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PRINCIPAL INVESTIGATOR: William H. McBride, Ph.D.

CONTRACTING ORGANIZATION: University of California
Los Angeles, California 90024

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14. ABSTRACT This effort is to determine if radiation affects presentation of prostate specific antigen (PSA) through endogenous and exogenous pathways by dendritic cells (DCs) and to devise novel strategies to translate radiation-induced cell death into the generation of tumor-specific immunity. The goal is to improve the therapeutic outcome from radiation therapy. Radiotherapy is normally thought of as being immune suppressive because it kills radiosensitive lymphocytes. Our hypothesis, in contrast, is that it also affects immune cell function and this has profound effects on the immune system and the development of anti-tumor immunity. We chose PSA as antigen for this study. However, because of the high risk nature of the experiments and the high PSA expression levels in prostate cancer patients that might interfere with its efficacy, we have also develop a back-up system using survivin as an antigen, since it also is overexpressed in prostate cancer. One aim of this proposal is to devise strategies to avoid radiation-induced immunosuppression and to translate radiation tumor cytotoxicity into beneficial tumor immunity with combination treatments of IL-3 and/or GM-CSF. Our studies on combined treatments of radiotherapy and IL-3, and on the effects of radiation on PSA presentation by DCs are presented, along with other milestones that have been attained.						
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Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	9
References.....	n/a
Appendices.....	10

Introduction

Ionizing radiation is generally considered to be immunosuppressive. However, not many studies have examined tumor antigen-specific responses following radiation therapy. Recently, we discovered a novel mechanism of radiation-induced immunosuppression based on induced dysfunction of antigen processing by dendritic cells (DCs). We proposed that this radiation-induced effect on DC is due to proteasome inhibition and/or the expression of immunosuppressive cytokines and related molecules by DCs. To overcome this radiation-induced immunosuppression, we plan to investigate the combined effects of radiation and cytokines such as IL-3 and/or GM-CSF. The final goal in this study is to translate radiation-induced tumor cell death into generation of tumor immunity in the hope of optimizing therapy for localized and disseminated prostate cancer. The aims are unaltered from the original submission.

Body

The statement of work for this year covers experiments aimed at studying the effects of radiation with PSA delivered to the endogenous antigen processing pathway of DCs by adenoviral vectors and to the exogenous pathway by pulsing DCs with PSA peptides. In addition, we have examined the impact of IL-3 on DC function. We have developed a treatment regimen with AdV-IL-3 combined with radiation therapy to examine this, as well as directly treating DCs. We have also developed a backup model using survivin as an antigen because of the high risk of focusing on one tumor antigen.

The model for our studies was based on our findings of the effects of radiation on processing of the MART-1 human melanoma antigen. However, there was no reason to believe that these findings would apply to other tumor antigens, in particular to prostate tumor antigens and we had no mechanistic insights into why irradiation was having its effects. To explore in more depths the effects of radiation on DCs, we examined if irradiated DCs were worse or better at inducing a mixed leukocyte reaction (MLR). In this assay, irradiated DCs are used to stimulate allogeneic splenocytes in vitro. As can be seen in Fig. 1, irradiated bone-marrow-derived DCs (BMDCs) grown by standard procedures are inferior stimulators in MLR in comparison to non-irradiated BMDCs. Flow cytometric data on surface expression of DC markers that might be responsible for the loss in activity gave no clue that loss of markers was responsible, and cell death did not seem to be involved. DCs do not proliferate when mature and therefore do not die following radiation exposure and cell death assays showed no marked loss of viability post radiation.

These studies supported the idea that we were examining a general phenomenon that might apply in prostate cancer. To test this, we studied the effect of radiation on processing of human PSA delivered to the endogenous pathway of DCs by adenoviral vectors (AdV-PSA) after 10 Gy radiation. Ten to 14 days after DC injection, spleens were harvested and the expression of IFN-gamma and IL-4 was measured by ELISPOT assays. As can be seen in Fig. 2, radiation slightly decreased the number of lymphocytes expressing IFN-gamma and IL-4. To study the effect on the exogenous pathway DCs humanized that express HLA-A2.1 were used since the epitopes expressed by purely murine DCs are not known. We used the human PSA-3 peptide that is known to be immunodominant in the context of HLA-A2.1 to pulse these cells and after irradiation,

injected them into mice. The number of IFN-gamma and IL-4 producing lymphocytes increased significantly in 10 Gy treated peptide-pulsed DC (Fig. 3). The data suggests that radiation suppresses DC to process endogenous PSA but enhances exogenous PSA-peptide. This is similar to what we have seen in MART-1 studies, however, the degree of inhibition of PSA processed endogenously is not as great. We think that this might be due to the difference in DC maturation status and characteristics of the PSA antigen. We will further investigate if this inhibition could be overcome by altering the maturation status by modifying the cytokine treatment and if the proteasome composition and/or function are affected.

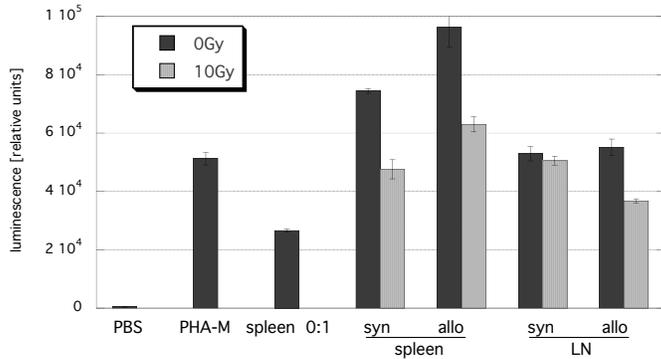


Fig.1 . Bone-marrow derived DCs were irradiated with 10Gy or left untreated and mixed with responders (splenocytes or lymphocytes) at a stimulator:responder ratio of 1:2. All responders were derived from C57Bl/6 mice. Non-stimulated splenocytes (spleen 0:1) were left untreated. PHA-M-stimulated splenocytes served as positive control. Cell viability was assessed in each sample after 3 days according to the overall ATP activity with the ATPlite assay.

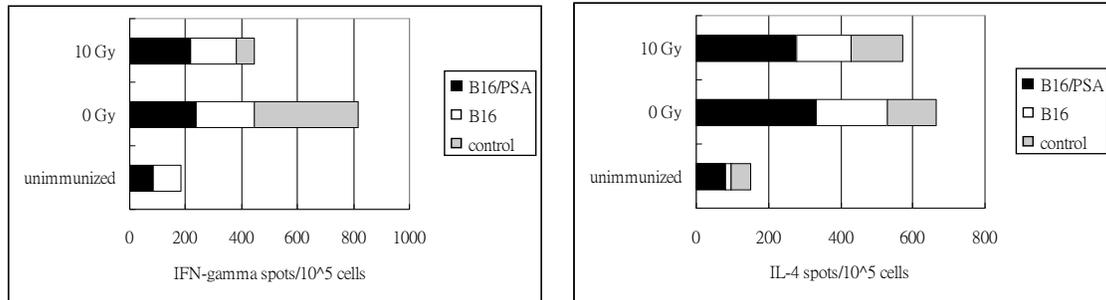


Fig. 2. Radiation effects on endogenous antigen processing by DCs. C57BL/6 mice were injected with DCs treated with or without 10 Gy and transduced with AdvPSA. Splens were harvested 10-14 days after DC immunization and restimulated with either B16 or B16/PSA or no stimulation. The production of IFN-gamma and IL-4 were assessed by ELISPOT.

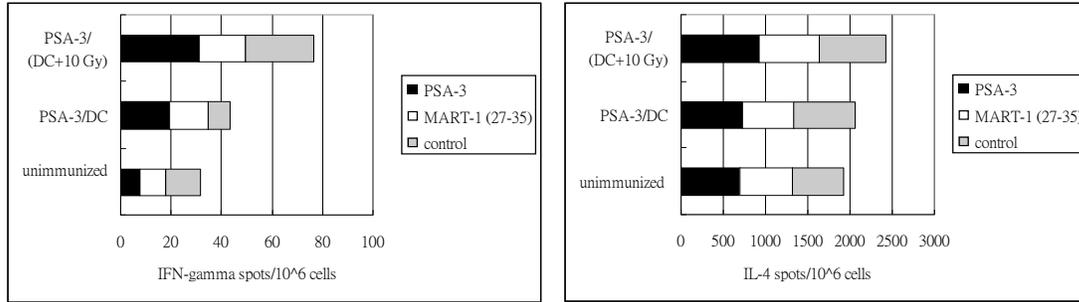


Fig. 3. Radiation effects on endogenous antigen processing by DCs. HLA-A2.1 mice were injected with DCs treated with or without 10 Gy and pulsed with PSA-3 peptide. Spleens were harvested 10-14 days after DC immunization and restimulated with either PSA-3 or non-specific peptide MART-1 (27-35) or no stimulation. The production of IFN-gamma and IL-4 were assessed by ELISPOT.

We have extensively sought the reason for the radiation-induced functional alterations in DCs. No significant changes of MHC class I, class II and co-stimulatory molecule expression were seen in irradiated DCs. We tested whether MHC class I stability is altered post irradiation. T2 cells are TAP-deficient human lymphoblast cells with empty MHC class I complexes on their surface that are inherently unstable. Pulsing these cells with peptides stabilizes MHC class I. Irradiation of 2 Gy and 10 Gy appears to further increase the levels of stable MHC I complexes over a 24 h period suggesting a stabilization effect by radiation (Fig. 4). This indicates that the radiation-induced changes are membrane associated but since the mechanism of radiation-induced stability of MHC is unknown, exploratory studies are needed to probe further.

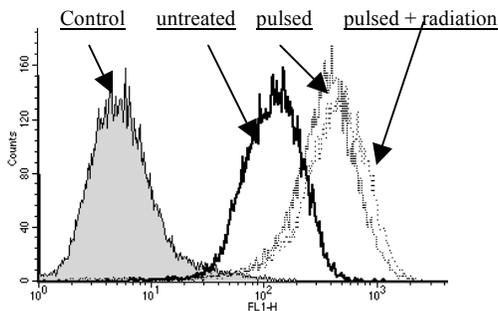


Fig. 4. Flow cytometric analysis of MHC class I on T2 cells. T2 cells were treated with 2 Gy and 10 Gy and tested for MHC class I expression 24 h after treatment. It seems that radiation increases the levels of stable MHC I complexes over a 24h period, an effect that is abolished in the presence of the Golgi inhibitor brefeldin A.

We have extensive data to show that IL-3 can alter tumor immunogenicity and we hypothesized that it might be able to overcome the radiation-induced immunosuppression mediated by effects on DCs. We have examined the effect of radiation on co-stimulatory molecules on IL-3-transduced DC (see last report) and the expression of MHC class II and CD86 was up-regulated after 10 Gy irradiation. Here we further tested the ability of AdvIL-3 transduced DC with or without radiation to protect mice from tumor challenge. We initially set up a pilot experiment in a MART model because the extent of immune suppression is greater, and we have to use a PSA-transfected tumor to perform the same experiments with PSA. We have now generated this tumor model and we will perform

the experiment in this year. However, as can be seen in Fig. 5, the survival rate of mice immunized with AdvIL-3/DC was higher than DC injected mice and the one with irradiated DC plus IL-3 performed better than the one without IL-3. The results suggest that the combined treatment of IL-3 and DC showed better therapeutic effect. Note, we did not observed much radiation-induced suppression in this experiment, probably because DCs were irradiated one day prior to AdVMART1 transduction, which may be too long and this will be limited to hours in the PSA experiment.

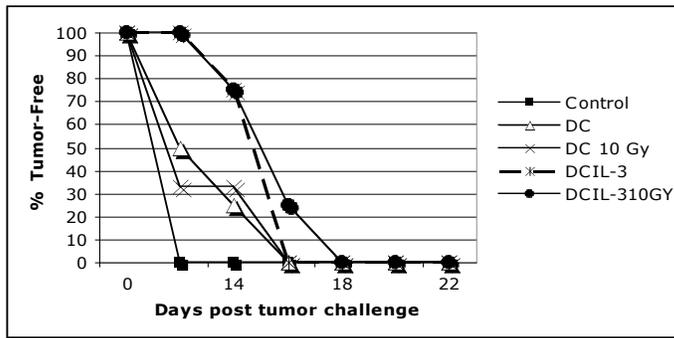


Fig. 5. Combined treatment of IL-3 and DC. Dendritic cells were transduced with AdvIL-3 (day 5), irradiated with 10 Gy (day 8), transduced with AdVMART1 (day 9) and harvested for injection (day 9). One week after immunization, C57BL/6 mice were challenged with B16 melanoma.

A. TNFR I

B. TNFR II

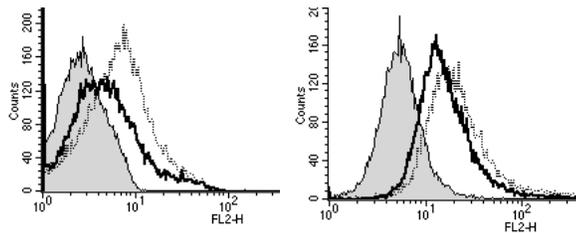


Fig. 6. Flow cytometric analysis of DC2.4 surface molecules 24 h after 10 Gy radiation. A. TNFR I B. TNFR II. Gray – unstained; Black - 0Gy; Dotted- 10Gy (24h)

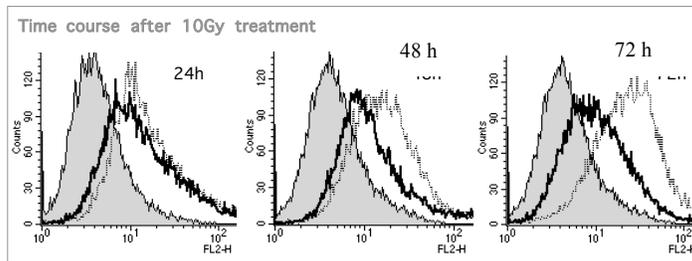


Fig. 7. Increase of surface TNFR I late after irradiation . DC2.4 (DC cell line) were irradiated with 10Gy and stained for surface TNFR I at 1day, 2 days and 3 days later. Lines: Gray: unstained control; Black line: stained control; Dotted line: irradiated DC.

Another hypothesis we have entertained is that radiation-induced cytokines or receptors might mediate this form of radiation-induced immune suppression. In previous study, 31 cytokines and receptors were tested in the media from irradiated and non-irradiated DCs. None of the immunosuppressive cytokines, IL-10 and TGF- β , was enhanced after 10 Gy irradiation. On the other hand, DCs released high levels of TNF receptor I and II (TNFR I and TNFR II) following irradiation. Although shedding of TNFR from the cell surface occurs constitutively, this is also induced during immune responses. It limits innate immune responses and may also be important for Th1 polarization. Using a DC cell line, we have shown that these receptors are up-regulated

following irradiation (Fig. 6 and Fig. 7). We have further tested the radiation effect on DC function using TNFR I and TNFR II knock-out mice by ELISPOT. Our initial data showed that loss of TNFR I seemed to confer superior antigen presenting function after radiation treatment compared to wild-type DCs, but as these data are still preliminary, they have to be repeated. However, currently, we postulate that radiation-induced TNFR I probably acts as a “brake” on immunity.

Because of the high risk of the proposed experiment and high background of natural PSA level in prostate cancer patient as mentioned in the last report, we chose to investigate an potential back-up antigen – survivin which is overexpressed in many prostate cancer patients. We continued to work with other labs in Europe to screen for survivin in patients treated with radiotherapy using tetramer assays. We also initiated developing a system with adenovirus expressing survivin (AdVsurvivin) as can be seen in Fig. 8. A survivin-specific T cell population could be detected in C57BL/6 mice. Although it is not part of our aims of the grant, these experiments will help guide those in this proposal by allowing us to look at responses to two antigens (PSA and survivin), within the one system and to see whether both are modulated in the same way simultaneously in the same DC cell population.

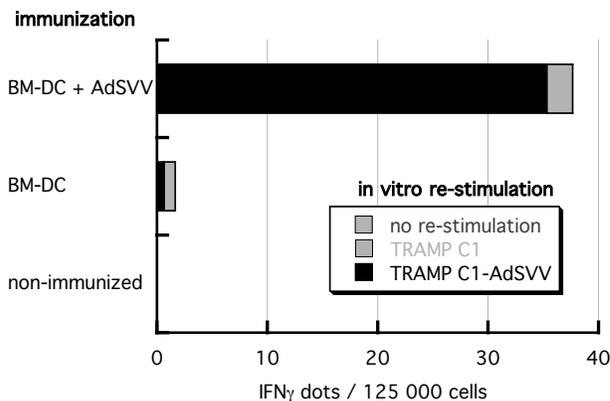


Fig. 8 . Survivin-specific T cell responses in mice. C57BL/6 mice were immunized with 5×10^5 bone marrow-derived DC that had been transduced or not with AdV-survivin. Spleens were harvested 7 days later and survivin-specific T cells enumerated by ELISPOT (IFN γ production) after in vitro stimulation with either TRAMP C1 cells or AdV-survivin transduced TRAMP C1.

Key Research Accomplishments

1. Study of the effects of IL-3 combined with radiation on DC function- 70% completed.
2. Development of a survivin system- 90% completed.
3. Study of the effects of radiation on endogenous processing of PSA by DC- 90% completed.
4. Study of the effects of radiation on PSA peptide presentation by DC- 90% completed.
5. Study of the effects of radiation and IL-3 and/or GM-CSF on PSA by prostate tumor cells- 40% completed.

Reportable Outcomes – Manuscripts and Abstracts

Liao, Y., C-C Wang, L.H. Butterfield, J.S. Economou, A. Ribas, W.S. Meng, K. S. Iwamoto and W.H. McBride: Local irradiation of murine melanoma affects the development of tumor-specific immunity. Submitted, 2007.

Tsai, C-H., J-H. Hong, K-F. Hsieh, H-W. Hsiao, W-L Chuang, C-C. Lee, W.H. McBride and C-S. Chiang: Tetracycline-regulated intratumoral expression of interleukin-3 enhances the efficacy of radiation therapy for murine prostate cancer. Cancer Gene Therapy 13:1082-1092, 2006.

Kachikwu, E, D. Schaeue, Y. Liao, and W. McBride. Effect of IL-3 on radiation-induced changes in maturation of dendritic cells. In: Abstracts of Papers for the 53rd Annual Meeting of the Radiation Research Society, Philadelphia, PA, 2006.

Reportable Outcomes – Presentations

Liao, Y and W. H. McBride. “A Novel Mechanism of Radiation-Induced Immune Suppression,” presented at the 53rd Annual Meeting of the Radiation Research Society, Philadelphia, PA, 2006.

McBride, William H. Invited Lecture: “Sense of Danger from Radiation,” Department of Radiation Oncology, New York University, NY, April 2006.

McBride, William H. Invited Speaker: “A Sense of Danger from Ionizing Radiation,” International Workshop on Non-Targeted and Non-Linear Effects of Ionizing Radiation, Edinburgh, Scotland, August 2006.

McBride, William H. Invited Lecture: “Effects of Radiation on the Immune System,” Department of Radiation Oncology, New York University, NY, September 2006.

Conclusions

This year has been spent continuing to investigate the mechanisms of radiation effects on DC function through the roles of processing PSA either endogenously or exogenously. These are novel findings and we are the only ones involved in these studies. We have initiated the study of in the animal model to examine the effects of IL-3 on DC in the hope of reversing radiation-induced immunosuppression. Although the preliminary results with DCs were not too impressive, based on many other experiments we have performed, we believe that utilizing IL-3 combined with radiation will be an effective strategy and that the effects on DCs will prove to be the key for this combination to work. We have had no changes in the directions of the proposed work in the grant, although we have set up a new system using survivin as a tumor antigen. This will allow us simultaneously to explore antigen presentation to 2 distinct antigens within the one system. It also will give insights into which would be the better for clinical translation. Doubts have been raised about PSA because of the high natural level of PSA in prostate

cancer patients. We have exerted a lot of effort in studying the radiation effects on antigen processing at the mechanistic level and have ended in a number of blind alleys. However, we now have all the tools in place to bring these studies to fruition in the next year.

References: n/a.

Appendices: 1 submitted manuscript, 1 published manuscript.

**Local irradiation of murine melanoma affects the development of tumor-specific
immunity**

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

*Department of Radiation Oncology, and ‡Department of Surgery, Division of Surgical Oncology, UCLA School of Medicine, Los Angeles, CA 90095; †Department of Medicine, University of Pittsburgh, UPCI, Pittsburgh, PA 15213; §Department of Pharmaceutical Sciences, Duquesne University, Pittsburgh, PA 15282; ¶Department of Radiation Oncology, Chang Gung Memorial Hospital, Taipei, Taiwan

Running title: Local irradiation affects the development of tumor-specific immunity

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Address correspondence to Dr. William H. McBride, Department of Radiation Oncology, UCLA David Geffen School of Medicine, B3-109 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90095-1714. Telephone: (310) 794-7051, FAX: (310) 206-1260, E-mail: wmcbride@mednet.ucla.edu

Abstract

Radiation therapy (RT) affects the immune system. In addition to killing radiosensitive immune cells, RT can induce functional changes in those that survive. Our recent studies showed that exposure of dendritic cells (DCs) to radiation *in vitro* influences their ability to present tumor antigen *in vivo*. Here we show that local RT of B16 melanoma tumors inhibits the development of systemic immunity to the tumor antigen MART-1. This inhibition could not be overcome by intratumoral injection of DCs transduced with adenovirus expressing human MART-1 (AdVMART1/DC) after RT, suggesting that tolerance might have developed. On the other hand, injection of AdVMART1/DC prior to irradiation could prevent radiation-induced inhibition from developing. These results suggest that local RT may block the generation of immunity to some tumors, possibly through induction of immune tolerance and that additional steps may be needed to prevent this and allow radiation-induced cell death to translate into the development of systemic immunity.

Introduction

Radiation therapy (RT) has been a primary cancer treatment for almost a century. It is widely accepted that even local RT alters the balance of circulating immune cells and this is often ascribed to the depletion of radiosensitive subsets of cells (1-3). However, recently attention has focused on radiation-induced functional changes in immune cells and on approaches to use radiation as an immunological adjuvant to enhance tumor control. Radiation is more than simply a silent killer of immune cells. It induces expression of a myriad of cytokines (e.g. TNF- α , IL-1 α , IL-1 β , IL-6, IL-8 and IL-10 (4-8)), up-regulates the expression of MHC class I (9) and the co-stimulatory molecules CD80 (10, 11) and CD86 (12), modulates the function of dendritic cells (DCs) to inhibit endogenous antigen processing while enhancing cross-presentation (13) .

Indeed, recent studies have revealed the value that might accrue from combining RT and immunotherapy in cancer treatment. In experimental models, DC-based vaccination against tumor-associated antigens appears to be superior when given with local tumor irradiation. (14-17). Boosting tumor immunity with gene therapy approaches showed a similar benefit from combination with RT (18-20). However, whether local RT *per se* promotes or inhibits systemic tumor immunity is less clear, although understanding this may be critical for the development of optimal strategies aimed at combining these two therapies.

In this study, we used a B16 murine melanoma tumor model to investigate the effect of local irradiation on systemic immunity. The B16 tumor expresses the murine homolog of the human MART-1 melanocyte lineage-specific tumor antigen (21, 22), which we used as a target antigen for monitoring responses. Local irradiation suppressed the weak

immune response that is normally generated in mice bearing B16 tumors and rendered these mice unable to respond to a subsequent injection of intratumoral MART-1 gene-modified DC (AdVMART1/DC). This suggests that local RT may on occasion not only decrease systemic tumor immunity but also block its generation in response to immunotherapy. Vaccination prior to tumor irradiation however could prevent radiation-induced inhibition, indicating that the timing of immunotherapy with respect to RT may be critical for a favorable outcome.

Materials and Methods

Mice and cell lines

Six-to 8-wk-old C57BL/6 mice were bred and maintained in the defined-flora AALAC-accredited Animal Facility of the Department of Radiation Oncology, UCLA. All experiment protocols were approved by the IACUC and the care of animals followed local and national guidelines. The C57BL/6 B16 melanoma and EL4 lymphoma cell lines were obtained from the ATCC (Manassas, VA) and maintained in DMEM medium (Mediatech, Herndon, VA) with 10% FBS (Sigma, St. Louis, MO) and 1% antibiotics-antimycotic solution (Mediatech, Herndon, VA). The EL4(MART-1) cell line, an EL4 transfectant carrying the MART-1 cDNA and neomycin resistance gene, was generated as described previously (23) and maintained in complete RPMI 1640 medium (Mediatech, Herndon, VA) with 0.5 mg/ml G418. 293 human embryonic renal cells (Qbiogene Inc., Carlsbad, CA) were used for amplification of adenoviral seed stocks.

Tumors were generated *in vivo* from 5×10^5 B16 tumor cells injected s.c. into the right thigh of mice. When the tumor size reached approximately 5-6 mm in diameter, tumors were treated with 0 or 10 Gy irradiation.

Irradiation

Mice were anesthetized with an i.p. injection of ketamine/xylazine (80 mg/4 mg/kg of mouse body weight; pentobarbital) and positioned in a Lucite jig with lead shielding the body and only the leg bearing tumor exposed for radiation treatment in the Gammacell 40 irradiator (Cs-137 source; Atomic Energy of Canada, Ltd., Ottawa, Canada) at a dose rate of approximately 67 cGy/min. Irradiation of cells was performed using the MARK-1-30

irradiator (Cs-137 source, J.L. Shepherd & Associates, San Fernando, CA) at a dose rate of 4.5 Gy/min. ELISPOT was used to measure MART-specific responses one week after radiation treatment.

Generation of bone-marrow-derived DCs

Murine DCs were generated from bone marrow cells as described (13, 23). Briefly, bone marrow from femurs was cultured overnight in RPMI 1640 containing 2% FBS and 1% antibiotics. Non-adherent cells were taken and resuspended in complete RPMI 1640 medium supplemented with 2 ng/ml murine GM-CSF and 10 ng/ml murine IL-4 (Biosource, Camarillo, CA) at $1-2 \times 10^6$ cells/ml. After 3-days of culture with cytokines, 80-90% of the medium was replaced with fresh medium containing GM-CSF and IL-4. Loosely adherent cells were harvested and used for experiments after 8 days in culture.

Adenovirus transduction of DCs

The E1-deleted replication defective adenoviral vector containing human MART-1 (AdVMART1) (23) was amplified in 293 cells, and purified by centrifugation for 2 h at 24,000 rpm in a 30%-60% sucrose gradient. The virus titer was determined using the tissue infectious dose 50 (TCID₅₀) methods as described in Quantum (Qbiogene, Inc.). Dendritic cells, irradiated (10 Gy) or unirradiated, were transduced with AdVMART1 at an M.O.I. of 100, washed and injected (5×10^5 cells) s.c. into the top of the inner leg of each mouse in a total volume of 100 μ l of PBS (13). In some experiments, mice were reimmunized ten days after the first injection. Splenocytes were harvested 7-14 days after immunization and ELISPOT was used to measure MART-specific responses.

ELISPOT assay

The expression of IFN- γ and IL-4 by individual lymphocytes was used to assess MART-1 specific immune responses, in an ELISPOT assay (13). Briefly, splenocytes were harvested on the indicated day after immunization, depleted of red blood cells in ammonium chloride buffered (ACK) solution (0.83% (w/v) NH_4Cl , 0.14% (w/v) KHCO_3 , 0.002% Na_2EDTA , pH 7.3) and restimulated with heavily irradiated (50 Gy) EL4 or EL4(MART-1) cells in the presence of 10 U/ml human IL-2 at 37°C for 48 h. Restimulated cells were added to anti-IFN- γ or anti-IL-4 antibody-coated MultiScreen-HA plates (Millipore, Bedford, MA) for a further 24-h period. Released cytokines were then detected by adding biotinylated anti-IFN- γ or anti-IL-4 antibody and horseradish peroxidase avidin D (1:2000 dilution; Vector Laboratories, Burlingame, CA). Red spots were developed by adding 0.4 mg/ml 3-amino-9-ethyl-carbazole (AEC tablets; Sigma, St. Louis, MO) in 0.05 M sodium acetate buffer (pH 5.0) and 0.012% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA). Spots were counted using an ImmunoSpot Image Analyzer (Cellular Technology Ltd., Cleveland, OH) at the Jonsson Comprehensive Cancer Center Core Facility, UCLA.

Results and Discussion

B16 tumors growing in C57BL/6 mice were irradiated when 5-6 mm in diameter with 10 Gy and MART-1-specific responses in the spleen were assessed 7 days later by ELISPOT assay. In repeated experiments, spleens from untreated tumor-bearing mice had reproducible, though relatively small, increases in the number of MART-specific IFN- γ -secreting cells, indicating that the tumor was weakly immunogenic (Fig 1). Interestingly, a non-curative dose of 10 Gy RT delivered to the tumor site abolished this splenic immune response, suggesting that systematic immunity was suppressed by local irradiation. This may not be the case for all tumors. For example, Lugade *et al.* (24) reported a different outcome. Local irradiation of ovalbumin gene-transfected B16 melanoma tumors increased the numbers of OVA-specific lymphocytes within the draining lymph nodes and tumors. Tsai *et al.* (20) showed a marginal increase in immunity after fractionated local RT of mouse prostate cancer TRAMP C1 tumors. Others have reported slightly decreased or unchanged immune responses after irradiation of C3 or MethA sarcoma (15). Whether radiation increases or decreases tumor-specific immune responses may depend upon the immunogenicity of the tumor or even the immunodominant epitope. Many animal tumor models are immunogenic and it has been known for decades that curative RT given to such tumors will result in systemic immunity that assists in achieving local and micrometastatic tumor control. However, most human tumors seem are weakly immunogenic. Our data suggests that under such circumstances RT might not often behave as a significantly powerful adjuvant to enhance the generation of biologically relevant levels of anti-tumor immunity.

Our model allowed us to investigate the potential mechanisms of RT-induced immunosuppression and whether these influence the generation of immunity to tumor vaccines, which has potentially important implications with respect to strategies aimed at combining immunotherapy with RT. Many types of immune suppressor cell populations have been described that could be activated by local RT, including regulatory/suppressor T cells with CD4⁺ (25, 26), CD8⁺ (27) and CD4⁻CD8⁻ (28) phenotypes, "null" or "natural" suppressor cells (29), as well as suppressive DC subsets (30, 31). On the other hand, whole body irradiation has been reported to eliminate radiosensitive CD8⁺ suppressor T cells and enhance rejection of immunogenic tumors (32). We have focused on the effect of RT on DCs. We demonstrated that *in vitro* irradiation of DCs can inhibit their ability to process MART-1 antigen through the endogenous pathway (13). This is reconfirmed in Figure 2. Dendritic cells were irradiated with 0 or 10 Gy prior to AdvMART infection, washed, and injected into mice. Two injections were given one week apart and spleens harvested for IFN- γ ELISPOT assays 10-14 days later. As shown in Figure 2, irradiation of DCs with 10 Gy prior to AdvMART1 transduction abolished their ability to generate MART-specific immune responses. This effect results from functional modification of the antigen processing pathway, rather than the cytotoxic effects of irradiation (13).

We then investigated if irradiated DCs are immunologically ineffective or actually switch off immunity to MART1 and/or induce tolerance. To test this, C57BL/6 mice were vaccinated with 5×10^5 irradiated (10 Gy) or non-irradiated DCs that had been transduced with AdvMART1. A second immunization was performed 10 days later with the same vaccine, and, in addition, mice treated with non-irradiated DCs received irradiated DCs

and *vice versa*. One week after the last immunization, MART-1 specific IFN- γ and IL-4 responses were assessed by ELISPOT assay (Fig. 3). Mice immunized with irradiated DCs showed the lowest IFN- γ (Fig. 3A) and IL-4 (Fig. 3B) responses. It did not matter whether irradiated DCs were given before or after non-irradiated DCs, responses were suppressed to the same extent, irrespective of the cytokine response that was measured. The findings suggests that irradiated AdvMART1/DCs are able to switch off MART-1-specific immunity and that both Th1 and Th2 T cell subset responses are affected. This confirms that loss of DC function is therefore an active process and not due to cell death. Tolerogenic DCs are normally associated with an immature phenotype and are known to play an important role in maintaining peripheral tolerance (33). As a result of insufficient co-stimulation, naïve T cells recognizing ligands on these immature DCs are deleted (34, 35). The concept that this is a two-way interaction was recently shown by Chang *et al.* (36) who observed that CD8⁺CD28⁻ T cells interfered with CD40-CD40L-mediated signaling and consequently prevented functional DC maturation. This scenario was also linked to inducible expression of inhibitory immunoglobulin-like transcript 3 (ILT3) and ILT4 on the DCs. Another mechanism by which specific tolerance can be induced by DCs is through capture of “self” antigens from dying cells (37). The mechanisms that confer immunosuppressive ability on DCs following radiation exposure seem somewhat different from the previous studies. So far, we have been unable to ascribe the inhibitory effect of radiation on DC function to a lack of expression of co-stimulatory molecules, despite the fact that phenotypic changes are evident post radiation exposure (13). Indeed, irradiation of DCs enhances their ability to present peptide antigen pulsed exogenously onto DCs (13) suggesting that antigen processing is the radiation target.

Radiation-induced inhibition of DC antigen processing may be part of a general mechanism that prevents the generation of autoimmunity following damage to self tissue. In a tumor setting, it seems plausible that locally irradiated DCs within the tumor microenvironment could switch off tumor immunity, which would have implications for the use of tumor vaccination in combination with RT. To test this, AdVMART1/DCs (5×10^5) were injected directly into B16 tumors and their ability to overcome the effects of 10 Gy given one day later was examined. Another group of tumors were irradiated (10 Gy) prior to administration of 5×10^5 intratumoral AdVMART1/DC. As controls, mice received either 10 Gy irradiation or intratumoral AdVMART1/DC injection alone.

As before, splenic responses that could be detected in control mice with growing B16 tumors (Fig. 4) were decreased by local tumor irradiation. Intratumoral injection of AdVMART1/DC generated high splenic T cell responses and this response was not influenced by RT given one day later. In contrast, local tumor RT one day prior to AdVMART1/DC injection blocked their immunostimulating effect. The number of MART-1 responsive lymphocytes in the spleens of mice receiving tumor irradiation prior to intratumoral AdVMART1/DC injection was as low as in the unimmunized mice. This suggests that tumor irradiation can block the response to vaccination whereas prior immunization may allow the immune system to resist the immunosuppressive effects of local RT. This may be because the intratumoral-injected DCs had already migrated to the lymph node and spleen by the time of irradiation, as has been shown by others (38, 39).

From a practical standpoint, our finding that AdVMART1/DC intratumoral vaccination was superior if given before RT and its effectiveness was abolished if given after RT may be important. Again, multiple mechanisms could be involved. The finding that non

antigen specific intratumoral DC administration, which has been clearly shown to enhance immune responses in several mouse models (14-16), had a better therapeutic outcome if the tumor was irradiated prior to intratumoral DC injection (15, 16) (39) might be a case in point. The outcome could also ultimately depend on properties of the tumor. Irradiation has multiple possible effects on the tumor microenvironment, such as up-regulating the expression of inflammatory mediators (e.g. COX2 and PGE2), heat shock proteins, immunomodulatory cytokines, adhesion molecules, co-stimulatory molecules, death receptors (e.g. Fas), and MHC class I molecules. For example, Chakraborty *et al.* (40) showed that local RT of colon adenocarcinoma combined with human carcinoembryonic antigen (CEA) vaccination was more effective because of radiation-induced up-regulation of Fas on tumor cells.

It is important to note that, in our model, *in vivo* intratumoral vaccination with AdvMART1/DC even one day prior to tumor irradiation resulted in resistance to radiation-induced immunosuppression, which gives hope that combining RT and immunotherapy with the aim of increasing control of local tumors and distant micrometastases is feasible even if RT is immunosuppressive and worth pursuing. However, if the combination is to be effective, provision of more effective "danger" signals than is provided by radiation alone may be required (41-43) and that the timing of immunization relative to irradiation may be critical. Finally, profiling the tumor-host relationship to identify tumor subsets that might respond differently to RT in terms of its effects on systemic immunity may be illuminating and instructive.

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Footnotes

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2. Address correspondence and reprint requests to Dr. William H. McBride, Department of Radiation Oncology, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095-1714. E-mail: wmcbride@mednet.ucla.edu
3. Abbreviations used in this paper: DC, dendritic cell; AdV, adenovirus; AdVMART1, adenovirus encoding MART-1 cDNA; AdVMART1/DC, AdVMART1 transduced DC; M.O.I., multiplicity of infection

Figure Legends

Fig. 1. Radiation suppresses B16 tumor immunity. C57BL/6 mice were implanted with viable B16 cells and irradiated with (B16 tumor + 10 Gy) or without 10 Gy (B16 tumor) when the tumor size reached approximately 5-6 mm (diameter). Mice without tumors and treatment were used as control. One week after the treatment, IFN- γ producing lymphocytes were detected by ELISPOT after 48-h restimulation with EL4(MART-1) (black), EL4 (white) or without restimulation (control; gray). Three mice were used in each group of different treatment. Results shown are the mean \pm 1SEM of triplicate data from one representative of three experiments.

Fig. 2. Ionizing radiation of DC inhibits generation of MART-1 specific immunity. Vaccination of C57BL/6 mice was performed twice, one week apart, with DCs treated with 0 or 10 Gy irradiation DC prior to AdvMART1 transduction or left unimmunized. IFN- γ expression by splenocytes was assessed 10-14 days after the last immunization and the splenocytes were restimulated *in vitro* for 48 h with EL4(MART-1) (black), EL4 (white) or without restimulation (control; gray). The results are the mean \pm 1SEM of triplicate data of one representative of three independent experiments.

Fig. 3. Radiation decreased immunity generated by AdvMART1/DC. C57BL/6 mice were treated with 5×10^5 irradiated (10 Gy) or non-irradiated AdvMART1/DC. A second immunization was performed 10 days later with 5×10^5 non-irradiated or irradiated AdvMART1/DC. One week after the last immunization, MART-1-specific (A) IFN- γ

and (B) IL-4 responses were assessed using ELISPOT assays. Splenocytes were restimulated for 48 h with EL4(MART-1) (black), EL4 (white) or without restimulation (control; gray). Mice immunized with irradiated DC showed reduced IFN- γ and IL-4 expression compared to AdVMART1/DC injected mice. MART-1-specific immune response generated by AdVMART1/DC was decreased by irradiated AdVMART1/DC whether given before or after AdVMART1/DC. Results are shown as mean \pm 1SEM of triplicate data of one representative of three independent experiments.

Fig. 4. Irradiation modulates the ability of local DCs to generate MART-1 specific responses. C57BL/6 mice were implanted with viable B16 cells and irradiated or injected intratumorally with AdVMART1/DCs (5×10^5) when the tumor size was approximately 5-6 mm in diameter. Six groups of mice were used in this study. (1) Mice without tumors or treatments. (2) Mice with tumors. (3) B16 tumors treated with 10 Gy irradiation. (4) Tumors injected with AdVMART1/DCs. (5) AdVMART1/DCs injected one day prior to local tumor irradiation (10 Gy). (6) Tumors irradiated (10 Gy) followed by AdVMART/DC injection. One week after the first treatment, IFN- γ producing lymphocytes were detected by ELISPOT after 48-h restimulation with EL4(MART-1) (black), EL4 (white) or left without restimulation (control; gray). Three mice were used in each group of different treatment. Results shown are the mean \pm 1SEM of triplicate data from one representative of two experiments.

Fig. 1.

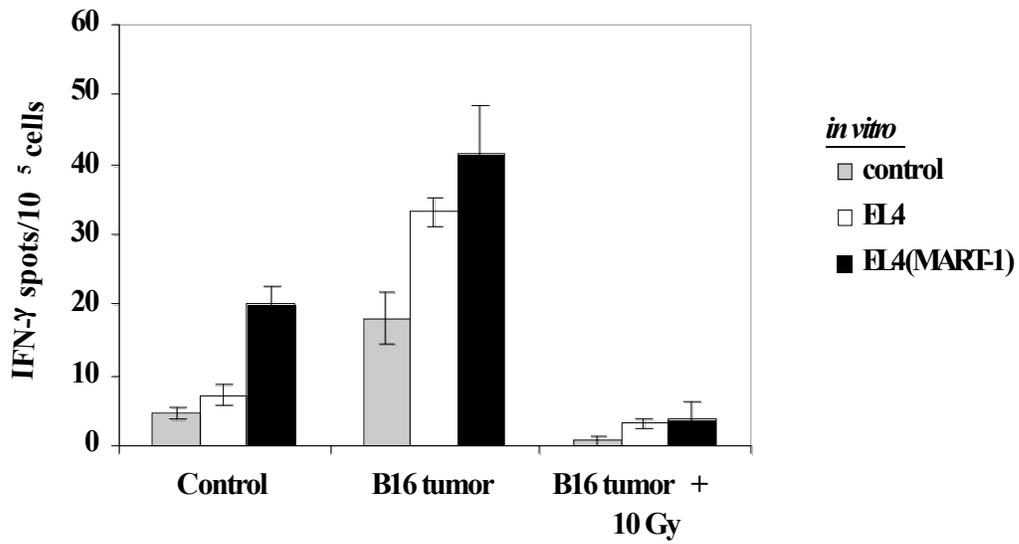


Fig. 2.

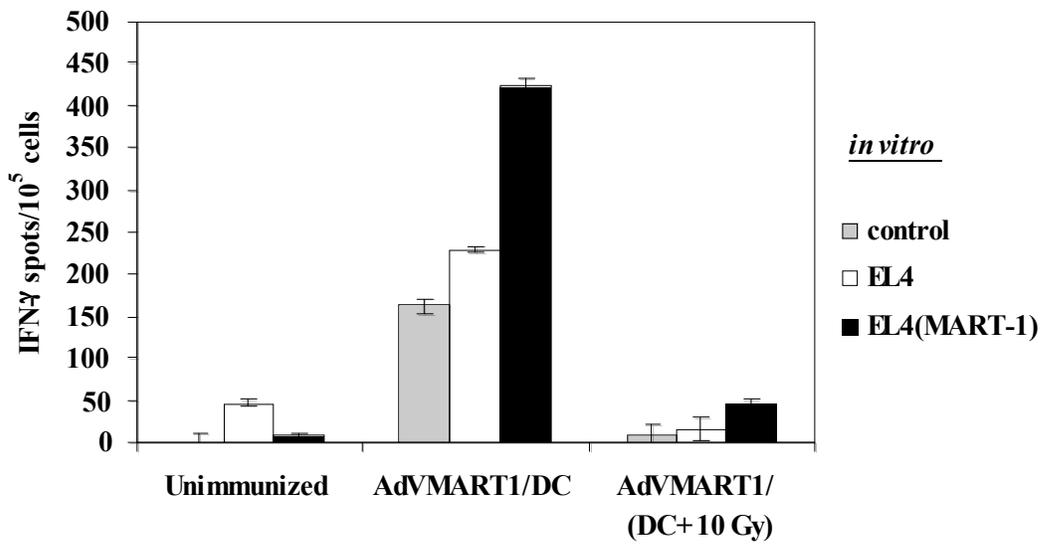


Fig. 3A.

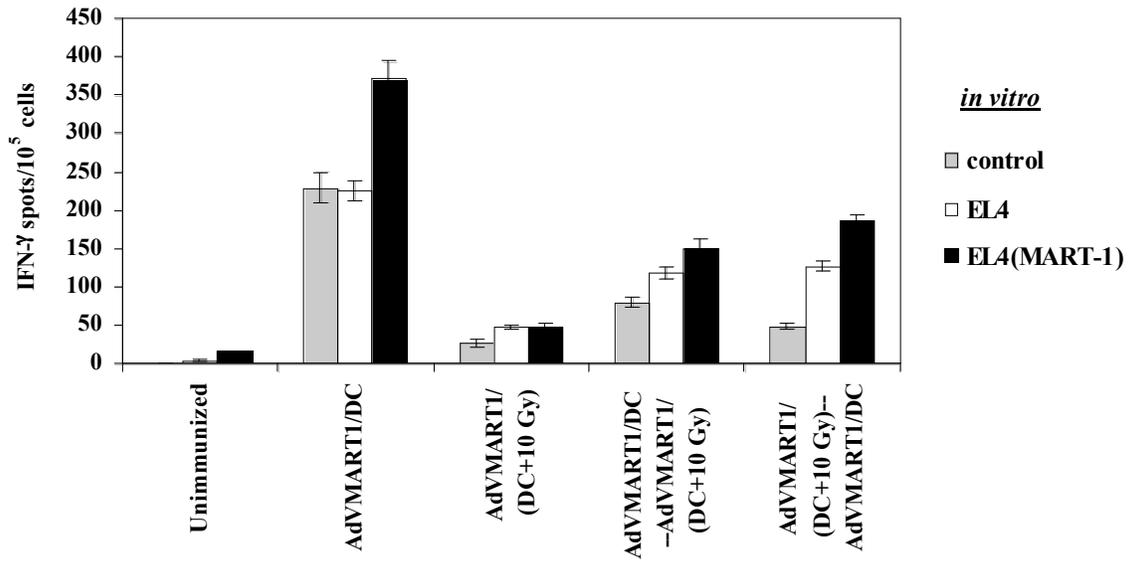


Fig. 3B.

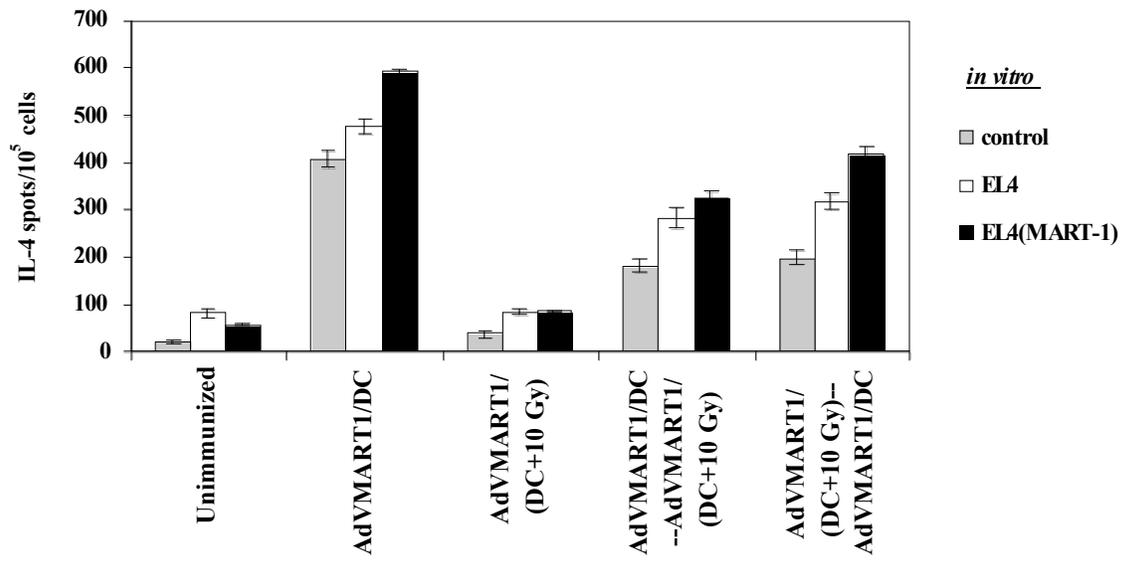
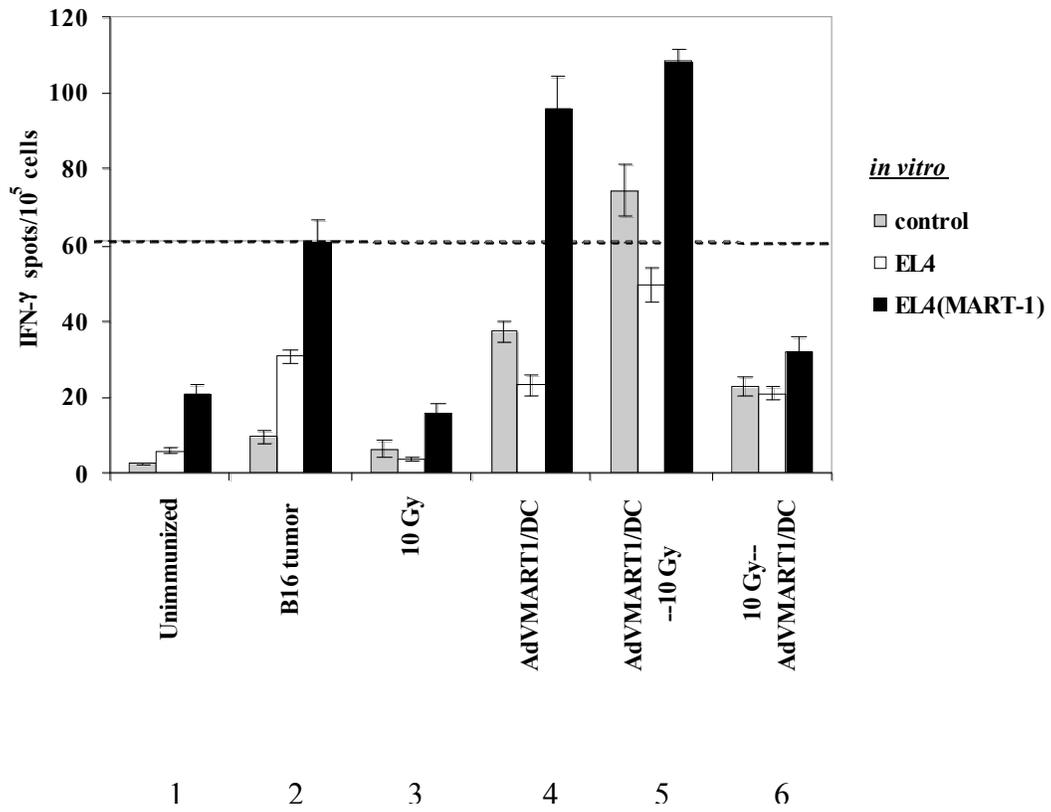


Fig. 4.



ORIGINAL ARTICLE

Tetracycline-regulated intratumoral expression of interleukin-3 enhances the efficacy of radiation therapy for murine prostate cancer

C-H Tsai¹, J-H Hong², K-F Hsieh¹, H-W Hsiao¹, W-L Chuang¹, C-C Lee², WH McBride³ and C-S Chiang¹

¹Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, Hsinchu, Taiwan; ²Department of Radiation Oncology, Chang Gung Memorial Hospital, Tao-Yuan, Taiwan and ³Department of Radiation Oncology, UCLA, CA, USA

The aim of this study was to investigate means of increasing the efficiency with which cancer cell death following local radiation therapy (RT) is translated into the generation of tumor immunity since, if this were to be achieved, it would be expected to enhance the rates of disease-free recurrence and survival. Our investigations centered around the use of interleukin-3 (IL-3), expressed intratumorally using an inducible adenoviral vector, to alter the immunogenicity of established murine TRAMP-C1 prostate cancer receiving a course of fractionated local RT (7 Gy per fraction per day for 5 days). Because high systemic levels of IL-3 can be associated with toxicity, a tetracycline-regulated gene delivery system was employed. The results show that while intratumoral IL-3 expression or RT alone caused a modest delay in TRAMP-C1 tumor growth, the combination was synergistic with 50% of mice being cured and developing a long-term, tumor-specific state of immunity. Immunological analyses performed on splenic lymphocytes demonstrated that, compared to RT or IL-3 alone, combined treatment significantly increased the number of tumor-specific IFN- γ -secreting and cytotoxic T cells. The study demonstrates that tetracycline-regulated IL-3 gene expression within tumors can enhance the immune response to prostate cancer and this can augment the efficacy of a course of RT without additional side effects.

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Keywords: IL-3; radiation; gene therapy; mice

Introduction

Radiation therapy (RT) is a front line treatment for human prostate cancer. While it is very effective in early stage disease, it is less able to deal with higher tumor burden and local RT does not impact on micrometastatic disease that has spread beyond the capsule. One current experimental approach to increasing local control is to increase the dose of radiation to the tumor using delivery techniques that better conform the field to fit the tumor site; however, this increases the risk of missing local microscopic extensions of disease and perhaps of long-term complications as a consequence of injury to adjacent normal tissues, normally rectum and bladder. Supplementing RT with a different adjunctive therapy such as

immunotherapy (IT) that would increase the probability of achieving local control while at the same time generating systemic antitumor immunity that might help eradicate minimal residual and micrometastatic disease is therefore an attractive option. The combination of RT with IT has been shown to be effective in preclinical models of renal adenocarcinoma,¹ prostate carcinoma,² and other tumors.³

The attraction of RT combined with IT is increased by findings that irradiation can modulate expression of several classes of genes in both murine and human cancers,⁴ such as Fas, MHC-1, ICAM-1, and MUC-1, that might enhance their ability to serve as immune targets.^{2,5–7} On the other hand, while there is evidence that RT generates ‘danger’ signals that might mature dendritic cells (DCs) to present tumor antigen^{8,9} there is no good evidence that this occurs in the clinic and such signals may be too weak to be effective. In a recent randomized phase II clinical trial study,¹⁰ addition of recombinant vaccine administration to standard RT in patients with clinically localized prostate cancer showed that vaccination was safe and the generation of a PSA-specific cellular immune response was enhanced following

Correspondence: Dr C-S Chiang, Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, 101 Sec 2, Kuang-Fu Road, Hsinchu 30013, Taiwan, Taiwan.

E-mail: cschiang@mx.nthu.edu.tw

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RT. We have shown in previous studies that expression of interleukin-3 (IL-3) gene within tumors can enhance the immunogenicity of even classically nonimmunogenic tumors,¹¹ without altering their intrinsic radiosensitivity, and this enhances the effects of radiation therapy (RT), allowing a long-term state of immunity to develop after the primary tumor regresses. At least two mechanisms of IL-3 action were proposed by us and others.^{11–15} One was by altering the phenotypic expression of cell adhesion molecules and of MHC class I by tumor cells. Another was by improving presentation of putative tumor antigens through maturation of DCs.

Recently, we demonstrated in a very preliminary study that adenoviral IL-3 gene delivery (Adeno-IL3) into murine prostate cancers (TRAMP-C2)¹⁶ could enhance the efficacy of RT. This study extends these findings mechanistically in a TRAMP-C1 model. Further, since long-term expression of IL-3 is associated with some hematological side effects, manifested in the mouse as splenomegaly, we have refined the adenoviral delivery system by employing a tetracycline-regulated vector.

Materials and methods

Mice, tumors, and tumor irradiation

C57BL/6J mice were purchased from the National Laboratory Animal Center, Taiwan. Male mice, 7- to 8-week old, were used for experiments. TRAMP-C1 tumors that developed in TRAMP transgenic mice¹⁷ and that are syngeneic to C57BL/6J mice were kindly provided by Dr NM Greenberg. Tumors were generated by inoculating 5×10^5 viable TRAMP-C1 cells into the right thighs of mice. After 10 days, when the tumors had reached 4–6 mm in diameter they were irradiated with 7 Gy per day for 5 consecutive days for a total dose of 35 Gy delivered to the tumor site using 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 diameters were measured in three mutually orthogonal dimensions at 2–3 day intervals with a vernier caliper and the mean values calculated to assess tumor growth until 12 mm was reached, when the mice were euthanized. The recommendations of the approved guide for the care and use of laboratory animals by the Institutional Animal Care and Use Committee (IACUC) of National Tsing Hua University, Taiwan, 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 varying 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). The surviving fraction was calculated by colonies counted/(cells seeded \times (plating efficiency of untreated control cells)).

In vitro gene transduction

The Adeno-X and Adeno-X-Tet-On expression systems (BD, Clontech, San Jose, CA; Cat. No. 631513 and 631508, respectively) were used as described by the manufacturer to introduce and express full-length IL-3 cDNA in cultured tumor cells (*in vitro*) and growing tumors (*in vivo/in situ*). The former adenovirus expression vector was used with a CMV promoter to drive constitutive expression of IL-3 or β -gal genes. The latter is a dual vector system where an Adeno-X-Tet-on virus produces rtTA (reverse tetracycline-controlled transactivator) in response to binding of tetracycline (or its analog, doxycycline (Dox)) to a Tet-responsive element (TRE),¹⁸ which controls expression of Adeno-X-TRE-IL3 or β -gal. The adenovirus preparation was enriched using an Adeno-X virus purification kit (Clontech; Cat. No. 631518) and titer determined by endpoint dilution assay using an Adeno-X Rapid Titer Kit (BD, Clontech).

The Tet-on system was tested using β -gal expression. TRAMP-C1 cells were cultured in eight-well chamber slides (Nalgen Nunc International, Rochester, NY) at a plating density of 1×10^5 cells per well. At 24 h after plating, varying concentrations of Adeno-X-Tet-on virus and Adeno-X-TRE- β gal virus were added. Immediately after infection, Dox (Sigma-Aldrich, St Louis, MO) was added in the final concentration of 1 μ g/ml. After 24 h, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature and expression assessed by staining for 1 h using an X-gal staining assay kit (Gene Therapy Systems Inc., San Diego, CA). For the IL-3 adenoviral vectors, mouse IL-3 protein was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Rockford, IL; Cat. No.: ENDEM-IL3).

In vivo gene transduction and tumor irradiation

Adv-X-Tet-On virus (1×10^7 IFU) and Adv-X-TRE-IL3 virus (1×10^7 IFU) or Adeno-X-TRE- β gal virus (1×10^7 IFU) was injected intratumorally into each of four quadrants of a tumor for a total volume of 60 μ l of PBS -1, 1, and 3 days following the first dose of a fractionated RT protocol (7 Gy/fraction for 5 days to a total dose of 35 Gy). Dox, or control PBS, was delivered by Alzet osmotic pumps (DURECT Corporation; Cat. No: 2002, Cupertino, CA) implanted subcutaneous (s.c.) under the skin of mice slightly posterior to the scapulae 1 h before the first injection of virus. The pumps contained 200 μ l of Dox at 1 mg/ml for secretion at a rate of 1 μ l per hour for 7 days. Pumps were not replaced after this time.

Immune response assays

ELISPOT was used to assess tumor-specific splenic lymphocyte responses. Spleens were taken 14 days after the last virus injection, or when the tumor reached 1 cm diameter, whichever was the sooner. Spleen cell preparations were depleted of red cells by osmotic lysis and lymphocytes restimulated by adding irradiated (35 Gy) TRAMP-C1 or B16 melanoma cells (ATCC number:

CRL-6745) at 25:1 responder-to-stimulator ratios for 48 h in the presence of 10 U/ml hIL-2 (BioSource International, Camarillo, CA). ELISPOT assays were performed in MultiScreen-HA96 plates (Millipore, Bedford, MA) coated with anti-IFN- γ or anti-IL-4 antibodies (BD Pharmingen, San Jose, CA). After washing and blocking with 10% fetal bovine serum/phosphate-buffered saline (FBS/PBS), restimulated splenocytes were added and incubated for 24 h. 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 incubated at room temperature for 45 min. Spots were developed by adding 150 μ l/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. The plate was kept in the dark for

20 min and spots were counted under a dissecting microscope.

Tumor-specific cytotoxic CD8 T lymphocytes were assessed in splenocyte preparations depleted of non-CD8 T cells using a cocktail of monoclonal antibodies (Cat. # 130-090-859, Miltenyi Biotech, Auburn, CA) bound to magnetic beads and passing them through a magnetic field of a MACS separator (Miltenyi Biotech). Cytotoxic function was analyzed using a CyToxiLux kit (OncoImmunin Inc., Gaithersburg, MD). Briefly, target (TRAMP-C1) or nontarget (B16 melanoma) tumor cells were fluorescently labeled with red dye and co-incubated with CD8 T cells. After 1 h, cells were resuspended in the presence of 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.

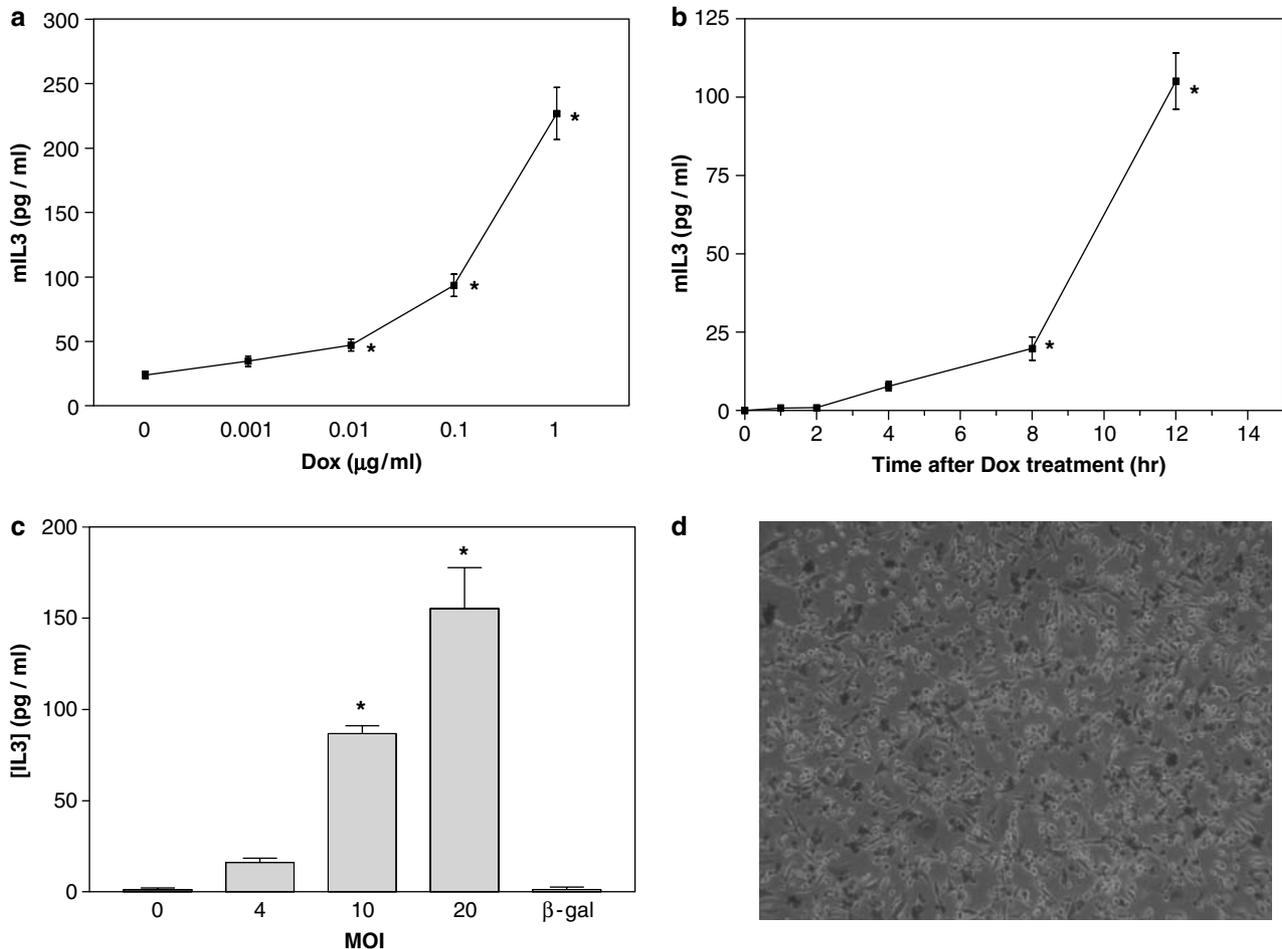


Figure 1 Variables involved in the expression of IL-3 by TRAMP-C1 cells into culture supernatants using the tet-on Adv-X-Tet-on/Adv-X-TRE-mIL3 system, as assessed by ELISA. Cells were cultured in 24-well plate at a plating density of 1×10^5 per well for 24 h before co-transfection and Dox addition. (a) The influence of Dox concentration on IL-3 expression at 24 h with an MOI for Adv-Tet-on and Adv-IL3 of 10. (b) Time dependent expression of IL-3 following Dox addition at 1 μ g/ml and with an MOI for both viruses of 10. (c) The influence of MOI on IL-3 expression at 24 h with Dox added at 1 μ g/ml (d) Expression of β -Gal in TRAMP-C1 cells 24 h after co-transfection with Adv-X-Tet-on (MOI = 10) and Adv-X-TRE- β gal viruses (MOI = 10) in the presence of 1 μ g/ml of Dox. Data and error bars represent the mean \pm 1 s.d. of three repeat assays. * $P < 0.05$ by ANOVA compared with control.

RT-PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Of total RNA, 2 μ g, was first reverse transcribed to cDNA by Omniscript reverse transcriptase (Quiagen, Valencia, CA). The primer pairs for ICAM-1 were (5' primer: 5'-AAGGGCTGGCAT TGTTCCTCTAA-3' and 3' primer: 5'-AGCGCAGCGG CAGGGTCT-3') and for β -actin were (5' primer: 5'-GGTGACGAGGCCAGAGCAA-3' and 3' primer: 5'-CCCGGCCAGCCAGGTC CAG-3').

Characterization of tumor-infiltrating host cells and tumor cell surface markers

Tumors were disaggregated into single cell preparations using dispase,¹¹ and host cell populations identified by flow cytometry using CyFlow (Partec, GmbH). Lymphocytes were identified by gating using splenocytes to define the appropriate FSC vs SSC region. Tumor-associated

macrophages (TAMs) were identified by fluorescein isothiocyanate (FITC)-conjugated-anti-CD11b (Mac-1) antibody (BD Pharmingen) following addition of Fc receptor blocking with 1 μ g/10⁶ cells of rat anti-mouse CD16/CD32 monoclonal antibody (Fc block BD Pharmingen).

Flow cytometry was also used to monitor the radiation-induced phenotype of *in vitro* cultured TRAMP-C1 tumor cells 4 h after 0 or 7 Gy irradiation. Cells were stained using FITC-conjugated monoclonal antibodies (BD Pharmingen) against murine H-2K^b (MHC-I, Cat. No. 553569), CD95 (Fas/APO-1 Cat. No. 554257), or CD54 (ICAM-1, Cat. No. 553252). FITC-Hamster IgG1 (Cat #: 553971) was used as isotype antibody control.

Statistical analysis of the data

Where indicated, the results of tests of significance are reported as *P*-values < 0.05 and are derived from analysis

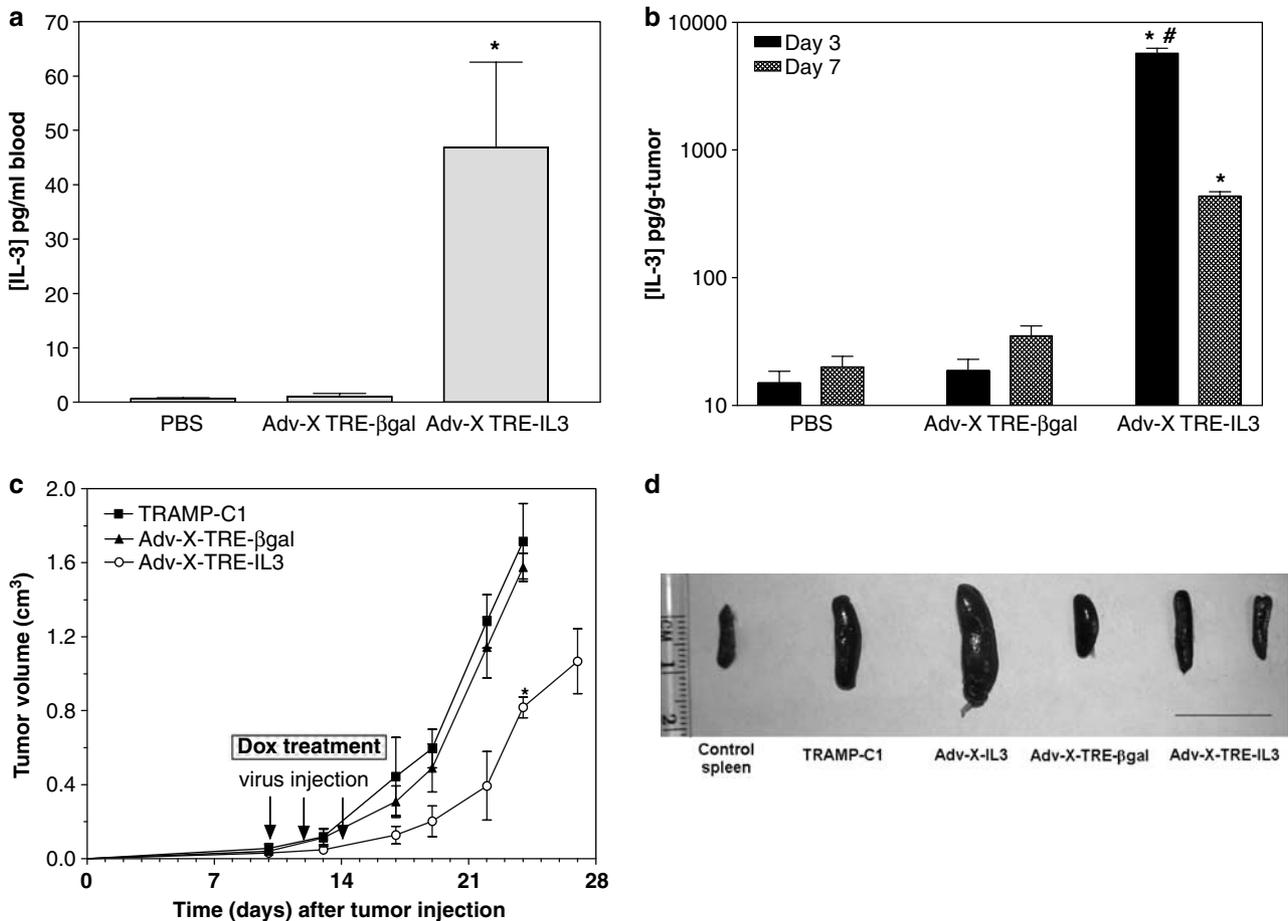


Figure 2 Assessment of the effects of intratumoral tet-on IL-3 gene therapy on *in vivo* 4–6 mm diameter established TRAMP-C1 tumors. Mice were given Dox by osmotic pump 24 h before three injections of 1×10^7 PFU Adv-X-Tet-on and Adv-X-TRE-IL3 viruses in 60 μ l every other day. (a) Levels of IL-3 in blood samples taken 12 h after the last virus injection, as measured by ELISA. (b) Levels of IL-3 within tumor samples taken at day 3 and 7 after the last virus infection, as measured by ELISA. (c) Regulation of IL-3 gene expression by Dox caused TRAMP-C1 tumor growth delay. Arrows indicate the times that virus was injected and the dotted rectangular area represents the time over which Dox (1 mg/ml) was administered by osmotic pump at a dose rate of 1 μ l/h. (d) Splenomegaly in mice bearing TRAMP-C1 tumors receiving Adv-X-IL3 viruses, and its absence in mice with tet-on Adv-X-TRE-IL3 gene therapy. Data and error bars represent the mean \pm 1 s.d. of three repeat assays with three (a and b) or 8 (c) mice. **P* < 0.05 by ANOVA compared with control. #: *P* < 0.05 by Student's *t*-test for day 3 and 7.

of variance (ANOVA) test or Student's *t*-test using a two-tailed distribution. The *P*-values were calculated using the GraphPad Prism software version 3.03 package (GraphPad Software Inc., San Diego, CA).

Results

Characterization of the tetracycline-induced adenovirus expression system in vitro

ELISA of supernatants from TRAMP C-1 cells infected *in vitro* was used to characterize some of the variables involved in production of IL-3 by the Adv-X-TRE-IL3/Adv-X-Tet-On gene transfer system (Figure 1). Addition of Dox from 0.01 up to 1 μg/ml to cells infected at an multiplicity of infection (MOI) of 10:1 led to IL-3 production that increased with time (Figure 1a, b) and there was no evidence of viral toxicity up to an MOI of 20:1 (Figure 1c). Regulation of gene expression by Dox was confirmed using the Adv-X-TRE-βgal virus (Figure 1d).

Influence of in vivo IL-3 expression on the growth of TRAMP-C1 tumor

The Tet-On gene transfer system was tested *in vivo* by injecting 10⁷ Adv-X-Tet-On particles along with an equal number of Adv-X-TRE-IL3 or Adv-X-TRE-βgal viruses into each of four quadrants of 4–6 mm diameter TRAMP-C1 tumors in 15 μl volumes per site. This was repeated three times every other day. Osmotic pumps that were designed to deliver Dox at a rate of 1 μg/μl per hour for 7 days were implanted s.c. in the mice one day before the first virus injection. This protocol was sufficient to produce measurable levels of IL-3 in serum (Figure 2a) and within tumors 3 and 7 days (Figure 2b) after the last injection of viruses, indicating that expression lasted for at least a week.

As can be seen in Figure 2c, switch-on of IL-3, but not β-gal, by Dox significantly delayed TRAMP-C1 growth compared with PBS-injected controls. Dox administration alone did not affect the rate of tumor growth (data not shown). Although elevated levels of IL-3 were found in the tumor up to 1 week, mice did not develop the splenomegaly (Figure 2d) that is generally found in mice bearing tumors constitutively expressing IL-3 either by adenovirus or retrovirus.¹⁹

The influence of IL-3 gene transfer on the tumor response to RT

The above experimental design was used to test the effect of IL-3 gene expression on the response of TRAMP-C1 tumors to RT *in vivo*. Adv-X-Tet-On/Adv-X-TRE-IL3 viruses were injected into 4–6 mm diameter TRAMP-C1 tumors on days –1, 1, and 3 days relative to the first dose of a fractionated RT protocol (total dose of 35 Gy delivered in 7 Gy/fraction for 5 days). The growth kinetics of the tumors in one of three repeated experiments, each of which gave similar findings, are presented in Figure 3a.

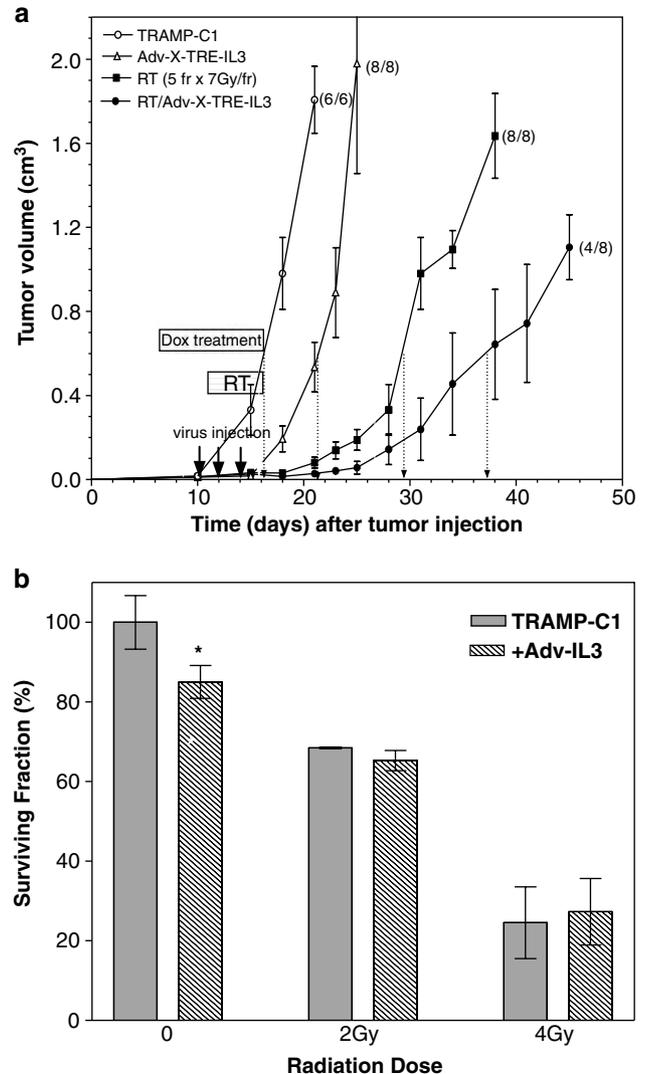


Figure 3 (a). The influence of intratumoral tet-on Adeno-X-TRE-IL3 gene therapy, as described in Figure 2, on TRAMP-C1 tumor response to fractionation radiation therapy. 1 × 10⁷ PFU Adeno-X-Tet-on and Adeno-X-TRE-IL3 viruses in 60 μl were given -1, 1, and 3 days (arrows) relative to the first of 5 daily 7 Gy doses of RT (solid line rectangular area). Dox was administered by osmotic pump for 7 days (dotted rectangular area) starting 1 day before virus injection. Parentheses represent the number of mice with tumors/the number of mice injected in one representative of three experiments. (b) The influence of Adeno-X-IL3 gene transduction at an MOI of 100 on the *in vitro* response of TRAMP-C1 cells to 0, 2, and 4 Gy irradiation. Irradiation was delivered 24 h after virus infection and the surviving fraction was measured by clonogenic assay. Adeno-X-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.

TRAMP-C1 is a fast growing tumor with a volume doubling time of 2 days. Dox-regulated IL-3 expression slowed growth, as did fractionated RT, causing 5 and 13 days of tumor growth delay, respectively. However, all tumors in these groups continued to grow progressively. In contrast, the combined treatment of RT with

Dox-regulated IL-3 gene expression extended tumor growth delay to 21 days and four out of eight mice tumors regressed completely with the mice remaining tumor-free for more than 2 months. Three of the mice that had rejected tumor were rechallenged by s.c. inoculation of 1×10^6 viable 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 *in vitro*. We confirmed this in TRAMP-C1 cells using the Adeno-X-IL3 vector system with the constitutive CMV promoter. TRAMP-C1 cells at 80% confluence were transduced with Adv-X-IL3 at an MOI of 100. Medium was collected one day later and IL-3 content, as measured by ELISA, was 213 ± 82 pg/ml/ 10^6 cells. Control TRAMP-C1 cells did not produce measurable IL-3. Cells were irradiated at this time and plated for clonogenic survival. Adeno-X-IL3 slightly decreased the plating efficiency of TRAMP-C1 (Figure 3b), but the response to 2–4 Gy was not affected. The observed *in vivo* radiation sensitivity of IL-3-transfected tumors in Figure 3a cannot therefore be ascribed to a change in intrinsic cellular radiosensitivity.

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

Several experiments were undertaken to determine if the immune system was responsible for the tumor regression following the combined RT plus Adv-X-TRE-IL-3 gene treatment. First, the change in the proportions of different cell types within TRAMP-C1 tumors was examined one day after last tumor irradiation with and without IL-3 gene therapy. RT significantly increased the intratumoral population of cells with FSC vs SSC flow cytometry profiles corresponding to lymphocytes from around 3 to 20% (data not shown). IL-3-treatment did not further increase the lymphocyte population. Macrophages, as detected by CD11b (Mac-1) staining, comprised 24.4% of the total cells in control tumor (Figure 4a). This increased to 29.5% in RT-treated tumor (Figure 4b). A similar increase was found for Adv-X-TRE-IL3-treated tumors (30.9%) (Figure 4c). The combined treatment of RT and Adv-X-TRE-IL3 further enhanced the percentage of Mac-1+ cells to 38.5% (Figure 4d). As Mac-1 is more highly expressed on infiltrating monocytes than mature macrophages (13), these results indicate that both RT and Adv-X-TRE-IL3 treatment results in infiltration of Mac-1+ cells from the periphery, and this was further enhanced when the two treatments were given.

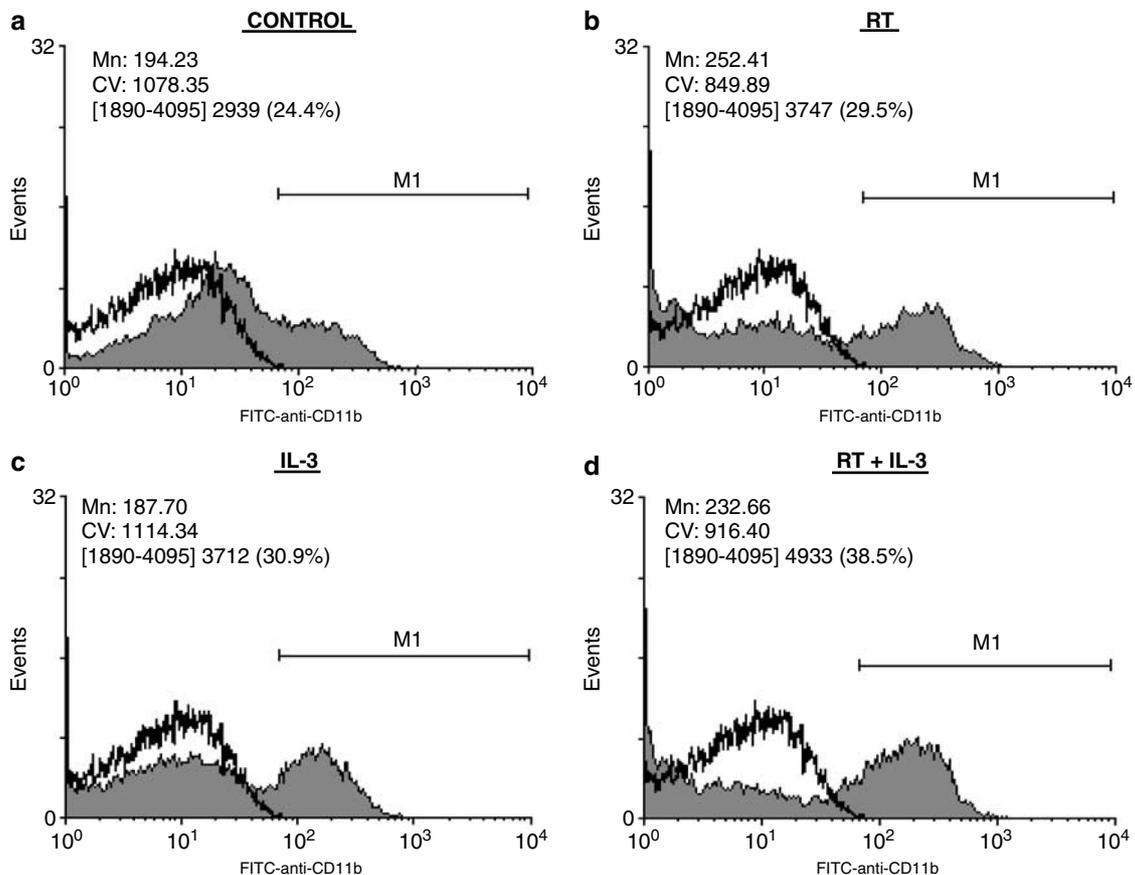


Figure 4 The change of FITC-anti-CD11b staining histograms for TRAMP-C1 tumor samples taken one day after last RT treatment. Tumors received same treatment protocol as described in Figure 3. (a) Control, (b) RT alone, (c) Adv-X-TRE-IL3 treatment, and (d) RT plus Adv-X-TRE-IL3 treatment.

The effects of short-term Dox-induced IL-3 and RT on tumor-specific T-cell responses were then assessed by ELISPOT (Figure 5) and CTL assay (Figure 6) using spleens taken from the mice 14 days after the last virus injection or when tumors reached 1 cm diameter, whichever was the sooner. Almost no immune response was detected by ELISPOT assay in mice bearing TRAMP-C1 tumors without treatment (Figure 5). Both intratumoral injection of Adv-X-Tet-on/Adv-X-TRE-IL3 virus and RT generated modest immune responses. In contrast, the combined treatment markedly increased tumor-specific T-cell responses. Intriguingly, whereas IL3 gene expression alone resulted in a predominantly IL-4 response, RT generated more IFN- γ producing cells, especially in the combined treatment group.

Cytotoxicity assays 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 (Figure 6d) than if they came from mice receiving either single treatment (Figure 6b, c) or PBS (Figure 6a). These responses were tumor-specific because responses did not occur when syngeneic B16 melanoma cells were used as stimulators in the ELISPOT assay (data not shown) or as target cells in CTL assay (Figure 6f).

The influence of IL-3 gene transfer and radiation on tumor phenotypes

We,^{11,12} and others,^{13,15} have previously shown that constitutive IL-3 gene expression can alter tumor pheno-

type, increasing MHC class I and CD44 expression. As radiation-induced changes in MHC class I, ICAM-1, and Fas expression by tumor cells irradiated *in vitro* also have been reported⁵⁻⁷ we examined expression of these molecules on TRAMP-C1 cells with and without Dox-regulated IL-3 expression 4h following a single *in vitro* dose of 0 or 7 Gy. Radiation was given 24h after Dox treatment. TRAMP-C1 cells express low levels of MHC class I, a moderate level of Fas, and no ICAM-1 (Figure 7a). Either irradiation or IL-3 production increased MHC class I (Figure 7b), ICAM-1 (Figure 7c), and Fas protein (Figure 7d) expression. The combined treatment further increased expression only for ICAM-1. The ability of irradiation to increase ICAM-1 expression was confirmed at the mRNA level by reverse transcription-polymerase chain reaction (RT-PCR) (not shown).

Discussion

This study shows that tetracycline-regulated intratumoral expression of IL-3 using an adenovirus vector potentiated the response of established murine prostate tumors (TRAMP-C1) to RT resulting in a significant number of complete regressions and the development of a state of long-term immunity. These data extend our studies using IL-3 retrovirus-transfected fibrosarcoma tumors¹¹ and Adv-IL3 with a constitutive promoter,¹⁶ as well as being consistent with results of others in other tumor models.^{1,20,21} The increased response to RT in this system was not due to IL-3-induced alteration in the intrinsic cellular radiosensitivity of the tumor cells. Rather, the evidence is overwhelming that the enhanced response was due to cooperative effects of RT with an IL-3 augmented tumor-specific immune response. Furthermore, we demonstrated that the side effects associated with production of persistent high levels of IL-3^{19,22} can be reduced by the tetracycline-regulated expression system.

RT alone increased intratumoral lymphocyte representation, however tumor-specific immune responses in the spleen were only modestly increased over unirradiated controls, as assessed by ELISPOT and CTL assays. The extent to which responses are generated may depend upon the model and where responses are assessed. For example, RT alone was recently shown to be able to stimulate intratumoral lymphocytes responsive to both a foreign model Ag and an endogenous tumor Ag in an OVA-expressing B16 melanoma tumor model.³ However, while RT seems able to generate 'danger' signals in general there is little to suggest that these are potent⁹ for the development of specific adaptive immunity. Immune stimulation may be additionally required and a recent randomized phase II clinical trial study,¹⁰ using a recombinant PSA vaccine combined with RT in patients with clinically localized prostate cancer has shown the promise of this strategy. Following RT, PSA-specific cellular immune responses to vaccination were enhanced. It would seem logical to take further steps to combine RT with tumor IT in the clinic.

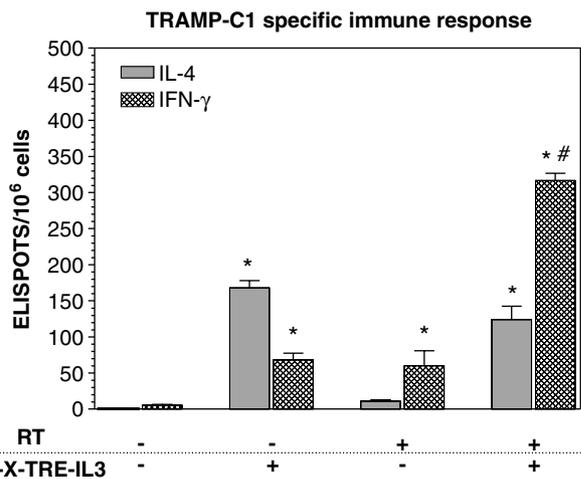


Figure 5 ELISPOT assays for immune responses in spleens of mice received different treatments and restimulated *in vitro* with irradiated TRAMP-C1 cells. TRAMP-C1 tumors growing *in vivo* generated few responsive T cells. Intratumoral expression of IL-3 increased responses, in particular of IL-4 producing cells while RT increased predominantly IFN- γ producing cells. Both these responses are however modest compared with the combined treatment, which had a predominantly IFN- γ producing profile. Data and error bars represent the mean \pm 1 s.d. of three mice from one representative experiment. * $P < 0.05$ by ANOVA test compared with control. #: $P < 0.05$ by Student's *t*-test comparing combined with single radiation treatment.

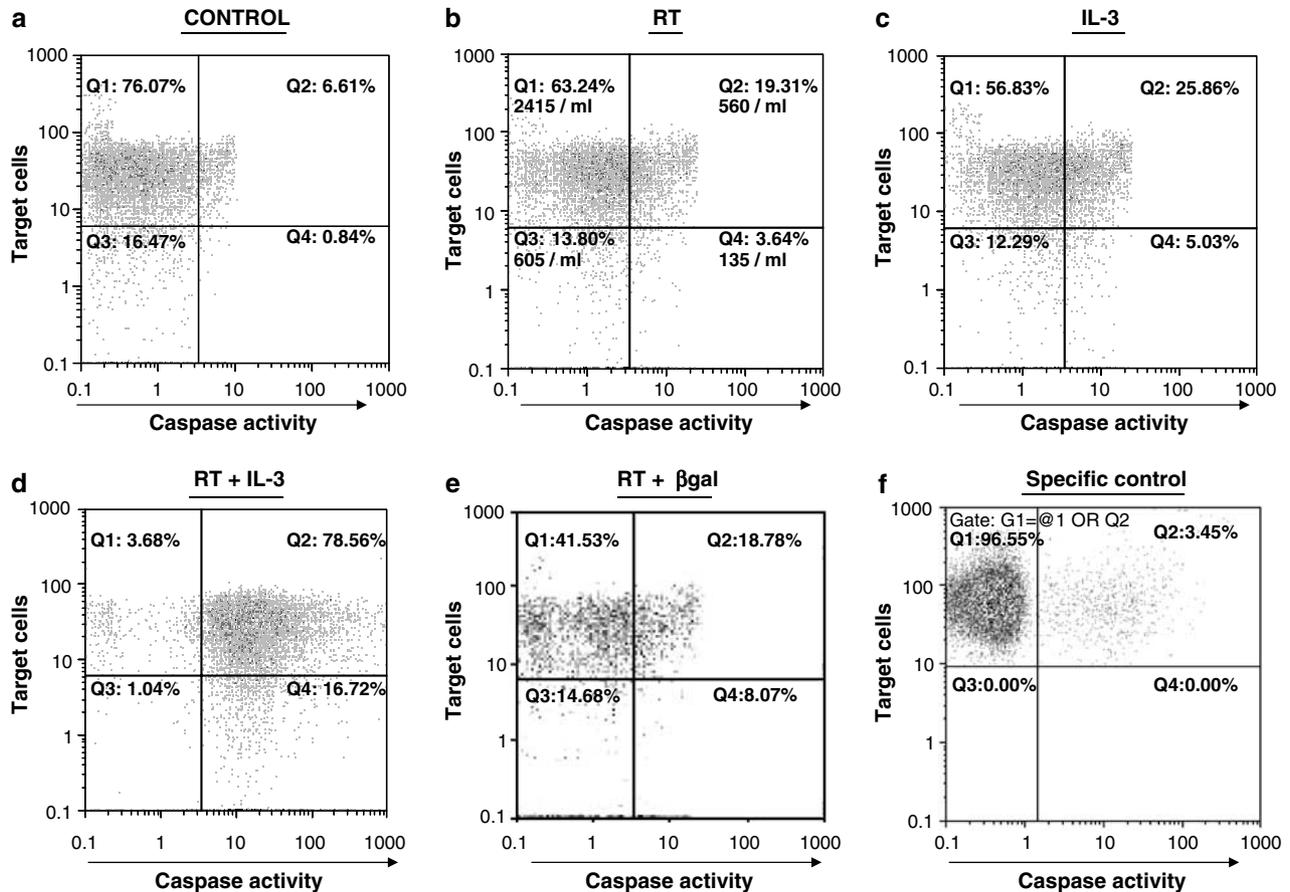


Figure 6 TRAMP-C1-specific CTL responses. CD8⁺ T cells were purified from spleens of mice as for Figure 5 and cocultured with irradiated TRAMP-C1 cells at an effector to target ratio of 10:1 for 1 h. CTL responses were detected by caspase activity induced within the TRAMP-C1 target cells using the CyToxiLux kit. The profiles shown represent CD8⁺ T cells from control mice (a), treated with RT (b), with IL-3 (c), with RT and IL-3 (d), and with β gal (e). To demonstrate that the CTL responses were tumor-specific, syngeneic B16 cells were used as target cells and the same assay performed (f).

We, and others, have examined the potential of IL-3 to act as adjuvant IT in combination with RT. As with other cytokine-based immunotherapeutic approaches, the mechanism may be complex.^{23,24} IL-3 is known to participate in determining the development and functional behavior of macrophages.²⁵ It can expand a subset of macrophages¹³ with increased expression of coaccessory molecules, including MHC class II molecules and IL-1.^{25,26} In cooperation with TNF- α , it stimulates growth of DCs, which are professional antigen presenting cells (APC).²⁷ It has been shown to enhance the presentation of OVA antigen by macrophage-like APC in the context of class I MHC to CD8⁺ CTL.^{15,28} Furthermore, it has been recently shown that IL-3 and IFN- β could prime DCs from peripheral blood mononuclear cells (PMBC) to induce antigen-specific CD8⁺ T cells in melanoma patients.^{29,30}

In this study, we found that even short-term intratumoral IL-3 production, switched on by Dox, could delay tumor growth, even though it could not cure mice of established tumors. Splenocytes isolated from these mice produced more IL-4 in response to *in vitro* stimulation

with irradiated TRAMP-C1 cells than controls, which is in consistent with knowledge of the function of IL-3 in enhancing IL-4 production.^{13,25,31,32} The mechanism behind this response may not necessarily be directly related to the generation of long-term immunity. It has been shown that recall antigen-specific IL-4 production by splenocytes in the presence of IL-3 can come from IL-3-activated mast cells.³² This indirect IL-4 pathway can potentially inhibit type 1 T-cell differentiation and may be insufficient to trigger the development of Th2 memory T cells. We have previously observed a basophilic response within IL-3 producing tumors.¹² In the current experiments we did not determine if the IL-4 producing cells were T cells or basophils but, perhaps more importantly, local RT delivered to IL-3 gene-transduced tumors enhanced IFN- γ producing Th1 and CTL responses in the spleen and seemed to redirect such responses away from an IL-4 pathway and towards a more potent Th1-type of immunity. Combined with results from our previous studies,^{11,23,24} there seems little doubt that the tumor regression generated by RT plus IL-3 gene therapy is due to a tumor-specific immune response.

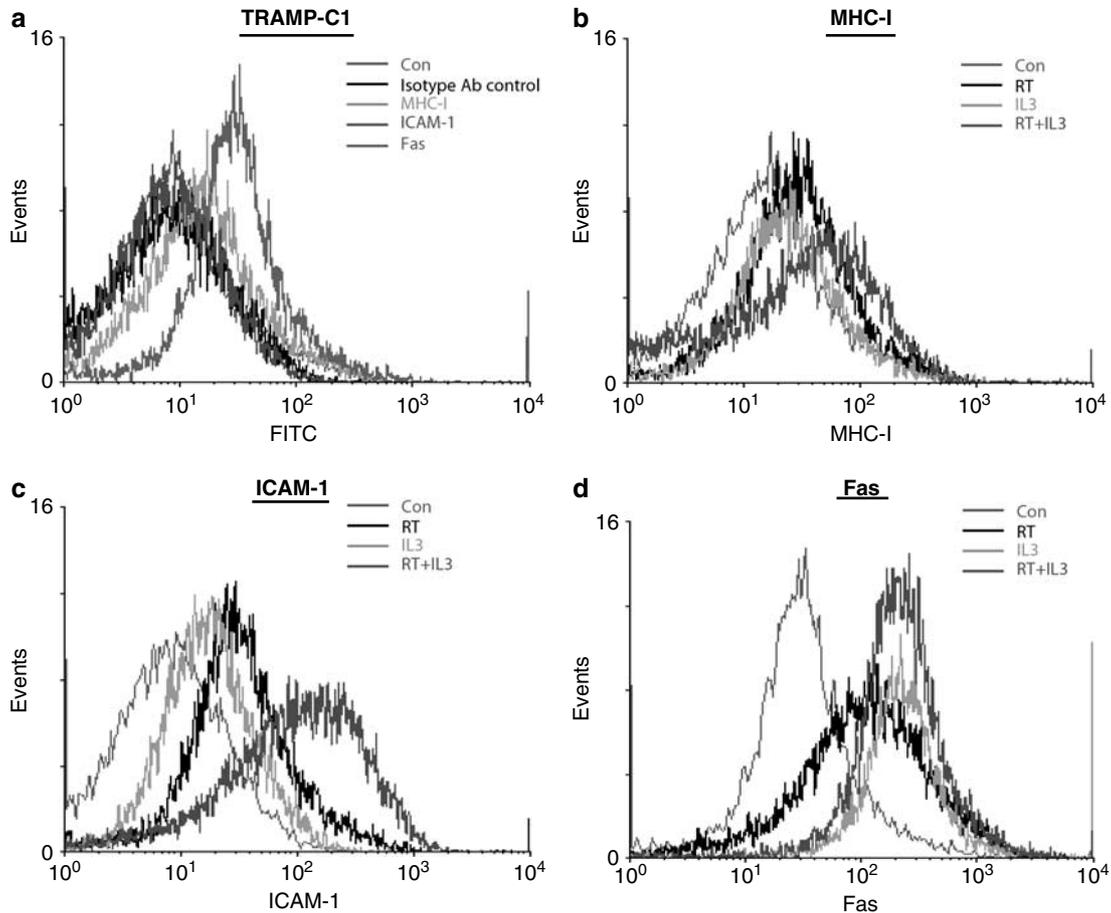


Figure 7 Flow cytometric examination of phenotypic changes following tet-on Adeno-X-TRE-IL3 treatment. (a) *In vitro* TRAMP-C1 cells express low level of MHC-I, ICAM-1, and Fas antigens. Changes of the expression of MHC-I (b), ICAM-1 (c), and Fas (d) molecules were examined 4 h after either 0 or 7 Gy irradiation in the presence or absence of Dox-regulated Adv-X-TRE-IL3 expression.

An important component of the tumor regression process following RT or IL-3 treatment, alone and in combination, may be infiltration of peripheral macrophages as evident by the increase in Mac-1 positive cells. TAMs are the major host cellular components within the tumor microenvironment (see review in Murdoch *et al.*³³), however, their roles in tumor growth remain controversial as they have variably been reported to be both growth promoting^{23,34} and tumoricidal.^{23,35} This may relate to the activation state of the TAMs. Infiltrating TAMs are also likely to be more cytotoxic than those that have been resident for some time.³⁶ The site within the tumor may also be relevant. A recent study by Ohno *et al.*,³⁷ showed that high numbers of TAMs in close contact with human endometrial cancers were associated with a high relapse-free survival rate, while the opposite was true if they were found in hypoxia or necrotic areas. It is possible that combining RT and IL-3 therapy alters the tumor microenvironment and redistributes or re-educates the TAMs to become tumoricidal. It may also be critical to know whether these infiltrating Mac1+ cells can develop into DC cells as reported in other models,^{14,29,30} which would assist in tumor antigen presentation.

While the effects of IL-3 and RT on host cells are obvious, a less obvious effect of both IL-3 and RT is on tumor cell phenotype. Part of the 'danger' response to irradiation is modification of cellular phenotype that may make cells more effective targets. In the same way, cytokines, such as interferon and IL-3, produced by T cells orchestrate dynamic changes in target cells that make them more sensitive to lymphocyte-mediated killing. Modification of these expression patterns on tumor cells by exogenous gene therapy or RT could therefore be important in enhancing the therapeutic benefit of IT.

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.¹² In one case, one fibrosarcoma cell line that was refractory to TNF- α treatment became sensitive after IL-3 gene transduction.²³ In addition to confirming some of these findings in this study 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 *et al.*⁵⁻⁷ They have proposed that radiation-induced tumor phenotype changes could enhance tumor cell susceptibility to antigen-specific CTL-mediated

killing. The mechanism that mediates this type of phenotypic switch by IL-3 is still unclear. In practical terms, it may in part counteract the radiation-induced suppression in function of DCs in the generation of MHC class I-mediated anti-tumor immunity that we recently reported.⁸ We are currently studying this possibility. The combined effects of IL-3 plus radiation may therefore both modulate antigen presentation and alter the tumor phenotype to result in enhancement of immune reactions.

In conclusion, we have demonstrated that the combination of RT and IL-3-based IT can cure mice of TRAMP-C1 tumors. Most notably, IFN- γ -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 IT is potentially a powerful combination that can increase the chances of achieving local, regional, and systemic control of cancer.

Acknowledgements

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