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Award Number: W81XWH-04-1-0126

TITLE: Radiation-Induce Immune Modulation in Prostate Cancer

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REPORT DATE: January 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20060503153

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE January 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Jan 2004 – 31 Dec 2004)

4. TITLE AND SUBTITLE
Radiation-Induce Immune Modulation in Prostate Cancer

5. FUNDING NUMBERS
W81XWH-04-1-0126

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
This proposal is designed to determine if radiation therapy inhibits presentation of prostate tumor antigens through the endogenous pathway by dendritic cells (DC) and to devise strategies to overcome this inhibition. This functional immune suppression following radiation therapy is not associated with cell killing but rather by interference with antigen processing. In this regard it is very different from the conventional view of immune suppression associated with lymphocyte sensitivity to radiation. The experiments will be performed within a humanized murine system for easy translation to the clinic. We chose to study responses to PSA, but given the high risk involved in these experiments and because PSA is so highly expressed in serum of patients with human prostate cancer, we decided to evaluate responses to a second antigen that is highly expressed in prostate cancer, survivin, as a back-up. The plan is to attempt to blunt radiation-induced immune suppression using IL-3 and/or GM-CSF to stimulate DC. Most of the first year has been spent developing reagents that can be used experimentally and refining protocols in particular for DC purification, which maximizes effects. This phase is now nearing completion, and we have initiated the murine experiments.

14. SUBJECT TERMS Radiation, dendritic cells, cytokines			15. NUMBER OF PAGES 87
			16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

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Introduction

Radiation therapy is known to be immunosuppressive. We have discovered a novel form of radiation-induced immune suppression that is based on dendritic cell (DC) dysfunction, which we believe is due to a defect in antigen processing caused by radiation-induced proteasome inhibition. The positive aspect of this mechanism is that it may be possible to reverse the process, and we plan to investigate the utility of IL-3 and/or GM-CSF in this regard. The overall goal is to better translate radiation-induced tumor cell killing into the development of a state of tumor immunity in the hope of increasing the effectiveness of this form of therapy in the cure of localized and disseminated prostate cancer. This has been the subject of a recent review (RO1) with another submitted for publication (attached – Demarian et al.)

Body

The statement of work for this year revolved around experiments to examine the effects of radiation on the immune response to PSA peptide-pulsed DC, on DC maturation, proteasome composition and function, on the development of protective immunity against tumors expressing PSA, and also developing an adenoviral vector to express PSA.

This has been a year spent in preparing the ground for future experiments by developing vectors and reagents as well as refining our experimental approach. Our hypothesis is that radiation affects DC function. One possibility is that this is due to a block in maturation of DC and/or an alteration in expression of co-accessory molecules such as CD86, CD80, and MHC class II, and/or an effect on proteasome function. The results of these experiments are critical to how we formulate hypotheses as to how to change radiation-induced DC inhibition. We have completed an extensive series of experiments investigating these effects, some of which were recently published in J. Immunol. In brief, irradiation does affect expression of co-accessory molecules, maturation status, and proteasome function in DC. CD86 and MHC class II expression, but not CD40 or CD80, are decreased, but the effect is not dramatic. DCs can still be stimulated to mature under the influence of CD40L and IFN-γ, and radiation does have an effect, but it is slight.

In the absence of evidence for an effect on co-accessory molecules, we have investigated the effects of irradiation on cytokine and cytokine receptor release using a multiplex assay (Fig 1). As can be seen the only major changes in cytokines released by DC following irradiation were in the soluble TNFR family. Since TGF-β, a major immunosuppressive cytokine was not included in this array, we examined the effects of irradiation on its release by DCs looking at active and total TGF-β released at various times after DC irradiation. As can be seen in Fig 2, the immunosuppressive effect of irradiation on DC function cannot be ascribed to increased TGF-β release.
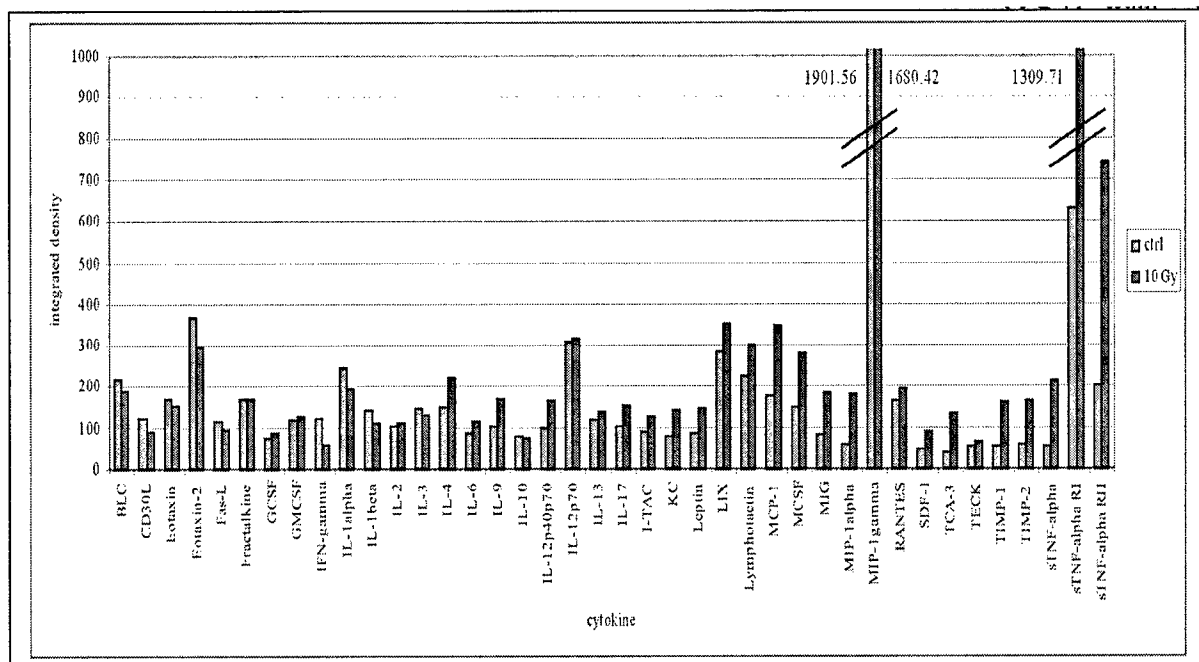
Fig 1: Murine DCs were generated in GM-CSF and IL-4 and irradiated with 0 or 10Gy. After 48hrs supernatents were harvested and levels of the indicated cytokines and receptors measured by ELISA.
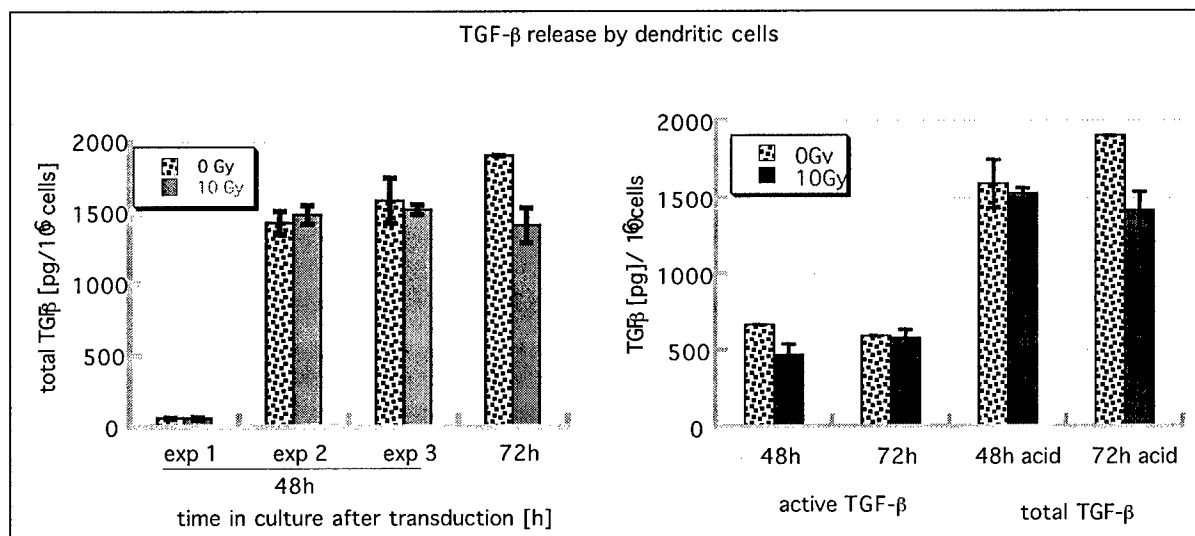

Fig2: DCs were established as for Fig 1 and supernatants tested for active and latent TGF-b at 28 and 72 hours after irradiation with 0 or 10Gy.

Detailed analyses of the results our data suggested that a small population of DCs might be preferentially affected by radiation rather than the whole population. This would be critical since we would have to investigate whether this population might preferentially present peptides, as opposed to processing antigen through the endogenous pathway. We therefore separated DC on Percoll gradients and determined which population is affected by radiation. Our preliminary data suggest that mature DCs were inhibited with respect to endogenous antigen presentation, but unexpectedly immature DC/macrophage

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subpopulations are not affected and their activity may be even increased (Fig 3). Defining these subpopulations and their responses to irradiation has therefore become a critical issue, and we have put the studies using PSA peptide-pulsed DCs on hold until it is resolved. If necessary, we will perform the experiments using subpopulations of DC, with and without irradiation. The peptide-pulsed studies will therefore be initiated in the New Year and be performed side-by-side with the AdV-based experiments.
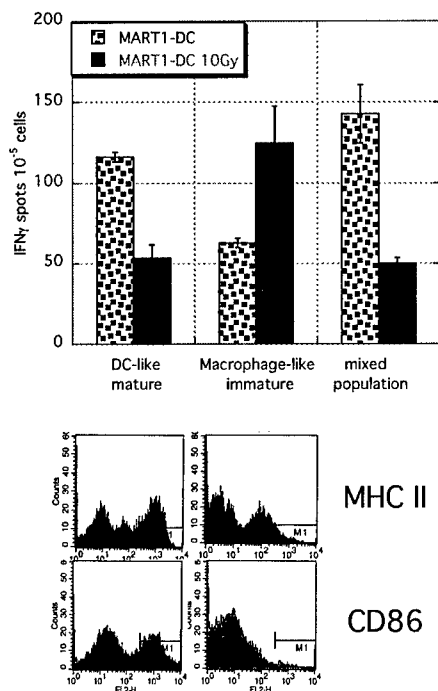

Fig 3: Dendritic cell populations were separated on Percoll gradients into mature and immature DCs as assessed by MHC class II and CD86 expression. These were infected with AdVMART and injected into mice s.c.. Ten days later the number of antigen-specific T cells were enumerated by ELISPOT.
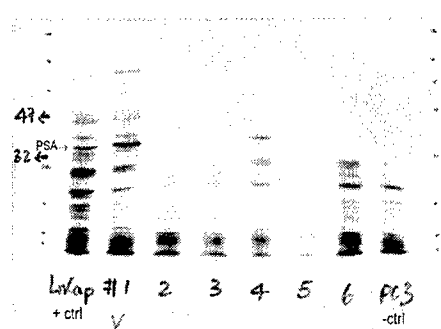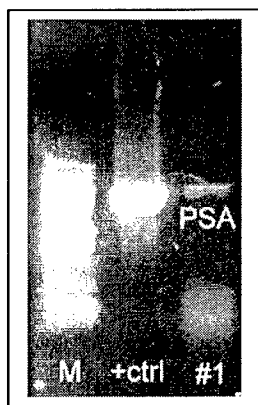

Fig 4: TRAMP-C1 cells expressing hPSA assessed by RT-PCR (left) and Western blot (clones 1 and 4 – right).

The experiments outlined in the grant involve a "humanized" mouse model. We have successfully bred HLA-A2.1 transgenic C57Bl/6 mice for this purpose and now have sufficient of these to perform the experiments. We have cloned human PSA and inserted it into TRAMP-C1 cells, as proposed (Fig 4). This has been injected into C57Bl/6 mice and we have shown that it grows in these mice. We have a high PSA expressing clone into which we have placed the HLA-A2.1 vector and a double-expressing clone was selected for testing. This tumor cell line unfortunately lost HLA expression with time and we have reengineered the vector to have a different selectable gene than neomycin resistance since TRAMP cells are already neo-resistant.

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This will act as a target for future in vivo experiments. We will be injecting this into the HLA-transgenics in the hope that it will grow and that we will be able to use this as our model for protection experiments, as planned. We are still developing the adenoviral PSA vector, but hope to have this successfully in place and virus stock expanded soon. We have also modified our AdV-IL-3 vector. We were discovered that this vector was rather more toxic than a control AdV vector and accordingly we developed a tet-inducible vector to express IL-3. While this is not critical at this stage in the experimental plan, it is critical for later in vivo experiments, since we have recently obtained data showing that the expression of IL-3 in an uncontrolled way in vivo might be associated with some toxicity. Our collaborators have used this vector in tumors growing in vivo and have shown that they can get controlled IL-3 expression using it (paper attached).

While constructing vectors and tranfected lines for use with to we used our MART system to explore our hypothesis that irradiated DCs may act as a tolerogenic signal for the immune system, as opposed to being simply "ignored." C57BL/6 mice were vaccinated with 5 × 10^5 irradiated (10 Gy) or non-irradiated DC that had been transduced with AdVMART1. A second immunization was performed 10 days later so that mice treated with non-irradiated DCs received irradiated DC and *vice versa*. One week after the last immunization, MART-1 specific IFN-γ and IL-4 responses were assessed using ELISPOT assays (Fig. 5). Mice immunized first with irradiated DCs showed reduced IFN-γ (Fig. 5A) and IL-4 (Fig. 5B) expression compared to mice injected first with non-irradiated transduced DCs, suggesting tolerance was induced. This
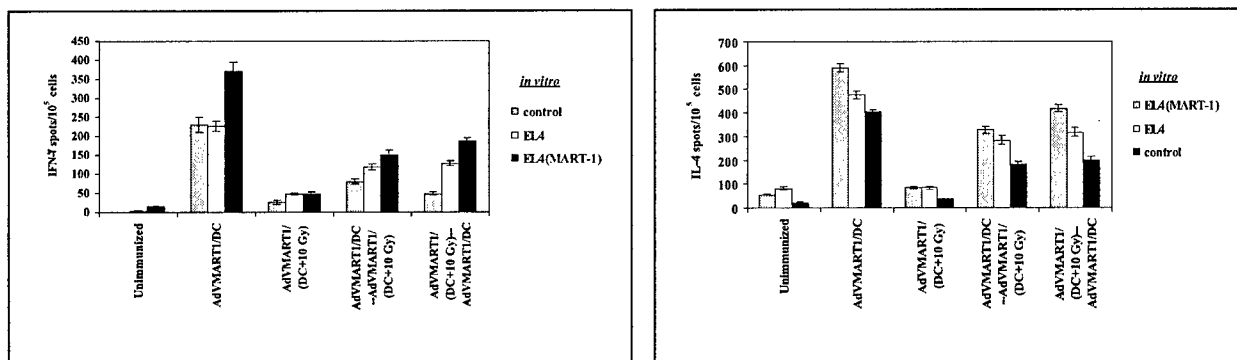
has important implications for the studies proposed in this grant.

There are no proposed changes to the Statement of Work. We have had to introduce minor modifications, such as DC separation techniques and an inducible vector as refinements to the experimental design. This has slowed progress a little, but not much. We also acknowledge that the experiments that are proposed are high risk and have initiated a development of a replacement antigen other than PSA, which may be difficult to investigate in humans because of the high natural PSA levels in prostate cancer patients. We have chosen survivin as the antigen and have developed an adenovirus-based system and peptide system that will serve as a back-up system if necessary. A high percent of prostate cancers over express this tumor-associated antigen. We also have initiated a collaboration that we hope will lead to clinical implementation of some of the outcomes of these experiments.

Key Research Accomplishments

1. Study of the effects of irradiation on DC co-accessory molecule expression – 90% completed.
2. Study of the effects of irradiation on DC maturation – completed.
3. Study of the effect of irradiation on proteasome structure and function, including immunoproteasome – 90% completed.
4. Isolation of different DC subpopulations and study of the effects of irradiation on these subpopulations – 75% completed.
5. Development of a murine prostate carcinoma cell line expressing hPSA – completed.
6. Development of a murine prostate carcinoma cell line expressing hPSA and HLA-A2.1 – 90% completed.
7. Development of an AdV-hPSA vector – 60% completed.
8. Development of an Ad-IL-3 vector with tet-inducible system – completed.
9. Breeding sufficient HLA-2.1 transgenics to complete the studies – completed.

Reportable Outcomes – Manuscripts and Abstracts

McBride, W.H., C-S. Chiang, J.L. Olson, C-C. Wang, J-H. Hong, F. Pajonk, G.J. Dougherty, K. Iwamoto, M. Pervan and Y-P. Liao: A sense of danger from radiation. Radiat. Res. 162:1-19, 2004.

Oh, Y-T., W-C. Chen, G.J. Dougherty and W.H. McBride: Adenoviral interleukin-3 gene-radiation therapy in a mouse prostate cancer model. Int. J. Radiat. Oncol. Biol. Phys., 59:579-583, 2004.

Liao, Y-P., C-C. Wang, L.H. Butterfield, J.S. Economou, A. Ribas, W.S. Meng, K.S. Iwamoto and W.H. McBride: Ionizing radiation affects human MART-1 melanoma antigen processing and presentation by dendritic cells. J. Immunol. 173:2462-2469, 2004.

Pervan, M. and W.H. McBride: Proteasome structures affected by ionizing radiation. Journal of Biological Chemistry, submitted 2004.

Pajonk, F. and W.H. McBride: The proteasome in cancer biology and therapy. In: Cancer Drug Discovery and Development: Proteasome Inhibitors in Cancer Therapy, J. Adams, ed. The Humana Press, Inc., New Jersey, 2004, pp. 111-121.

Chiang, C-S., C.L. Hsieh, W.C. Liu, Y.T. Oh, W.H. McBride and J.H. Hong: Combing IL-3 gene immunotherapy with radiotherapy for prostate cancer. In: Abstracts of Papers for the 95th Annual Meeting of the American Association for Cancer Research, Orlando, Florida, 2004.

Pervan, M. and W.H. McBride: Mechanisms of proteasome inhibition by ionizing radiation. In: Abstracts of Papers for the 95th Annual Meeting of the American Association for Cancer Research, Orlando, Florida, 2004.

Liao, Y.-P., L. Butterfield, W. Meng, A. Ribas and W.H. McBride: Radiation modulates tumor antigen presentation by dendritic cells. In: Abstracts of Papers for the 95th Annual Meeting of the American Association for Cancer Research, Orlando, Florida, 2004.

Pervan, M. and W.H. McBride: Radiation targets within the proteasome. In: Abstracts of Papers for the 51st Annual Meeting of the Radiation Research Society, 2004.

Chiang, C.-S., C-H. Tsai, W-C. Liu, K-F. Hsieh, F-H. Chen, W.H. McBride and J-H. Hong: Enhancement of radiotherapy for murine prostate cancer by regulation of interleukin-3 gene expression using an adenovirus tetracycline turn-on system. In: <u>Abstracts of Papers for the 96th Annual Meeting of the American Association for Cancer Research</u>, Anaheim, California, 2005.

<u>Reportable Outcomes – Presentations</u>

2/4/04
University of California, Riverside
Environmental Toxicology Program, Riverside, CA
Invited Seminar: "The Proteasome as a Senor of Stress"

3/17/04
UCLA Department of Dentistry
Monthly Seminar, Los Angeles, CA
Invited Speaker: "The Proteasome as a Target for Cancer Therapy"

6/27-6/30/04
American Statistical Association Conference on Radiation and Health
Radiation in Realistic Environments: Interactions between Radiation and Other Risk Modifiers, Beaver Creek, CO
Invited Speaker: "The Proteasome and Radiation"
Session Discussant: "Interactions of Radiation with Genetic Factors"

9/20-9/22/04
Pharmacology & Therapeutics Department
Roswell Park Cancer Institute, Buffalo, NY
Invited speaker: "Radiation Effects Antigen Presentation to the Immune System"

10/23-10/28/04
23rd Annual ESTRO Meeting, Amsterdam, The Netherlands
Abstract presentation: "The Proteasome as a Redox-Sensitive Target for Radiation Effects"

<u>Conclusions</u>

This has been a year of reagent development and refinement of approach. The major finding was the fact that subpopulations of DC appear to respond differently to irradiation. This was unexpected and exciting, although it delayed implementation of certain other studies. This situation will be resolved shortly, and we will be able to move ahead. We have had no changes in the directions outlined in the Statement of Work, although we have instituted fall-back positions as we approach the high-risk experiments. We are optimistic that these experiments will lead to a "so what" scenario in which we can assess the effects of radiation therapy for prostate cancer in terms of the tumor-specific response. We anticipate a loss of reactivity as DC becomes tolerogenic more that immunogenic and that we may be able to overcome this form of immune suppression using cytokines, such as IL-3 and GM-CSF.

Appendices

One published manuscript and one abstract are in the appendix, as are 2 submitted publications.

Liao, Y-P., C-C. Wang, L.H. Butterfield, J.S. Economou, A. Ribas, W.S. Meng, K.S. Iwamoto and W.H. McBride: Ionizing radiation affects human MART-1 melanoma antigen processing and presentation by dendritic cells. J. Immunol. 173:2462-2469, 2004.

C. S. Chiang, C. H. Tsai, W. C. Liu, K. F. Hsieh, F. H. Chen, W. H. McBride and J. H. Hong: Enhancement of radiotherapy for murine prostate cancer by regulation of interleukin-3 gene expression using an adenovirus tetracycline turn-on system. 2005 AACR meeting in Anaheim, CA.

C. S. Chiang*, C. H. Tsai*, K. F. Hsieh*, H. L. Hsieh*, W. C. Liu*, W. H. McBride[+], and J. H. Hong[#] Immune enhancement of radiation therapy for murine prostate cancer by tetracycline-regulated intratumoral expression of interleukin-3. Submitted.

Sandra Demaria, M.D.[*^], Nina Bhardwaj, M.D, Ph.D.[@], William H. McBride, Ph.D. [‡], and Silvia C. Formenti, M.D.[#] Combining radiotherapy and immunotherapy: a revived partnership. Submitted.

Ionizing Radiation Affects Human MART-1 Melanoma Antigen Processing and Presentation by Dendritic Cells[1]

Yu-Pei Liao,* Chun-Chieh Wang,*¶ Lisa H. Butterfield,‡ James S. Economou,† Antoni Ribas,†
Wilson S. Meng,§ Keisuke S. Iwamoto,* and William H. McBride[2]*

Radiation is generally considered to be an immunosuppressive agent that acts by killing radiosensitive lymphocytes. In this study, we demonstrate the noncytotoxic effects of ionizing radiation on MHC class I Ag presentation by bone marrow-derived dendritic cells (DCs) that have divergent consequences depending upon whether peptides are endogenously processed and loaded onto MHC class I molecules or are added exogenously. The endogenous pathway was examined using C57BL/6 murine DCs transduced with adenovirus to express the human melanoma/melanocyte Ag recognized by T cells (AdVMART1). Prior irradiation abrogated the ability of AdVMART1-transduced DCs to induce MART-1-specific T cell responses following their injection into mice. The ability of these same DCs to generate protective immunity against B16 melanoma, which expresses murine MART-1, was also abrogated by radiation. Failure of AdVMART1-transduced DCs to generate antitumor immunity following irradiation was not due to cytotoxicity or to radiation-induced block in DC maturation or loss in expression of MHC class I or costimulatory molecules. Expression of some of these molecules was affected, but because irradiation actually enhanced the ability of DCs to generate lymphocyte responses to the peptide MART-1$_{27-35}$ that is immunodominant in the context of HLA-A2.1, they were unlikely to be critical. The increase in lymphocyte reactivity generated by irradiated DCs pulsed with MART-1$_{27-35}$ also protected mice against growth of B16-A2/K^b tumors in HLA-A2.1/K^b transgenic mice. Taken together, these results suggest that radiation modulates MHC class I-mediated antitumor immunity by functionally affecting DC Ag presentation pathways. *The Journal of Immunology,* 2004, 173: 2462–2469.

E xposure to ionizing radiation often leads to immunosuppression, although enhancement of immunity has been reported under certain circumstances, in particular with lower doses. Radiation-induced immunosuppression is most often attributed to lymphocyte killing, while immunoenhancement has been ascribed to elimination of a radiosensitive population of suppressor T cells (1). In this study, we report that ionizing radiation affects the function of dendritic cells (DCs),[3] which are powerful, relatively radioresistant APCs (2, 3) that initiate naive T cell-mediated immune responses.

DCs are required to undergo phenotypic and functional changes following Ag capture that culminate in their transition to mature APCs before they can generate immunity (4–6). DC maturation is associated with loss of endocytic/phagocytic receptors and gain in expression of MHC class I and II, CD11a, CD40, CD54, CD58, CD80, CD83, and CD86 molecules (7, 8). The gain in costimulatory molecules promotes their ability to activate Ag-specific T

cells and diminishes their ability to induce tolerance to self Ags (4, 9). Danger signals, including those generated by pathogens and proinflammatory cytokines, promote the maturation process (10–12). Because radiation induces the expression of a number of potential danger signals in tissues (13), its impact on DC function is of interest with respect to the generation of antitumor immunity and autoimmunity, both of which could affect the outcome of radiation therapy for cancer.

Peptides can be loaded onto MHC class I molecules by different mechanisms. In general, antigenic peptides from endogenously synthesized protein result from proteasomal degradation and are loaded onto MHC class I molecules in the endoplasmic reticulum (14–16). In contrast, exogenous peptides can bind to MHC class I molecules directly or are cross-presented (17–19). We have recently shown that radiation inhibits proteasome function in a number of different cell types (20, 21), leading to the possibility that Ag processing might be differentially affected depending on the pathway that is used.

We chose to use human melanoma Ag recognized by T cells (hMART-1) as a tumor Ag because we have previously studied its presentation when processed by the endogenous pathway and when loaded as an exogenous peptide in both murine and human DC systems (22–26). hMART-1, a human melanocyte lineage-specific protein, is expressed by 75–100% of melanomas depending on their clinical stages (27). It is frequently recognized by CTLs from HLA-A2.1 patients (28, 29). This is a common HLA haplotype (30) for which immunodominant epitopes have been defined (29). Several groups (31, 32) have used hMART-1 as a target for immunotherapy of melanoma, including DC-based strategies (33, 34).

In this study, we report the effects of radiation on DC processing and presentation by the endogenous pathway and an exogenous loading of hMART-1 peptide. For the former, replication-deficient adenovirus was used to express the entire hMART-1 (AdVMART1) Ag. For the latter, the MART-1$_{27-35}$ peptide (the HLA-A2.1-restricted immunodominant epitope) was chosen. The results

suggest that radiation modulates antitumor immunity by affecting DC function.

Materials and Methods
Mice

C57BL/6 mice (H-2^b) were purchased from The Jackson Laboratory (Bar Harbor, ME). HLA-A2.1/K^b transgenic mice (α1 and α2 HLA-A2.1 domains/α3 K^b domain) on a C57BL/6 background were kindly provided by Dr. L. Sherman (35) (The Scripps Research Institute, La Jolla, CA). Mice were bred and maintained in a strict defined-flora, pathogen-free environment in the American Association of Laboratory Animal Care-accredited Animal Facilities of Department of Radiation Oncology, University of California (Los Angeles, CA). Experiments used 6- to 8-wk-old female mice, and all local and national guidelines for the care of animals were adhered to.

Radiation

Cells were irradiated using a MARK-1-30 irradiator (Cs^{137} source; J. L. Shepherd & Associates, San Fernando, CA) at a dose rate of ~4.5 Gy/min.

Cell lines

The B16 melanoma and EL4 lymphoma cell lines, both of C57BL/6 origin, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The 293 human embryonic renal cells were used for amplification of adenoviral seed stocks. M202 is a MART-1-positive human melanoma cell line (23). Cell lines were maintained in vitro in DMEM (Mediatech, Herndon, VA) with 10% FBS (Sigma-Aldrich, St. Louis, MO) and 1% antibiotics-antimycotic solution: 10,000 IU penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B (Mediatech). An EL4 transfectant carrying the MART-1 cDNA and neomycin resistance gene, EL4(MART-1), was generated, as described previously (25), and maintained under constant G418 selection (0.5 mg/ml) in RPMI 1640 medium (Mediatech) with 10% FBS.

Generation of bone marrow-derived DCs

DCs were generated from murine bone marrow cells, as described by Inaba et al. (36), with modifications (24). Bone marrow cells were obtained from femurs and tibias (day 0) and cultured overnight in RPMI 1640 with 2% FBS and 1% antibiotics. Nonadherent cells were resuspended in RPMI 1640 supplemented with 2 ng/ml murine GM-CSF and 10 ng/ml murine IL-4 (BioSource International, Camarillo, CA), 10% FBS, 1% antibiotics, and 50 μM 2-ME (Sigma-Aldrich) at 1–2 × 10^6 cells/ml in a flask or 24-well plate. On day 4, 80–90% of the medium was removed, and the cells were fed fresh medium containing cytokines. After 8 days in culture, loosely adherent cells were harvested and used for experiments.

AdV transduction of DCs

The E1-deleted replication-defective adenoviral vector containing hMART-1 (AdVMART1) has been described previously (24). It was amplified in 293 cells, purified by centrifugation for 2 h at 24,000 rpm in a 30–60% sucrose gradient. DCs were transduced with AdVMART1 (multiplicity of infection = 100) at 37°C for 2 h in a total volume of 1 ml of RPMI 1640 medium with 2% FBS. Infection was terminated by adding 9 ml of RPMI 1640 with 10% FBS. Transduced DCs produce full-length hMART-1 protein and express MART-1 peptide on the cell surface (23). AdVMART1/DCs were kept at 37°C for an additional 20 min (24) and washed three times, and 5 × 10^5 cells were injected s.c. into each mouse in a volume of 100 μl of PBS. For assessment of the ability of DCs to stimulate lymphocyte responses, mice were immunized twice (1 wk apart). For AdVMART1/DC in vivo protection studies, they were immunized once.

RNA analysis of MART-1 expression

Total RNA was extracted from cells using TRIzol (Invitrogen Life Technologies, Carlsbad, CA) and treated with DNase 1 (amplification grade; Invitrogen Life Technologies), according to manufacturers' instructions. Each RNA sample was reverse transcribed using Qiagen OneStep RT-PCR kit (Qiagen, Valencia, CA) with primer pairs for MART-1: 5'-TTG GCA GAC GTC TAG ACT CGC TGG CTC TTA AGG T-3' and 5'-CCT CTT TCT CTC TAG ACC TGT GCC CTG ACC CTA CAA-3' (24).

Flow cytometric analyses

Cells were stained with relevant FITC- and PE-labeled Abs and analyzed using a FACSCalibur flow cytometry system (BD Biosciences, Mountain View, CA). The following mAbs were used: FITC anti-H-2K^b (mouse

MHC class I), FITC anti-HLA-A2 (human MHC class I), FITC anti-I-A^b (mouse MHC class II), PE anti-CD80 (B7-1), FITC anti-CD40, and PE anti-CD86 (B7-2) (all from BD Pharmingen, San Diego, CA). FITC anti-mouse CD83 was purchased from BioCarta (San Diego, CA).

IFN-γ and CD40L treatment

Bone marrow-derived DCs were generated, as described above, but on day 6 cells were irradiated in the flask and on day 8, 0.1 μg/ml mouse CD40L (R&D Systems, Minneapolis, MN) and 0.1 μg/ml IFN-γ (BioSource International) were added with fresh RPMI 1640 medium with 10% FBS and 1% antibiotics. After 48 h, DCs were harvested, and cell surface molecule expression was analyzed by flow cytometry.

Peptide synthesis and pulsing of DCs

hMART-1$_{27-35}$ (AAGIGILTV), human α-feto-protein$_{485-493}$ (CIRHEMTPV), and human tyrosinase$_{1-9}$ (MLLAVLYCL) epitopes were synthesized at the University of California Peptide Synthesis Facility using standard F-moc strategies. DCs were pulsed with 25 μg/ml peptide in 1 ml of serum-free RPMI 1640 at 37°C. After 4-h incubation, cells were washed, and 5 × 10^5 DCs in 100 μl of PBS were injected s.c. into each animal. Mice were immunized twice (1 wk apart) for in vivo protection studies, but once for assessment of lymphocyte responses.

Generation of a B16-A2/K^b cell line

A B16-A2/K^b cell line was developed by dual transfection of B16 cells with the plasmids pSV2-neo (ATCC) and pA2K^b (pSV2 backbone with α1 and α2 domains of HLA-A2.1 and the α3 transmembrane and cytoplasmic region of K^b; a generous gift from Dr. L. Sherman) (37). Transfection was performed using Tfx-20 reagents (Promega, Madison, WI), according to the manufacturer's instructions. Briefly, B16 cells were cultured in a 24-well plate. On the next day, DNA (0.5 μg of pSV2-neo and 0.5 μg of pSV2-A2K^b per well) was diluted in serum-free DMEM, mixed with Tfx-20 reagent (2:1 reagent-to-DNA ratios), added to the plate after removal of the medium, and incubated at 37°C for 1 h. Transfection was terminated by adding DMEM with 10% FBS to the cells. Positive clones were selected in G418 and screened for HLA-A2 expression by flow cytometry using fluorescent Abs (FITC anti-HLA-A2; BD Pharmingen). The B16-A2/K^b cell line was maintained in the presence of 0.5 mg/ml G418.

Measurement of immune function by ELISPOT

ELISPOT was used to assess MART-1-specific splenic lymphocyte responses in immunized mice. Splenocytes were harvested on day 10–14 after immunization and depleted of RBC by treatment with ammonium chloride buffer (0.83% (w/v) NH$_4$Cl, 0.14% (w/v) KHCO$_3$, 0.002% Na$_2$ EDTA, pH 7.3). MultiScreen-HA plates (Millipore, Bedford, MA) were coated with anti-IFN-γ or anti-IL-4 Ab (BD Pharmingen). For the AdVMART1/DC study, cells were first restimulated with irradiated (50 Gy) EL4 or EL4(MART-1) (25:1 responder-to-stimulator ratios) in the presence of 10 U/ml hIL-2 (BioSource International) in a flask for 48 h. After washing and blocking plates with 10% FBS/PBS, restimulated splenocytes were added and incubated for an additional 24 h. For the MART-1$_{27-35}$-pulsed DC study, lymphocytes were added to the Ab-coated plate directly with 30 μg/ml peptides and 10 U/ml hIL-2 and incubated for 48 h. After incubation, plates were washed and incubated at 4°C with biotinylated anti-IFN-γ or anti-IL-4 Ab (BD Pharmingen). HRP avidin D (Vector Laboratories, Burlingame, CA) diluted 1/2000 in blocking buffer (10% FBS/PBS) was added, and the plates were incubated at room temperature for 45 min. Spots were developed by adding 150 μl/well substrate buffer containing 0.4 mg/ml 3-amino-9-ethyl-carbazole (AEC tablets; Sigma-Aldrich) in 0.05 M sodium acetate buffer (pH 5.0) and 0.012% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) and counted using an ImmunoSpot Image Analyzer (Cellular Technology, Cleveland, OH).

In vivo protection studies

A total of 5 × 10^5 AdVMART1/DCs was administered s.c. in the left inner thigh of C57BL/6 mice. B16 tumor challenge (1 × 10^6 cells s.c. in 0.1 ml into the right thigh) was performed 10–14 days later using a single-cell suspension prepared from tumor growing in syngeneic mice, as described (24), with the following modification. Viable cells were isolated from minced tumors by treatment with 4.8 mg/ml Dispase II solution (Roche, Mannheim, Germany) for 40 min with stirring. After washing three times, cells were resuspended in PBS for injection.

A total of 5 × 10^5 MART-1$_{27-35}$ peptide-pulsed DCs was injected s.c. in the left inner thigh of HLA-A2.1/K^b transgenic mice twice with a 1-wk interval. B16-A2/K^b tumor cells (5 × 10^5), isolated from progressively
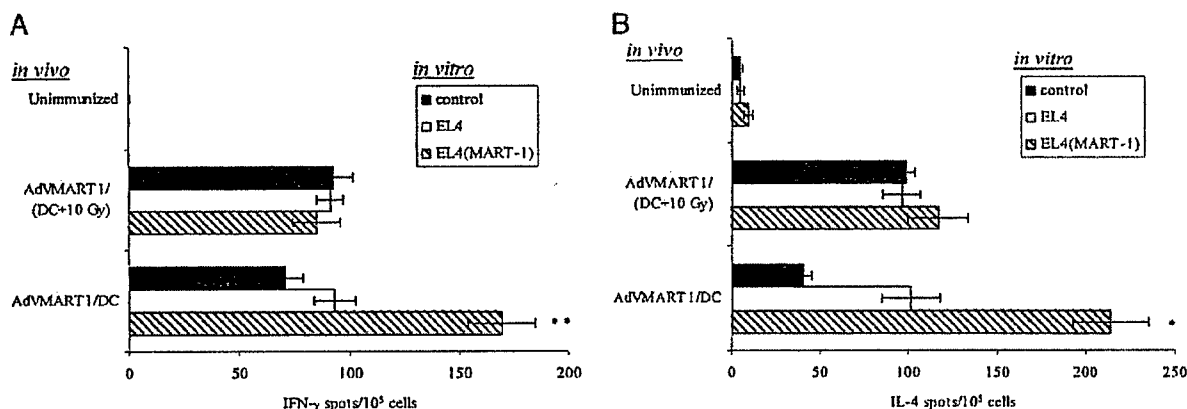
FIGURE 1. Irradiation of DCs inhibits their ability to generate MART-1-specific lymphocyte responses. C57BL/6 mice were immunized s.c. twice, 1 wk apart, with 5×10^5 DC treated with 0 or 10 Gy irradiation before AdVMART1 transduction, or were left unimmunized. Splenocytes were harvested 10–14 days after the last immunization and were restimulated in vitro for 48 h with EL4 (□), or EL4(MART-1) (▨), or without restimulation (control; ■). ELISPOT assays for activated splenocytes producing IFN-γ (A) and IL-4 (B) were performed. Nonspecific background values are common in mice injected with DC (26). The results are the mean $\pm$ 1 SEM of triplicate data of one representative of three independent experiments (*, $p < 0.05$ and **, $p < 0.01$; t test).

growing tumors, as described earlier (24), were injected s.c. in the right thigh 10 days later.

Tumor sizes were measured three times per week using calipers and presented as mean tumor volume (mm³) using the following formula: $4\pi/3 \times (width/2)^2 \times (length/2)$.

Proteasome function assay

DCs were treated with 0 or 10 Gy irradiation and/or with 100 nM PS-341 for 3 h (a kind gift from Millenium Pharmaceuticals, Cambridge, MA). Proteasomes were extracted after treatment, as described previously (21). Chymotrypsin-like activity was measured using 100 μM SucLLVY-7-amido-4-methylcoumarin (Sigma-Aldrich) as fluorogenic substrate. Fluorescence (excitation, 380 nm; emission, 460 nm) was monitored every minute for 30 min using a TECAN Spectrafluor reader (Salzburg, Austria). Protein concentrations were determined by Bio-Rad protein assay (BioRad, Hercules, CA).

Results

Effect of irradiation on the ability of DCs to process and present hMART-1 after AdVMART1 transduction

To determine whether irradiation affected presentation of Ag processed through the endogenous pathway, DCs (day 8) were irradiated or left nonirradiated, transduced immediately afterward with AdVMART1, and tested for their ability to generate MART-1-specific T cell responses following injection into syngeneic mice. Spleens were harvested 10–14 days after two immunizations 1 wk apart, and T cell responses were assessed by ELISPOT after 48 h of in vitro restimulation (Fig. 1).

The number of responsive T cells in the spleens of unimmunized mice was very low, while lymphocytes from mice immunized with AdVMART1/DC responded specifically to in vitro MART-1 restimulation. In three independent experiments, irradiation was shown to abrogate the ability of AdVMART1/DC to generate MART-1-specific T cell responses ($p < 0.01$; t test). This was true for both IFN-γ (Fig. 1A)- and IL-4 (Fig. 1B)-producing lymphocyte responses. The number of lymphocytes activated after immunization of mice with irradiated AdVMART1/DC was no greater than if mice were injected with untransduced DC (data not shown) or if there were no in vitro MART-1 stimulation.

Loss of Ag-presenting function by DC following irradiation was not due to reduced efficiency of AdVMART1 transduction, as assessed by RNA analysis for MART-1 (Fig. 2) or by immunohistochemistry (data not shown). It was also not due to radiation

cytotoxicity. The viability of irradiated and nonirradiated DC was identical on injection, being in excess of 80% in all experiments, as assessed by trypan blue exclusion. Furthermore, culturing DCs for up to 48 h in vitro showed no difference in viability between irradiated and nonirradiated cells, presumably because cell proliferation was minimal, and therefore radiation damage was not expressed.

Irradiation affects protective antitumor immunity induced by AdVMART1/DC vaccination

Because irradiation of DC abrogated their ability to generate MART-specific immunity, we investigated whether this would translate into loss of protection against tumor growth. We have previously shown that AdVMART1/DC immunization of mice prevented or slowed growth of B16 melanoma cells (25) due to the fact that B16 melanoma cells express mMART, which has 68.8% homology with hMART (38). Injection of AdVMART1/DC completely protected mice against B16 challenge (Fig. 3). Irradiated (10 Gy) AdVMART1/DC had lost this protective ability. Tumors grew at a similar rate as in control unimmunized or in mice injected with untransduced DCs.

Effect of irradiation on expression of DC surface costimulatory molecules

A possible explanation for the loss of DC function following irradiation would be decreased expression of MHC and/or costimulatory molecules. To test this hypothesis, DCs were irradiated (day
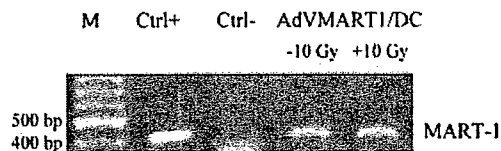
FIGURE 2. Analysis of MART-1 mRNA expression in radiation-treated DCs. Irradiated (10 Gy) or nonirradiated DCs were transduced with AdVMART1 at a multiplicity of infection of 100. RT-PCR of MART-1 mRNA was assayed 24 h after transduction. Irradiation had no effect on MART-1 expression (~400 bp). M202, expressing MART-1, was used as a positive control (Ctrl⁺). The negative control (Ctrl⁻) was DC without AdVMART1 transduction. M, 100-bp size marker.
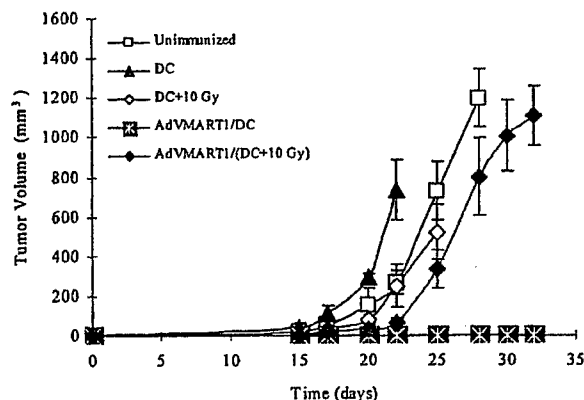
FIGURE 3. Irradiation of DCs abrogates AdVMART1-induced protection of mice against growth of B16 melanoma cells. C57BL/6 mice were injected once with 5 × 10⁵ irradiated (10 Gy) or nonirradiated DCs transduced with AdVMART1. A total of 10⁶ B16 cells was injected 10–14 days after DC immunization. Tumor size was measured three times per week. Results are the mean volume ± 1 SEM of five mice in each group.

8) and analyzed by flow cytometry for MHC class I, MHC class II, CD80, and CD86 expression. In a series of six experiments, MHC class I expression was not changed 24 h after irradiation. CD86 and MHC class II expression was marginally reduced by 2 or 10 Gy irradiation whether mean fluorescence (Fig. 4A) or percentage of positive cells (Fig. 4B) was the endpoint. Similar results were found when we analyzed expression 48 h after irradiation (data not shown). In addition, there was no significant change in expression of any of the molecules immediately after irradiation (data not shown).

Another possible mechanism for radiation-induced loss in DC function would be failure of DCs to mature after irradiation. To test this hypothesis, DCs were cultured from bone marrow cells in the presence of GM-CSF and IL-4, but on day 6, cells were treated with 10 Gy or left unirradiated. We chose day 6 because it has been shown that a small portion of mature DC appears on day 8 (39) and to be better able to assess any effect of irradiation on the maturation process. Maturation was induced by addition of 0.1 μg/ml CD40L and 0.1 μg/ml IFN-γ on day 8. Expression of costimulatory molecules was assessed 48 h later as mean fluorescence (Fig.
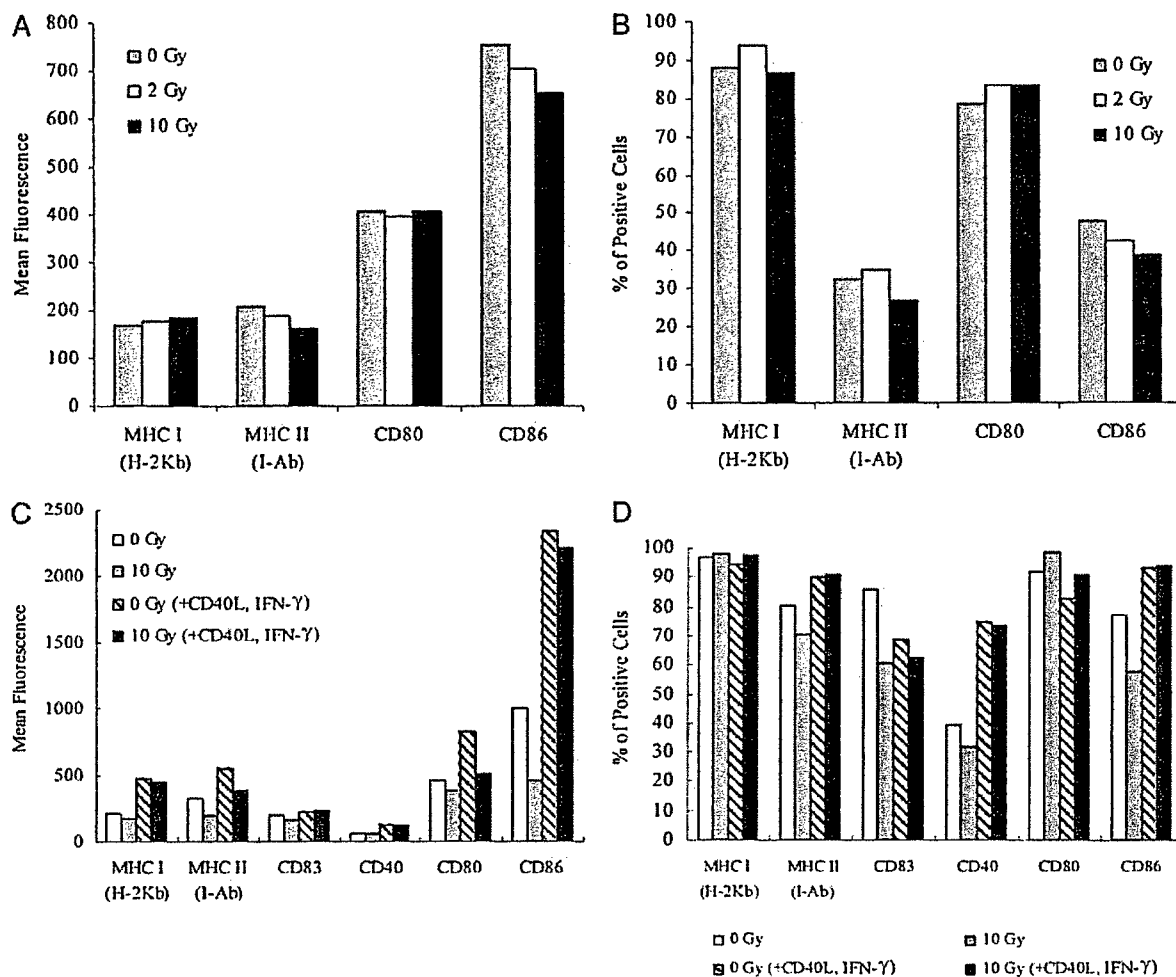

FIGURE 4. Flow cytometric analyses of DC costimulatory molecules. DCs were harvested (day 8), irradiated, and incubated for 24 h. Flow cytometric analysis of MHC I (H-2Kᵇ), MHC II (I-Aᵇ), CD80, and CD86 molecules is shown as mean florescence (A) and percentage of positive cells (B). In another experiment, DCs were irradiated on day 6, harvested on day 8, and treated with 0.1 μg/ml CD40L and 0.1 μg/ml IFN-γ for 48 h. Flow cytometric analyses of mean fluorescence (C) and percentage of positive stained cells (D) are shown using FITC-conjugated anti-MHC I and II, CD40 and CD83 Abs, and PE-conjugated anti-CD80 and anti-CD86 Abs.

FIGURE 5. Irradiation enhances the ability of MART-1_{27-35}-pulsed DCs to generate MART-specific lymphocyte responses. HLA-A2/K^b mice were unimmunized or immunized (s.c.) with 5×10^5 irradiated (10 Gy) or nonirradiated DCs that had been pulsed with MART-1_{27-35}. Splenocytes were harvested after 10–14 days and restimulated in vitro with MART-1_{27-35} (▨), or nonspecific α-feto-protein$_{485-493}$ peptide (☐), or left without restimulation (control; ■). ELISPOT assays were performed to assess the frequency of splenocytes producing IFN-γ at 48 h. MART-1-specific responses from mice immunized with irradiated DC were increased significantly compared with the nonirradiated DC group ($p < 0.001$; t test). In each DC-injected group, the number of MART-1_{27-35}-specific IFN-γ spots was higher than that of nonspecific stimulation (*, $p < 0.05$ and **, $p < 0.01$; t test). Results are the mean spots ± 1 SEM of triplicate data of one representative of three independent experiments.
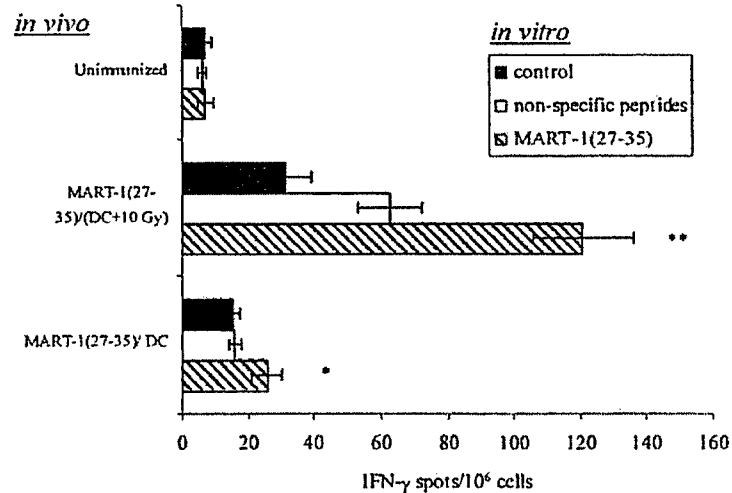
4C) and percentage of positivity (Fig. 4D). In a series of four experiments, CD40L and IFN-γ treatment of unirradiated DCs induced expression of MHC class I, MHC class II, and CD86. Irradiated DCs had marginally decreased expression of MHC class II, CD83, and CD86 in the absence of maturation signals, in agreement with the results in Fig. 4, A and B, but irradiation did not blunt the response to IFN-γ and CD40L.

It can be concluded that although radiation does affect expression of costimulatory molecules with and without the presence of maturation signals, its influence is unlikely to be sufficient to account for the loss in Ag presentation that we observed.

Effect of irradiation on the ability of DCs to present MART-1_{27-35} following exogenous peptide pulsing

We examined whether presentation of exogenously loaded Ag would also be affected by DC irradiation. Irradiated and nonirradiated HLA-A2.1/K^b DCs were pulsed with MART-1_{27-35} peptide. We have shown previously that this strategy could be used to generate tumor-specific MHC class I-restricted T cell responses in both mice and humans (40, 41). In contrast to the irradiation effects on the Ag presentation ability of AdVMART1/DC, in three independent experiments, ELISPOT assays of spleen cells from immunized HLA-A2.1/K^b transgenic mice showed that irradiation of DCs resulted in a significant increase in MART-1_{27-35}-specific IFN-γ-producing T cell responses over nonirradiated DCs ($p < 0.001$; t test) (Fig. 5). In this experiment, there was a high background nonspecific response in irradiated DC-injected group, which is not uncommon in DC-injected mice; however, the difference between the MART-1-specific and nonspecific response was significant in each experiment ($p < 0.05$; t test).

Generation of a B16-A2/K^b cell line

The effect of irradiation on the ability of peptide-pulsed DCs to present the HLA-A2.1-restricted epitope MART-1_{27-35} and to generate protective antitumor immunity was next evaluated. To do this, we first transfected the poorly immunogenic B16 murine melanoma tumor with the gene for the chimeric MHC class I HLA-A2 molecule (A2/K^b). Flow cytometric evaluation identified stable transfectants of B16 expressing A2/K^b (referred to as B16-A2/K^b). Greater than 90% of cells were A2/K^b positive as compared with untransfected cells (data not shown). A2/K^b expression remained constant for at least 1 mo without G418 selection, although as a precaution cells were normally maintained in G418. Compared

with the growth of B16 in C57BL/6 mice, B16-A2/K^b tumors in HLA-A2/K^b mice were slower to develop and take was more variable, but growth was progressive and relatively fast in those mice that did develop tumors (data not shown).

Irradiation enhances the ability of MART-1_{27-35} peptide-pulsed DCs to protect mice against challenge with B16-A2/K^b tumors

Mice were immunized twice with irradiated or nonirradiated DCs pulsed with MART-1_{27-35} peptide. Ten days after the last immunization, animals were challenged s.c. with viable B16-A2/K^b tumor cells. Mice injected with irradiated (10 Gy), peptide-pulsed DCs showed greater tumor growth delay than comparable mice injected with MART-1_{27-35}-pulsed DCs or unimmunized mice (Fig. 6). This in vivo protection study together with the previous
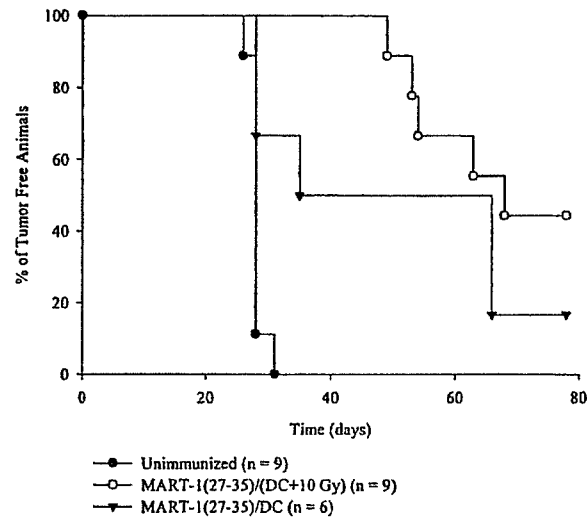
FIGURE 6. Irradiation of DCs before pulsing with MART-1_{27-35} enhances their ability to generate protective immunity against B16-A2/K^b tumor challenge. HLA-A2/K^b mice were immunized twice at a weekly interval with or without 10 Gy irradiated MART-1_{27-35}-pulsed DCs. Ten days after the last immunization, mice were challenged with viable B16-A2/K^b tumor cells. The percentage of mice that were tumor free with time is presented as a Kaplan-Meier plot ($p < 0.0001$ between the groups; log rank test).
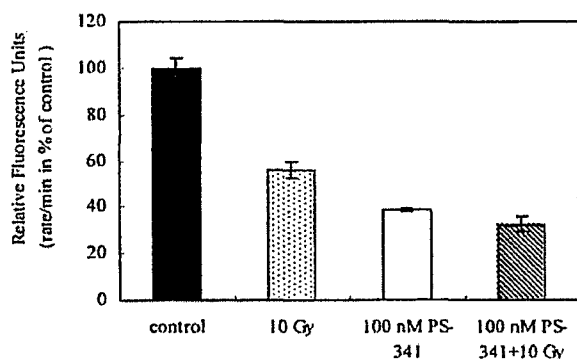
FIGURE 7. Irradiation (10 Gy) of DCs inhibits proteasome activity, as does treatment with the reversible proteasome inhibitor, PS-341, or combined treatment of 100 nM PS-341 (3 h), followed by irradiation. Chymotrypsin-like activity was measured in cellular extracts by the rate of degradation of fluorogenic substrate. Results are shown as percentage of relative fluorescence units of corresponding control cells.

data on the immune responses of mice injected with irradiated and MART-1-pulsed DCs (Fig. 5) confirm irradiation enhances the ability of peptide-pulsed DCs to stimulate immunity.

Irradiation affects DC proteasome activities

The proteasome plays an important role in protein degradation and the generation of immunogenic peptides for MHC class I presentation. Because we have shown that irradiation inhibits proteasome function in tumor cells (21), we hypothesized this might also occur after irradiation of DC, and this might account for the loss of the ability of irradiated AdVMART1/DCs to present Ag. To investigate this hypothesis, we extracted proteasomes from DCs after treatment with 10 Gy radiation and/or 100 nM reversible proteasome inhibitor, PS-341 (3 h). Proteasomal activities were analyzed by fluorogenic assay. In repeated experiments, proteasome activity was down-regulated in DCs treated with irradiation and/or 100 nM PS-341 (Fig. 7). Radiation-induced inhibition was not complete,

but varied $\sim$50–60% of normal in keeping with findings from our other studies (21). This is also true for combined treatment of irradiation and PS-341, which did not further inhibit proteasome function.

To determine whether proteasome inhibition could modulate Ag presentation by the endogenous pathway and exogenously loaded DCs in the same way as was seen for radiation, DCs were treated with 100 nM PS-341 for 3 h before AdVMART1 transduction or MART-1$_{27-35}$ peptide pulsing and injected into wild-type or HLA-A2.1/K^b transgenic mice as before. Lymphocytes expressing IFN-γ, as measured by ELISPOT, were >50% reduced if the DCs had been treated with PS-341 before AdVMART1 transduction (Fig. 8A). In contrast, the number of IFN-γ-producing lymphocytes in spleens of mice immunized with PS-341-treated MART-1-pulsed DCs was increased compared with untreated DCs (Fig. 8B). Taken together, these results suggest that inhibition of proteasome function with PS-341 affects the ability of DCs to present peptides in a manner similar to irradiation, and this depends on how the Ags are generated.

Discussion

The immunosuppressive effects of ionizing radiation are well known and are generally ascribed to killing of radiosensitive lymphocytes. In contrast, even local radiotherapy can be immunosuppressive (42, 43), leading one to wonder whether mechanisms other than cell death might participate. Furthermore, there is little evidence that radiation-induced cell death translates efficiently into the development of tumor-specific immunity, suggesting Ag presentation might be compromised, although few studies have examined this topic in any depth. In this study, we describe the effects of irradiation on DC function as it relates to MHC class I-restricted Ag presentation. DCs are not particularly sensitive to the cytotoxic effects of radiation, as is shown in this study, presumably because they are largely nonproliferative and, unlike lymphocytes, are relatively resistant to radiation-induced apoptosis.

We have shown that radiation can have divergent effects on DC function depending on the pathway that is being used. Immune
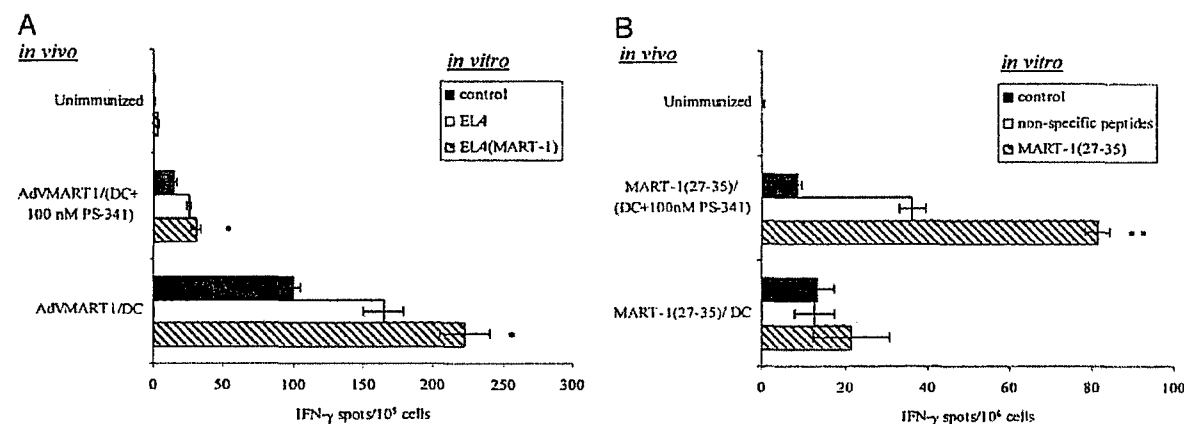
FIGURE 8. Proteasome inhibition by PS-341 affects the ability of DCs to generate MART-1-specific lymphocyte responses. *A*, C57BL/6 mice were immunized twice with DC treated with 100 nM PS-341 for 3 h before AdVMART1 transduction or were left unimmunized. DCs (5 × 10^5) were injected s.c. into C57BL/6 mice twice with a weekly interval. Splenocytes were harvested 10–14 days after the last immunization and were restimulated in vitro for 48 h with EL4 (□), or EL4(MART-1) (▨), or without restimulation (control; ■). IFN-γ-producing lymphocytes were detected by ELISPOT (*, $p <$ 0.05; t test). *B*, HLA-A2/K^b mice were unimmunized or immunized (s.c.) with 5 × 10^5 irradiated (10 Gy) or nonirradiated DC that had been pulsed with MART-1$_{27-35}$. Splenocytes were harvested after 10–14 days and restimulated in vitro with MART-1$_{27-35}$ (▨), or nonspecific TYP$_{1-9}$ peptide (□), or left without restimulation (control; ■). ELISPOT assays were performed to assess the frequency of splenocytes producing IFN-γ at 48 h (**, $p <$ 0.01; t test). Results are the mean number of spots ± 1 SEM of triplicate data of one representative of three independent experiments.

responses to hMART-1 produced endogenously from an adenoviral vector were decreased in DCs irradiated before transduction, as was their ability to generate protective immunity against tumor growth. We are not aware of many studies on the effect of ionizing radiation on Ag presentation. However, markers of Langerhans cells, which are skin DCs, have been reported to be lacking late after irradiation in humans (3) and mice (44). Several mechanisms could be responsible, but the finding that low dose irradiation of allografts often decreases their immunogenicity (45, 46) is hard to explain other than by an effect on functional Ag presentation by passenger leukocytes.

We found that expression of MHC class II and CD86 on DCs decreased marginally over a 24-h culture period after irradiation, although viability was not significantly affected. Maturation of DCs under the influence of CD40L and IFN-γ was also not greatly affected after irradiation. Radiation-induced alteration in MHC class I molecule expression has been reported in the B16 melanoma cell line. H-2D^b expression was enhanced, implying that irradiated tumor cells may be more susceptible to MHC class I-restricted CTLs (47). The costimulatory molecule CD80 (B7.1) was also found to be up-regulated by radiation of B cell lymphoma (48) and myeloid leukemic cells (49). We observed no consistent change in MHC class I, CD80, or CD40 expression by DCs after irradiation in four separate experiments and under a variety of experimental conditions. The discrepancies may be due to differences in the pathways activated by radiation in different cell types.

Radiation has been reported to alter tumor Ag expression. Ciernik et al. (50) showed ionizing radiation enhanced killing of fibrosarcoma cells by increasing expression of tumor-associated mutant p53 epitopes, although the mechanistic basis for this effect is unclear. Our attempts to measure MART-1 expression by AdVMART1/DCs by immunohistochemistry showed no effect of irradiation. In short, we have evidence that radiation can affect DC phenotype, but the magnitude of the effects is small and unlikely to account for loss of the ability of irradiated DCs to present endogenous Ag.

In addition, perhaps the best argument against phenotypic changes being responsible for the observed immunosuppressive effect of radiation is the finding that presentation of an MHC class I-restricted exogenous peptide was enhanced in irradiated DC, as was the ability of these DCs to generate protective immunity. The fact that irradiation of DCs enhanced responses to exogenous MART-1$_{27-35}$ peptide Ag and decreased responses to endogenously processed MART-1 argues strongly against a radiation-induced deficit in presentation per se and the phenotypic changes being critical to the observed altered responsiveness. Furthermore, it suggests that radiation switches the direction of immunity away from endogenous Ag processing toward exogenous peptide presentation pathways.

Our working hypothesis is that the mechanism underlying these effects is at the level of the proteasome. Proteolytic cleavage by the proteasome is in most cases essential for production of antigenic peptides for loading onto MHC class I molecules. Several studies have demonstrated that proteasome inhibitors block proteasome degradation processes, inhibiting MHC class I Ag presentation (51, 52), something we demonstrate in this study for AdVMART1 using the proteasome chymotryptic inhibitor PS-341. We have shown that the proteasome is a direct target for radiation in tumor cells, resulting in a decrease in function by ~50–60% (21). In this study, we show the same for DCs following irradiation, and it seems highly possible that this affects endogenous Ag processing. In keeping with the suggestion that the proteasome is involved in the effects we observed of irradiation on DC function, PS-341 had the same enhancing effect on presentation of MART-1$_{27-35}$ peptide

by pulsed DCs as did radiation. Although the hypothesis that radiation affects Ag presentation by affecting proteasome function has yet to be proved, it seems possible that proteasome inhibition could enhance peptide presentation by preventing processing of endogenous self Ags, freeing MHC class I molecules for more efficient exogenous peptide loading. Wiertz et al. (53) showed that the proteasome inhibitor, lactacystin, interfered with the recycling of MHC class I molecules. It seems possible that by inhibiting proteasome activity and the recycling of MHC class I molecules, irradiation might increase the $t_{1/2}$ of peptide/MHC class I complexes on the cell surface.

The major argument against the proteasome hypothesis is the fact that we have shown that inhibition of proteasome functions at doses as low as 25 cGy (21). We have no data on the effect of such low doses of radiation on DC function; however, our preliminary data suggest that, while a single clinically relevant (2 Gy) fraction of radiation enhances peptide presentation, less consistent effects are seen with endogenously processed Ag. Further studies over a wider range of single and fractionated doses might elucidate whether the dose-response relationships for effects on proteasome inhibition and Ag presentation are similar. Alternative hypotheses might involve radiation-induced costimulatory cytokine molecules such as TNF-α (54, 55), IL-1 (56), IL-6 (57, 58), and IL-10 (59).

These studies add further evidence to the emerging notion that radiation has much more to offer than being a powerful cytotoxic agent. It has profound effects in changing cellular biology. Radiation has definitive immunodulatory properties and is a potential adjuvant for cancer immunotherapy, if we knew how to best harness its cytotoxic potential to the generation of tumor-specific immunity. Several studies with tumor irradiation followed by DC administration showed enhanced antitumor effects in mouse models (60, 61). Within an appropriate danger milieu, DC vaccines might recruit tumor Ags more efficiently, and local infiltrating DCs might up-regulate their abilities for Ag processing and presentation following irradiation treatment. DCs may serve as targets for immunomodulation in combination with radiotherapy for cancer. One might expect that providing more effective danger signals than is provided by radiation (62–65) alone might overcome some of its inhibitory effects and increase the generation of immunity by tumor cells, allowing increased control of local and micrometastatic disease. An appropriate understanding of the effect of radiation on DC function may therefore allow for new strategies in cancer therapy.

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Enhancement of radiotherapy for murine prostate cancer by regulation of interleukin-3 gene expression using an adenovirus tetracycline turn-on system. C. S. Chiang*, C. H. Tsai*, W. C. Liu*, K. F. Hsieh*, F. H. Chen*, W. H. McBride⁺, and J. H. Hong# *: Dep. of Atomic Science, National Tsing Hua University, Hsinchu 30013, Taiwan; +: Dep. of Radiation Oncology, UCLA, CA 90095; #: Dep. of Radiation Oncology, Chang Gung Memorial Hospital, Tao-Yuan 30033, Taiwan.

Abstract:
This study aimed to examine whether the efficacy of radiotherapy (RT) for prostate cancer could be enhanced by a tetracycline-regulated IL3-based immunotherapy for prostate cancer. To this end, mice bearing s.c. TRAMP-C1 prostate tumors were treated with intratumoral (i.t.) injections of Adv-Tet-On-IL3 virus in combination with local fractionated tumor irradiation (7 Gy per fraction per day for 5 days). The expression of IL-3 gene within tumors was regulated by the delivery of tetracycline analogue, doxycycline, using an osmotic pump. The regulated IL-3 gene expression did not cause the splenomegaly, a major side effect caused by long period of intratumoral IL-3 gene expression. Adv-Tet-On-IL3 viruses or RT administration alone caused a modest delayed TRAMP-C1 tumor growth. Adv-Tet-On-IL3 administration combined with RT inhibited TRAMP-C1 tumor growth in a synergistic manner. Splenocytes from mice treated with i.t. Adv-Tet-On-IL-3 contained significantly more tumor-specific IL-4 secreting T cells compared with control or RT alone groups. However, splenocytes from mice treated with Adv-Tet-ON-IL3 plus RT contained significantly more tumor-specific IFN-gamma-secreting T cells compared with control or RT alone groups. Moreover, T cells isolated from these mice had better CTL response against TRAMP-C1 cells than T cells from single treatment or control groups. The study demonstrates that tetracycline regulated IL-3 gene expression can be used to enhance prostate cancer response to radiotherapy with tolerable side effects. It also demonstrates that combining IL-3 gene immunotherapy with radiotherapy can switch the anti-tumor immune response from a Th2 to a Th1 response.

Immune enhancement of radiation therapy for murine prostate cancer by tetracycline-regulated intratumoral expression of interleukin-3[1]

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Running title: Combining radiation therapy and immunotherapy

Key words: radiation, prostate, immunotherapy, interleukin-3, adenovirus

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Abstract:

This study aimed to examine whether the efficacy of radiotherapy (RT) for prostate cancer could be enhanced by anti-tumor immune responses generated through tetracycline-regulated intratumoral IL3 gene expression. To this end, mice bearing s.c. TRAMP-C1 prostate tumors were injected intratumorally (i.t.) with Adv-X Tet-On/Adv-X TRE-IL3 viruses and treated with local fractionated tumor irradiation (7 Gy per fraction per day for 5 days). The expression of IL-3 within tumors was regulated by the delivery of the tetracycline analogue, doxycycline, using an osmotic pump. IL-3 expression or RT administration alone caused a modest delay in TRAMP-C1 tumor growth, but the combination inhibited TRAMP-C1 tumor growth in a synergistic manner and cured 50% mice. Cured mice were resistant to re-challenge with viable TRAMP-C1 cells, indicating the development of long-term immunity. Immune analysis showed that, compared to RT or IL-3 alone, RT plus IL-3 treatment significantly increased the number of tumor-specific IFN-gamma-secreting T cells in the spleen. Moreover, these T cells were more cytotoxic for TRAMP-C1 cells than T cells from single treatment or control groups. The study demonstrates that tetracycline-regulated IL-3 gene expression can be used to enhance prostate cancer response to radiotherapy with minimal side effects. It also demonstrates that combining radiotherapy with IL-3 gene immunotherapy can enhance the anti-tumor immune response to an IFN-gamma-directed pathway and re-direct the IL-3-elicited-immune pathway.

Introduction:

Radiation therapy (RT) is one of the major treatments for human prostate cancer. In an attempt to increase local control, one approach that is currently being studied is to utilize higher doses of radiation. However, this carries with it an increased risk of long-term complications as a consequence of injury to adjacent normal tissues, normally rectum and bladder neck, or, if the size of the field is restricted, a risk of missing local microscopic extensions of the disease. Supplementing radiation with an adjunctive therapy that would increase control of local disease without increasing normal tissue reactions would be a major step forward in the treatment of cancer of the prostate and other disease sites.

Recently, several studies have shown that radiation therapy can modulate expression of several classes of genes in both murine and human cancers (1) and that these might enhance their ability to serve as targets for adjunctive immunotherapy (IT) (2-4). Therefore, combining RT with immunotherapy is an attractive means to increase the probability of achieving local control while at the same time generating a state of systemic anti-tumor immunity that might be able to help eradicate minimal residual and micro-metastatic disease.

We have shown in previous studies that expression of IL-3 genes within tumors can enhance the efficiency of radiotherapy by promoting the generation of immunity even to classically non-immunogenic tumors (5), without altering the intrinsic radiosensitivity of tumors. A long-term state of immunity develops after the primary tumor regresses. Two mechanisms of IL-3 action were proposed by us and others (5-9). One was by altering the phenotypic expression of cell adhesion molecules, including MHC class I. Another was by improving presentation of putative tumor antigens. The combination of RT and IT has

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been shown to be effective in other preclinical models of renal adenocarcinoma (10) and prostate carcinoma (11).

Recently, we demonstrated that intra-tumoral Adv-IL3 administration could enhance the efficacy of radiotherapy for murine prostate cancer (TRAMP-C2) (12). However, long-term expression of IL-3 is associated with some hematological side effects, manifested in the mouse as splenomegaly. In order to minimize these side effects, in this study we used a tetracycline-regulated adenovirus to express IL-3 in transgenic adenocarcinoma mouse prostate (TRAMP-C1) tumors and combined this with treatment using fractionated doses of radiation. IL-3 expressing tumors were more sensitive than parental tumors to irradiation *in vivo,* but not *in vitro*, and a high level of systemic immunity developed with minimum side effects. Interestingly, analysis of the IL-3-elicited tumor-specific T cell response profiles showed that radiation therapy caused an increase in IFN-gamma producing cytotoxic T cells and appeared to alter the balance of the IL-3-directed anti-tumor immunity.

Materials and Methods:

Mice, tumors, and tumor irradiation: C57BL/6J mice were purchased from the National

Laboratory Animal Center, Taiwan.   Seven-eight week old male mice were used for

experiments.   TRAMP-C1 tumors that developed in TRAMP transgenic mice (13) that

are syngeneic to C57BL/6J mice were kindly provided by Dr. Greenberg, N.M.   Tumors

were generated by inoculating 5 x 10^5 viable TRAMP-C1 cells into the right thighs of

mice.   Tumors 4 - 6 mm in diameter were irradiated by 6 MV x-rays from a linear

accelerator with a dose rate of 2.3 Gy/min and a 1.5 cm bolus on the surface, with the rest

of body shielded.   Tumor growth was determined by measuring three mutually

orthogonal tumor diameters at 2-3 day intervals with a vernier caliper and calculating the

mean values.   This measurement was performed until tumors reached 12 mm in

diameter, when mice were euthanized.   The recommendations of the approved guide for

the care and use of laboratory animals by the Commission of Chinese National

Laboratory Animal were followed at all times.

   In vitro irradiation of cells was performed using a cobalt source in the Nuclear

Science and Technology Development Center, National Tsing Hua University, Taiwan,

with a dose rate of 1.1 Gy/min.   Clonogenic survival after irradiation was assessed by

plating various cell numbers in 100 mm diameter Petri dishes and counting colonies of

greater than 50 cells on day 12 after staining by Giemsa (Sigma-Aldrich, St. Louis, MO).

In vitro gene transduction: The BD Adeno-X and Adeno-X Tet-On expression systems

(Clontech, San Jose, CA; Cat. No. 631513 and 631508, respectively) were used to

introduce and express the full length cDNA of mIL-3 into cultured tumor cells (*in vitro*)

and growing tumors (*in vivo/in situ*), as described by the manufacturer's protocol (BD,

Clontech, San Jose, CA).   The former is an adenovirus expression vector using CMV as

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the promoter to constitutively drive the expression of IL-3 or β-gal gene. The latter is a dual vector system where an Adeno-X Tet-on virus is used to produce rtTA that controls expression of Adeno-X-TRE-IL-3 or β-gal. The adenovirus titer was enriched by BD Adeno-X virus purification kit (BD, Clontech, San Jose, CA; Cat. No. 631518) and determined by endpoint dilution assay using an Adeno-X^{TM} Rapid Titer Kit (BD, Clontech, San Jose, CA). IL-3 gene expression was examined by RT-PCR and protein production by a mouse IL-3 ELISA kit (Endogen, Rockford, IL; Cat. No.: ENDEM-IL3). The system was tested using β-gal assay. Cells were cultured in eight-well chamber slides (Nalgen Nunc International, Rochester, NY) at a plating density of 1x 10^5 per well. Twenty-four hours after plating, the cells were exposed to graded dose of Adeno-X Tet-on virus and Adeno-X TRE-β gal virus. Twenty-four hours after infection, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and stained by the X-gal staining assay kit (Gene Therapy Systems Inc, San Diego, CA) for 1 hr.

In vivo gene transduction: Adv-X Tet-On virus (1x10^7 ifu) and Adv-X TRE-IL3 virus (1x10^7 ifu) or Adeno-X TRE-βgal virus (1x10^7 ifu) in 60 µl of PBS were injected intra-tumorally into tumors for 3 days (-1, 1, and 3 day following RT or every other days for 3 days without RT). Virus solution was equally injected into each of four quadrants of a tumor. Alzet Osmotic pumps (DURECT Corporation; Cat. No: 2002, Cupertino, CA) were used to deliver doxycycline (Dox) (Sigma-Aldrich, St. Louis, MO). They were implanted subcutaneous in the back of mice slightly posterior to the scapulae by following the instruction of manufacture procedures (DURECT Corporation, Cupertino, CA). The pumps contained 200 µl of doxycycline solution at the dose of 1 mg/ml and drugs were secreted at a rate of 1 µl per hour for 7 days. All mice including control animal were implanted with the Alzet Osmotic pumps one hour prior to the injection of

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first virus solution.

<u>Immune Response Assays:</u> ELISPOT was used to assess tumor-specific splenic lymphocyte responses. Splenocytes were harvested from experimental mice, depleted of RBC by lysis, and re-stimulated in a flask for 48 h with 10U/ml hIL-2 (BioSource International, Camarillo, CA) plus irradiated (35 Gy) TRAMP-C1 cells (25:1 responder-to-stimulator ratios) to measure tumor-specific responses or syngeneic B16 melanoma cells (ATCC number: CRL-6745) to assess non-specific responses. MultiScreen-HA96 plates (Millipore, Bedford, MA) were coated with anti-IFN-γ or anti-IL-4 antibodies (BD Pharmingen, San Jose, CA). After washing and blocking plates with 10% FBS/PBS, re-stimulated splenocytes were added and incubated for an additional 24 h. After incubation, plates were washed and incubated at 4°C with biotinylated anti-IFN-γ or anti-IL-4 Ab (BD Pharmingen, San Jose, CA). HRP avidin D (Vector Laboratories, Burlingame, CA) diluted 1/2000 in blocking buffer (10% FBS/PBS) was added, and the plates were incubated at room temperature for 45 min. Spots were developed by adding 150 µl/well substrate buffer containing 0.4 mg/ml 3-amino-9-ethyl-carbazole (AEC tablets; Sigma-Aldrich, St. Louis, MO) in 0.05 M sodium acetate buffer (pH 5.0) and 0.012% hydrogen peroxide. The plate was kept in the dark for 20 minutes. The spots that developed were counted under a dissecting microscope.

Tumor-specific cytotoxic function of CD8 T lymphocytes was assessed. Splenocytes were harvested from experimental mice and depleted of RBC by lysis. CD8 T cells were isolated by depletion of non-CD8 T cells using a cocktail of monoclonal antibodies bound to magnetic beads and passing them through a magnetic field of a MACS separator (Miltenyi Biotech, Auburn, CA). The cytotoxicity reaction

was analyzed using a CyToxiLux kit (OncoImmunin, Inc, Gaithersburg, MD). Briefly, target (TRAMP-C1) or non-target (B16 melanoma (ATCC number: CRL-6745)) tumor cells were fluorescently labeled with red dye and co-incubated with CD8 T cells. After 1 hr co-culture, cells were re-suspended in a solution containing a fluorogenic caspase substrate. Following incubation and washing, samples were analyzed by flow cytometry (CyFlow®, Partec, GmbH) for the increased green fluorescence in dying cells. The percentage of double positive cells was used as the index of cytotoxicity.

RT-PCR analysis: Total RNA was isolated from cells using Trixol reagent (Invitrogen, Carlsbad, CA). Two μg of total RNA was first reverse transcribed to cDNA by Omniscript reverse transcriptase (Quiagen, Valencia, CA). The primer pairs for ICAM-1 (5' primer: 5'-AAGGGCTGGCATTGTTCTCTAA-3' and 3' primer: 5'-AGCGC-AGCGGCAGGGTTCT-3') and β-actin (5' primer: 5'-GGTGACGAGGCCCAGAGC-AA-3' and 3' primer: 5'-CCCGGCCAGCCAGGTCCAG-3') were used in PCR reaction.

Characterization of tumor-infiltrating host cells and tumor cell surface markers: The host cell populations present within dispase-elicited (5) single-cell digests of control and treated tumors were identified by flow cytometry using CyFlow® (Partec, GmbH). The tumor composition was examined by FSC vs SSC dot plot. Cells (R1 region of Fig. 4A) were gated using the smallest FSC of lymphocytes as the threshold. The FSC vs SSC region for lymphocyte population (R2 region of Fig. 4A) was obtained by running splenocytes of a normal spleen using the same flow cytometry setting. The tumor-associated macrophages were identified by FITC-conjugated-anti-CD11b (Mac-1) antibody (BD Pharmingen, San Jose, CA). The procedures for antibody staining followed the manufacturer's (BD Pharmingen) suggested protocol and the Fc-mediated non-specific binding was blocked by adding 1 μg/10^6 cells of rat anti-mouse CD16/CD32

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monoclonal antibody (Fc block™, BD Pharmingen, San Jose, CA) prior to the addition of FITC-conjugated antibody.

For characterizing the change of tumor cell phenotype following RT, cultured tumor cells were irradiated with 0 or 7 Gy. Four hours after irradiation, cells were harvested and stained by FITC-conjugated-monoclonal antibody (BD Pharmingen, San Jose, CA) against murine H-2K^b (MHC-I, Cat. No. 553569), CD95 (Fas/APO-1 Cat. No. 554257), or CD54 (ICAM-1, Cat. No. 553252). They were then analyzed by CyFlow® (Partec, GmbH).

Results:

1. Characterization of the tetracycline-induced adenovirus expression system in vitro.

To characterize the Adv-Tet-on-IL3 system in TRAMP C-1 cells, the production of IL-3 protein with time after addition of varying doxycycline (Dox) concentrations, and at various multiplicities of adenovirus infection (MOI), was determined by ELISA (Fig. 1). Addition of Dox from 0.01 up to 1µg/ml to TRAMP cells infected at an MOI of 10:1 led to IL-3 production that increased with time (Fig 1A, B) and there was no evidence of cytotoxicity even with an MOI up to 20:1 (Fig 1C). Regulation by Dox was confirmed using Adv-X TRE-βgal (Fig. 1D).

2. Influence of in vivo IL-3 expression on the growth of TRAMP C-1 tumor.

Adv-X Tet-On (10^7 particles) along with an equal number of Adv-X TRE-IL3 or Adv-X TRE β-gal viruses were injected into each of four quadrants of 4-6 mm diameter TRAMP-C1 tumors in 15 µl volumes per site. This was repeated 3 times every other day. The efficacy of virus infection was monitored by examining X-gal staining in the tumors (data not shown). In addition, IL-3 levels were measured in serum and tumors by ELISA. The data showed that this protocol was sufficient to produce measurable levels of IL-3 in serum (Fig 2A) and within tumors on day 3 and 7 (Fig. 2B), and indicated that expression lasted for at least a week following virus infection. Although elevated levels of IL-3 were found in the blood up to 1 week, mice did not develop splenomegaly (data not shown), which is found in mice bearing tumors constitutively expressing IL-3 (14). Dox administration alone did not affect the rate of tumor growth (data not shown), and therefore only the data for tumor growth in Dox-treated mice are presented. As can be seen (Fig 2C), switch-on of IL-3, but not β-gal, by Dox significantly delayed tumor growth, compared with PBS-injected controls.

3. The influence of IL3 gene transfer on the tumor response to radiotherapy:

To test the effect of IL-3 gene expression on tumor response to radiotherapy *in vivo*, Adv-X Tet-On/Adv-X TRE-IL3 viruses were injected on days –1, 1, and 3 of a course of fractionated radiation delivered daily at 7 Gy/fraction for 5 days. The growth kinetics of the tumors in one of three repeated experiments is presented in figure 3A. TRAMP-C1 is a fast growing tumor with a doubling time of 2 days. Dox-regulated IL-3 expression slowed growth, as did fractionated radiotherapy, causing 5 and 13 days of tumor growth delay, respectively, but with all tumors continuing to grow progressively. The combined treatment of radiotherapy and Dox-regulated IL-3 gene expression extended the growth delay to 21 days and, furthermore, in four out of 8 mice tumors regressed completely and mice were tumor-free for more than 2 months. Three of the mice that had rejected tumor were re-challenged by s.c. inoculation of 1 x 10^6 of TRAMP-C1 cells and all resisted the re-challenge (data not shown), indicating that long-term immunity had developed.

We had shown previously that IL-3 gene transfection of fibrosarcoma cells did not alter their intrinsic radiosensitivity. We confirmed this in TRAMP-C1 cells using an Adv-X-IL3 adenoviral vector system with a constitutive CMV promoter. TRAMP-C1 cells at 80 % confluence were transduced with Adv-X-IL-3 at an MOI of 100. Cells were irradiated one day after transfection. Prior to irradiation, the medium was collected for the measurement of IL-3 by ELISA and the cell number was counted after trypsinzation. TRAMP-C1 cells infected with Adv-X-IL3 just prior to the irradiation produced IL-3 at the level of 213 ± 82 pg/ml/10^6 cells in 24hrs. IL-3 gene transfer slightly altered the plating efficiency of TRAMP-C1 as measured by clonogenic assay (Fig. 3B), but the response to 2 and 4 Gy of radiation delivered on day 1 was not affected. The observed *in vivo* radiation sensitivity of IL-3-transfected tumors can not therefore be

ascribed to a change in intrinsic radioresponsiveness.

Flow cytometry was used to determine changes in the composition of tumors following RT +/- IL-3. When the whole tumor population was examined by FSC vs SSC dot plot, it was found that RT significantly increased the population of cells corresponding to the profile of lymphocytes (R2 region of Fig. 4A), from around 3 % to 20%. IL-3-treatment did not further increase the lymphocyte population. Analysis of FITC-anti-CD11b stained macrophages (Fig. 4B) showed that they were 24.4 % of the total cells (R1 region of Fig. 4A), which increased to 29.5% one day after RT, which was similar to that in Adv X TRE IL-3 (30.9%) treated tumors. The combined treatment of RT and Adv-X-TRE-IL3 further enhanced the percentage of Mac-1+ cells to 38.5%. These changes were more dramatic (Fig. 4C) if the lymphocyte population (R2 region of Fig. 4A) was gated out of the total cells (R1 region of Fig. 4A). Since Mac 1 is more highly expressed on infiltrating cells than mature macrophages (13), these results indicate that RT and AdvX-TRE-IL3 treatments both result in the infiltration of Mac-1+ cells from the periphery, and this was further enhanced when the two treatments were given.

We (5, 6), and others (7, 9), have previously shown that IL-3 expression can alter tumor phenotype, as can irradiation. Since radiation-induced changes in MHC Class I, ICAM-1, and Fas expression have been reported (2-4), we examined expression of these molecules on TRAMP-C1 cells with and without Dox-regulated IL-3 expression 4 hours following a single *in vitro* dose of 0 or 7 Gy. TRAMP-C1 cells *in vitro* expressed low levels of MHC class I, a moderate level of Fas, and no ICAM-1 (Fig 4D). A single dose of 7 Gy radiation or switch on of IL-3 production increased the expression level of MHC class I (Fig. 4D_a), ICAM-1 (Fig. 4D_b), and Fas proteins (Fig. 4D_c). For ICAM-1, the combined treatment further increased expression. The ability of 7 Gy radiation to

increase ICAM-1 expression was confirmed at the mRNA level by RT-PCR (Fig 4D_d).

4. The immune response following combined RT and IL-3 gene therapy:

We have previously shown that intratumoral IL-3 gene expression can enhance tumor immunity. To examine whether the combined effect of short-term tetracycline-induced IL-3 with RT shown in figure 3 could be ascribed to the generation of tumor-specific immunity, we used ELISPOT to measure T cell responses in the spleen of mice received different treatments. The splenocytes were divided into two groups, one for ELISPOT assay (Fig. 5) and one for CTL assay (Fig. 6). Almost no immune response was detected in mice bearing TRAMP-C1 tumors without treatment. Intratumoral injection of Adv-X Tet-on/Adv-X TRE-IL-3 vectors or RT generated modest immune responses. However, treatment with RT plus Adv-X Tet-on/Adv-X TRE-IL-3 vectors markedly increased tumor-specific T cell responses. Furthermore, whereas the adenoviral IL-3 expressing vectors alone resulted in a predominantly IL-4 response, RT generated more IFN-γ producing cells, especially in the combined treatment group. Furthermore, cytotoxicity assays (Fig. 6) showed that CD8+ positive T cells isolated from the same splenocytes as used in figure 5 were more cytotoxic towards TRAMP-C1 cells *in vitro* if mice received the combined RT plus IL-3 treatment than if they came from mice receiving either single treatment or PBS. These responses are tumor-specific because the immune response did not occur when the syngeneic B16 melanoma cells were used as the stimulator in ELISPOT assay (data not shown) or the target cells in CTL assay (Fig. 6F)

Discussion:

This study shows that tetracycline-regulated intratumoral expression of IL-3 using an adenovirus vector potentiated the response of a murine prostate tumor (TRAMP-C1) to radiation therapy (RT) and promoted the development of tumor-specific long-term immunity. The intrinsic *in vitro* radiosensitivity of the tumor cells was not affected by the adenovirus infection or the autocrine effects of IL-3, although both IL-3 expression and RT affected the phenotype of the TRAMP-C1 cells *in vitro*. Rather, the evidence is overwhelming that the enhanced response to RT is due to co-operation with an IL-3 augmented tumor-specific immune response. These data extend our studies using IL-3-transfected fibrosarcoma tumors (5) and Adv-IL-3 with a constitutive promoter (12), as well as being consistent with results of others in other tumor models (10, 15, 16). Furthermore, we demonstrated that the side effects associated with production of persistent high levels of IL-3 (14, 17) can be reduced by the tetracycline-regulated expression system.

The results confirm that while radiation can cause tumor growth delay and increase lymphocyte population in irradiated tumors, its cytotoxic action and lymphocyte recruitment do not result in the generation of strong specific systemic anti-tumor immune reactions as assessed by ELISPOT and CTL assays. This is in spite of an obvious increase in the intratumoral lymphocyte population after RT, but it is not surprising as radiation is generally considered to be a weak danger signal (18) for the development of specific adaptive immunity.

The mechanism by which IL-3 enhances the efficacy of radiation therapy may be complex. IL-3 is known to participate in determining the development and functional behavior of macrophages (19). It can expand a subset of macrophage (7) with increased

expression of co-accessory molecules, including MHC class II molecules and IL-1 (19, 20). In cooperation with TNF-α it stimulates growth of dendritic cells, which are professional antigen presenting cells (APC) (21). It has been shown to enhance the presentation of OVA antigen in the context of class I MHC to CD8+ CTL (9, 22).

This study showed that RT or IL-3 treatment alone resulted in the infiltration of peripheral macrophages as evident with the increase of Mac-1 positive population. This effect was even more evident when RT and IL-3 treatments were combined. Within the tumor microenvironment, macrophages are the major cellular components (see review in (23)). However, their roles in tumor growth remain controversial as they can display both a growth promoting (24) as well as a tumoricidial phenotype (25). A recent study by Ohno, S. et al (26) showed that the high contents of TAMs in close contact with cancers were associated with high relapse-free survival rate, while the high contents of TAMs in hypoxia or necrotic areas were a hazard to relapse-free survival. It is possible that combining RT and IL-3 therapy alters the tumor microenvironment and re-distributes and re-educates the TAMs to become tumoricidial. The re-location of TAMs following RT is currently under investigation.

Another aspect of IL-3 action is to alter tumor cell phenotype. We have previously shown that IL-3 gene expression increases the expression of MHC class I, CD44, ICAM-1, and TNFR expression by fibrosarcoma cells (6). Even cells that were refractory to TNF–α treatment became sensitive. In addition to confirm these findings using TRAMP-C1 cells, we further found that IL-3 induced Fas antigen expression, as did RT. The effect of RT is similar to recent findings by Chakraborty, M. et al (2-4). They have proposed that radiation-induced tumor phenotype changes, such as Fas, MHC-1, ICAM-1, and MUC-1 gene expression, could enhance tumor cell susceptibility to antigen-specific

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CTL-mediated killing. The mechanism that mediates this type of phenotypic switch is still unclear. In practical terms it may in part counteract the radiation-induced suppression in function of dendritic cells in the generation of MHC class I-mediated anti-tumor immunity we recently reported, where RT did not increase expression of immunologically functional co-accessory molecules and, if anything, decreased it (27). We are currently studying whether IL-3 can overcome radiation-induced suppression of DC. The combined effects of IL-3 plus radiation may therefore both modulate antigen presentation and alter the tumor phenotype to result in the enhancement of immune reactions.

In this study, we found that the switch-on of IL-3 production by doxycycline delayed tumor growth, but couldn't cure mice of established tumors. Furthermore, the splenocytes isolated from those mice produced IL-4 in response to *in vitro* stimulation with irradiated TRAMP-C1 cells. This is in consistent with knowledge of the function of IL-3 in enhancing IL-4 production (7, 19, 28, 29). However, it may not be the whole story. It has been shown that recall Ag-specific IL-4 production by splenocytes can be due to IL-3-activated mast cells (29) and this indirect IL-4 pathway is insufficient to trigger the development of Th$_2$ memory T cells. It could also potentially inhibit type 1 T cell differentiation. We have observed a basophilic response within IL-3 producing tumors previously (6). In the current experiments we did not determine if the IL-4 producing cells were T cells or basophils but, in any event, local RT delivered to the tumor seems to enhance IFN-γ producing Th$_1$ and CTL responses in the spleen and may even redirect such responses away from an IL-4 pathway and towards a more potent Th1-type of immunity.

In conclusion, we demonstrated that the combination of RT and IL-3-based

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immunotherapy can cure mice of TRAMP-C1 tumors. Most notably, IFN-gamma-directed CTL immune pathway following radiation therapy was enhanced in the presence of IL-3. This is a feasible approach to enhance the efficacy of prostate cancer therapy with minimum side effects. RT and immunotherapy is a powerful combination that can increase the chances of achieving local, regional, and systemic control of cancer.

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Figure legends:

Fig. 1. (A) Time dependent expression of IL-3 following Dox administration. The MOIs for both viruses were 10 and the concentration of doxycycline was 1µg/ml. (B) The influence of Dox concentration on IL-3 expression. The MOI of Adv-Tet-on and Adv-IL-3 were both 10. (C) Influence of MOI on IL-3 expression. After 24hr incubation with Dox (1µg/ml), medium was harvested and assayed for IL-3 expression by ELISA kit. (D) Expression of β-Gal in TRAMP-C1 was under the regulation of doxycylcine. TRAMP C-1 cells were co-transfected by Adv-X-Tet-on (MOI=10) and Adv-X TRE-βgal viruses (MOI=10). After 24hr incubation with Dox (1µg/ml), cells were fixed and stained with X-gal.

Fig. 2.   Measurement of IL-3 production *in vivo* and its effect on tumor growth.   (A) The concentration of IL-3 in blood samples taken 12 hr after the last virus injection, as measured by ELISA.   (B) The concentration of IL-3 within tumor samples taken at day 3 and day 7 after the last virus infection, as measured by ELISA. (C) Regulation of IL-3 gene expression by Dox caused TRAMP C-1 tumor growth delay.

Fig. 3.   (A) The influence of Adv-X-TRE-IL3 gene transduction on tumor response to fractionation radiation therapy.   Tumors 4 ~ 6 mm in diameter were injected with 1 x 10^7 pfu Adv-X-Tet-on and Adv-X- TRE-IL3 viruses in 60 µl at -1, 1, and 3 days following 5 x 7 Gy of radiation therapy.   The Dox (1mg/ml) was released at a dose rate of 1µl/hr by the osmotic pump for 7 days.   The pump was implanted s.c. in the backs of mice 1 hr prior to the first injection of viruses.   (B) The influence of Adv-IL3 gene transduction on the *in vitro* response of TRAMP-C1 cells to 0, 2, and 4 Gy irradiation.

Irradiation was delivered 24hrs after virus and the surviving fraction was measured by a conventional clonogenic assay. Adv-IL3 reduced the plating efficiency of TRAMP-C1 cells, but did not alter their response to irradiation. *: $p < 0.05$ by Student's T test

Fig. 4. (A): Flow cytometric analyses cell populations (FSC vs SSC dot plot) from TRAMP-C1 tumors 1 day after the last RT treatment show that RT or RT plus Adv-X-TRE-IL3 treatments increase a population of cell corresponding to the profile of lymphocytes (R2 region). (B): The FITC-anti-CD11b staining histograms show that RT or Adv-X-TRE-IL3 treatment increases the proportion of Mac-1 positive cells compared to controls and this is increased further by combined RT and Adv-X-TRE-IL3 treatments. (C): These changes were more dramatic if the lymphocyte population (R2 region) was gated out of the total cells (R1 region) (D): Flow cytometric analyses of *in vitro* TRAMP-C1 cells show alterations in expression of MHC-I (a), ICAM-1 (b), and Fas (c) molecules 4 hours after either 0 or 7Gy, Adv-X-TRE-IL3, or combined treatment. Increased expression of ICAM-1 by RT was confirmed by RT-PCR (d).

Fig. 5. ELISPOT assays for immune responses in spleens of mice received different treatments and re-stimulated *in vitro* with irradiated TRAMP-C1 cells. TRAMP-C1 tumors growing *in vivo* generate few T cell responses. Intra-tumoral expression of IL-3 increases responses, in particular of IL-4 producing cells while RT increases predominantly IFN-γ producing cells. Both these responses are however modest compared with the combined treatment which has a predominantly IFN-γ producing profile. The specific immune response was confirmed by that the irradiated syngenic B16 cells as stimulators did not generate significant T cell responses (data not shown)

Fig. 6. TRAMP-C1 specific CTL responses. CD8+ T cells were purified from spleens of mice as for Fig 5 and co-cultured with irradiated TRAMP-C1 cells at an effector to target ratio of 10:1 for 1 hour. CTL responses were detected by caspase activity induced within the TRAMP-C1 target cells using the CyToxiLux$^{®}$ kit. The profiles shown represent CD8+ T cells from control mice (<u>A</u>), treated with RT (<u>B</u>), with IL-3 (<u>C</u>), with RT and IL-3 (<u>D</u>), and with βgal (E). To demonstrate that the CTL responses were tumor-specific, syngenic B16 cells were used as target cells and the same assay performed (<u>F</u>).

Figures:

Fig. 1

Fig. 2

Fig. 3

A

B

26

Fig. 4A

Fig. 4B

CONTROL
Mn: 194.23
CV: 1078.35
[1890-4095] 2939   (24.4 %)
M1
Events
10^0 10^1 10^2 10^3 10^4
FITC-anti-CD11b

RT
Mn: 252.41
CV: 849.89
[1890-4095] 3747   (29.5 %)
M1
Events
10^0 10^1 10^2 10^3 10^4
FITC-anti-CD11b

IL-3
Mn: 187.70
CV: 1114.34
[1890-4095] 3712   (30.9 %)
M1
Events
10^0 10^1 10^2 10^3 10^4
FITC-anti-CD11b

RT + IL-3
Mn: 232.66
CV: 916.40
[1890-4095] 4933   (38.5 %)
M1
Events
10^0 10^1 10^2 10^3 10^4
FITC-anti-CD11b

28

Fig. 4C

CONTROL
Mn: 192.97
CV: 1084.25
[1890-4095] 2749 (23.9 %)
M1
Events
FITC-anti-CD11b

RT
Mn: 258.64
CV: 831.90
[1890-4095] 3568 (41.8 %)
M1
Events
FITC-anti-CD11b

IL-3
Mn: 189.29
CV: 1106.04
[1890-4095] 3598 (31.3 %)
M1
Events
FITC-anti-CD11b

RT + IL-3
Mn: 237.79
CV: 898.84
[1890-4095] 4655 (53.9 %)
M1
Events
FITC-anti-CD11b

29

Fig. 4D

Fig. 5

TRAMP-C1 specific immune response

Combining radiotherapy and immunotherapy: a revived partnership.

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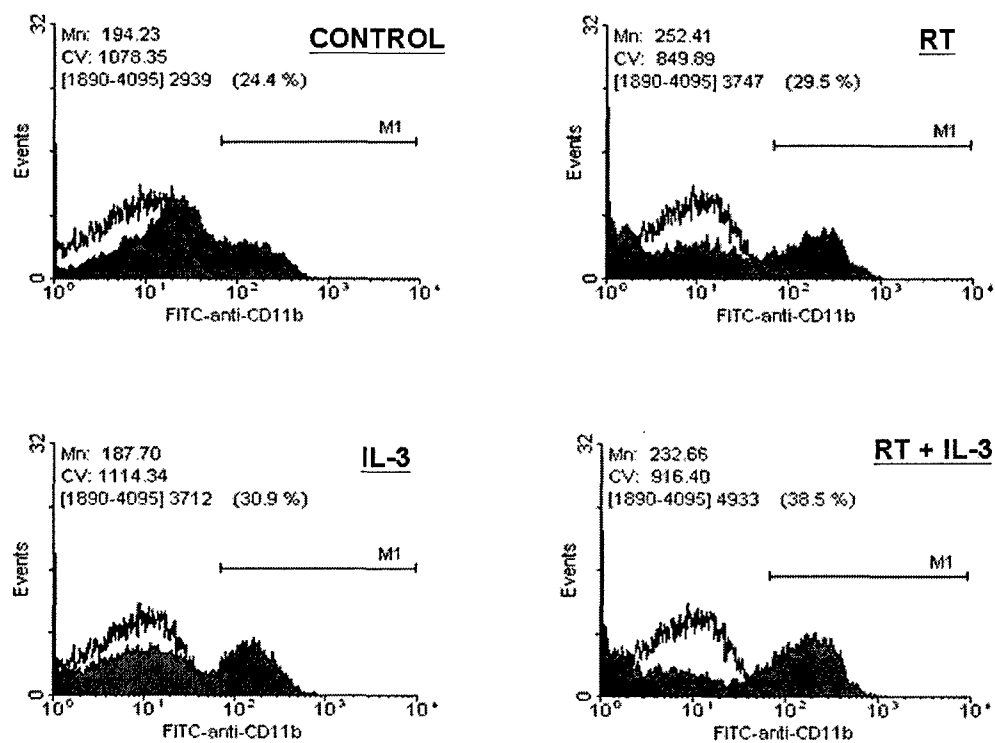
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RUNNING TITLE: Local radiation and immunotherapy.

CONFLICT OF INTEREST NOTIFICATION PAGE:

There are no potential conflicts of interest involving any of the authors.

Abstract.

Ionizing radiation therapy (RT) is an important local modality for the treatment of cancer. The current rationale for its use is based largely on the ability of RT to kill the cancer cells by a direct cytotoxic effect. Nevertheless, considerable evidence indicates that RT effects extend beyond the mere elimination of the more radio-sensitive fraction of cancer cells present within a tumor at the time of radiation exposure. For instance, a large body of evidence is accumulating on the ability of RT to modify the tumor microenvironment and generate inflammatory or danger signals. This may have far reaching consequences on the response of a patient to treatment, especially if radiation-induced tumor cell kill were to translate into the generation of effective anti-tumor immunity. Although much remains to be learned about how radiation can impact tumor immunogenicity, data from pre-clinical studies provide the proof of principle that different immunotherapeutic approaches to induce anti-tumor immunity can be combined with RT to enhance its therapeutic benefit. Conversely, RT could reveal a useful tool to combine with immunotherapy.

This article will briefly summarize for the clinician the relevant knowledge on tumor immunity, including tumor-associated antigens (TAA), antigen presenting cells, and effector mechanisms. In addition, the experimental evidence supporting the contention that RT can be used as a tool to induce anti-tumor immunity is discussed, and a new approach to radio-immunotherapy of cancer is proposed.

3

Introduction.

Conventional cytotoxic therapies like radiation and chemotherapy have been generally viewed as immunosuppressive. However, advances in the understanding of the mechanisms that regulate the development of anti-tumor immunity, as well as improved knowledge of the complex effects of radiation on tissues [1], have stimulated a revived interest in the possibility of combining radiation and immune-based therapies to achieve a better local and systemic tumor control [2].

The concept that the immune system can distinguish the neoplastic from the normal self has been proposed almost a century ago [3]. Since William Coley started treating patients at the end of the 19th century with bacterial toxins, there have been waves of enthusiasm for immunotherapy for treatment of cancer. The introduction of cytokines, in particular interleukin-2 (IL-2), for cancer treatment was a major clinical effort that had modest success. Until recently, however, these efforts have been hampered by a lack of molecular definition of tumor antigens, a means of delivering them effectively, and a sensitive and reliable way to measure responses. This situation changed with the molecular cloning of human tumor-associated antigens that could be recognized by T cells, the ability to culture powerful antigen presenting cells in the form of dendritic cells (DC), and to assess immune responses to specific tumor epitopes using tetramer and ELISPOT assays [4]. These advances allied to the development of genetically modified mouse models have led to a deeper understanding of the interactions between cancer and the immune system of the host [5]. The available experimental evidence supports the hypothesis that once tumors have become clinically apparent their immunogenicity has been modified by the selective pressure of the immune system, resulting in the growth of tumors that are characteristically poorly immunogenic, being able to escape immune detection and/or to actively inhibit immune

effectors [5]. Furthermore, it is clear that, although T cells become tolerant to many self antigens in the thymus, which depletes the pool that might react to cancer, tolerance to many self components is actively maintained in the periphery by several mechanisms. For example, immature DC presenting self antigens to T cells are tolerogenic. This peripheral tolerance can be broken by maturation of DC in local sites, leading to the belief that T cells can respond to "self" antigens on tumors, something for which there is now considerable evidence [6]. The recognition of the fact that the host can break a state of tolerance that has developed to its own tumor offers many possibly effective immunotherapeutic strategies, some being currently tested in clinical trials.

In this paper, the basic knowledge about the interactions between tumors and the immune system, and the mechanisms that regulate the activation of cell-mediated immunity will be briefly reviewed, as will, evidence for a possible role of radiation therapy in enhancing overall tumor immunogenicity and homing of effector immune cells to the tumor site. Strategies for combining the use of ionizing radiation and immunomodulators are proposed.

1. Tumor Antigens.

The antigen specificity of T and B cells, i.e., their ability to recognize with extreme specificity the subtle differences that occur in normal cells upon infection or transformation, is one of the major appeals of immunotherapy. Truly tumor-specific antigens (TSA) are rare. They can arise from point mutations [7] or other genetic alterations specific to a given tumor or group of tumors, such as fusion proteins generated by translocations [8], or sometimes from alterations in post-translational modification [9]. Most of the tumor antigens that are targets for the immune system are more properly defined as tumor associated antigens (TAA) (Table 1). This definition

includes antigens that are not mutated but are differentially expressed by neoplastic and normal cells, either in time, quantity, location or cellular context, resulting in a preferential or exclusive recognition of the tumor by the immune system. For example, carcinoembryonic antigens are normally expressed only during embryonic development [10], p53 and HER-2/neu are overexpressed in some cancer cells [11, 12], and a growing family of Cancer Testes (CT) antigens are expressed only in male germ cells, and sometimes placenta and fetal ovary [13]. TAA with a tissue-restricted expression can be legitimate targets for immunotherapy, especially when the tumor arises from non-essential tissues, such as differentiation antigens expressed by melanoma [14], and prostate cancer [15]. A special class of TAA is derived from oncogenic viruses associated with some types of cancer, such as human papilloma virus E6 and E7 proteins in cervical cancer, and Epstein-Barr virus-derived antigens in lymphomas [16, 17]. Importantly, TAA-specific T cells are frequently detected in the peripheral blood and within the tumor of cancer patients [13]. Tumor-infiltrating lymphocytes have on many occasions been used to define TAA that have then been successfully cloned. Obviously, these are by themselves ineffective at causing tumor regression and the aim of immunotherapy is to effectively boost and harness these existing resources to convert them into an effective anti-tumor response.

2. Antigen Presentation.

a. Dendritic cells.

In the last decade the crucial role played by the innate immune system, and in particular by DC in determining T cell activation has been better understood. DC are lineage-negative, bone marrow-derived mononuclear cells found in blood and many peripheral tissues (reviewed in [18]). They can be broadly divided into myeloid or plasmacytoid DC (MDC and PDC, respectively)

based on phenotypic, morphological and functional differences. MDCs are comprised of additional subsets, e.g. Langerhans cells of the epidermis, and dermal or interstitial DC. PDC are the major interferon-alpha (IFNα) producing cells in the body and play a role in mediating anti-viral and tumor-specific immune responses. MDC in particular are capable of capturing antigens with high efficiency by phagocytosis, macropinocytosis, and adsorptive endocytosis mediated by an array of receptors. Antigens acquired both endogenously (i.e., synthesized within the DC cytosol), or exogenously (acquired from the extracellular environment) are processed into peptides, which are loaded onto Major Histocompatibility Complex class I and II (MHC I and II) molecules and transported to the cell surface for recognition by antigen-specific T cells.

DC most efficiently capture antigens as "immature" cells. The terminal process of differentiation termed "maturation" transforms DCs from poorly immunostimulatory cells specialized for antigen capture into cells specialized for T cell stimulation. This process is accompanied by cytoskeletal reorganization, loss of adhesiveness, acquisition of cellular motility with development of characteristic cytoplasmic extensions or "veils", migration to lymphoid tissues, reduced phagocytic uptake, and enhanced T cell activation potential [18]. Mature DCs can secrete chemokines and cytokines that attract other immune cells and activate resting T cells. Maturation can be induced by "danger signals" which warn the resting DC of the presence of pathogens, inflammation or tissue injury [19]. These signals are transmitted through receptors on DCs that recognize host-derived inflammatory molecules such as CD40 ligand (CD40L), TNFα, IL-1, IL-6 and IFNα. Microbes and damaged host tissues release molecules which ligate important transmembrane receptors related to Drosophila Toll protein known as Toll-like receptors (TLRs) [20]. Activation of DC through these receptors induces their maturation, thereby enhancing their antigen presenting qualities.

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b. Cross-presentation of cell associated antigens.

One of the unique features of DC is their capacity to process captured exogenous antigens. These include apoptotic and necrotic virus-infected or tumor cells, immune complexes, opsonized tumor cells, heat shock proteins (HSPs), DNA or RNA encoded antigens, organisms, e.g. bacteria, viruses, virus-like particles, exosomes, soluble proteins [21] and even "bits" of live cells "nibbled off" by phagocytes, onto MHC I [22]. These phenomena, termed "cross-presentation," permits DC to elicit CD8^{+} as well as CD4^{+} T cell responses to exogenous antigens. Several antigen uptake receptors direct the targeting of exogenous antigens to DC, including FcRs, C-type lectins, scavenger receptors, integrins and heat shock protein receptors [19].

Cross-presentation is considered to be a major mechanism by which tumor antigens are presented to T cells. TAA can access DCs through the uptake of cellular components, in particular apoptotic cells or their fragments (also termed bodies), antibody opsonized tumor cells, necrotic cells and possibly proteins and nucleic acids released from dying tumor cells. Apoptosis, a physiological form of cell death, occurs during normal tissue turnover, during embryogenesis and following infection or inflammation of tissues. The safe disposal of apoptotic cells by surrounding phagocytes including DC and macrophages, prevents the activation of bystander cells and tissue damage following the release of cellular constituents. Indeed, Huang et al. have shown that mouse DC subsets can constitutively acquire apoptotic cells in the intestine and transport the ingested material to mesenteric lymph nodes [23].

Cells undergoing apoptosis are characterized by altered distribution of membrane lipids and exposure of modified carbohydrates on the plasma membrane enabling rapid recognition of apoptotic cells by specific receptors on phagocytes. The uptake of apoptotic cells by phagocytes under normal conditions fails to elicit clinically significant autoimmune responses, as this

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process renders the phagocytes immunosuppressive and induces "cross-tolerance" in contrast to cross-priming [24]. A similar mechanism may occur in tumor settings where DC having captured dying tumor cells, but not exposed to a maturation stimulus, may induce tolerance rather than immunity. In fact, apoptotic cells, when delivered to DCs together with inflammatory signals (or "danger" signals) are an excellent source of antigen for priming of effector T cells. Several studies have examined the effects of DC pre-pulsed with apoptotic cells and administered in vivo to mice, and currently, to humans (reviewed in ref. [25]). Significantly, the efficiency of cross-presentation of cell-associated antigens is substantially greater (many hundred fold) than cross-presentation of soluble antigen [26].

Necrotic cells may also be sources of tumor antigens for DC and while simultaneously providing maturation signals. Secondary necrosis can ensue when there is failure of clearance of apoptotic cell death [27]. Exposure of immature DC to necrotic but not apoptotic cells, (including tumor cells), both in vitro and in vivo, results in their maturation [28]. Moreover, cells rendered necrotic by freeze-thawing were shown to have adjuvant activity when injected in vivo as they enhanced the T cell responses to co-injected antigens [29, 30]. The endogenous adjuvant activity was heightened if the cells were first stressed by radiation, indicating that injury can modulate this effect. In situ induction of tumor cell death can enhance tumor immunity [31]. It is likely that the injury and death incurred by tumor cells in these cases enhances the immune response through the cross-presentation of tumor antigens.

The relative contribution of apoptosis *versus* necrosis to cross-presentation remains unknown but when monitored in vitro, the efficiency of antigen presentation is not dramatically different [32]. Endogenous factors that are released from or are associated with necrotic cells may account for the ability of necrotic cells to activate DC (reviewed in [19]). Examples include

immunostimulatory self DNA that binds TLR9, self ssRNA that stimulates TLR7 and TLR8, secondary structures of mRNA that activate TLR3, and HSP that stimulate TLR4. Recently, uric acid was identified as another factor associated with cell death and activation of DCs [33]. And lastly, the immune system is alerted to massive cell death not only by factors released from dying cells, but also by factors emanating from disruption of tissue architecture e.g. fibrinogen [34], such as oligosaccharides of hyaluronan [35], EDA-containing fibronectin [36] and heparan sulfate proteoglycan that stimulate phagocytes. Therefore, the induction of necrosis in vivo may not only be accompanied by the release of self antigens, but also inflammatory factors which may enhance the immune response. Some of these factors may facilitate the coordination and generation of spontaneous anti-tumor immune responses.

Although, the prevailing view is that apoptotic cells induce tolerance when captured by DC, it is worth pointing out that there are examples in the literature where apoptotic cells are immunostimulatory (reviewed in [25]). For example, immunization with apoptotic cells or in situ induction of tumor cell apoptosis [31, 37, 38] induced T cell responses in vivo. The discrepancies between the different results are likely to be due to the cell type tested and apoptosis pathway induced [39].

c. Nature of the antigens transferred to DC in cross-presentation and role of DC maturation.

The form of cell associated antigens being cross presented from dying cells could include HSPs-associated proteins, native proteins [40], peptides [41] or other consituents. The relative contribution of each, which may depend upon the system/antigen being studied, remains to be established. It is generally considered that maturation signals are essential to convert cross-tolerance to cross-priming [42]. Signals from virus or bacteria infected apoptotic cells (e.g. dsRNA,

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and inflammatory cytokines such as TNF alpha, type I IFNs) can provide the maturation stimuli (reviewed in [19]). In the case of tumors, however, unless there is extensive necrosis and/or release of endogenous adjuvants that activate DC, the end result may be cross-tolerance vs. cross-priming. Radiation in the form of UV light, at least in vitro, induces apoptosis and promotes the uptake of cells by DC [43]. Ionizing radiation as discussed below, not only heightens the expression of MHC molecules on tumor cells but also leads to their death. Thus a combined approach of inducing cell death by RT, in concert with delivery of a maturation signal to tissue DC, can lead to the priming or enhancement of anti-tumor responses [44].

3. Effector mechanisms.

Although antibodies directed against abnormally expressed or activated receptors on cancer cells (e.g., trastuzumab, or Herceptin) [45], have a role in the therapy of cancer , in general antibodies against most TAA are not very effective at causing tumor regression. Non-specific effector mechanisms also operate in a cancer setting, such as NK, NKT cells, macrophages and neutrophils. However, the evidence overwhelmingly indicates that T cell-mediated immunity is far more effective, in particular in terms of tumor cell killing (reviewed in ref. [46]). Much is known about the mechanisms of activation of T cells and how they cause tumor cell lysis.

Specific T cells bind through their T cell receptors (TCR) to a molecular complex composed of MHC-encoded glycoproteins called in humans Human Leukocyte Antigens (HLA) that are loaded with a short antigenic peptides derived from partially degraded proteins. CD8+ T cells recognize MHC I molecules that have antigenic peptides of 8 to 10 amino acids in length that are derived from intracellular proteins by proteasome cleavage [47]. CD4+ T cells recognize MHC II molecules with bound peptides of 15 to 20 amino acids in length derived from

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extracellular proteins that are processed throught the endocytic compartment [48]. Whereas MHC I

molecules are widely expressed in the organism, MHC II expression is normally restricted to

cells of hematopoietic origin capable of functioning as antigen-presenting cells (APC) such as

macrophages, dendritic cells, and B cells. Since activated T cells have to recognize and kill

cancer cells that, for the most part, will not express MHC II, it is not surprising that cytolytic

CD8+ T cells (CTL) are the main effectors against cancer cells [49], whereas CD4+ T cells play a

role in induction and maintenance of the CD8+ T cell response mainly by providing help via the

production of cytokines [50].

While antigen expression in the context of MHC I molecules, for example TAA by tumor

cells, is sufficient for their recognition by activated CD8+ effector T cells, it is insufficient to

activate naïve CD8+ T cells. In order to become activated T cells need not only the signals

mediated by engagement of the TCR but also co-stimulatory signals. The latter are provided by

interaction of CD28 on the T cell with CD80 and CD86 co-stimulatory molecules on the DC

(Figure 1). In addition, activation of CD4+ T cells to produce IL-2, and release of IL-12 by DC

are all required for development of an effective CTL response.

Following activation, it takes about 48-72 hours for CD8+ T cells to differentiate into a

CTL, a process that requires the expression of effector molecules capable of inducing the death

of target cells. CTL use two major independent pathways to kill, mediated through release of

cytotoxic granule contents, and by ligation of death receptors, respectively (reviewed in

reference [51]). TCR-mediated recognition of a target cell triggers degranulation of the CTL and

release of effector molecules perforin and granzymes into a cleft formed between the two cells.

Granzymes enter the target cell by endocytosis and are released into the cytoplasm by the

activity of the pore-forming protein perforin. Granzyme B triggers apoptosis mainly by cleavage

of Bid and caspase activation through the mitochondrial pathway, whereas granzyme A causes single-strand DNA breaks and apoptosis by a slower pathway. Alternatively, CTL express the ligands for death receptors such as Fas, TNFR, and TRAIL-R and can trigger apoptotic death of the target cells expressing them. Overall, the ability of CTL to use multiple pathways to kill cancer cells contributes to their effectiveness as anti-cancer effectors.

4. Regulation of T cell activation.

Activated T cells are powerful effectors that can destroy cells. Since anti-cancer responses are in many cases directed against self antigens, the aim of immunotherapy may be construed as an attempt to develop a directed pathogenic autoimmune response and will always carry with it a risk of autoimmunity. In fact this is sometimes seen in patients responding to immunotherapy of melanoma when they develop vitiligo associated with tumor regression [52]. It is therefore not surprising that multiple mechanisms are in place to regulate T cell activation and that these mechanisms will also regulate attempts at tumor immunotherapy.

The process of T cell activation requires an orderly sequence of events. As described above, after antigen acquisition in peripheral tissues, DC migrate to lymphoid organs such as lymph nodes and spleen where they interact with naïve and quiescent memory T cells. In order to be able to optimally stimulate T cells, DC require to undergo "maturation", and in its absence DC instead induce tolerance to the captured antigens [53, 54]. The functional state of DC therefore plays a crucial role in activation of T cell-mediated immunity. Dysregulation of DC maturation and function has been reported in tumor-bearing patients and in experimental mouse models, and is recognized as an important mechanism of suppression of anti-tumor immunity [55-57].

Another important resource for maintaining peripheral tolerance and controlling T cell activation lies in a recently defined T cell subset, namely regulatory CD4+CD25+ T-cells (T-reg) [58]. Depletion of T-reg cells can cause organ-specific autoimmunity, and also can induce rejection of some tumors [59]. Noticeably, the efficacy of cancer vaccines is enhanced when T-reg are depleted [60].

Finally, T cell activation is regulated also at the level of the effector T cells themselves. One of the better understood mechanisms is that mediated *via* the CTLA-4 molecule, which is up-regulated on the surface of T-cells during the early stages of activation. CTLA-4 down-regulates T-cell responses by competing with CD28 for binding to co-stimulatory molecules (reviewed in reference [61]). In physiological conditions, CTLA-4–mediated inhibition is important for the maintenance of peripheral tolerance. However, in conditions of suboptimal APC function such as in tumor-bearing hosts it is an obstacle to the development of effective anti-tumor immunity [62, 63].

4. Effects of ionizing radiation on the immune system.

Ionizing radiation has a well-established ability to kill cancer cells, and other cells within the tumor stroma, including endothelial cells and intratumoral lymphocytes [64]. Tumor cells killed by RT should be a very good source of antigens for DC uptake and presentation to T cells [65] (Figure 1). Understanding this process has important implications for the effects of RT on development of anti-tumor immunity. The possibility of using RT to promote tumor antigen-presentation by DC has been explored by us and others in pre-clinical studies showing that anti-tumor immunity can be elicited *in vivo* when tumor irradiation is combined with administration of DC or a DC growth factor to increase DC numbers in tumor-bearing mice [66-68]. However, no

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direct evidence that RT on its own is able to enhance tumor immunity is currently available. As mentioned above, optimal activation of T cells by DC presenting tumor-derived antigens can be achieved only in the presence of inflammatory or "danger" signals. Danger signals are generated upon radiation exposure, although their nature remains largely undefined [1]. Pro-inflammatory cytokines IL-1β and TNFα can be induced by radiation, and may act to signal danger [69, 70]. In addition, production of other inducers of DC maturation such as prostaglandin E2 is upregulated in tumor cells following radiation [71, 72]. Overall, the available in vivo data support the hypothesis that RT may provide at least some of the necessary maturation signals. In vitro, irradiation of DC does not block their ability to undergo maturation in response to appropriate signals. However, irradiated DC, even though they are not killed, do lose some of their ability to process antigens and generate anti-tumor T cell-mediated immunity [73].

Other effects of RT may influence the effector phase of the anti-tumor immune response. RT can up-regulate death receptors such as Fas/CD95, HLA class I antigens, and co-stimulatory molecules on certain tumor cells, and this may enhance their tendency to either die or be recognized [2, 74-80]. Radiation-mediated up-regulation of Fas on mouse colon adenocarcinoma cells has been shown to sensitize tumor cells to killing by anti-tumor CTL adoptively transferred or elicited by vaccination of mice with recombinant Pox viruses [79, 81]. In this system, improved killing was mediated by Fas on tumor cells being cross-linked by Fas-Ligand on T cells. It will be interesting to determine whether the same mechanisms can play a role in other tumor models.

Finally, radiation has complex effects on the tumor microenvironment and vessels that have been shown to facilitate homing of both antigen presenting and effector T cells to the tumor [67, 82]. Homing may be facilitated by radiation-induced inflammatory signals, and by changes in

extracelluar matrix proteins, and in the expression of adhesion molecules by endothelial cells [83-86].

5. Pre-clinical studies of radiotherapy in combination with immunotherapy.

While clearly the potential is present for RT to generate anti-tumor immunity, the evidence that it does so in the clinical situation is lacking. However, strategies tested in recent pre-clinical studies have shown some promise, by combining radiation with some of the approaches recently developed in the immunotherapy field [87].

a. Strategies based on cytokines.

Fifteen years ago Cameron et al. [88] showed that local tumor irradiation could be successfully combined with the T-cell growth factor IL-2 and/or adoptive transfer of tumor-infiltrating lymphocytes (TIL) to obtain a synergistic anti-tumor effect. Unfortunately, the toxicity of systemic IL-2 administration has limited its clinical application. Since then, many other cytokines have been characterized and shown to induce powerful anti-tumor effects [87]. Among them, IL-3 and IL-12 have been tested in combination with radiation.

IL-3 can expand hematopoietic precursors and enhance antigen presentation by DC [89]. The intratumoral expression of IL-3 in a mouse fibrosarcoma and prostate carcinoma increased the tumor response to radiation by eliciting anti-tumor immunity [90, 91]. Irradiation was also shown to increase the response of non-transduced tumors to anti-tumor immunity elicited by systemic vaccination with IL-3 gene transduced tumor cells [92]. This supports the hypothesis that local radiation enhances the susceptibility of solid tumors to immune-mediated destruction, perhaps by facilitating the penetration and/or function of DC and effector T cells.

IL-12 is secreted by activated DC and is required for the development of effective anti-tumor T cell-mediated immunity [93]. The combination of IL-12 and local radiation was tested in a poorly immunogenic mouse fibrosarcoma model [94]. Intratumoral delivery of an adenoviral vector encoding IL-12 combined with fractionated RT improved both, local and systemic tumor control via local anti-angiogenic effects of IL-12, and IL-12 elicited anti-tumor immune responses, respectively. A similar approach was used to induce the expression of IL-12 and the costimulatory molecule CD80 in the poorly immunogenic 4T1 mammary carcinoma and B16 melanoma models[95]. In both tumors the growth delay was significantly better when RT and adenoviral-mediated gene transduction were used in combination. The therapeutic effect was mediated by T and NK cells, in addition to other detected effects, such as inhibition of angiogenesis.

b. Strategies based on dendritic cells.

Fms-like tyrosine kinase receptor 3 ligand (Flt3-L), is a growth factor that stimulates production of DC and has been shown to induce anti-tumor immunity to several mouse tumors, although its effects as a single agent are limited to early and more immunogenic tumors [96, 97]. The first study to test the combination of Flt3-L with local RT employed the Lewis lung model of metastatic carcinoma [66]. When Flt3-L was administered following the ablation of the primary tumor by high dose (60 Gy) local RT, lung metastases were inhibited and disease-free survival enhanced compared to that of mice treated with RT or Flt3-L alone. Importantly, the anti-metastatic effect required T cells, since it was not observed in *nude* (T cell deficient) mice. These results provide preliminary evidence in support to the hypothesis that RT-induced tumor cell death can release antigens for DC to present to T cells. The high single dose of radiation used in this study limits its clinical applicability, in addition to the fact that the intrinsic tumor

immunogenicity could explain these responses. Nevertheless, these studies provide initial proof of principle, and stimulated our group to further investigate whether more clinically relevant radiation doses could be used to elicit systemic anti-tumor immunity in combination with Flt3-L. We used the mouse mammary carcinoma 67NR, a moderately immunogenic syngeneic tumor. A radiation dose sufficient to cause growth delay of the irradiated tumor, in this case 2Gy, was able to induce a systemic anti-tumor effect only in combination with Flt3-L administration. Inhibition of tumor growth outside of the field of radiation was specific and required T cells, confirming that it was immune-mediated [68].

Other groups have used a slightly different approach based somehow on the same hypothesis, that RT can free tumor-derived antigens for DC uptake and presentation. Nikitina *et al.*, [67] used *in vitro* bone marrow-derived DC to be injected i.v. and s.c. around the tumor following local irradiation, whereas Teitz-Tennenbaum *et al.* [98] used intratumoral injection of DC. In both cases, the administration of DC after RT was able to induce a potent anti-tumor immune response.

c. Radiation and antibody-mediated blockade of CTLA-4.

As mentioned above, one of the main obstacles to the success of immunotherapy is the fact that the immune system is tolerant to antigens on growing tumors. Therefore, strategies aimed at breaking this tolerance have become a main focus of tumor immunotherapy [99]. Monoclonal antibody-mediated blockade of the CTLA-4 molecule on T cells was shown to be sufficient to elicit effective anti-tumor immunity to relatively immunogenic tumors by facilitating tumor-specific T cell activation [62]. For poorly immunogenic tumors, CTLA-4 blockade was effective if used in combination with vaccination with irradiated tumor cells modified to produce GM-CSF [100-102]. In a pre-clinical model of metastatic breast cancer, the mouse 4T1 adenocarcinoma, we

tested the combination of local RT and CTLA-4 blockade. Similarly to other poorly immunogenic tumors, 4T1 primary tumor growth and metastatic spread were not affected by CTLA-4 blockade alone. However, radiation in combination with CTLA-4 blockade was able to induce a CD8+ T cell mediated anti-tumor response capable of inhibiting the metastases outside the field of radiation and extending the survival of the mice [103]. These results indicate that, at least in some cases, radiation directed to the primary tumor can increase *in situ* the tumor immunogenicity and potentially become an alternative to vaccination with irradiated tumor cells. The relative simplicity and low cost of this approach make it an attractive candidate for translation into the clinic.

6. Future directions.

Traditionally considered an immune-suppressive treatment modality, ionizing radiation has started to reveal its potential to enhance immunity. However, the role of radiation as an independent immune-enhancer remains under investigation. While encouraging preclinical data are emerging it is still difficult to translate them to the clinic. Several reasons justify this delay. First of all, the exact mechanisms for this new application of RT remain quite elusive. They are likely to be context-dependent and relative to the degree of tumor immunogenicity. Literally, no clinical data are available to indicate what is the optimal radiation dose/technique and fractionation to optimize its application as a form of immunotherapy. In fact, scant data are available with regards to the specific radiosensitivity of the different cellular components of the immune system. Original studies describing the metachronous effect of RT on circulating immune cells are available, but desperately need to be revisited and updated to reflect the

enormous progress made by immunology. Specifically, modern accurate monitoring of changes in the circulating immune cells during standard fractionated radiotherapy are warranted.

Conversely, the rapid parallel growth of tumor vaccine strategies makes it compelling to explore a renewed partnership, especially in tumor settings where chemotherapy and radiation have failed and immunotherapy is showing promising initial results [104, 105]. Therefore, although it is premature to use RT as an immunomodulator outside of its scope as a cytocidal agent, the combination of radiotherapy with some immunomodulators shown to be effective in pre-clinical studies could be tested in the clinic. For instance, clinical trials assessing the efficacy of CTLA-4 blockade in combination with some types of vaccination are ongoing and have shown some promise [106, 107]. The ability of local RT and CTLA-4 blockade to activate anti-tumor T cells could be explored in the setting of metastatic disease, and both local and systemic (outside of the field of radiation) responses could be monitored. In such setting it would be possible to gather preliminary evidence as to whether RT used to control tumor growth also works as an "in situ vaccination".

In conclusion, more investigations about a potentially novel application of ionizing radiation as a component of immunotherapy are warranted. If clinical efficacy is demonstrated it could open a completely new approach to cancer management, with the advantage of using an established modality and treatment equipment commonly available in the community.

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FIGURE LEGENDS.

Figure 1. A model for the role of ionizing radiation in promoting cross-presentation of TAA and activation of anti-tumor T cells. It is well-established that dendritic cells (DC) can efficiently uptake tumor associated antigens (TAA) from apoptotic and necrotic tumor cells and present them to both CD4+ and CD8+ cytolytic T cells (CTL), a process termed cross-presentation. By killing tumor cells ionizing radiation can promote this process. In the presence of adequate "danger signals" that induce DC maturation and up-regulation of co-stimulatory molecules CD80 and CD86, tumor-specific T cells are activated to produce pro-inflammatory cytokines and become effectors capable of killing the tumor cells. Recognition and killing of tumor cells by CTL may be further enhanced by the radiation-induced up-regulation of Fas and/or MHC I molecules on the tumor cells.

Table 1. Examples of human tumor-associated antigens recognized by T cells*.

Category	Gene‡	Tumor expression
Cancer Testis	BAGE	Melanoma, myeloma, lung, bladder and breast carcinoma
	GAGE-1	Melanoma, myeloma, lung, bladder, prostate and breast carcinoma, esophageal and head/neck SCC, sarcoma
	MAGE-A1	Melanoma, myeloma, lung, bladder, prostate, colorectal and breast carcinoma, esophageal and head/neck SCC, sarcoma
	NY-ESO-1	Melanoma, myeloma, lung, bladder, prostate, and breast carcinoma, esophageal and head/neck SCC, sarcoma
Differentiation	Gp100	Melanoma
	Melan-A/MART-1	Melanoma
	Prostate-specific antigen	Prostate carcinoma
	Mammoglobin-A	Breast carcinoma
Overexpressed	Alpha-fetoprotein	Hepatocellular carcinoma and yolk-sac tumors.
	HER-2/neu	Melanoma, ovarian, gastric, pancreatic and breast carcinoma
	P53	Esophageal, gastric, colon, pancreatic, and other carcinomas
Mutated (shared) ‡	K-ras	Pancreatic and colorectal adenocarcinomas
	TRP-2/INT2	Melanoma, high grade gliomas

*This table lists only some examples of the more common tumor antigens identified. For references about individual antigens listed and for a comprehensive review see Novellino et al.[108].

‡Mutated antigens are tumor-specific. However, few mutations common to more than one patient and sometimes more than one tumor type have been identified. These mutations are usually crucial in the process of neoplastic transformation.
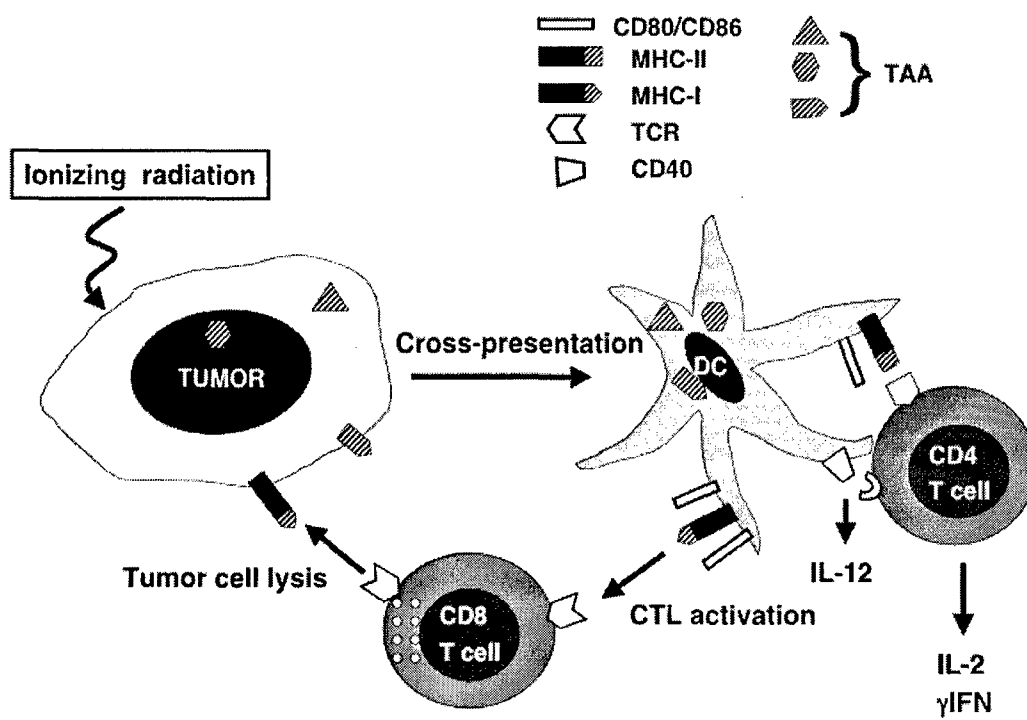
FIGURE 1.

Fig.6

A: CONTROL — Caspase activity (x-axis) vs Target cells (y-axis); Q1:76.07%, Q2:6.61%, Q3:16.47%, Q4:0.84%

B: RT — Caspase activity vs Target cells; Q1:63.24% 2415/ml, Q2:19.31% 560/ml, Q3:13.80% 605/ml, Q4:3.64% 135/ml

C: IL-3 — Caspase activity vs Target cells; Q1:56.83%, Q2:25.86%, Q3:12.29%, Q4:5.03%

D: RT + IL-3 — Caspase activity vs Target cells; Q1:3.68%, Q2:78.56%, Q3:1.04%, Q4:16.72%

E: RT + βgal — Caspase activity vs Target cells; Q1:63.67%, Q2:8.73%, Q3:14.53%, Q4:3.07%

F: Specific control — Caspase activity vs Target cells; Gate: G1=Q1 OR Q2; Q1:96.55%, Q2:3.45%, Q3:0.00%, Q4:0.00%