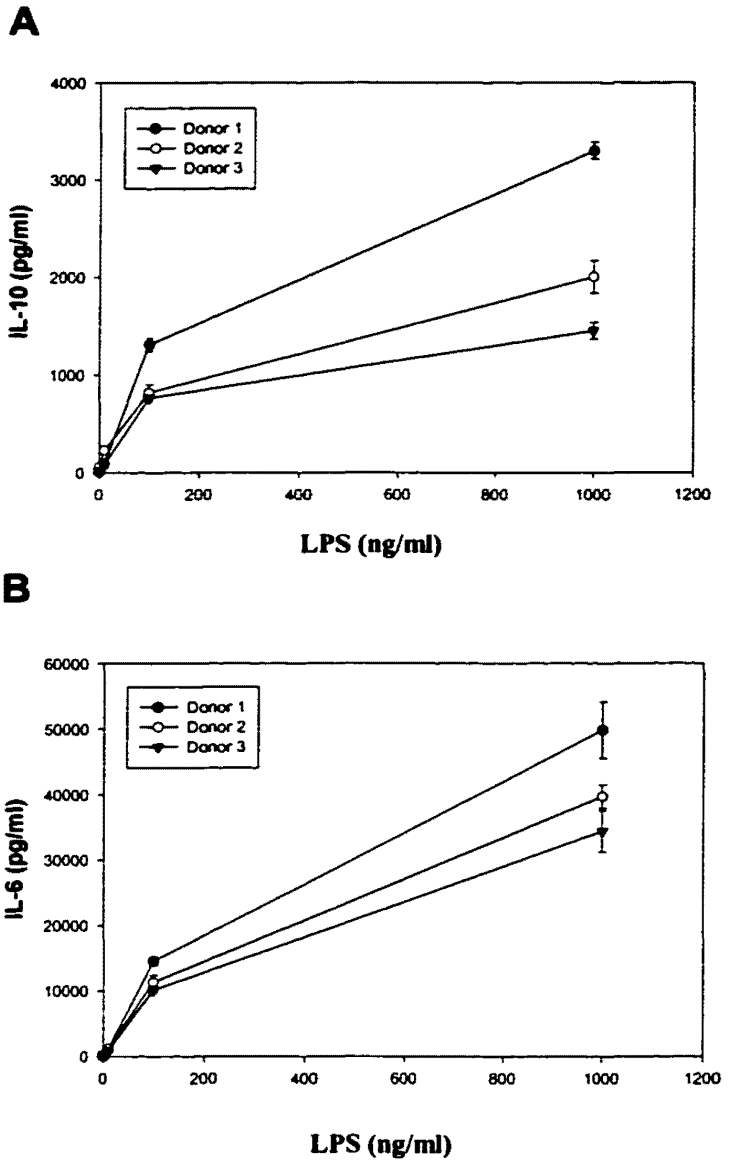

Figure 16. LPS dose response curves for IL-10 and IL-6 secretion by monocytes. Monocytes (0.4 x 10^6/ well) from three adult donors seeded into 96-well tissue culture dishes were treated with concentrations of *E. coli* K235 LPS ranging from 0.01 to 1000 ng/ml. Cell supernatant was harvested 24 hours after treatment and IL-10 **(A)** and IL-6 **(B)** protein were measured by quantitative ELISA.

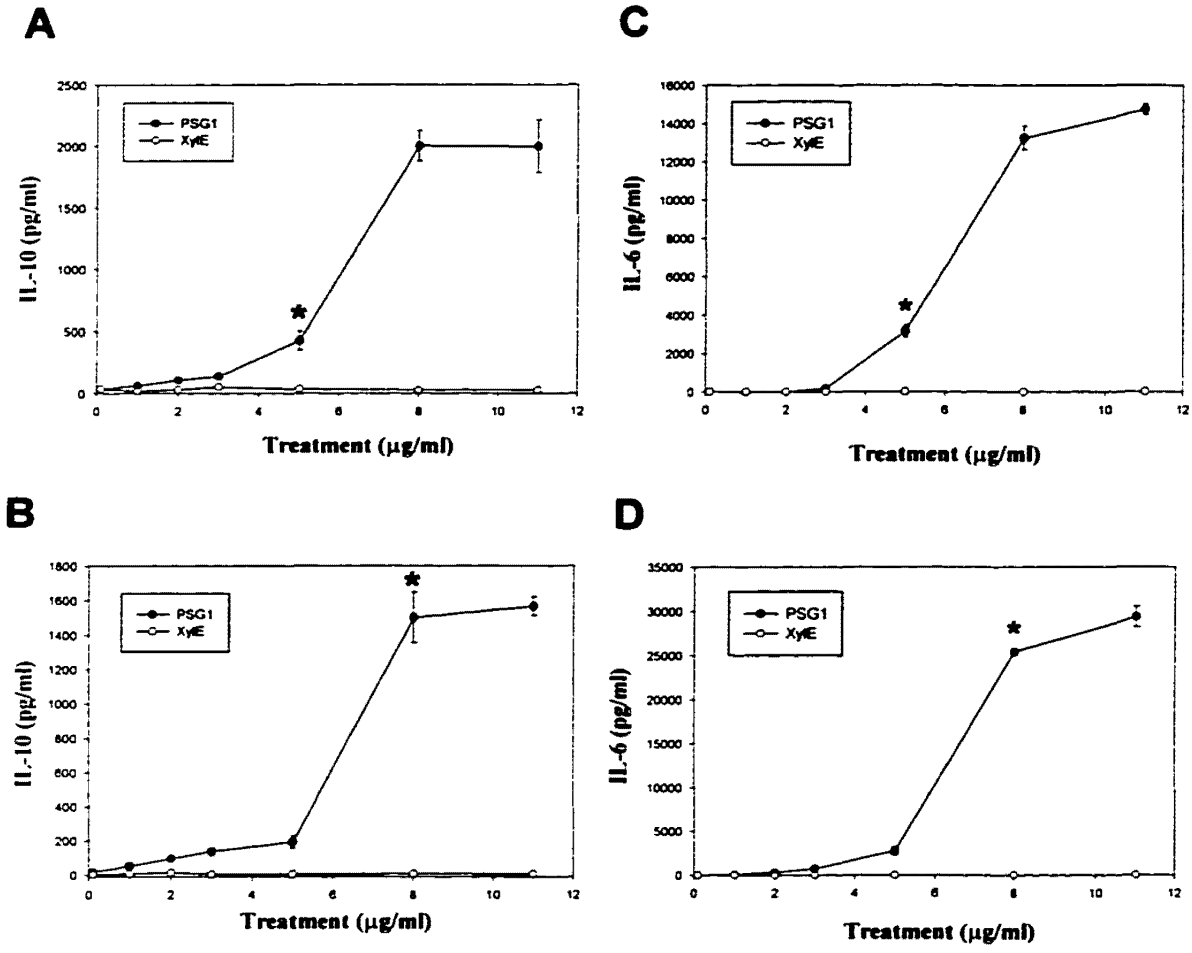

Figure 17. PSG1 dose response curves for IL-10 and IL-6 secretion by monocytes. Monocytes (0.4 x 10^6/ well) obtained from two adult donors were treated in 96-well tissue culture dishes with concentrations of GST-PSG1 or GST-XylE ranging from 0.1 to 11 µg/ml. Cell supernatant was harvested 24 hours after treatment, and IL-10 (**A** and **B**) and IL-6 (**C** and **D**) secretion were measured by quantitative ELISA. PSG1 induced dose-dependent secretion of IL-10 and IL-6 compared to XylE control protein. The doses at which PSG1 first induced significant amounts of IL-10 and IL-6 are indicated by asterisks. Significance was determined using two-way ANOVA and Student-Newman-Keuls test.

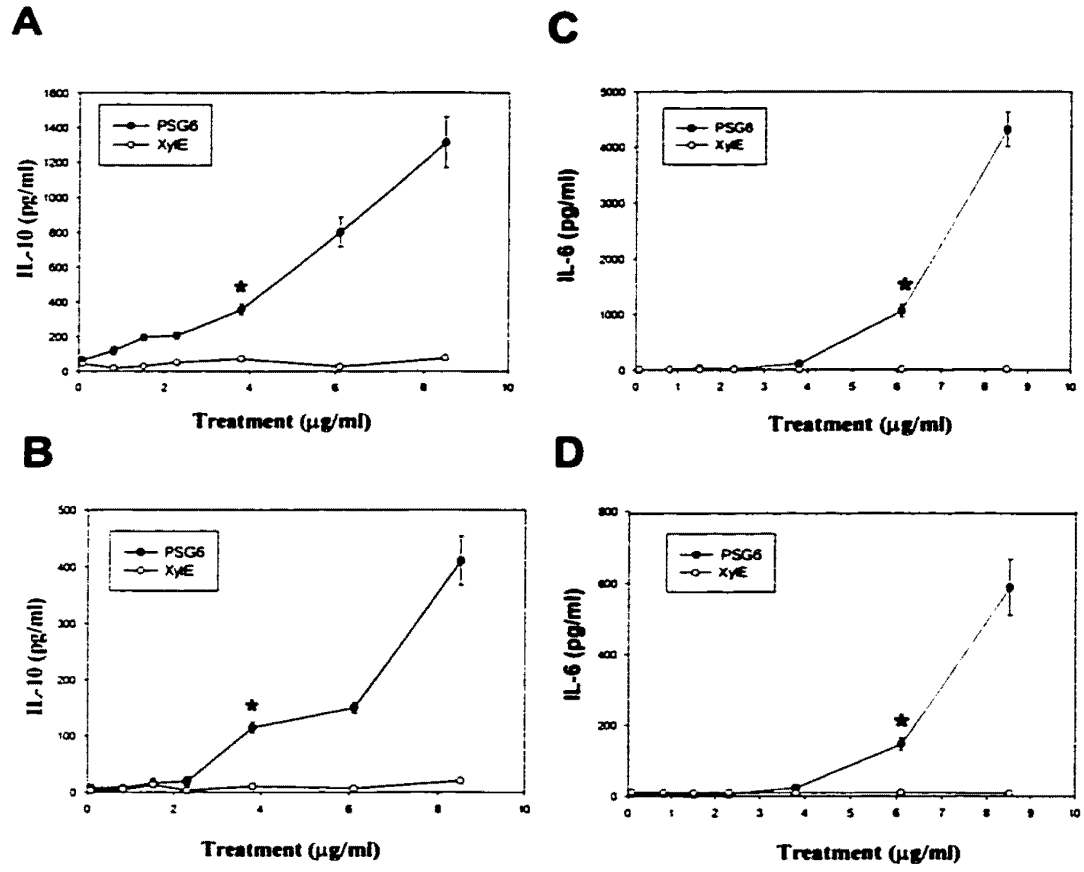

Figure 18. PSG6 dose response curves for IL-10 and IL-6 secretion by monocytes. Monocytes (0.4 x 10^6/ well) obtained from two adult donors were treated in 96-well tissue culture dishes with concentrations of GST-PSG6 or GST-XylE ranging from 0.08 to 8.5 μg/ml. Cell supernatant was harvested 24 hours after treatment, and IL-10 (**A** and **B**) and IL-6 (**C** and **D**) secretion were measured by quantitative ELISA. PSG6 induced dose-dependent secretion of IL-10 and IL-6 compared to XylE control protein. The doses at which PSG6 first induced significant amounts of IL-10 and IL-6 are indicated by asterisks. Significance was determined using two-way ANOVA and Student-Newman-Keuls test.

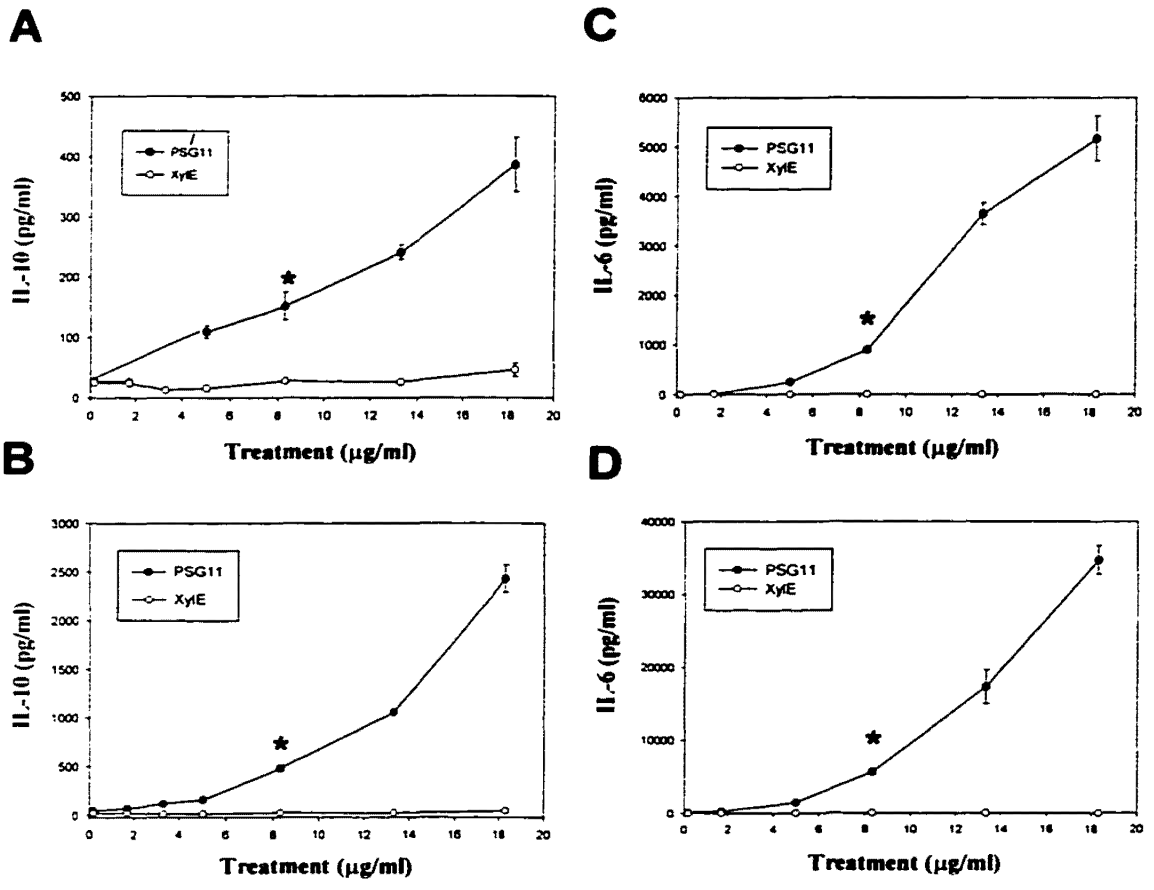

Figure 19. PSG11 dose response curves for IL-10 and IL-6 secretion by monocytes. Monocytes (0.4 x 10^6/ well) obtained from two adult donors were treated in 96-well tissue culture dishes with concentrations of GST-PSG11 or GST-XylE ranging from 0.17 to 18 μg/ml. Cell supernatant was harvested 24 hours after treatment, and IL-10 (**A** and **B**) and IL-6 (**C** and **D**) secretion were measured by quantitative ELISA. PSG11 induced dose-dependent secretion of IL-10 and IL-6 compared to XylE control protein. The doses at which PSG11 first induced significant amounts of IL-10 and IL-6 are indicated by asterisks. Significance was determined using two-way ANOVA and Student-Newman-Keuls test.

concentrations of recombinant protein used in these experiments (8.5 μg/ml or 110 pmol/ml, and 18.3 μg/ml or 247 pmol/ml, respectively), but IL-6 and IL-10 induction increased in a dose-dependent manner in all experiments. PSG6 induced significant secretion of IL-10 by monocytes at 3.8 μg/ml, and significant IL-6 secretion at 6.1 μg/ml of recombinant protein compared to GST-XylE. Maximum IL-10 and IL-6 protein secretion induced by PSG6 in two different donors was 1314 pg/ml and 4320 pg/ml, respectively. PSG11 induced significant IL-10 secretion by monocytes at 8 μg/ml, and the maximum IL-10 secretion induced by PSG11 was 386 pg/ml or 2430 pg/ml in two donors. PSG11 induced significant IL-6 secretion at 8 μg/ml, and the maximum IL-6 protein was 5 - 34 ng/ml.

In summary, minimum concentrations of GST-PSG protein between 3.8 and 8 μg/ml induced statistically significant amounts of IL-10 and IL-6 secretion by human monocytes compared to control GST-XylE at 24 hours after treatment, and a dose-dependent relationship was seen when monocytes were treated with increasing amounts of GST-PSGs. At the highest concentrations tested (8.5 – 18.3 μg/ml), PSGs induced IL-10 secretion comparable to 10-1000 ng/ml LPS, and IL-6 values (except for one PSG6 experiment) were in the same range. At term, human PSG concentrations in maternal serum reach 200-400 μg/ml, with presumably much higher amounts at the maternal-fetal interface where PSGs are produced. Therefore we believe that the magnitude of the PSG induction of IL-10 and IL-6 secretion by monocytes is likely to be physiologically relevant. However, it is acknowledged that the presence of multiple cytokines, hormones and other immune mediators in vivo may potentiate or decrease the effect of PSGs on monocytes.

Time course of PSG Induction of IL-10 and L-6

We determined the kinetics of IL-10 and IL-6 secretion by monocytes after induction by recombinant PSGs. Monocytes were treated with 12 μg/ml GST-PSG or GST-XylE, and cell supernatant was analyzed for the presence of IL-10 or IL-6 at time points from 2 to 22 hours after treatment (Figure 20). PSG6 and PSG11 induced significant secretion of IL-10 at 13 hours after treatment compared to XylE, while PSG1 induced significant secretion only at 22 hours. Also, at 22 hours the induction by individual PSGs was significantly different, with PSG1 inducing the highest amount of IL-10. PSG6 and PSG11 induced significant secretion of IL-6 as early as 6 hours after treatment compared to XylE, and PSG1 induced significant secretion only at 10 hours or later. As was observed in the dose response curves, where

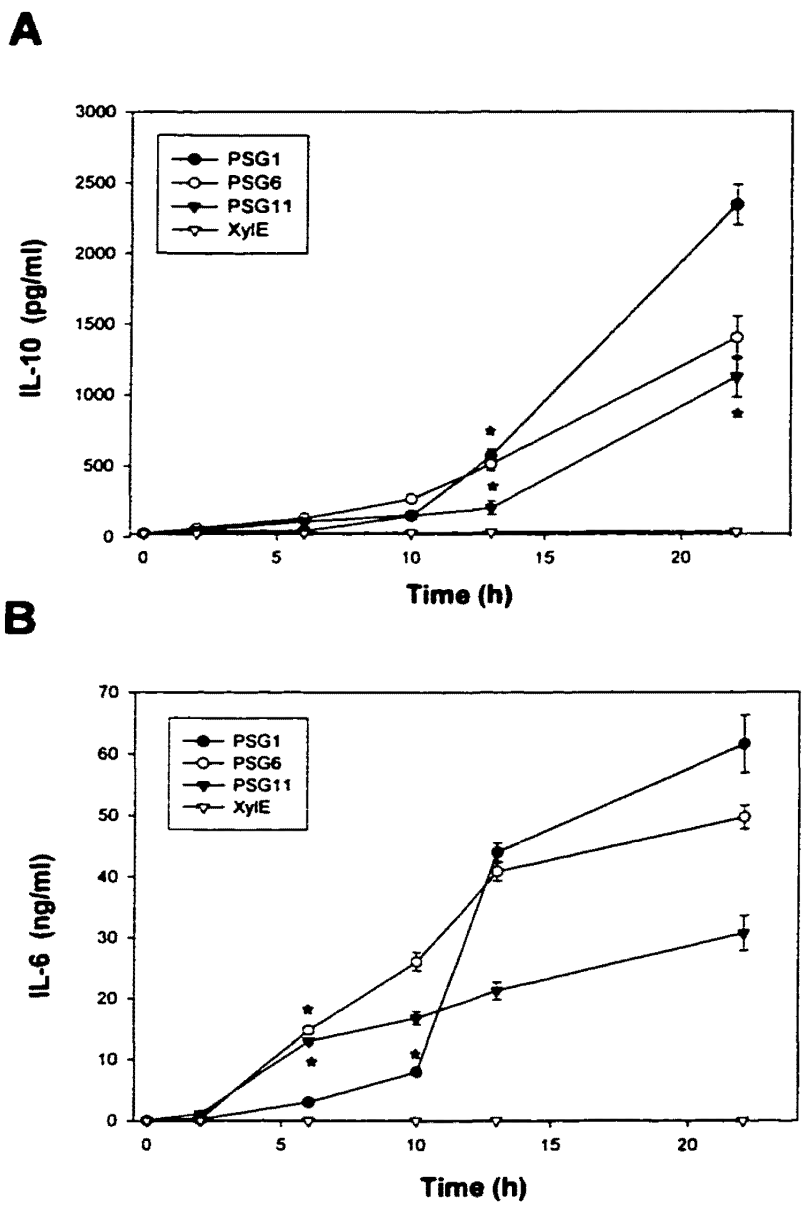

Figure 20. Time course of recombinant PSG induction of IL-10 and IL-6. Monocytes (0.4 x 10^6/ well) obtained from two adult donors were treated in 96-well tissue culture dishes with 12 µg/ml of GST-PSG1, GST-PSG6, GST-PSG11 or GST-XylE. Cell supernatant was harvested 24 hours after treatment, and IL-10 (**A**) and IL-6 (**B**) secretion were measured by quantitative ELISA. PSG6 and PSG11 induced significant amounts of IL-10 13 hours after treatment compared to control XylE, while PSG1 induced significant secretion only at 22 hours. PSG6 and PSG11 induced significant amounts of IL-6 at 6 hours, while PSG1 induced significant amounts of IL-6 only at 10 hours or later. At the later time points PSG1 induction of both IL-10 and IL-6 was greater than that of PSG6 and PSG11.

the PSG1-induced secretion of IL-10 and IL-6 plateaued and the other PSGs did not, the kinetics of PSG1 induction of IL-10 and IL-6 differed from that of PSG6 and PSG11. At early time points after treatment, PSG1 induction was lower than PSG6 and PSG11, but at the later time points PSG1 induction surpassed that of PSG6 and PSG11. As mentioned above, there was no significant secretion of IL-10 before 4 hours after treatment, indicating that PSG induction of IL-10 is not inhibiting early secretion of inflammatory cytokines by monocytes, although it could reduce secretion of IL-6 13 hours after treatment.

PSG6 Induces an Increase in IL-10 and IL-6 mRNA

Since cytokine protein is not stored in the cell, cytokine synthesis and secretion is generally determined by the amount of cellular mRNA. Cytokine mRNA levels are largely controlled by the rate of gene transcription, because cytokine mRNA is usually unstable due to the presence of destabilizing AU-rich elements in the 3'UTR. To determine if PSG induction of IL-10 and IL-6 protein secretion was preceded by an increase in mRNA, we treated monocytes with PSG6, and measured amounts of specific mRNA 5 hours after treatment by semi-quantitative RT-PCR (Figure 21).

PSG6 induced a 19-fold increase in IL-10 mRNA compared to the XylE control, and a 9-fold increase in IL-6 mRNA at 5 hours after treatment. Monocytes were also treated with the translation inhibitor cycloheximide to determine whether protein synthesis was necessary for the increase in cytokine mRNA observed after treatment with PSG6. Addition of cycloheximide decreased PSG6 induction of IL-6 mRNA by 80%, indicating that the PSG6-induced increase in IL-6 mRNA requires protein synthesis, even though significant induction of IL-6 protein secretion was seen as early as 6 hours, and IL-6 was rising before the 6 hour time point (Figure 20). Addition of cycloheximide increased amounts of IL-10 mRNA after treatment with either control XylE or PSG6. Cycloheximide alone (1 μg/ml) has been shown to induce an increase in IL-10 mRNA by human PBMCs through both mRNA stabilization and an increase in transcription (Stordeur et. al., 1995). In contrast, cycloheximide inhibits LPS induction of IL-6 mRNA (Wang et. al., 1994). The late upregulation of IL-10 synthesis by monocytes in response to PSGs (13-22 hours) implies a requirement for protein synthesis. Some studies have suggested that early production of TNF-α in response to LPS is involved in the later synthesis of IL-10 by monocytes (Platzer et. al., 1995, Meisel et. al., 1996). However, the fact that monocytes treated with both PSG6 and cycloheximide produced significantly more IL-10 mRNA than the XylE plus cycloheximide treated cells indicates that a

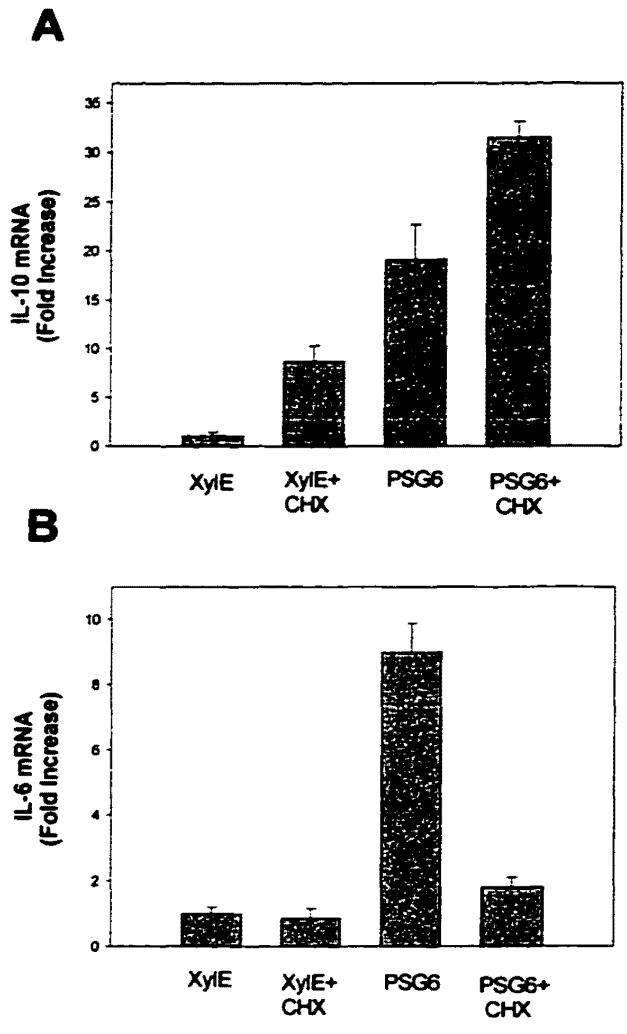

Figure 21. PSG6 induces an increase in IL-10 and IL-6 mRNA at 5 hours post-treatment. Monocytes (3.5×10^6/ well) were seeded into 12-well tissue culture dishes and treated in triplicate with 10 μg/ml GST-PSG6 or GST-XylE with or without 5 μg/ml cycloheximide (CHX). Five hours after treatment, total RNA was harvested from each well and amounts of IL-10 **(A)** and IL-6 **(B)** mRNA were analyzed by RT-PCR and Southern blot. The hybridization signal was quantitated using a Storm 860 PhosphorImager. A fragment of the housekeeping gene, GAPDH, was amplified as a control for the amount of total RNA isolated from each treated well. IL-10 and IL-6 mRNA values were normalized by dividing by the GAPDH value. The amount of mRNA is expressed as fold increase over the XylE control value. PSG6 induced a 19-fold increase in IL-10 and a 9-fold increase in IL-6 mRNA. Cycloheximide inhibited the increase in IL-6 mRNA, but potentiated the increase in IL-10 mRNA after treatment with PSG6. Significance ($p < 0.05$) was determined using one-way ANOVA and Student-Newman-Keuls test.

PSG6-mediated increase in IL-10 mRNA was probably occurring in the presence of cycloheximide. Further experiments need to be done to clarify this issue. In summary, PSG6 induction of IL-10 and IL-6 protein secretion by monocytes appears to occur through an increase in specific mRNA, although whether the mechanism is an increase in transcription or a decrease in mRNA stability (or both) was not determined.

PSG6 Induction of IL-10 and IL-6 Requires Protein Tyrosine Kinases, Protein Kinase C and cAMP

We investigated the intracellular signals involved in PSG-stimulated IL-10 and IL-6 secretion by monocytes, using a strategy of treatment with PSG6 and specific inhibitors or activators for signal transduction pathways together with measurement of cytokine secretion. To examine whether increased protein tyrosine phosphorylation mediates the functional responses of monocytes to PSGs, the effect of inhibiting tyrosine phosphorylation on PSG6-induced secretion of IL-6 and IL-10 was determined. Herbimycin A is an effective inhibitor of most tyrosine kinases (IC_{50} 900 nM), but does not inhibit the Ser/Thr kinases PKA and PKC, or phospholipase C (Fukazawa et. al., 1991). Herbimycin A blocks in vivo activity of the src family PTKs (Fukazawa et. al., 1991) through irreversible binding to thiol groups (SH) of the kinases. Herbimycin A (800 nM) has also been shown to be an effective inhibitor of LPS-induced protein tyrosine phosphorylation in mouse (Weinstein et. al, 1992) and human (Weinstein et. al., 1993) macrophages, as well as an inhibitor of IL-10 (Meisel et. al., 1996) and IL-6 (Beaty et. al., 1994) mRNA and protein production. Herbimycin A at 500 nM almost completely inhibited LPS-induced IL-10 expression by human PBMC (Meisel et. al., 1996). Monocytes were treated with 500 nM Herbimycin A in the presence of 12 μg/ml PSG6 or XylE to determine whether tyrosine kinases are important in PSG responses. Secretion of IL-10 and IL-6 at 24 hours after treatment was measured by quantitative sandwich ELISA (Figure 22).

The cAMP-dependent protein kinase A (PKA) is implicated in IL-10 gene expression. Compounds that elevate intracellular cAMP levels or directly activate PKA increase IL-10 expression in response to LPS (Tineke et. al., 1995, Platzer et. al., 1995). The reported effects of elevated cAMP on IL-6 expression are contradictory. In one report, specific inhibition of PKA reduced IL-6 expression by human monocytes, indicating that PKA activity may contribute to IL-6 induction by LPS (Geng et. al., 1993). Rolipram, an antidepressant drug, increases intracellular cAMP levels by exerting a selective inhibition of

phosphodiesterase (PDE) type IV (Beavo and Reisfeld, 1990). There are five classes of PDE isoenzymes, and PDE IV is known as the "cAMP-specific" PDE, because its affinity for cAMP is much greater than its affinity for cGMP. This isoenzyme plays an important role in regulating cAMP levels in monocytes after LPS treatment (Kambayashi et. al., 1995, Eigler et. al., 1998). Rolipram (IC_{50} 0.8 μM) partially inhibits IL-6 secretion by mouse macrophages (Kambayashi et. al., 1995), and rolipram (1 μM), but not other specific inhibitors of PDE isoenzymes, increases LPS-induced IL-10 mRNA and protein production by both human (Eigler et. al., 1998) and mouse macrophages (Kambayashi et. al., 1995). Monocytes were treated with 1 μM rolipram in the presence of 12 μg/ml PSG6 or XylE to examine how increases in intracellular cAMP, brought about through specific inhibition of monocyte PDE IV, affect PSG6 induction of IL-6 and IL-10 secretion (Figure 22).

(Rp)cAMPS, a specific PKA type I and type II inhibitor, binds to the cAMP site on the holoenzyme and blocks the conformational change in the PKA regulatory subunits, thus preventing them from dissociating from the catalytic subunits. (Rp)cAMPS is the Rp diastereoisomer of cAMP phosphorothioate, and has a K_I of 8-11 μM for PKA inhibition. (Rp)cAMPS (300 μM) decreases IL-10 production induced by LPS or cAMP elevating agents (Eigler et. al., 1998), indicating that activation of protein kinase A is necessary for cAMP-mediated enhancement of IL-10 mRNA levels. To examine whether PKA activity is necessary for PSG-mediated IL-6 and IL-10 secretion, monocytes were treated with 300 μM (Rp)cAMPS and 12 μg/ml PSG6 or XylE, and secretion of IL-6 and IL-10 was measured at 24 hours (Figure 22).

Protein kinase C (PKC) is required for IL-10 induction by LPS-stimulated human monocytes (Geng et. al., 1993, Gross et.al., 1993, Meisel et. al., 1996), and is important in IL-6 expression in non-monocytic cells (Sehgal et. al., 1987, Gross et. al., 1993). To investigate whether PKC activity is required for PSG induction of IL-6 and IL-10 secretion, monocytes were treated with the specific PKC inhibitor Calphostin C. The IC_{50} value of Calphostin C for PKC is 0.05 μM (Kobayashi et. al., 1989). Concentrations as high as 50 μM only slightly inhibit cAMP-dependent protein kinase (Kobayashi et. al., 1989). The N-terminal half of PKC is a regulatory domain, which contains binding sites for 1,2-diacylglyceride or phorbol ester, phosphatidylserine, and calcium, while the C-terminal half is the catalytic domain that contains two ATP binding sites. The mechanism of action of Calphostin C is competition for

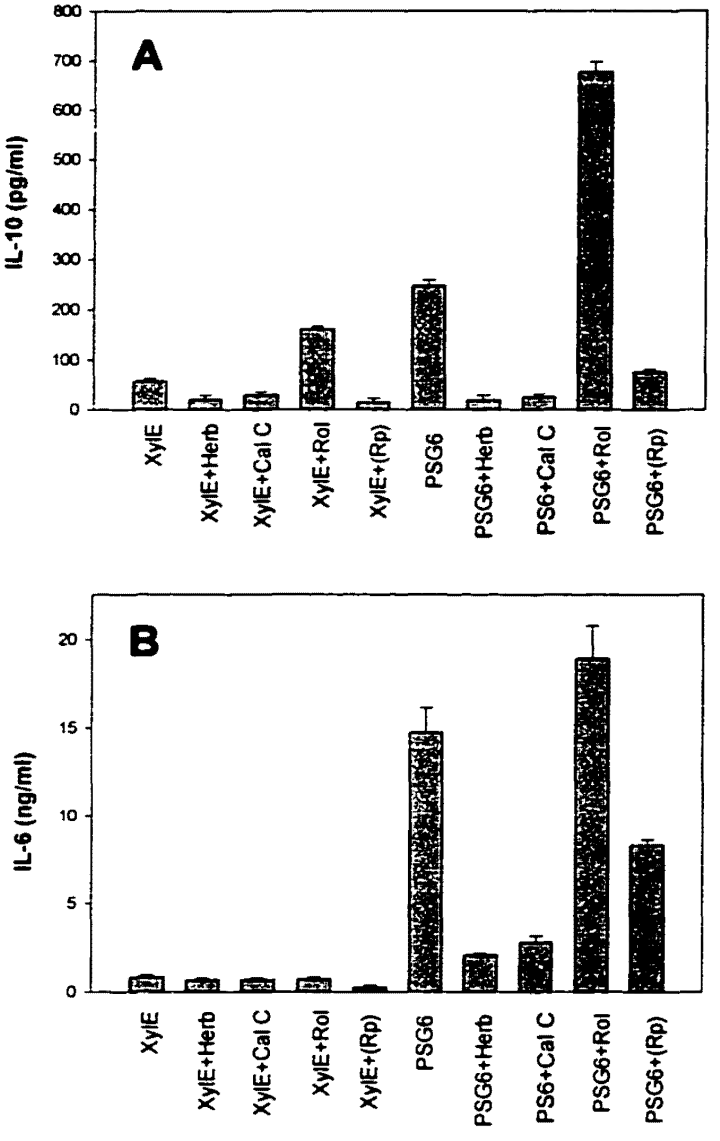

Figure 22. PSG 6 induction of IL-10 and IL-6 secretion involves protein tyrosine kinases, protein kinase C and PKA. Monocytes (0.4 x 10^6/well) in 96-well tissue culture dishes were treated with 12 μg/ml GST-PSG6 or GST-XylE with or without either 500 nM Herbimycin A (Herb), 1 μM Calphostin C (Cal C), 1 μM rolipram (Rol), or 300 μM (Rp)cAMPS (Rp), and IL-10 and IL-6 secretion at 24 hours after treatment was measured by quantitative sandwich ELISA. Monocytes were also treated with each inhibitor alone or DMSO alone as controls (data not shown). Herbimycin A, Calphostin C and (Rp)cAMPS significantly inhibited PSG6 induction of IL-10 and IL-6, while the cAMP elevating agent rolipram increased both IL-10 and IL-6 secretion induced by PSG6.

the binding site for diacylglycerol/phorbol ester in the PKC regulatory domain. Interaction with the regulatory domain, in contrast to the kinase domain (which is similar in various protein kinases), allows greater specificity of inhibition. PKC inhibitors such as staurosporine, which directly inhibit the catalytic domain, also inhibit cAMP-dependent protein kinase. Monocytes were treated with 1 μM Calphostin C in the presence of 12 μg/ml PSG6 or XylE, and secretion of IL-6 and IL-10 was measured at 24 hours (Figure 22).

PSG6 induction of IL-10 secretion by monocytes was inhibited 92% by treatment with Herbimycin A, 90% by Calphostin C and 69% by (Rp)cAMPS. Rolipram increased PSG6 induction of IL-10 by 2.7 fold. These signal transduction inhibitors and activators had similar inhibitory or enhancing effects on the (low) induction of IL-10 by the XylE control. Herbimycin A (85%), Calphostin C (81%) and (Rp)cAMPS (43%) also significantly inhibited PSG6 induction of IL-6 secretion by monocytes. Rolipram increased IL-6 production by PSG6 by 28%. None of the inhibitors/activators had a significant effect on XylE induction of IL-6 secretion, which was very low. In general, IL-6 secretion was less sensitive to inhibition or enhancement by the chemicals used. The results indicate that protein tyrosine kinases, protein kinase C and protein kinase A are required for PSG6 induction of IL-10 and IL-6 by monocytes, and allow us to hypothesize that these kinases mediate PSG signal transduction. Elevation of cAMP and activity of PDE type IV are important in PSG6 induction of IL-10, as has been shown for LPS. The effect of increasing cAMP (by rolipram) or inhibiting PKA (by RpcAMPS) on IL-6 secretion was less pronounced than their effects on IL-10 secretion. The fact that IL-10 inhibits monocyte secretion of IL-6 complicates the analysis of the results because PSG6 induces significant amounts of IL-10 by 13 hours after treatment. For example, by increasing IL-10 production, rolipram may thereby decrease IL-6 synthesis to some extect, although the signal pathway for IL-6 induction may involve cAMP. Similarly, the inhibition of IL-10 secretion by (Rp)cAMPS may reduce the inhibition of IL-6 secretion by this compound at the later time points when significant IL-10 is produced in response to PSG6. cAMP elevating agents have been reported to both increase (Nakamura et. al., 1998) and decrease (Strassmann et. al., 1994) IL-6 production by macrophages. Our results indicate that levels of cAMP and activity of PKA are involved in PSG6 induction of IL-6.

Monocytes were also treated with each inhibitor alone or with DMSO (dimethyl sulfoxide) alone to assess the effects of these chemicals on cytokine secretion. (Herbimycin A and Calphostin C were dissolved in DMSO.) In addition, monocytes were treated with PSG6 plus DMSO to determine if DMSO inhibited PSG6 induction of cytokines. None of the inhibitors induced IL-6 or IL-10 secretion, and DMSO did not decrease PSG6 induction of IL-6 or IL-10. At the tested concentrations the inhibitors had no effect on cell viability as assessed by trypan blue exclusion assay of the recovered cells

PSG6 N-domain Induction of IL-10 and IL-6 Synthesis by Monocytes

In addition to the experiments regarding the effect of PSGs on macrophage function, we wanted to preliminarily characterize the monocyte receptor-binding region within PSGs. To this end, we examined whether the PSG6 N-domain alone could mediate the effect of PSG6 on IL-10 and IL-6 induction by human monocytes. The N-domain is implicated in PSG function (Rutherford et. al., 1995). Amino acid sequence variation between PSG family members is clustered in the N-terminal domain, in regions analogous to the hypervariable regions of Igs. Although PSG proteins may lack one or more of the IgC-like domains, all PSGs identified in adult tissues contain an N-terminal IgV-like domain that is thought to be critical to function. In addition, a peptide including the RGD motif from PSG11(s) was shown to bind cells of the promonocyte lineage (Rutherford et. al., 1995). Moreover, the N-domain of mouse PSG18 mediates an increase in LPS-induced secretion of IL-10 and IL-6 by peritoneal macrophages (J. Wessells, 1999 doctoral thesis, USUHS). We treated monocytes with a PSG6 N-domain GST fusion protein produced using the baculovirus expression system, and measured IL-10 and IL-6 protein secretion compared to full-length PSG6. Monocytes were treated with 8 μg/ml GST-PSG6N, GST-PSG6 or GST-XylE. Cell supernatants were harvested at 24 hours after treatment, and IL-10 and IL-6 protein secretion were examined by ELISA (Figure 23). Dose response curves for PSG6N induction of IL-10 and IL-6 were also performed and compared to the GST-XylE curve (Figure 23).

Monocytes treated with GST-PSG6N secreted significant levels of IL-10 and IL-6 into the culture medium as compared to GST-XylE. Thus, the N-terminal domain of PSG6 is sufficient for induction of these cytokines by monocytes. GST-PSG6N induced approximately twice as much IL-10 and IL-6 as the same amount of full-length PSG6. The use of equal quantities (μg) of PSG6 and PSG6N results in 1.6 times more PSG6 N-domain protein molecules added to the cells. Therefore, if most of the activity of

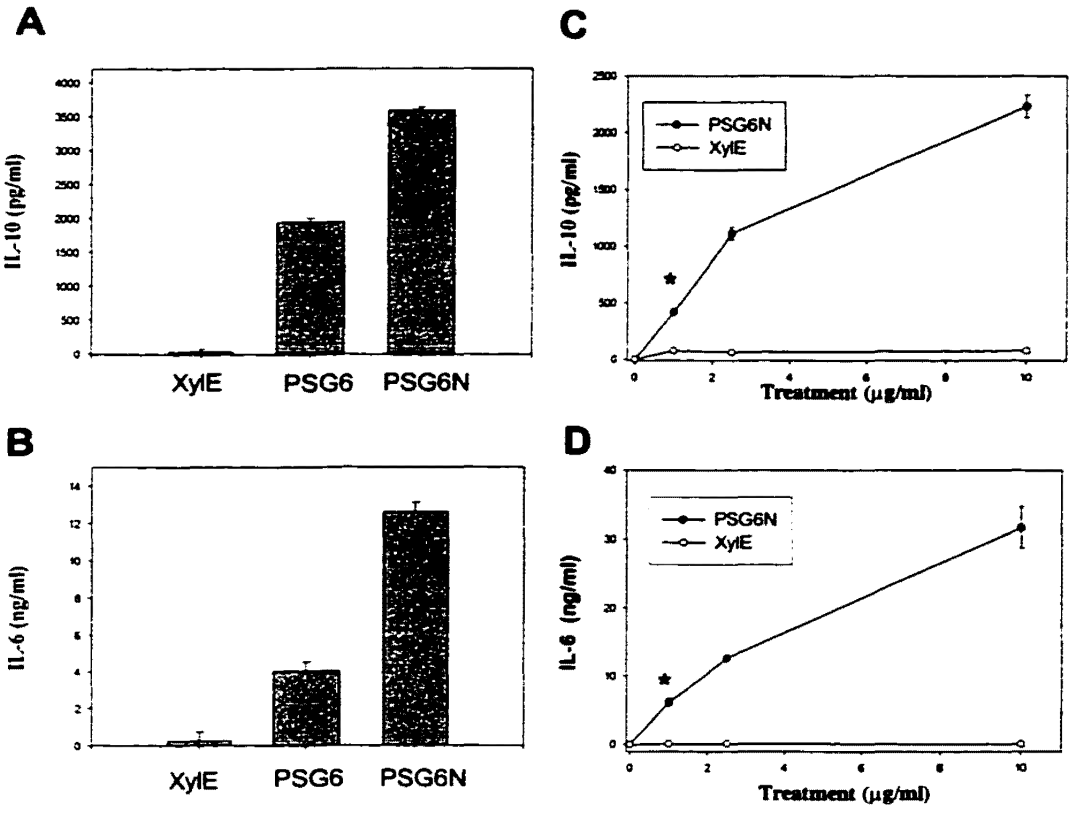

Figure 23. PSG6N induces secretion of IL-10 and IL-6 by monocytes. **(A and B)** Monocytes (0.4 x 10^6/well) in 96-well tissue culture dishes were treated with 8 µg/ml GST-PSG6, GST-PSG6N or GST-XylE, and IL-10 **(A)** and IL-6 **(B)** secretion at 24 hours was measured by ELISA. PSG6N induced significant secretion of both IL-10 and IL-6, indicating that the N-terminal domain alone was sufficient for induction of cytokines through binding to the monocyte PSG receptor. **(C and D)** Monocytes were treated with 1, 2.5 and 10 µg/ml GST-PSG6N or GST-XylE, and cell supernatant was analyzed for the presence of IL-10 **(C)** and IL-6 **(D)** at 24 hours. PSG6N induced IL-10 and IL-6 secretion in a dose dependent manner, with significant induction of both cytokines at 1 µg/ml (indicated by asterisks).

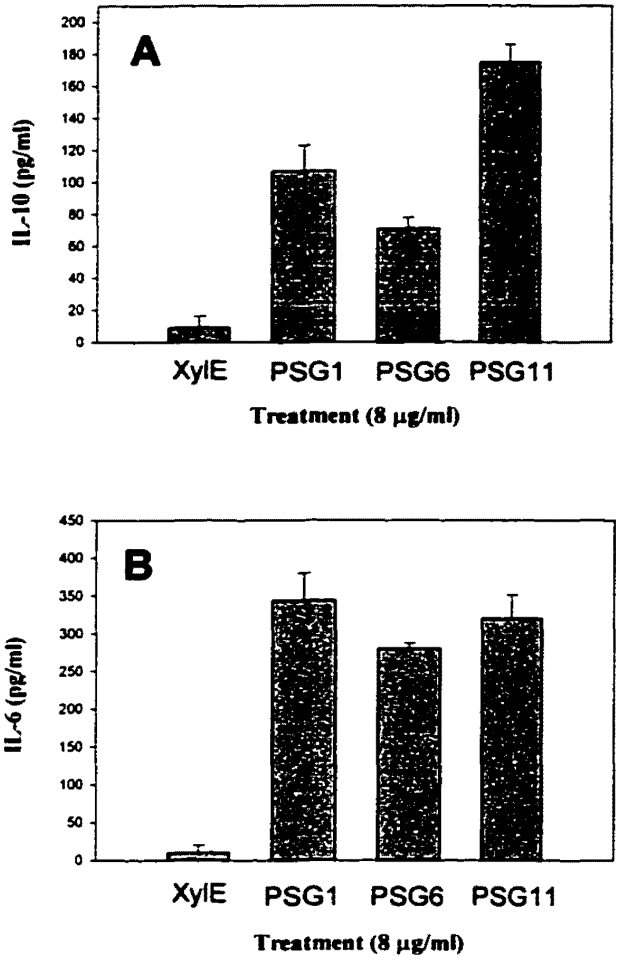

Figure 24. Human recombinant PSG fusion proteins induce IL-10 and IL-6 secretion in a mouse macrophage cell line. RAW 264.7 cells (1 x 10^6) were seeded into wells of a 24 well tissue culture dish, and treated with 8 µg/ml GST-PSG1, 6, 11, or GST-XylE in 300 µl total volume. Twenty-four hours after treatment, cell supernatant was collected, and murine IL-10 **(A)** and IL-6 **(B)** production was measured by ELISA. PSG1, PSG6 and PSG11 induced significant secretion of IL-10 and IL-6 by the mouse macrophage cell line RAW 264.7.

PSGs resides in the N-domain, it would be predicted that PSG6N would result in greater secretion than equal amounts of full-length PSG6. GST-PSG6N induced IL-6 and IL-10 in a dose-dependent fashion. One μg/ml PSG6N induced significant levels of IL-10 and IL-6 compared to control GST-XylE, and the dose response curves were beginning to plateau at 10 μg/ml. Significance for IL-10 and IL-6 secretion by full-length PSG6 was observed at higher concentrations, between 4 and 6 μg/ml (Figure 18). We would expect PSG6N induction to be greater than full-length PSG6 at the same concentration in μg, and the use of monocytes from different donors may also affect the dose at which significance occurs due to different numbers of PSG receptors. We conclude that the N-terminal domain of PSG6 (and probably PSG1 and PSG11) binds to the monocyte PSG receptor. These experiments did not address the question of whether the RGD or RGD-related motif, located at a conserved position within the N-domain, is involved in receptor binding. This question could be examined by competition with N-terminal peptides for PSG receptor binding or through mutational studies.

Cross-Species Induction of Macrophage Cytokine Secretion by PSGs

Since mouse and human PSGs consist of similar structures of repeated Ig-related domains that contain RGD-like motifs in the N terminal domain, are expressed mainly by the placenta, and members of both families induce IL-10 and IL-6 secretion by monocytes/macrophages, we wondered whether PSGs had cross-species activity. We tested this idea by treating a mouse macrophage cell line, RAW 264.7, with recombinant human PSG1, 6, and 11. At 24 hours after treatment cell supernatant was harvested, and murine IL-10 and IL-6 protein production were measured by ELISA (Figure 24).

GST-PSG1, 6, and 11 induced significant secretion of IL-10 and IL-6 protein by the mouse cell line RAW 264.7 compared to the GST-XylE control. The mouse PSG18 N-domain recombinant protein also induced IL-10 and IL-6 secretion by human monocytes (data not shown). It is apparent that mouse and human PSG proteins and their receptors can have a degree of similarity that allows cross-species macrophage receptor binding and activation. It will be interesting to determine whether the cross-reactivity of human PSGs is mediated through the presentation of a solvent-exposed RGD-like sequence by the similar Ig fold structure of the N terminal domains in these proteins.

IV. Discussion

Pregnancy-specific glycoproteins are a family of highly conserved, secreted proteins produced abundantly by the placenta (Bohn and Sedlacek, 1975, Lin et. al., 1974). Abnormally low levels of PSGs in maternal serum have been correlated with important complications of pregnancy including spontaneous abortion, intrauterine growth retardation, and fetal hypoxia (Masson et. al., 1983, Hertz and Schultz-Larsen, 1983, Gordon et. al., 1977, Tamsen et. al., 1983, MacDonald et. al., 1983). Although PSGs were discovered in 1970, no definitive function for these proteins has been shown. Various studies have suggested an immune system function for PSGs. The fetus is semi-allogeneic, expressing both maternal and paternal antigens, and as such, is considered an allograft that should be rejected by the mother according to standard immunological theory (Medawar, 1953). Explanations for the lack of fetal rejection include the absence of classical MHC class I and ciass II molecule expression by fetal tissues in contact with maternal blood, and therefore lack of presentation of fetal antigens to alloreactive maternal T cells (Sunderland et. al., 1981, Hunt et. al., 1988), and the presence of an antiinflammatory Th2-like immune environment in the uterus (Wegmann et. al., 1993). The uteroplacental unit produces a large array of cytokines that function in intercellular communication between maternal and fetal cells, and convincing evidence exists that antiinflammatory mediators such as IL-10, TGF-β, PGE2, and progesterone protect the fetus from damage by inflammatory cells (LGLs and macrophages). In both human and mouse studies, dysregulation of cytokine production during pregnancy has been shown to negatively affect pregnancy outcome (Tezabwala et. al., 1989, Chaouat et. al., 1990, Brabin and Brabin, 1992, Tangri and Raghupathy, 1993, Chaouat et. al., 1995, Hill et. al., 1995, Marzi et. al., 1996, Clark et. al., 1998, Arnold et. al., 1999).

It is likely that the feto-placental unit itself orchestrates the creation of the proper immune environment necessary for fetal survival, and experimental evidence supports this contention. Placental trophoblast is a key regulator of cytokine production during pregnancy (Robertson et. al., 1994). The placenta is known to synthesize factors that modify the maternal metabolism and uterine environment to benefit the fetus. Pregnancy-specific glycoproteins are products of the placenta produced in large quantities throughout pregnancy. We postulated that the PSGs may mediate expression of one or more immunoregulatory molecules, as part of the feto-placental control of the local immune environment.

We investigated the ability of three human recombinant PSGs to regulate the production of immune mediators such as IL-10, TNF-α, and IL-6 by macrophages in vitro. The capability to synthesize a large number of regulatory cytokines in response to microbial and immune signals places the macrophage at a central position in uteroplacental cytokine networks. We showed that PSGs induce specific and dose-dependent production of IL-10 and IL-6 by human blood monocytes, but not TNF-α, IL-1β or IL-12. The N-terminal Ig-variable-like domain of PSG6 was sufficient for induction of IL-10 and IL-6, suggesting that this domain mediates the interaction of PSGs with their receptor. We also showed that increased IL-10 and IL-6 secretion by monocytes was accompanied by an increase in specific mRNA for these cytokines. In preliminary investigation of the signal transduction pathways involved in PSG-mediated cytokine secretion by monocytes, we observed that protein tyrosine kinases, protein kinase C and cAMP elevation are involved in PSG induction of IL-10 and IL-6 secretion.

Mouse Pregnancy-specific Glycoproteins

In addition to investigation of the function of human PSG proteins, we have begun to develop a mouse model for PSG function by cloning murine PSG cDNAs. Mouse and human PSG genes show structural similarites. Both families are members of the CEA gene family and the immunoglobulin superfamily of genes. Mouse and human PSGs consist of repeated Ig-like domains. While human PSGs have one N-terminal Ig variable-like domain and several Ig constant-like domains with a short C-terminal domain, the mouse PSGs have 2-3 variable-like domains, only one constant-like domain, and no C-terminal domain comparable to that of human PSGs. Both families contain a RGD or RGD-related sequence motif at a conserved position within the N-terminal domain. Mouse and human recombinant PSG proteins are functionally similar in that they both induce IL-10 and IL-6 secretion by monocyte/macrophage cells. In addition, human PSGs induce cytokine secretion by a mouse macrophage cell line and mouse PSG18N induces IL-10 and IL-6 secretion by human monocytes. These observations indicate that PSG function is highly conserved. Although the human PSG gene family has been studied extensively, little is known about mouse PSG proteins and cDNA structures. The establishment of an animal model in the genetically well-characterized mouse will facilitate in vivo experiments to elucidate of the role of PSGs in mouse pregnancy, and should lead to predictions of the function of human PSGs.

We sequenced two novel mouse PSG cDNAs, PSG23 and PSG28, and determined that, similar to the expression of other murine PSGs, transcripts encoding these proteins were expressed exclusively by placenta. Human PSG mRNAs are expressed by endometrium, testes, intestine, PMNs, and monocytes, and protein production has been demonstrated for testes and intestine. In published studies, mouse PSG17-19 were expressed exclusively by placenta, PGS16/bCEA is expressed in brain, and CEA6 is a pseudogene. Zimmermann et. al. (unpublished data) have shown that all murine PSG mRNAs except CEA6 and PSG28 were coordinately expressed by placenta in increasing amounts during gestation. PSG28 may have been undetectable using the degenerate oligonucleotide primers in that study. Since we isolated PSG28 from placental RNA, it is clear that PSG28 is also expressed by placenta. The future identification of possible extraplacental sites of expression for novel PSG transcripts in the mouse will provide the basis for studying the role of these proteins in non-placental tissues.

Murine PSG23 and PSG28 cDNAs were shown to have structures similar to other known mouse PSGs. Both PSG23 and PSG28 code for a 34 amino acid leader peptide, three IgV-like domains, N1, N2 and N2, and an IgC-like A domain at the C termius. The N2 and N3 domains are preceded by hydophobic partial leader-like sequences of 9-12 amino acids, which is a characteristic of mouse PSGs. Both cDNAs contained 7 potential N-linked glycosylation sites within the IgV-like domains. PSG23 has an RGD-related motif, HGE, at a conserved position in the N-terminal domain, where the positively charged amino acid histidine replaces the positively charged arginine, and glutamic acid replaces the very similar acidic amino acid aspartic acid. PSG28 has a QGE sequence motif at a comparable position, where glutamine replaces arginine.

The mouse PSG18 N-terminal domain has recently been shown to induce IL-10 and IL-6 mRNA expression by peritoneal macrophages, and IL-10 and IL-6 protein secretion by the macrophage cell line RAW 264.7 (J. Wessells, 1999 doctoral thesis). This mouse PSG protein contains the RGD-like sequence motif, RGE, within the N domain. A peptide from human PSG11 containing the RGD motif has been reported to bind to cells of the monocytic cell lineage, and PSG11 and PSG6, which also contains an RGD, are shown in this study to induce IL-10 and IL-6 protein secretion by human monocytes. Human PSG1 has a KGD sequence motif, where the basic amino acid arginine is replaced by the basic amino acid lysine. PSG1 was also shown to induce both IL-10 and IL-6 secretion by monocytes in vitro, indicating that the

RGD sequence is not essential for PSG function. PSG induction of uterine macrophage IL-10 may have an important role in maintaining an anti-inflammatory immune environment at the feto-placental interface. Since some functions of individual human PSGs are redundant (three human PSGs induced monocyte IL-10 and IL-6 secretion), it will be important to determine whether mouse PSG23 (HGE) and PSG28 (QGE), whose N-terminal domain sequence motifs differ more substantially from the canonical RGD than does PSG18, can induce macrophage production of IL-10 and IL-6.

The establishment of a mouse model for PSG function will significantly contribute to the detemination of PSG function in vivo. The cloning and expression analysis of murine PSG23 and PSG28 cDNAs brings us closer toward this goal. A complete mouse model for PSG function will require identification of the cDNA sequences for the remaining mouse PSGs (PSG20-22, 24-27, and PSG29), and determination of the sites and temporal patterns of their expression. Functional experiments on mouse PSG17 and PSG19 are currently being done in Dr. Dveksler's laboratory, and preliminary data indicates that PSG17 also induces IL-10 and IL-6 secretion by macrophages. An antibody to mouse PSGs, which is also being produced in our laboratory, will allow measurement of the concentration of PSG protein in maternal serum during normal and abortion-prone mouse pregnancy, the size of serum PSGs, and identification of extraplacental sites of protein expression. In humans, maternal serum PSG levels are very high, reaching 200-400 μg/ml at term, and indicating their importance in pregnancy.

An antibody to murine PSGs will also allow experiments to determine how neutralization of PSGs affects specific cytokine production by uterine and placental tissues in vivo. In a mouse model of recurrent spontaneous abortion (RSA), inflammatory cytokine production by placenta is increased, and IL-10 injection prevents the abnormally high incidence of abortion (Chaouat et. al., 1995). In future experiments, the question of whether administration of PSGs can decrease abortion in the mouse model of recurrent abortion, with a concomitant decrease in production of inflammatory cytokines, and an increase in IL-10 production at the maternal-fetal interface, can be addressed.

The production of PSG-deficient mice is not practical, due to the large number of PSG genes, but transgenic mice constitutively expressing a PSG gene are currently being produced by our laboratory. These mice could be mated and back-crossed with mice of the abortion-prone mating phenotype to study PSG affects on RSA, and with a mouse strain that is a model for multiple sclerosis to study PSG effects on

this autoimmune inflammatory disease. PSG transgenic mice could also be used to examine whether constitutive expression of PSG protein affects the normal development of a polarized Th1 or Th2 type response to specific pathogens.

In Vitro Functional Studies of Human PSG1, PSG6 and PSG11

To study PSG function, we synthesized three different human recombinant PSGs using the baculovirus insect cell expression system. Although human PSGs are highly similar in amino acid sequence (85%), they are differentially expressed by the human placenta, and may have the same, different, or even opposing activities during pregnancy. Multiple PSGs are present during pregnancy, and may bind the same receptor(s) to produce additive, synergistic or antagonistic effects. The similarity between PSG protein sequences, the large number of PSG family members, and the fact that some PSGs have allelic variants that are pseudogenes, suggests that at least some PSG functions may be redundant. PSG1 gene products predominate in human placenta and pregnant serum (Streydio and Vassart, 1990, Rutherford et. al., 1995). PSG1 was shown to enhance the growth and maturation of mouse embryos in cocultures of embryos with CHO cells expressing recombinant PSG1 (Wu et. al., 1999). PSG6 mRNA is also highly expressed in human placenta (Pan et. al., 1994), although it was not detected in placenta by Northern blot in one study (Zimmermann et. al., 1989). PSG6 is also preferentially expressed by hydatidiform mole (Leslie et. al., 1990). PSG11s is expressed by placenta throughout pregnancy (Streydio and Vassart, 1990, Pan et. al., 1994). Peptides from the N-terminal domain of PSG11 have been shown to bind to cells of the promyelocytic lineage (Rutherford et. al., 1995), suggesting a route by which PSGs may modulate macrophage function. Recently, reduced endometrial expression of PSG11, and to a lesser extent PSG1, was reported to be associated with RSA (Arnold et. al., 1999). In the same study PGS11s, but not PSG1a, enhanced the secretion of IL-10 by LPS-activated monocytes, and induced IL-10 protein secretion (at low levels of less than 30 pg/ml) by two monocytic cell lines (Arnold et. al., 1999). PSG1a is an alternatively spliced form of PSG1 that differs from PSG1d in the last four amino acids of PSG1a, and PSG1d has an additional 7 amino acids at the extreme C terminus. Both PSG1a and PSG1d are expressed throughout pregnancy by placenta (Streydio and Vassart, 1990). PSG6 and PSG11 contain an RGD motif within the N-terminal domain, and PSG1 (KGD) does not. We examined the role of human PSG1(d), PSG6(r), and PSG11(s) as immunomodulators in vitro via their effects on cytokine production by human monocytes.

Recombinant human PSG proteins, produced as N-terminal GST fusions, were secreted into infected insect cell supernatant, and were purified using affinity chromatography. The GST-XylE control fusion protein was isolated from insect cell lysate and also purified by glutathione affinity chromatography. Both a rabbit polyclonal antiserum and a monoclonal antiserum to human PSGs (BAP-1) detected the full-length recombinant PSG proteins in Western blot. These antibodies did not detect GST-XylE or proteins from mock-infected insect cell supernatant, indicating their specificity for PSGs. An anti-GST antibody detected the recombinant PSG and XylE control proteins in immunoblots. PSG proteins are highly glycosylated, with carbohydrate making up to 30% of their mass. Most unglycosylated PSGs are predicted to be 37-49 kD in size, but gel filtration and SDS-PAGE show major bands at 110, 72, 64 and 54 kD, indicating a high level of glycosylation for human PSGs in vivo. Although the recombinant PSG proteins produced by the baculovirus system were probably glycosylated, as shown by the increased apparent MW in gel electrophoresis, and precipitation by the lectin concanavalin A, the extent of glycosylation was not as high as that of serum PSGs. Glycosylation may function to extend the half-life of PSGs in serum. Because the recombinant PSGs induced functional responses by monocytes, it is unlikely that glycosylation plays a role in their function, but this remains to be determined.

Recombinant PSG1, PSG6 and PSG11 Induce Monocyte Secretion of IL-10 and IL-6

Our results showed that treatment with human PSG1, PSG6 and PSG11 resulted in a significant induction of IL-10 and IL-6 secretion by monocytes compared to the control GST-XylE. The specificity of PSG protein induction of these cytokines was shown by the fact that removing PSG protein from insect cell supernatant by immunoprecipitation significantly reduced monocyte production of IL-10 and IL-6 to 10-15% of control. PSG1, PSG6 and PSG11 induced dose-dependent secretion of IL-10 and IL-6, indicating that PSG cytokine induction by monocytes is a specific biological effect of PSG protein. The amount of IL-10 and IL-6 secretion induced by PSGs varied widely when monocytes from different donors were used. This result was expected since it is known that the capacity to produce cytokines varies between individuals (Eskdale et. al., 1998), and recent illness in monocyte donors or other factors affecting blood monocyte phenotype can affect cytokine production. Although PSG1 consistently induced higher amounts of IL-10 and IL-6, so far the data indicate that PSG function may be highly redundant. Gene duplication that resulted in multiple PSGs with very similar sequences may have occurred to increase PSG production

during pregnancy. Until the functions of other PSG family members are determined, and more detailed information regarding amounts and temporal patterns of individual PSG protein expression are available, this theory cannot be tested.

The PSG1 dose response curves reached a maximum at 8 μg/ml PSG protein, while the PSG6 and PSG11 curves did not. PSG1 may be a partial agonist of the PSG6/PSG11 receptor. If PSG6 and PSG11 have less affinity for the PSG receptor, they may require higher concentrations to achieve full receptor occupancy. Partial agonists produce a lower receptor response at full occupancy than full agonists. A ligand can have strong affinity for a receptor and yet not efficiently induce the active conformation (for example some receptor antagonists have higher affinity for their receptor than agonists). Since maximum responses for PSG6 and PSG11 induction of IL-10 and IL-6 were not reached, it is impossible to say whether PSG6 and PSG11 have a higher maximum response than PSG1. Different numbers of PSG receptors expressed on donor monocytes could theoretically affect the dose at which the maximal response occurs. Donors with a higher number of PSG receptors can achieve a maximum response at a lower dose, because the same number of active receptors can be generated at a lower dose. (Binding of receptor and ligand depend on the amount of both components of the complex.) However, our results indicate that different individuals may have similar numbers of PSG receptors because PSG1 induced a maximal response at 8 μg/ml in four different donors. Since PSG1 also had slightly different kinetics of cytokine induction, lagging behind the other two PSGs early in the response, and then surpassing them at 13 hours, it may be that PSG1 is binding to a different receptor.

The N-terminal Ig Variable-like Domain Mediates PSG Receptor Binding

It has been suggested that PSG proteins bind to cell-surface integrins, because of the presence of an RDG sequence motif in the N-terminal domain (Rebstock et. al., 1993). This motif is found in several ECM proteins where it has been shown to bind to integrins (Ruoslahti and Pierschbacher, 1987). PSG1(d) protein does not have an RGD sequence motif in the N-terminal domain like PSG6 and PSG11. Instead it has a KGD motif, where arginine is replaced by another basic amino acid, lysine. A similar replacement is seen in the N-terminus of three mouse PSGs that have the related sequence motif HGE, (containing a different basic residue), at a conserved position. The RGD motif is located in a predicted solvent-exposed loop in the N-terminal domain. Because all three PSGs examined in this study induced cytokine secretion

by monocytes, we conclude that either the RGD motif is not necessary for binding the PSG receptor, that KGD can substitute for the RGD, or that PSG1 is binding to a different receptor. However, it was demonstrated that the N-domain was important in PSG function, as has been hypothesized based on the presence of this domain in all alternatively spliced PSGs produced by adult tissues. Recombinant protein consisting of only the N-terminal Ig variable-like domain of PSG6 was sufficient for induction of IL-10 and IL-6 by monocytes. PSG6N also showed a dose-dependent induction of these cytokines. It is likely that PSG6 and PSG11 induction of IL-10 and IL-6 is mediated by binding of the N-domain to the monocyte PSG receptor.

Human PSGs Selectively Induce IL-10 and IL-6 Secretion by Monocytes

That PSG treatment did not simply activate monocytes was shown by the fact that PSGs did not induce inflammatory cytokines like TNF-α, IL-1β and IL-12. These cytokines are normally produced by macrophages in an inflammatory response to microbial products, which is followed by later IL-10 secretion that downregulates the inflammatory activity of macrophages. PSGs did not induce statistically significant amounts of TNF-α or IL-1β compared to the XylE sup control, but some TNF and IL-1β secretion was observed after treatment with PSGs and XylE sup. Apparently components of insect cell supernatant (perhaps FBS) were responsible for this effect. XylE sup also induced low amounts of IL-10 and IL-6. Therefore, it is possible that components of insect cell supernatant in the PSG protein samples acted to prime monocytes to respond to PSGs in some way. For example, TNF-α induces macrophage synthesis of IL-10 mRNA (Platzer et. al., 1995). Priming of macrophages in vivo by IFN-γ, GM-CSF or other factors commonly occurs as macrophages are induced to become activated, in a step-wise fashion, for specific effector functions (see below). The uterine environment contains many immune modulatory factors that induce specific uterine macrophage phenotypes. It seems reasonable that this phenotype would respond to PSGs more efficiently than blood monocytes.

Interleukin 12, also called natural killer cell stimulatory factor (NKSF), is a 70 kD heterodimeric cytokine composed of disulfide-linked 40 kD (p40) and 35 kD (p35) subunits encoded by separate genes (Wolf et. al., 1991). IL-12 is an important regulator of the effector phase of cell-mediated immune responses. IL-12 induces production of the inflammatory cytokines IFN-γ and TNF-α by T cells and NK cells (Kobayashi et. al., 1989, Chan et. al., 1991), enhances cytotoxic activity of NK and T cells (Chehimi

et. al., 1992), directly induces and synergizes with IL-2 in generation of LAKs (Stern et. al., 1990), and induces proliferation of activated T and NK cells (Gately et. al., 1991, Perussia et. al., 1992). IL-12 is the most potent NK cell stimulator known, synergizing with IL-2 to induce NK cell production of IFN-γ. IL-12 promotes the development of the Th1 phenotype (Bradley et. al. 1995, Magram et. al., 1996), and inhibits the development of human Th2-like IL-4-producing T cells (Manetti et. al., 1993).

Monocytes, macrophages and dendritic cells are the principal sources of IL-12 (Trinchieri, 1995, Muller et. al., 1995). Whole bacteria strongly induce IL-12 in human monocytes (7 ng/ml by 10^6 cells), and LPS and other bacterial and viral products are also efficient inducing stimuli (D'Andrea et. al., 1992, Cleveland et. al., 1996, Ballas et. al., 1996). NK cells, T cells and B cells do not express IL-12 (Wolf et. al., 1992, Guery et. al., 1997). IL-10 inhibits the production of IL-12 p40 mRNA and protein secretion induced by LPS, and this is an important mechanism by which IL-10 inhibits Th1 cell development and downregulates inflammatory immune responses (D'Andrea et. al., 1993, Kubin et. al., 1994). In fact, IL-12 has been shown to induce T cell production of IL-10, which then inhibits IL-12 synthesis by APCs in a negative feedback loop (Van der Pouw Kraan, 1995). TGF-β, IL-4 and PGE2 also inhibit IL-12 production by APCs (Trinchieri, 1995, Van der Pouw Kraan et. al., 1995). Because of its effects on Th1 type T cell differentiation, IFN-γ production, and activity of NK cells, cytotoxic T cells and LAKs, IL-12 would be expected to be harmful to pregnancy.

Tumor necrosis factor (TNF) is a primary mediator of immune regulation and has both proinflammatory and cytotoxic effects. TNF-α exists in two forms; a 26 kD transmembrane pro-TNF and a 17 kD secreted form. Pro-TNF on the plasma membrane is proteolytically cleaved in the extracellular domain by an MMP-like protease called TNF-converting enzyme, or TACE, to release mature TNF (Kriegler et. al., 1988, Black et. al., 1997). The transmembrane form of TNF can mediate cytotoxicity through cell-cell contacts (Kriegler et. al., 1988). Secreted TNF is a homotrimer (51 kD) held together by noncovalent interactions between C termini (Smith and Baglioni, 1987). TNF is produced by a wide range of cells including monocytes, NK cells, B and T cells, granulocytes, mast cells, osteoblasts, smooth muscle cells, and keratinocytes. Macrophages produce TNF in response to viruses, bacterial and parasitic products, and cytokines such as IL-1, IL-2, IFN-γ, GM-CSF and TNF itself. IFN-γ significantly enhances TNF synthesis by macrophages (Philip and Epstein, 1986). TNF production by macrophages is inhibited

by TGF-β, PGE2, IL-4, IL-6, IL-10 and corticosteroids, among other factors. TNF induces PGE2 production (Dayer et. al., 1985, Elias et. al., 1987), and cAMP elevating agents, including PGE2 and rolipram, inhibit TNF-α synthesis by human mononuclear cells, even in the absence of significant levels of IL-10 (Sinha et. al., 1995, Semmler et. al., 1993, Eigler et. al., 1998).

TNF-α is a major mediator of host responses to Gram-negative bacteria, and is critical to host control of infection. At low concentration TNF is a local regulator of leukocyte and endothelial cells. It has angiogenic activity and causes vascular endothelial cells to express adhesion molecules for leukocytes, which results in accumulation of leukocytes at sites of inflammation. TNF also activates inflammatory leukocytes (especially neutrophils) to kill microbes. It induces acute phase protein synthesis directly and through its induction of IL-1 and IL-6. TNF was originally characterized as a protein with antitumor properties (Carswell et. al., 1975). In this regard TNF is both directly cytotoxic and activates the antitumor activity of the immune system (Feinman et. al., 1987). TNF-α is also an important immunoregulator. TNF-α enhances T cell responses by increasing the T cell proliferative response to antigen, IL-1 receptor expression, and induction of IFN-γ (Scheurich et. al., 1987). TNF-α also stimulates accessory, cytotoxic and secretory effector functions of macrophages (Philip and Epstein, 1986).

Although large amounts of TNF are detrimental to pregnancy, TNF-α and its receptors are normally expressed by uterine and placental tissues, and probably have a role in normal reproduction. In the mouse model of spontaneous abortion, fetal demise is strongly correlated with increased placental production of Th1 cytokines, including TNF-α (Tangri and Raghupathy, 1993). However low amounts of this cytokine may be beneficial during pregnancy via effects on cellular growth and differentiation, and uterine tissue repair and remodeling to accommodate the growing embryo. Human endometrial epithelia and stroma produce TNF-α, which may be regulated by female steroid hormones, and is proposed to function in modulation of cell growth and viability of the cycling endometrium (Hunt et. al., 1992, Terranova et. al., 1995). TNF is also produced by human placental cytotrophoblast (Li et. al., 1994, Yang et. al., 1993) and syncytiotrophoblast (Chen et. al., 1991), and trophoblast contains TNF receptors (Yelavarthi and Hunt, 1993). TNF has been shown to decrease proliferation of rat trophoblast (Hunt et. al., 1989), and to promote apoptosis of human villous trophoblast (Yui et. al., 1994). The invasive nature of trophoblast resembles that of tumor cells, and one function of uterine TNF may therefore be to control

trophoblast proliferation and invasion through induction of trophoblast differentiation or apoptosis. TNF has been shown to induce human trophoblast release of hCG via induction of IL-6 (Eades et. al., 1988, Li et. al., 1992). The lack of induction of monocyte TNF secretion by PSGs supports the notion that PSGs do not induce an inflammatory macrophage phenotype, but instead, through secretion of IL-10, downregulate macrophage inflammatory activity.

Interleukin-1 is a multifunctional cytokine with a major role in innate immunity and inflammation. The major source of IL-1 is activated macrophages. IL-1 production by macrophages is triggered by diverse microbial products, such as LPS, by contact with CD4+ T cells, or by TNF or IL-1 itself (Schlinder et. al., 1990a). IL-1 is also produced by many other cells, including epithelial and endothelial cells, B and T lymphocytes, and NK cells. IL-1 exists is two forms, IL-1α and IL-1β, each consisting of a 17 kD mature protein, but products of two distinct genes. A third member of the IL-1 family is the IL-1 receptor antagonist, IL-1RA, which is also produced in response to endotoxin (Arend, 1993). Both IL-1 proteins bind to the same receptors and their activities are similar, but human monocytes produce predominantly IL-1β. IL-1 is synthesized as a 31 kD precursor without a leader sequence, and processing of IL-1α and IL-1β to the mature form requires proteases (Black et. al., 1988). IL-1β is active only in the 17 kD mature form (Jobling et. al., 1988).

The primary function of IL-1 is as a mediator of the inflammatory response to infection and trauma. Like TNF, IL-1 increases leukocyte migration to sites of inflammation, via increased vascular adhesion, increases leukocytes ability to kill microbes, has angiogenic activity, and at high concentration, causes fever and induction of acute phase proteins, but is not capable of causing death. An important function of IL-1 is induction of IL-6 synthesis (LeMay et. al., 1990). Although IL-1 (α and β) is produced by cytotrophoblast (Paulesu et. al., 1991, Stephanou et. al., 1995), and seems to have a role in embryonic development, IL-1 is not essential for pregnancy, as shown by studies using mice deficient in IL-1β, ICE or IL-1RI (Kuida et. al., 1995). IL-1 induces human trophoblast release of hCG and placental lactogen in a pathway involving increased trophoblast production of IL-6 (Masuhiro et. al., 1991, Stephanou and Handwerger, 1994). IL-1β stimulates IL-1 and IL-6 synthesis by trophoblast, and, in a negative regulatory loop, IL-6 inhibits IL-1 expression by cytotrophoblast (Stephanou et. al., 1995). IL-1 is present in amniotic fluid during the third trimester of pregnancy, and has been shown to induce production of PGE2 by human

amnion and decidua, and thus may function in initiation of labor (Romero et. al., 1989b, Mitchell et. al., 1990). IL-1 also stimulates growth of rat trophoblast (Hunt et. al., 1989). LPS induction of IL-1 mRNA synthesis by human trophoblast may be an important mechanism in control of intrauterine infection (Kauma et. al., 1992).

Specific induction of both IL-10 and IL-6, and not other inflammatory cytokines, has been demonstrated for another protein, HIV Nef (Brigino et. al., 1997, Chirmule et. al., 1994), showing that such activity is not unprecedented. IL-10 can both prevent development of inflammatory macrophages and inhibit an ongoing inflammatory response. We did not investigate whether PSGs could downregulate the secretion of inflammatory cytokines by LPS-activated macrophages, although experiments did indicate that simultaneous treatment with LPS and PSGs synergistically upregulated IL-10 production by monocytes at 24 hours after treatment (data not shown).

Increased Monocyte Cytokine Secretion Induced by PSGs is Associated with an Increase in Specific mRNA Expression

Since cytokine production is generally regulated by the amount of specific mRNA, we examined whether PSG6 induced an increase in IL-10 and IL-6 mRNA levels in monocytes. At five hours after treatment, PSG6 induced a 19-fold increase in IL-10 mRNA and a 9-fold increase in IL-6 mRNA compared to the XylE control. Whether the mRNA increases were due to increased transcription and/or increased mRNA stability was not determined. Cycloheximide inhibited the increase in IL-6 mRNA induced by PSG6, indicating that protein synthesis is required for PSG-mediated increases in IL-6 mRNA. Addition of cycloheximide increased amounts of IL-10 mRNA after treatment with XylE or PSG6. Low spontaneous production of IL-10 mRNA occurs in human PBMCs, and treatment with cycloheximide (1 μg/ml) further enhances IL-10 production from 30 minutes to 24 hours after treatment (Stordeur et. al., 1995). Removal of FCS from culture media diminishes basal IL-10 transcription in monocytic cells (Lehmann, 1998). The effect of cycloheximide on levels of IL-10 mRNA was shown to involve both mRNA stabilization and an increase in transcription in experiments utilizing actinomycin D, and from nuclear run-on assays (Stordeur et. al., 1995). Cycloheximide also increases steady-state levels of IL-6 mRNA in human fibroblasts (Zhang et. al., 1988), and TNF-α mRNA in rat astrocytes via an increase in mRNA transcription and stability (Chung et. al., 1992). However in human PBMCs, cycloheximide (5 μg/ml) inhibits LPS-induced IL-6

mRNA levels (Wang et. al., 1994). If transcription of these cytokines is under the control of repressor proteins with a high turnover rate, cycloheximide may act by inhibiting the synthesis of the repressor proteins, allowing transcription to occur. (For example, the transcription factor NF-κB is activated by cycloheximide treatment, perhaps through inhibition of the synthesis of IκB.) An AUUUA repeat motif, found in the 3' UTR of some cytokine (TNF, IL-6) and other immediate early gene mRNAs, is thought to regulate mRNA stability through binding to trans-acting factors that facilitate mRNA deadenylation and degradation (Caput et. al., 1986). IL-10 mRNA contains 6 AUUUA motifs in the 3'UTR (Moore et. al., 1990). Cycloheximide may also exert its effects on cytokine gene expression by inhibition of synthesis of such RNA binding factors. The use of alternative protein synthesis inhibitors, such as anisomycin, might show whether protein synthesis is required for PSG-mediated increases in IL-10 mRNA. The late production of IL-10 induced by PSGs (significance at 13-22 hours) suggests a requirement for protein synthesis.

PSG-induced IL-10 Secretion by Monocytes is Upregulated Later than IL-6 Secretion

IL-10 inhibits the expression of IL-6 by human monocytes via inhibition of transcription (Wang et. al., 1994) and/or enhancement of mRNA degradation (Takeshita et. al., 1996). Twenty ng/ml IL-10 added 4 hours or less after treatment with 80 ng/ml LPS inhibits TNF-α induction completely and inhibits IL-6 secretion by greater than 90% (Wang et. al., 1994). The highest measured amounts of secreted IL-10 induced by GST-PSG was 2.4 ng/ml, and IL-6 was as high as 61 ng/ml, so inhibitory effects of PSG-induced IL-10 on IL-6 secretion may not have been seen. A time course for PSG induction of IL-10 and IL-6 was done to further characterize the monocyte response to PSGs. PSGs induced significant secretion of IL-10 between 13 and 22 hours after treatment, and IL-6 between 6 and 10 hours. If significant amounts of IL-10 are not produced early in the response to PSGs (before 4 hours after treatment), IL-10 will not prevent macrophage secretion of IL-6 protein, but the higher levels of IL-10 produced later in the response may have reduced IL-6 secretion. To investigate this further, treatment with anti-IL-10 antibody together with PSG protein would be necessary.

Signal Transduction Pathways Involved in PSG Induction of Monocyte IL-10 and IL-6 Expression

We investigated the intracellular signals involved in PSG-mediated IL-10 and IL-6 secretion by monocytes. As a starting point we examined pathways that are known to be activated in LPS induction of

cytokine secretion. Activation of monocytes by LPS results in a burst of proinflammatory cytokine secretion (TNF, IL-1 and IL-6) that is followed by delayed secretion of anti-inflammatory mediators, such as IL-1 receptor antagonist (IL-1RA) and IL-10 (de Waal Malefyt et. al., 1991b). Monocyte activation occurs through LPS binding to CD14, a 55 kD glycoprotein attached to the macrophage plasma membrane by GPI linkage (Wright et. al., 1990). In mouse macrophages LPS-bound CD14 associates with a transmembrane molecule called Tlr-4 (Toll-like receptor-4) that provides a signal transduction function (Poltorak et. al., 1998). The signal transduction events that follow LPS binding are incompletely understood. It is known that LPS treatment results in a rapid activation of PTKs (protein tyrosine kinases) (Weinstein et. al., 1991, Beaty et. al., 1994), PKC (protein kinase C) (Chung et. al., 1992, Shapira et. al., 1994), and MAPKs (mitogen-activated protein kinases) (Weinstein et. al., 1992, Liu et. al., 1994b, Geppert et. al., 1994).

The most important target of macrophage-derived cytokines is the macrophage itself. Macrophages are only transiently activated (Poste and Kirsh, 1979) and both negative and positive feedback regulation by macrophage secretory products control macrophage activity. Cytokines such as IL-1 and TNF-α have been shown to induce TNF-α in human monocytes (Philip and Epstein, 1986). TNF and IL-1 also stimulate IL-1 production by macrophages (Bachwich et. al., 1986, Schlindler et. al., 1990a), and induce IL-6 synthesis by fibroblasts (Content et. al., 1985, Van Damme et. al., 1987). In human monocytes IL-1 has been shown to induce IL-6 production (Bauer et. al., 1986, Gross et. al., 1993). In a negative regulatory loop, IL-6 inhibits the secretion of TNF and IL-1 (Aderka et. al., 1989, Schlindler et. al., 1990b). Some studies indicate that IL-10 gene expression after LPS stimulation depends on earlier production of TNF and PGE2 (Platzer et. al., 1995, Meisel et. al., 1996). LPS-induced PGE2, IL-10 and TGF-β regulate LPS responsive genes resulting in downregulation of inflammatory functions of the macrophage or "macrophage deactivation" (Strassmann et. al., 1994, Bogdan et. al., 1991, Tsunawaki et. al., 1988, Vodovotz et. al., 1993). IL-10 inhibits PGE2 production by monocytes in a negative feedback loop (Mertz et. al., 1994), but TGF-β1 has been shown to enhance mouse macrophage production of IL-10 in response to LPS (Maeda et. al., 1995).

Protein Tyrosine Kinases

Enzyme mediated phosphorylation and dephosphorylation of cellular proteins is a principal mechanism by which external signals regulate intracellular responses like gene expression. LPS increases protein tyrosine phosphorylation in both murine (Weinstein et. al., 1991) and human macrophages (Weinstein et. al., 1993), which is detectable by 5 minutes, maximal at 15-30 minutes, and declines after 60 minutes post-treatment (Weinstein et. al., 1991, Beaty et. al., 1994). Stefanova reported that the PTK $p56^{lyn}$ is coupled to the LPS receptor CD14 in human monocytes, and that LPS transiently (5-60 min.) stimulates activity of the src family PTKs $p56^{lyn}$, $p58/64^{hck}$ and $p59^{c\text{-}fgr}$ (Stefanova et. al., 1993). Beaty et. al. (1994) showed that LPS induced both hck and lyn kinase activity in human monocytes before the onset of TNF-α production (at 1 hour), and that this activity was inhibited by the PTK inhibitor Herbimycin A. Inhibition of PTKs by pretreatment with Herbimycin A blocks LPS-induced human monocyte secretion of TNF-α, IL-1, IL-6 and IL-10 suggesting that PTK activation is essential for production of these cytokines (Stefanova et. al., 1993, Geng et. al., 1993, Shapira et. al., 1994, Beaty et. al., 1994, Meisel et. al., 1996). Herbimycin A inhibits TNF-α and IL-6 mRNA accumulation in human monocytes after LPS treatment, indicating a pretranslational mechanism of action (Shapira et. al., 1994, Beaty et. al., 1994). Although Herbimycin A inhibits LPS-induced tyrosine phosphorylation and TNF-α secretion by human macrophages, it does not strongly inhibit PMA (phorbol 12-myristate 13-acetate) induced tyrosine phosphorylation (Weinstein et. al., 1991). Thus, Herbimycin A does not inhibit all PTKs, and LPS and PMA activate different PTKs. IL-10 expression (both mRNA and protein) by monocytes requires both an early and a late (at five hours) PTK activity (Meisel et. al., 1996).

The MAPK Pathway

Signalling through the MAPK pathway subsequent to PTK activation is implicated in inflammatory cytokine secretion by macrophages after LPS stimulation (Dong et. al., 1993, Reimann et. al., 1994). MAPKs may be involved in expression of TNF-α, IL-1 and IL-6. The p42/p44 (ERK) MAPK family is associated with signal transduction by growth factors including the ras/raf-1 signal pathway. Activated MAPK phosphorylates and activates nuclear transcription factors that regulate immediate early gene induction. Activation of MAPKs requires both tyrosine and threonine residue phosphorylation. Targets of LPS-induced PTK activity in murine macrophages include two isozymes of MAPK (Weinstein

et. al., 1992). $p42^{MAPK}$ is tyrosine phosphorylated after LPS treatment in murine (Novogrodsky et. al., 1994) and human macrophages (Liu et. al., 1994b). Liu et. al. (1994b) showed that both p42 and p44 MAPKs are activated after LPS stimulation of human monocytes, and induction of IL-1β mRNA occurs coincident with MAPK activation. LPS activation of the raf-1/MAPK pathway stimulates TNF and IL-1 synthesis, and is downstream of tyrosine kinase activity (Geppert et. al., 1994, Reimann et. al., 1994). p42/p44 MAPK activity after LPS treatment peaks at 40 minutes in mouse renal resident macrophages.

Protein Kinase C

PKC is a calcium-activated phospholipid (phosphatidylserine) dependent Ser/Thr protein kinase that is activated and translocated to the cell membrane downstream of phosphoinositide hydrolysis. Phosphatidylinositol- 4,5-bisphosphate (PIP_2) is cleaved by a specific phospholipase C into diacylglyceride (DAG), and inositol-1, 4, 5-trisphosphate, both of which act as second messengers. Inositol trisphosphate elevates intracellular Ca^{2+} levels by binding to a receptor on the endoplasmic reticulum (er) that results in liberation of calcium from internal er stores. DAG forms a complex with Ca^{2+}, phosphatidylserine and PKC at the plasma membrane, and acts by increasing the affinity of PKC for Ca^{2+}, allowing the enzyme to become fully activated without a net increase in Ca^{2+} concentration. Phorbol esters, such as PMA, are structurally similar to DAG and activate some PKC isoenzymes directly. However membrane DAG is only transient while PMA is not easily degraded. Thus PMA is sometimes used to activate and then deplete cellular PKC activity through induction of prolonged association of PKC with the membrane, resulting in enzymatic degradation of PKC. PKC activity is also controlled by auto- and transphosphorylation, including phosphorylation by tyrosine kinases (Ron and Kazanietz, 1999). There are ten PKC isoenzymes (α, βI, βII, γ, δ, ε, η, θ, ι and ζ), four of which require Ca^{2+} for activation. The PKC isoenzymes δ, ε, θ, and η are activated by DAG but not by Ca^{2+}. The ζ and ι isozymes are unresponsive to both Ca^{2+} and DAG. The N-terminal half of PKC is a regulatory domain, which contains distinct regions that bind 1,2-diacylglyceride or phorbol ester, phosphatidylserine, and calcium (in the α, β, and γ isoforms), while the C-terminal half is the catalytic domain that contains two ATP binding sequences. Most cell types have more than one subspecies of PKC, with the α-subspecies found to be the most widely distributed. The βII, δ, and ζ subspecies are also found in many tissues. A mouse macrophage cell line was shown to constitutively express PKC βII, ε, and ζ isoenzymes (Fujihara et. al., 1994). The acidic myristoylated protein MARCKS

is a specific substrate of PKC that has been implicated in secretion and membrane trafficking, cell motility and regulation of the cell cycle. LPS induces MARCKS synthesis in macrophages, and treatment with PMA results in phosphorylation of MARCKS (Aderem, 1992).

LPS initiates hydrolysis of PIP_2 in murine macrophages (Prpic et. al., 1987), and an early PKC activity is required in LPS-induced production of TNF-α, IL-1β and IL-10 by human monocytes (Geng et. al., 1993, Gross et. al., 1993, Shapira et. al., 1994, Meisel et. al., 1996). Treatment with PMA alone can induce TNF-α and IL-1 mRNA in human monocytes (Geng et. al., 1993). Pre-treatment with the PKC inhibitors calphostin C or H-7 strongly inhibits LPS-induced IL-10 production by human monocytes, but PMA does not induce IL-10 mRNA or protein (Meisel et. al., 1996). Thus, in contrast to TNF and IL-1 induction, PKC activation alone is not sufficient for IL-10 induction. Since PKC inhibitors completely suppressed TNF-α mRNA upregulation by LPS, PKC is involved in LPS signalling upstream of TNF-α transcription (Shapira et. al., 1994). LPS-induced stimulation of PKC reaches a maximum at 30 minutes and returns to baseline at 60 minutes (Shapira et. al., 1994). Although tyrosine phosphorylation peaks before PKC activity, PKC activity is not suppresssed by PTK inhibitors (Shapira et. al., 1994), indicating that PKC activity is independent of PTK activation.

The role of PKC in signal transduction leading to IL-6 secretion is controversial. Small amounts of LPS in culture media, and the macrophage activation state may alter the activity of PKC, and may thus provide a basis for contradictory experimental results. In addition, at high concentration, LPS may directly bind the phosphatidylserine site of PKC (Ellis et. al., 1987). Human fibroblast expression of IL-6 mRNA is enhanced by diacylglycerols (activation of PKC) and the calcium ionophore A23187, and in these cells PKC inhibitors suppress IL-1α and TNF-α induction of IL-6, suggesting a PKC-dependent pathway for IL-6 induction (Sehgal et. al., 1987). In the epidermoid carcinoma cell line Hep-2, IL-6 is induced in a PKC-dependent pathway (Gross et. al., 1993). However, the induction of IL-6 in human monocytes by IL-1 and LPS is reportedly PKC-independent; IL-6 mRNA is not induced by PMA, and staurosporine, a PKC inhibitor, does not inhibit IL-6 mRNA increase (Gross et. al., 1993, Geng et. al., 1993). Some evidence suggests that LPS signalling may involve a calcium-independent PKC isoform (Liu et. al., 1994b), or a DAG-independent pathway for PKC activation (Shapira et. al., 1994). If a DAG-independent PKC isoform

is involved in IL-6 upregulation, PMA would not induce IL-6. Thus, a requirement for PKC activity in IL-6 induction by LPS is not completely ruled out.

Protein Kinase A Activation and cAMP Elevation

The second messenger cAMP has important modulating effects on immune functions. An increase in intracellular cAMP generally results in inhibition of IL-2 (Paliogianni et. al., 1993), IL-12 (Tineke et. al., 1995), TNF (Eigler et. al., 1998), IL-1 (Kambayashi et. al., 1995) and IFN-γ (Munoz et. al., 1990), and induces upregulation of IL-4 (Novak and Rothenburg, 1990), IL-5 (Munoz et. al., 1990), IL-6 (Zhang et. al., 1988) and IL-10 (Eigler et. al., 1998) expression in human and mouse T cells and macrophages. This suggests that gene expression of Th1 and Th2 type cytokines is differentially regulated by cAMP. Elevation of cAMP has also been associated with the suppression of macrophage activation. Intracellular levels of cAMP depend on the relative level of activity of adenylate cyclase and cyclic nucleotide phosphodiesterase (PDE). Adenylate cyclase converts AMP to cAMP, and cyclic nucleotide PDE hydrolyzes the 3'-phosphoester bond converting cAMP to 5'-AMP.

The arachidonic acid metabolite, PGE2, participates in macrophage autoregulation of inflammatory activity via elevation of cAMP. PGE2 is produced in response to LPS, at least in part through induction by TNF (Dayer et. al., 1985, Bachwich et. al., 1986), and it suppresses macrophage activation via stimulation of adenylate cyclase and elevation of intracellular levels of cAMP (Bonney et. al., 1980), resulting in protein kinase A activation (Yamamoto and Suzuki, 1987). There are two PKA isozymes, type I (PKA-I) and type II (PKA-II), which have identical catalytic subunits, but differ in their regulatory subunits. Cyclic AMP binds to sites on the inactive tetrameric PKA holoenzyme causing it to dissociate into regulatory and active catalytic subunits, which can phosphorylate serine and threonine residues.

Cyclic AMP elevation via PGE2 suppresses LPS-induced TNF-α secretion in rat (Renz et. al., 1988) and mouse macrophages (Spengler et. al., 1989). IL-6 secretion in mouse macrophages is also inhibited by PGE2 treatment (Strassmann et. al., 1994). Additionally, cyclic AMP elevating agents such as dibutyryl cAMP, forskolin and cholera toxin suppress LPS-induced expression of TNF-α mRNA and protein in mouse peritoneal macrophages (Spengler et. al., 1989, Tannenbaum and Hamilton, 1988). Nonspecific phosphodiesterase inhibitors, like theophylline and pentoxifylline, increase total cellular

cAMP and suppress LPS-induced TNF-α production, but not IL-1β (Endres et. al., 1991) or IL-6 (Waage et. al., 1990) synthesis in human monocytes.

In contrast, PGE2 increases IL-10 synthesis by LPS-stimulated human (Tineke et. al., 1995) and mouse monocytes (Strassmann et. al., 1994), and experiments utilizing anti-IL-10 antibody indicate that the increase in IL-10 is largely responsible for the inhibition of TNF-α and IL-6 secretion by PGE2 treatment in mouse macrophages (Strassmann et. al., 1994). Human monocyte IL-12 production induced by LPS is inhibited almost 100% by PGE2 and other cAMP elevating agents, while IL-6 secretion was inhibited only by 40% (Tineke et. al., 1995). The inhibitory effect of PGE2 on IL-12 secretion was independent of PGE2-mediated enhancement of IL-10 induction, but the inhibition of IL-6 was shown to be due to IL-10 (Tineke et. al., 1995). Other cAMP elevating agents, such as iloprost and Bt_2cAMP, also stimulate LPS-induced IL-10 secretion in human macrophages (Platzer et. al., 1995). Kambayashi et. al. demonstrated that inhibition of the PDE type IV isozyme alone causes an increase in LPS-induced IL-10 production, and partially inhibits secretion of IL-6 by mouse macrophages (Kambayashi et. al., 1995). PDE type IV inhibitors also increased IL-10 production by LPS-stimulated human monocytes (Eigler et. al., 1998). Thus, IL-10 induction involves the cAMP pathway, and the PDE type IV isoenzyme is critical in regulation of cAMP levels after LPS stimulation of monocytes.

The reported effects of increased cAMP on IL-6 production are contradictory, perhaps due to cAMP enhancement of IL-10 production (Platzer et. al., 1995). Cyclic AMP may directly induce both IL-6 and IL-10, followed by downregulation of IL-6 secretion by IL-10. Inhibition of PKA by the specific inhibitor H89, results in reduction in IL-6 mRNA and protein in human monocytes at 24 hours (Geng et. al., 1993), indicating a role for PKA in IL-6 induction. Cyclic AMP elevating agents increase IL-6 promoter activity at one hour post-treatment, as measured with IL-6 reporter constructs, and increase production of IL-6 in LPS-stimulated rat resident renal macrophages (Nakamura et. al., 1998). PGE2 strongly induces IL-6 production by rat osteoblasts in a pathway demonstrated to involve increases in cAMP and activity of PKA (Millet et. al., 1998). In human fibroblasts IL-6 mRNA synthesis is triggered by an increase in intracellular cAMP (Zhang et. al., 1988). The human IL-6 gene contains a cAMP response element (CRE) and serum response element (SRE), as well as binding sites for NF-κB, NF-IL6, AP-1, AP-2, and Sp1 (Ray et. al., 1988). Deletion and mutation analysis of the IL-6 gene promoter showed

that the CRE was important in angiotensin II-induced IL-6 expression, and gel shift assays indicated an increase in CREB binding to CRE after angiotensin II treatment in rat smooth muscle cells (Funakoshi et. al., 1999).

Intracellular Calcium

An increase in intracellular calcium levels, brought about by either electroporation or by treatment with the calcium-specific ionophore ionomycin, has been shown to induce both increased IL-10 mRNA and protein expression in a monocytic cell line (Lehmann and Berg, 1998). It is not known whether changes in calcium concentrations after LPS stimulation play a role in IL-10 induction. However, HIV-1 Nef protein induces an increase in IL-10 (Brigino et. al., 1994) and IL-6 (Chirmule et. al., 1994) mRNA and protein by human PBMCs, and IL-10 secretion by Nef was shown to involve an increase in calcium, and not the PKA (cAMP) or PKC pathways (Brigino et. al., 1997). Like PSGs, HIV Nef did not induce other cytokine mRNA, such as that of IL-4, IL-5, IL-12, or IFN-γ (Brigino et. al., 1997).

Monocyte IL-10 Secretion can be Induced by Proinflammatory Mediators of the Early Response to LPS

IL-10 synthesis after LPS stimulation of monocytes may be induced in part by earlier immune mediators (TNF and PGE2) released in response to LPS. Platzer et. al. reported that TNF-α (greater than 100 pg/ml) induced IL-10 mRNA but not protein synthesis by human monocytes (Platzer et. al., 1995). Treatment with a monoclonal antibody specific for TNF-α reduced IL-10 induction by LPS up to 60% (Platzer et. al., 1995). These investigators also showed that cAMP elevating agents (PGE2, iloprost and BtcAMP) induced IL-10 mRNA synthesis. Subsequently it was demonstrated that concomitant stimulation with TNF-α and cAMP elevating agents, both downstream mediators of LPS signalling, results in IL-10 mRNA and protein production by human PBMC (Meisel et. al., 1996). The pathway downstream of TNF-α and cAMP involves activation of PTKs, perhaps accounting for the reported requirement of a late PTK activity in IL-10 induction (Meisel et. al., 1996). The 5' noncoding region of the human IL-10 promoter contains a cAMP responsive element (Platzer et. al., 1995). Taken together, these results suggest that LPS induces an early secretion of proinflammatory cytokines like TNF, which is followed by prostaglandin synthesis and increases in intracellular cAMP (which inhibits TNF-α production in a feedback loop).

Together these two mediators (TNF and cAMP) induce IL-10 synthesis which downregulates the secretion of all proinflammatory cytokines (de Waal Malefyt et. al., 1991b).

PSG-induced IL-10 and IL-6 Secretion Involve Activation of Tyrosine Kinases

Because of the above described roles for protein tyrosine kinases, protein kinase C, protein kinase A, and cAMP in LPS induction of monocyte cytokines secretion, we examined the effects of a PTK inhibitor, a PKC inhibitor, a PKA inhibitor and a cAMP elevating agent on PSG6 induction of IL-10 and IL-6 by monocytes. We did not investigate whether MAPKs or increases in calcium concentrations are involved in PSG signal transduction. Herbimycin A has been shown to be an effective inhibitor of LPS-induced protein tyrosine phosphorylation in mouse (Weinstein et. al, 1992) and human (Weinstein et. al., 1993) macrophages, as well as an inhibitor of IL-10 (Meisel et. al., 1996) and IL-6 (Beaty et. al., 1994) mRNA and protein production by human monocytes. Herbimycin A at 500 nM almost completely inhibited LPS-induced IL-10 expression by human PBMC (Meisel et. al., 1996). Treatment with Herbimycin A inhibited PSG6 induction of monocyte IL-10 secretion by 92% and IL-6 by 85%, indicating that protein tyrosine kinase activation is required for PSG-stimulated IL-10 and IL-6 synthesis. It is possible that the PSG receptor itself is a tyrosine kinase or that it is associated with a tyrosine kinase that becomes activated upon PSG binding to its receptor.

Protein Kinase C Activation Plays a Role in PSG-mediated IL-10 and IL-6 Secretion

PKC is required for IL-10 induction by LPS-stimulated human monocytes (Geng et. al., 1993, Gross et. al., 1993, Meisel et. al., 1996) and is important in IL-6 expression in non-monocytic cells (Sehgal et. al., 1987, Gross et. al, 1993). To investigate whether PKC activity is required for PSG induction of IL-6 and IL-10 secretion, monocytes were treated with the specific PKC inhibitor Calphostin C. Calphostin C, unlike some other PKC inhibitors, targets the regulatory domain of the enzyme, and therefore does not inhibit other Ser/Thr protein kinases like PKA. Treatment with Calphostin C inhibited PSG6-induced IL-10 secretion by 90% and IL-6 by 81%, suggesting that PKC activity may be required in PSG6 signalling.

Protein Kinase A Activation and cAMP Elevation are Required for IL-10 and IL-6 Secretion Induced by PSGs

The cAMP-dependent protein kinase A is implicated in IL-10 gene expression. Compounds that elevate intracellular cAMP levels, specifically inhibit PDE type IV, or directly activate PKA increase IL-10

expression in response to LPS (Tineke et. al., 1995, Platzer et. al., 1995, Eigler et. al., 1998). As discussed above, the reported effects of elevated cAMP on IL-6 expression are contradictory. For example, PGE2, which activates adenylate cyclase, decreases IL-6 production in mouse and human monocytes, by upregulation of IL-10 secretion (Strassmann et. al., 1994, Tineke et. al., 1995). In contrast, cAMP increases IL-6 expression by rat renal macrophages (Nakamura et. al., 1998), rat osteoblasts (Millet et. al., 1998), rat smooth muscle cells (Funakoshi et. al., 1999) and human fibroblasts (Zhang et. al., 1988). Rolipram increases intracellular cAMP levels by selectively inhibiting PDE type IV (Beavo and Reisfeld, 1990). PDE IV has been shown to be the major PDE isozyme responsible for catabolizing cAMP in monocytes (Torphy and Undem, 1991). Rolipram partially inhibits IL-6 secretion by mouse macrophages (Kambayashi et. al., 1995), and increases LPS-induced IL-10 mRNA and protein production by human macrophages (Eigler et. al., 1998). Concentrations of rolipram of 1 μM more than double the amount of IL-10 secretion induced by LPS in human monocytes (Eigler et. al., 1998). In our experiments, rolipram increased PSG6 induction of IL-10 by 2.7 fold, and increased IL-6 by 28%. It is possible that cAMP upregulation of IL-10 synthesis reduced the stimulatory effect of cAMP on IL-6 induction in these experiments. However, the results suggest that elevations in cAMP, via specific PDE type IV inhibition, participate in both IL-10 and IL-6 secretion induced by PSG6 in monocytes.

To confirm that the cAMP pathway was involved in PSG-mediated secretion of IL-10 and IL-6, we used a specific PKA inhibitor (Rp)cAMPS. (Rp)cAMPS has been shown to decrease IL-10 production induced by LPS or cAMP elevating agents (Eigler et. al., 1998). Treatment with (Rp)cAMPS significantly inhibited PSG6 induction of IL-10 (69%), and IL-6 (43%) secretion. Although the inhibition of IL-6 was less pronounced, this may have been the result of the reduction in IL-10 secretion caused by (Rp)cAMPS. We hypothesize that the cAMP pathway, and specifically PKA and PDE type IV activity, are important in PSG signalling resulting in IL-10 and IL-6 gene expression.

Although the above experiments do not conclusively prove the involvement of PTKs, PKC and PKA in PSG6 signalling, since activity of these kinases may function in other downstream events leading to IL-10 and IL-6 secretion, they do provide a basis for future investigation. For example, candidate tyrosine kinases could be specifically inhibited by chemicals, expression of dominant negative proteins, or by antisense methods. Phosphorylation of known kinase activating residues could be examined by

immunoblot, or in vitro kinase assays could be performed after PSG treatment of cells. Importantly, the measurement of IL-10 and IL-6 mRNA levels after treatment with kinase inhibitors will more directly show that specific kinases have a role in PSG signal transduction.

CandidateTranscription Factors for PSG Signal Transduction

We did not examine transcription factors involved in PSG-mediated upregulation of IL-10 and IL-6, but NF-κB, NF-IL6, AP-1 and CREB may be involved in this process. Binding of LPS to CD14 results in activation of nuclear translocation of NF-κB, which is an important transcription factor for cytokine gene expression, including IL-2 (Shibuya et. al., 1989, Hoyos et. al., 1989), TNF-α (Lenardo and Baltimore, 1989), IL-1β (Kopp and Ghosh, 1995), IL-12 p40 (Baeuerle and Henkel, 1994) and IL-6 (Shimizu et. al., 1990, Libermann and Baltimore, 1990). LPS activation of NF-κB in human monocytes has been reported to depend on protein tyrosine phosphorylation and to some extent on PKA activity, but not PKC (Geng et. al., 1993). The human IL-10 gene contains NF-κB binding sites (Eskdale et. al., 1997), as does the IL-6 gene promoter (Ray et. al., 1988). LPS stimulation of human monocytic cell lines resulting in IL-6 secretion is accompanied by increased transcriptional activity of NF-κB (Takeshita et. al., 1996). In transfected human cell lines the κB regulatory element alone has been reported to be sufficient for the inducibility of IL-6 by IL-1β or TNF-α (Merola et. al., 1995). TNF-α is also a strong inducer of the transcription factor NF-κB (Vilcek and Lee, 1991), and may enhance IL-6 and IL-10 synthesis through this mechanism. IL-10 has been shown to inhibit LPS-induced human monocyte cytokine secretion by inhibiting the level of transcription of IL-1β, IL-6 and TNF-α (Wang et. al., 1994). Wang and colleagues report that IL-10 inhibition is mediated, at least in part, through suppression of NF-κB activity, but not NF-IL6, AP-1, AP-2, Sp1 or CREB, in human monocytes (Wang et. al., 1995). Results of several studies suggest that inhibition of NF-κB may be sufficient for inhibition of monocyte inflammatory cytokine synthesis. Thus, inhibition of NF-κB by IL-10 provides a mechanism for its inhibition of IL-6 and its own transcription.

NF-IL6, also known as C/EPBβ (CCAAT/enhancer binding protein-β), is involved in IL-6 gene expression, as well as expression of TNF and IL-1β (Isshiki et. al., 1990). The human IL-6 promoter contains an NF-IL6 binding site that has been reported to be essential for the inducibility of the IL-6 gene.

The IL-10 5' gene flanking region also contains the NF-IL6 recognition sequence (Eskdale et. al., 1997). NF-IL6 activity is induced at the mRNA level, as well as by Thr phosphorylation by MAP kinases. Both PKA and PKC phosphorylate a serine residue in the NF-IL6 activation domain, which enhances its transactivation (Trautwein et. al., 1994). NF-IL6 activity is upregulated by LPS in human monocytic cell lines concomitant with IL-6 expression (Takeshita et. al., 1996). Although both IL-4 and IL-10 inhibit IL-6 secretion, neither acts via downregulation of NF-IL6 activity (Wang et. al., 1995, Takeshita et. al., 1996).

The cAMP/PKA pathway activates the transcription factor AP-1 via phosphorylation of the inhibitory factor, inhibitory protein 1 (IP-1), which dissociates from the AP-1 dimer, allowing AP-1 to bind DNA. AP-1 is a transcription factor formed by either homodimerization or heterodimerization of members of the jun family of proteins (c-jun, junB, and junD), or heterodimerization of jun with members of the fos family (c-fos, fosB, fra-1, and fra-2). AP-1 recognizes the TPA response element (TRE), which is found in the promoter of many PMA-responsive genes. Members of the AP-1 family also can bind the CRE motif. LPS and TNF-α are strong inducers of AP-1 (Vilcek and Lee, 1991, Trepicchio et. al., 1997). The human IL-10 promoter contains a TATA box, glucocorticoid, serum and cAMP response elements, and NF-IL6, NF-κB, AP-1, AP-2 and SP1 sites (Kube et. al., 1995, Eskdale et. al., 1997). The human IL-6 promoter also contains AP-1 and AP-2 sites. IL-10 expression induced by cAMP may depend upon AP-1 activation by these factors. IL-10 inhibits LPS-induced IL-6 expression by human monocytes by reducing IL-6 mRNA levels (de Waal Malefyt et. al, 1991, Wang et. al., 1994). In one study, IL-10 inhibited AP-1 binding activity in human monocytes, which was accompanied by reduced mRNA expression of components of the AP-1 complex, c-fos and c-jun (Doktor et. al., 1996). Inhibition of AP-1 and NF-κB may be a mechanism whereby IL-10 suppresses IL-6 and its own expression.

The cAMP pathway may also activate IL-6 and IL-10 expression through the cyclic-AMP-responsive element binding protein (CREB). CREB is a substrate of PKA that binds and activates an enhancer containing the CRE consensus sequence. CREB heterodimerizes with another CRE binding protein, activating transcription factor 1 (ATF-1). Both the human IL-6 and IL-10 genes contain CRE enhancers. There are four putative CRE elements (CRE 1-4) in the human IL-10 promoter. Stimulation of the human monocytic cell line THP-1 with a cAMP analogue results in IL-10 production and CREB-1/ATF-1 binding to CRE1 and CRE4, and luciferase reporter assays showed that CRE mediated 50% of the

cAMP-dependent activity of the IL-10 promoter (Platzer et. al., 1999). In addition, three AP-2 sites are bound when the IL-10 promoter is activated by cAMP (Platzer et. al., 1999).

Tyrosine kinase and PKA activity, shown to be involved in PSG-mediated increases in IL-10 and IL-6, may induce translocation of NF-κB into the nucleus and binding to promoter NF-κB sites in the IL-10 and IL-6 genes. PKA and PKC may phosphorylate NF-IL6, resulting in its activation and binding to the IL-10 and IL-6 promoters. PSG-induced elevation of cAMP may result in AP-1 and/or CREB binding to their respective sites in the IL-10 and IL-6 promoters. The specific transcription factors that selectively result in IL-10 and IL-6 gene expression after PSG signalling need to be determined.

Cross-species Induction of IL-10 and IL-6 by Mouse and Human PSGs Indicates that PSG Function may be Highly Conserved

In addition to the experiments on human PSG immunomodulatory function, we examined whether human PSG protein could induce cytokine secretion by mouse macrophages. Mouse and human PSGs are structurally similar and members of both families have now been shown to induce IL-10 and IL-6 secretion by monocyte/macrophage cells. We demonstrated that human PSGs could indeed induce secretion of these cytokines by a macrophage cell line in vitro, although the level of secretion was much lower than that induced in human monocytes. Mouse PSG18 N-domain protein induced both IL-10 and IL-6 secretion by human monocytes (data not shown). These results indicate that mouse and human PSG protein and their receptors are similar, and that PSG function is highly conserved between these two species.

A Potential Role for Placental PSGs in Macrophage Regulation and Control of Inflammatory Responses in the Uterus During Pregnancy

The uteroplacental unit produces a large array of regulatory molecules that assist in the implantation and development of the embryo and the placenta. Cytokines have recently been recognized to function in intercellular communication between maternal and fetal cells required for these complex processes. The highly versatile macrophage cell is believed to play an important part in this process, because of its ability to both synthesize and respond to cytokine signals. In addition to providing a critical line of defense against microorganisms, macrophage activity, in conjunction with cytokines secreted by the placenta, participates in formation of the local immune environment that allows for tolerance of the fetal allograft and actively promotes fetal growth. Most cytokines believed to be important at the uteroplacental

interface are growth factors or products of the innate immune system. T cell products like IL-2 and IL-4 appear to play only a minor role during pregnancy. There is an influx of immune system cells into the uterus during pregnancy, consisting mainly of macrophages and NK-like cells (Vince et. al., 1990). Macrophages produce colony stimulating factors (CSFs), such as GM-CSF, which have been shown to enhance growth of human trophoblast in culture (Loke et. al., 1992). In addition, macrophages are major producers of cytokines like TGF-β and IL-10, which inhibit release of cytokines known to be harmful to pregnancy (like TNF), and suppress cytotoxic activity of NK cells, LAKs and macrophages themselves.

Control of monocyte/macrophage activity is critical during pregnancy. The deleterious effects of Th1 cytokines on the fetus is mainly a function of their induction of cytotoxic macrophage, NK cell and LAK activity. Because of their ability to significantly influence pregnancy in both positive and negative ways, and their central role in cytokine networks, we chose to evaluate the effects of human PSGs on cytokine secretion by human monocytes. In our experiments we used freshly isolated human blood monocytes from healthy donors at the NIH blood bank that were isolated by centrifugal elutriation, a process known not to activate monocytes. Adherent cells were grown in serum-free media and remained in culture for about 24 hours, which is less time than is needed for macrophage generation. Culturing of monocytes in the absence of serum does not result in a macrophage phenotype, although expression of some genes may be altered, and adherence may prime monocytes to differentiate in response to appropriate biological signals. Various cytokines including IL-4, (te Velde et. al., 1988), CSF-1 (Becker et. al., 1987), GM-CSF and G-CSF (Geissler et. al., 1989), and IL-3 (Young et. al., 1990) induce differentiation of human monocytic cells to macrophage-like cells in culture, with varying effects on surface antigen expression and inducible effector functions. No growth factors or differentiating agents were used in these experiments, since each of these agents, or combinations of agents, induces relatively undefined macrophage phenotypes with altered capacity to produce cytokines and other factors upon stimulation. Because different differentiation and activation states can be mutually exclusive, and the precise region-specific differentiation factors and phenotype of uterine macrophages is unknown, we chose to use undifferentiated, non-activated monocytes for our study. (In experiments where FBS was included in macrophage medium, or when monocytes were cultured for up to seven days, PSGs did induce IL-10 and IL-6 secretion [data not shown]). It is acknowledged that the presence of diverse cytokines and hormones in the uterine

environment during pregnancy would likely affect the ability of macrophages to respond to PSGs. Ideally, uterine tissue macrophages from placentas would provide the best in vitro experimental system for testing PSG effects on macrophage cytokine secretion, but these cells are not readily available.

An antiinflammatory environment at the maternal-fetal interface has been shown to exist and to be beneficial during pregnancy, and there is evidence that the fetal unit itself regulates the development of this environment through trophoblast production of anti-inflammatory mediators (PGE2, TGF-β, IL-10) and steroidal hormones. PSGs are synthesized by trophoblast in increasing amounts during gestation and their absence results in abortion in mice and monkeys. PSG production by trophoblast may be one mechanism by which the fetus creates an antiinflammatory environment (Figure 25). PSGs induce monocyte secretion of IL-10 in vitro, and we hypothesize that PSGs will have a similar effect on trophoblast cells. In culture, IL-10 is secreted in substantial amounts by human and mouse placenta, and PSG production by trophoblast could be responsible for IL-10 synthesis in an autoregulatory circuit. PSG induction of secretion of the anti-inflammatory cytokine IL-10 by uterine macrophages could help polarize the immune response to the Th2-like type via inhibition of IL-12 and IFN-γ synthesis by macrophages, and T and NK cells, respectively (Figure 25). IL-10, if produced in sufficient amounts, would also downregulate macrophage production of inflammatory factors known to be harmful to pregnancy (TNF), and inhibit macrophage NO production, which can directly kill trophoblast. IL-10 also decreases the generation of activated cytotoxic cells (CTLs, NK cells, LAK, and macrophages) in the uterus, which are the major effector cells responsible for fetal damage and abortion. In regard to allograft rejection, IL-10 is known to inhibit T cell activity and antigen presentation by macrophages. IL-10 would therefore function to prevent fetal rejection. Although very large amounts of IL-10 secretion by monoyctes in response to PSGs was not observed in our experiments, the concentrations of PSGs used were low compared to those produced by the placenta and found in maternal serum. Also, macrophages of uterine-specific phenotypes may be primed by PSG receptor upregulation or other means, to secrete more IL-10 and IL-6 than blood monocytes. In consequence, we believe that in vivo the amounts of IL-10 could be substantial, especially if other types of

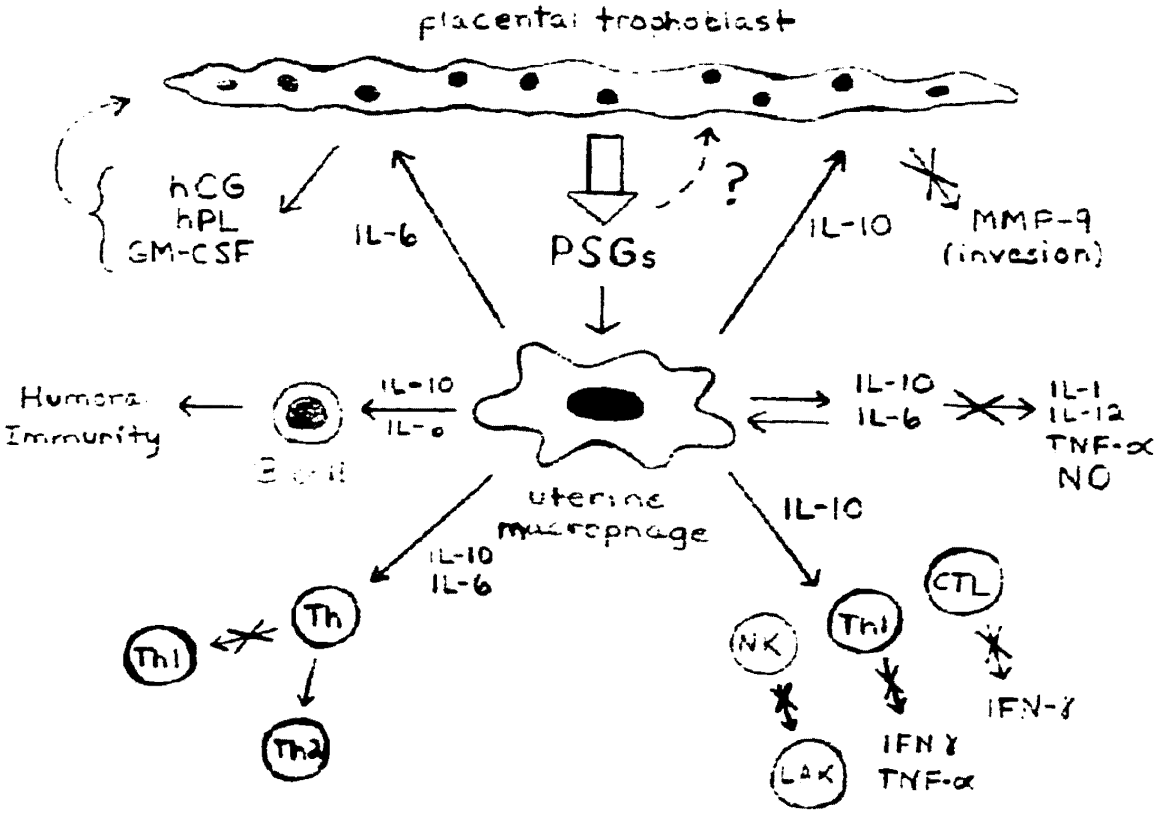

Figure 25. Schematic diagram of potential PSG effects on uterine placental cytokine networks. PSGs secreted by placental trophoblast may bind to cell surface receptors on uterine macrophages and induce release of IL-10 and IL-6. IL-6 stimulates trophoblast to secrete hCG and GM-CSF, which act as trophoblast growth factors. IL-10 acts on trophoblast to inhibit MMP-9 production and trophoblast invasion in vitro. IL-1 and IL-6 produced by macrophages enhance B cell activity and the humoral immune response, perhaps with systemic effects on autoimmuntity. In an autocrine loop, IL-10 and IL-6 inhibit macrophage secretion of IL-1, TNF-α, IL-12 and NO, resulting in inhibition of inflammation. IL-6 and IL-10 may promote naïve precursor T cell (Th) differentiation to a Th2-like immunoreactivity that is beneficial to pregnancy. IL-10 inhibits NK cell development to lymphokine activated killer cells (LAKs) through downregulation of IFN-γ synthesis, and inhibits inflammatory cytokine secretion by Th1-type T cells and cytotoxic T cells (CTLs). Black lines with Xs indicate inhibition.

cells, not examined in this study, respond to PSGs by producing IL-10.

High Levels of PSGs in Maternal Serum May Affect Systemic Immunity During Pregnancy

Alterations in the local immune system during pregnancy can have systemic effects, as demonstrated by the decreased severity of cell-mediated inflammatory autoimmune disease, the increase in humorally-mediated autoimmune disease, and the decrease in resistance to some pathogens associated with pregnancy. Conversely, certain systemic immune responses, like a strong Th1-like response to pathogens, imperils pregnancy in humans and mice (Brabin and Brabin, 1992, Krishnan et. al., 1996). Placental production of PSGs may play a role in the systemic effects of pregnancy on autoimmunity and microbial infection. In addition to induction of spontaneous abortion in normal and abortion-prone mice, high levels of TNF-α have been associated with intrauterine infection resulting in preterm labor. PSG production by placenta, and subsequent macrophage synthesis of IL-10 and IL-6, may aid in downregulating TNF production and strong Th1 responses to pathogens to maintain pregnancy. The presumed function of HIV Nef protein's specific induction of both IL-10 and IL-6 secretion by PBMCs is to suppress Th1 responses and CMI (Brigino et. al., 1997). PSG synthesis by trophoblast may even be upregulated by microbial components such as LPS during infection. Trophoblast can respond to LPS, and this bacterial component induces trophoblast synthesis of IL-10 (Roth et. al., 1996). Since PSGs are in such high concentration in maternal serum, PSGs could also affect severity of systemic autoimmune disease. In fact, increased levels of PSGs in serum during pregnancy have been correlated with improvement in rheumatoid arthritis, an inflammatory autoimmune disease (Fialova et. al., 1991). If antiinflammatory properties of PSGs can be demonstrated in vivo, treatment with these proteins might be used in certain autoimmune diseases and other chronic inflammatory conditions. A mouse model for PSG function should be very useful in this regard because certain strains of mice can be induced to mimic important aspects of autoimmune diseaeses like multiple sclerosis.

PSGs May Participate in Control of Placental Invasion

PSG-induced IL-10 may also function in another critical process during pregnancy, the control of placental invasion of the uterine wall. We showed that PSGs induce monocyte production of IL-10, and uterine macrophages may be one important target of IL-10 activity, but trophoblast also secretes and expresses receptors for IL-10. IL-10 inhibits trophoblast invasiveness in vitro through downregulation of

synthesis of the metalloproteinase MMP-9, which has been shown to be essential to trophoblast invasion (Roth and Fisher, 1999). Trophoblast capacity to invade endometrial stroma decreases considerably during gestation. PSGs, which induce IL-10 secretion, increase in concentration during pregnancy. It is possible that one function of PSG-induced IL-10 involves the downregulation of trophoblast ability to further invade the uterus as gestation continues (Figure 25). However, PSGs are synthesized in large quantity by placental tissue in choriocarcinoma and invasive mole, which are highly invasive pathological conditions. Also preeclampsia and intrauterine growth retardation are associated with shallow trophoblast invasion, and have been correlated with low serum levels of PSGs, suggesting that PSGs might promote invasion. Because putative functional effects of PSGs on control of trophoblast invasion inferred from correlative studies are contradictory, experiments should be done to examine this important issue. If PSGs are shown to enhance invasion, PSG neutralization might be used prophylactically to treat patients with hydatidiform mole before invasive mole is diagnosed. Conversely, PSG treatment could be used to enhance invasion and maternal-fetal exchange in cases of preeclampsia and intrauterine growth retardation.

Possible Roles for PSG Induction of IL-6 Secretion by Macrophages

PSG induction of IL-6 secretion by monocytes may play an important role in pregnancy. PSGs could function in IL-6-mediated upregulation of hCG and hPL release by trophoblast (Nishino et. al., 1990, Stephanou and Handwerger, 1994). Since hCG supports cytotrophoblast growth, IL-6 may be acting indirectly as a growth factor for trophoblast. IL-6 has also been shown to induce GM-CSF (Sachs et. al., 1989), another growth factor for trophoblast and a suppressor of fetal resorption in abortion-prone mice (Chaouat et. al., 1990). Although IL-6 is commonly thought of as an inflammatory cytokine because it is induced in response to infection and trauma, IL-6 deficient mice exhibit increased severity of inflammation in response to LPS (Xing et. al., 1998). IL-6 inhibits synthesis of TNF-α, IL-1 (Aderka et. al., 1989, Schlindler et. al., 1990b) and IFN-γ (Rincon et. al., 1997), indicating that though IL-6 has important functions in immune responses to infection, it may also act to downregulate the inflammatory response. In addition, IL-6 increases prostaglandin production by human decidua (Mitchell et. al., 1991), another possible antiinflammatory effect of this cytokine. Significantly, IL-6 has been reported to induce T cell IL-4 synthesis and differentiation to the Th2 subtype in the mouse (Rincon et. al., 1997). All of the above activities of IL-6 would either promote fetal growth or help to prevent immune-mediated fetal rejection.

IL-10 and IL-6 Have Some Functional Similarities

The induction of both IL-10 and IL-6 by PSGs is interesting because these proteins have several similar functional properties. Both inhibit the synthesis of the inflammatory cytokines TNF-α and IL-1 by macrophages, and inhibit production of IFN-γ by T cells (Fiorentino et. al., 1989, Rincon et. al., 1997). IL-10 inhibits development of Th1 type T cell development (Bejarano et. al., 1992), and, in the mouse, IL-6 induces differentiation to the Th2 phenotype (Rincon et. al., 1997). Both cytokines are important stimulators of B cell growth, differentiation and immunoglobulin secretion (Kishimoto 1989, Go et. al., 1990). IL-10 enhances M-CSF-mediated growth and differentiation of human monocytes (Hashimoto et. al., 1997), and IL-6 induces differentiation of human myeloid cell lines (Onozaki et. al., 1989). Therefore, these two cytokines can downregulate inflammation, produce a bias toward a Th2 immune response, enhance antibody production, and perhaps participate in monocyte maturation to a uterine-specific phenotype. We can speculate that the observed shift to a Th2-like environment in the uterus, and its systemic immune effects on pregnancy, which result in amelioration of inflammatory autoimmune diseases and increased severity of antibody-mediated autoimmune diseases, may be brought about in part through PSG induction of these cytokines (Figure 25). Inflammatory immune responses and specifically the cytokines TNF-α, IL-2 and IFN-γ are known to induce fetal resorption in the mouse, and are strongly associated with abortion in women. If the effect of PSGs on production of an antiinflammatory environment in vivo are confirmed, treatment with these proteins could be used in cases of recurrent spontaneous abortion of no known recognized cause, and where an immune system etiology is suspected.

Possible Functions for PSGs in Non-placental Human Tissues

Human tissues other than placenta, including fetal liver, endometrium, salivary gland, testes, intestine, and myeloid cells express PSG mRNAs, and PSG protein expression has been demonstrated in testes, intestine and fibroblast cell lines. Cell surface binding of PSGs to human granulocytes and monocytes has been demonstrated by immunofluorescence. Human PSG treatment enhanced white blood cell recovery after bone marrow transplant into irradiated mice (Blomberg et. al., 1998), suggesting a role for PSGs in blood cell growth regulation. The expression of PSG mRNAs by fetal liver, the site of fetal hematopoiesis, and cell-surface binding by granulocytes and monocytes is consistent with a growth regulatory role for PSGs in these tissues. PSG induction of monocyte IL-6 secretion and the demonstrated

ability of IL-6 to enhance proliferation of hematopoietic stem cells suggests a possible mechanism for PSG growth regulatory effects on blood cells. The expression in testes and intestine may reflect an antiinflammatory function of PSGs. The interior of the seminiferous tubules of the testes is protected from the immune system by the blood-testis barrier, because sperm-specific antigens can induce a deleterious immune response in males. IL-10 deficient mice develop chronic intestinal inflammation due to an uncontrolled immune response to enteric antigens (Kuhn et. al., 1993). It is possible that production of PSGs in these two tissues, resulting in IL-10 synthesis by resident target cells, helps prevent such harmful immune responses. Reduced endometrial expression of PSGs has been associated with increased incidence of recurrent spontaneous abortion (Arnold et. al., 1999). PSG production by cycling endometrium, and subsequent IL-10 and IL-6 secretion by target uterine cells, may function in preparing the uterus for the presence of the embryo via downregulation of the inflammatory response to paternal/fetal antigens and the trauma associated with implantation. Lack of expression of PSG11 by uterine endometrium has been associated with RSA (Arnold et. al., 1999).

Future Directions for PSG Research

Identifying the PSG receptor and characterizing its expression by monocytes and other cells such as trophoblast are the next goals of our laboratory. The presence of an RGD motif in the N-terminal domain of most PSGs has suggested that these proteins may bind cell-surface integrin receptors. Also, the binding of a PSG11 N-domain peptide containing the RGD motif to monocytic cells, and demonstration of competition of binding by RGD-containing peptides is evidence that the RGD motif may mediate PSG receptor binding. Recently, the T cell cytokine Eta-1 was shown to bind a mouse macrophage integrin receptor and induction of macrophage secretion of IL-12 through this receptor was blocked by a GRGDS peptide, but not a GRADS peptide (Ashkar et. al., 2000). These results show that RGD-mediated binding to integrin receptors can induce signal transduction leading to macrophage cytokine secretion.

Trophoblast cells have been compared to macrophages because of morphological and functional similarites between these two cell types. Trophoblast cells produce cytokines (IL-1, IL-4, IL-6, IL-10, TGF-β, and TNF), form syncytia, are phagocytic, and respond to LPS with increased production of IL-10. Trophoblast cytokine production is thought to regulate maternal responses to the presence of the fetus. In the mouse model of RSA, fetal resorption is associated with increased placental production of TNF-α and

IFN-γ, and decreased placental synthesis of IL-10. Human trophoblast produces substantial quantities of IL-10 in culture (Cadet et. al., 1995, Roth et. al., 1996). Trophoblast also contains receptors for many cytokines, indicating that cytokines regulate trophoblast function. IL-1, IL-6 and TNF induce trophoblast release of hCG, and IL-10 inhibits trophoblast production of MMP-9 and ECM invasion (Roth and Fisher, 1999). GM-CSF enhances trophoblast growth, and INF-γ and TNF inhibit trophoblast proliferation. PSGs have been shown to bind to the surface of trophoblast (Horne et. al., 1976a, Tatarinov et. al., 1976, Lin and Halpert, 1976). We hypothesize that trophoblast expresses receptors for PSGs, and that PSGs will mediate trophoblast synthesis of immune mediators. Autoregulation of trophoblast function by PSGs might turn out to be a primary and critical function of these proteins.

In conclusion, pregnancy-specific glycoproteins appear to directly regulate aspects of the innate immune system, which could have imporant effects on the outcome of pregnancy. Cells of the innate immune system (macrophages and NK cells) are important targets of feto-placental regulatory mechanisms and cytokine dysregulation is implicated in female infertility. The results from this study support previous theories regarding a role for PSGs as modulators of the immune system, and indicate that more research on the effects of PSGs during pregnancy will benefit the field of reproductive biology.

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