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13. ABSTRACT (Maximum 200 Words) Substantial progress has been made in creating a simple, feasible method to induce an anti-cancer immune response in prostate cancers or metastases by manipulating the regulation of the immune response. Cancer cells are transformed into antigen presenting cells by inducing of the MHC class II molecules and suppressing the co-induced immunoregulatory Ii protein with antisense or siRNA methods. The resulting anticancer immune response is profound, curing up to 80% of mice with established prostate tumors, transplanted into their normal prostates. The important achievement of this past year has been to design, synthesize and validate two second generation reagents, for mice and humans, that are more potent, simpler to use, and set the stage for definite preclinical validation in mice, and with human tumors in tissue culture. Substantial improvement on the reagent of the original grant proposal was achieved by a) tripling the expression of the reverse gene construct which suppresses Ii protein expression, and b) by both chemical and plasmid genetic forms of small inhibitory RNA, which achieve the same end, but with surprising potency. These substantial technical advances (under Tasks 1 and 2) set the stage for advancement toward clinical trials (when justified by data from next year's effort).				
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INTRODUCTION

We have created a novel prostate cancer immunotherapy by converting cancer cells into antigen presenting cells (APC) *in vivo*. By presenting endogenous tumor antigens, such cells induce a potent T-helper cell-mediated immune response, which helps CD8+ T cells to eradicate residual tumor and micrometastases. We convert tumor cells into APC by inducing MHC Class II molecules and then suppressing the immunoregulatory Ii protein, which normally blocks the antigenic peptide binding site of MHC Class II molecules at synthesis in the endoplasmic reticulum from binding of endogenous antigenic peptides that have been transported into that compartment. The therapeutic phenotype is therefore MHC Class II+/Ii- tumor cells. By creating such MHC Class I+/II+/Ii- phenotype, tumor cells simultaneously present endogenous tumor antigens through both MHC Class I and II to activate both CD4+ and CD8+ T cells, generating a very potent tumor cell vaccine. Prior to this grant we had proved the principle that *in situ* intratumor suppression of Ii protein in cells also rendered MHC class II molecules-positive was a potent therapeutic. In the first year of this grant we have optimized and validated a more potent Ii suppression reverse gene construct containing 3 reverse genes of Ii protein. However, the major advance in this past year was to adapt methods of small inhibitory RNA (siRNA) to inhibit Ii protein expression. The field of siRNAs is moving very swiftly and we conceived of adapting this method to our objective in the original proposal without emphasizing it. The initial results were excellent, leading to potent synthetic oligonucleotide siRNA dimers, and genetic constructs suitable for murine and human cells. These results are presented, will be published and appear to be eliciting interest by major pharmacological oncology firms toward additional preclinical development.

REPORT BODY

Task 1: Clone CIITA cDNA gene into three-copy Ii-RGC(-92,97) and verify the induction of MHC Class II+/Ii- phenotype *in vitro* and *in vivo* (Months 1 through 6).

- a. Construction of plasmid containing triple Ii-RGC and CIITA. We have constructed a three-copy Ii-RGC(-92,97) which is most effective in Ii-suppression. We will clone CIITA gene into this plasmid to generate pBudCE4.1/CIITA/Ii-RGC(-92,97)₃ which contains EF-1a-CIITA, CMV-Ii-RGC(-92,97), RSV-Ii-RGC(-92,97), and UbC-Ii-RGC(-92,97).
- b. Verify the induction of MHC Class II+/Ii- phenotype in RM-9 cells by pBudCE4.1/CIITA/Ii-RGC(-92,97)₃ *in vitro* and *in vivo*. RM-9 cells will be transfected with pBudCE4.1/CIITA/Ii-RGC(-92,97)₃ and selected with antibiotics. The resulting lines will be assayed by fluorescent staining and FACS analysis. *In vivo* transfection will be done by injecting RM-9 tumor nodules with pBudCE4.1/CIITA/Ii-RGC(-92,97)₃ and followed by immunohistostaining with antibodies to MHC Class II and Ii.

1. Induction of MHC class II and Ii in DU145 human prostate tumor cells. DU145 is a well-studied, human prostate tumor cell line. It is a MHC class II-negative/Ii-negative tumor line. In order to effect Ii protein suppression in a MHC class II molecule-positive tumor, we have to first generate MHC class II+/Ii+ DU145 tumor cells. This effort mimics our requirement to work with MHC class II-negative, Ii protein-negative human prostate tumors within patients. Since

MHC class II transactivating factor (CIITA) is the master inducer of MHC class II molecules and Ii protein, we have used a plasmid containing a gene encoding the CIITA transactivating protein, to transfect the tumor cells, to act on regulatory elements preceding the respective structural genes to induce the expression of MHC class II molecules and Ii protein. We used the gene gun-mediated method to deliver CIITA into cells. The genes are coated uniformly on ultra-small gold beads, to insure quantitative delivery per cell and a constant ratio of CIITA to Ii-RGC per cell. For each cartridge (one cartridge per shooting), 0.5 mg of 1 μ m gold microparticles was used. Briefly, the indicated amount (5-20 mg for 10-40 cartridges) of gold microparticles was suspended by sonication in 100 μ l of 0.05 M spermidine. The indicated amount of total DNA at a concentration of 1 μ g/ml in endotoxin-free water was added and sonicated; 100 μ l of 1 M CaCl₂ was subsequently added dropwise. This gold-DNA mixture was washed 3 times using 250 μ l of 100% ethanol and finally re-suspended with the indicated amount of 100% ethanol (1 ml for producing 17 coated 0.5-inch cartridges). The coated cartridges were stored at 4°C with desiccant prior to use. For transfecting DU145 cells, 10⁶ cells in 20 ml medium was plated onto a tissue culture dish in about 1 cm diameter circles, which were subjected to a gene gun shooting with one 0.5-inch cartridge using a helium pressure of 350 psi. After culturing, cells were stained with anti-MHC class II (FITC-conjugated anti-human HLA-DR) and anti-Ii monoclonal antibodies (anti-human CD174 plus FITC-conjugated anti-mouse IgG) and analyzed by flow cytometry to determine the percentage of MHC class II+/Ii- cells. **Figure 1** shows the FACS profile of a representative experiment. One observes that more than 50% of DU145 cells have been induced to MHC class II molecules and Ii protein at 0.5 μ g of CIITA/cartridge. The express of MHC class II and Ii is CITA dose-related.

2. Construction of Ii suppressing genetic constructs: Ii-RNAi. We previously constructed effective Ii-RGCs (Ii reverse gene constructs), which effectively inhibited Ii expression in many murine tumor cells. Likewise we previously constructed and validated active human Ii-RGCs. However, recent reports (in particular since the submission of the grant proposal) have shown that RNAi technology is possibly a more effective and reliable method to silence specifically expression of a given gene. Therefore, we constructed the Ii-RNAi to suppress human Ii expression in prostate cancer cells. However, since the DU145 human prostate cancer cell line is MHC class II-negative and Ii negative, we did the initial testing the Raji MHC class II+/Ii+

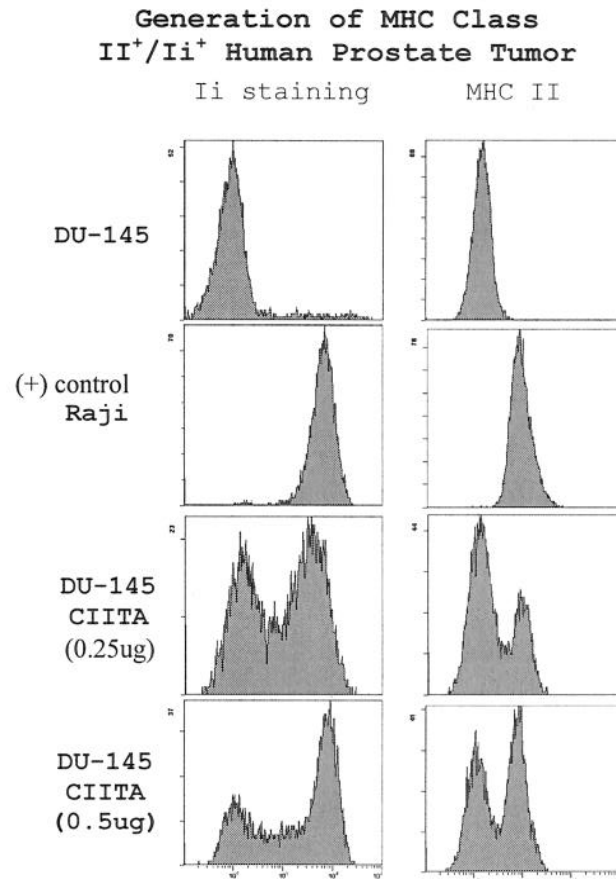


Figure 1. MHC class II and Ii protein induction by CIITA gene transfection. Gene gun method was used to deliver the DNA.

lymphoma cell line. Ten Ii-RNAi expression constructs were engineered in the pSuppressorAdeno plasmid (Imgenex, CA), following standard molecular biology techniques and instructions of the manufacturer. The Ii-RNAi sequences were designed according to either a computer algorithm of Imgenex or by inspection by our scientists. Raji cells were used for testing the Ii suppressing activity of these Ii-RNAi constructs. Three out of the ten Ii-RNAi constructs were significantly active in suppressing Ii protein expression. **Figure 2** shows that the three Ii-RNAi constructs significantly inhibited Ii expression in about 40-50% of cells (reflecting the transfection efficiency) without affecting expression of MHC class II molecules. Raji cells were transfected *in vitro* with the Ii-RNAi constructs using gene gun delivery. Cells were then cultured for 18-24 hours and stained with anti-Ii and anti-HLA-DR antibodies and analyzed by flowcytometry for Ii and MHC class II expression. The active constructs will be used for optimization of the Ii suppression in the experiments planned in DU145 prostate cells, which are underway, below.

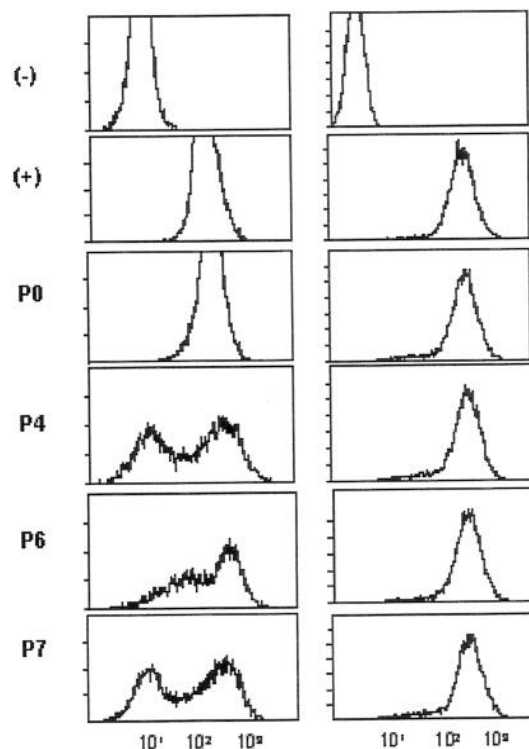


Figure 2. Ii inhibition in Raji cells. Cells were stained with anti-human Ii (left) and HLA-DR antibodies (right). P0 is empty plasmid control

3. Continuing work for the induction of MHC

class II+/Ii- phenotype in DU145 human prostate cancer cells. DU145 cells will be gene gun-transfected with CIITA plus Ii-RNAi constructs. We have shown in Raji cells that p4 plus p7 give an additive effect in Ii inhibition. We will use 0.5 mg of CIITA plus 0.5, 1.0, 1.5, 2.0, and 2.5 μ g (one cartridge can be coated with 3.0 μ g of DNA according to our experience) of p4+p7 (half:half). Cells will be then incubated for 18, 40, and 64 hr and then stained with anti-HLA-DR and Ii antibodies. The FACS analysis will be performed to evaluate the staining profile. The reason for using 1:1 to 1:5 ratios of CIITA versus Ii-RNAi is that in B16 cells, we have to use 4 times more Ii-RGC to suppress Ii in co-transfection experiments. Once we have demonstrated the effective use of CIITA plus an siRNA construct, we will return to the original goal of putting both genes into one plasmid, as described in the original Task 1. We expect to have then a much more potent product for the completion of the projected studies.

4. Inhibition of murine invariant chain (Ii) in bone marrow-derived dendritic

Cells. In parallel to the above efforts to create effective siRNAs for use in human cells, we have pursued the design, synthesis and validation of similar reagent for murine cells. The bulk of our Task 2 is to validate our reagents in murine models. We have successfully demonstrated the inhibition of Ii in murine cells using synthetic and expressed siRNAs for the purpose of enhancing the presentation of MHC class II epitopes in antigen presenting cells. These experiments have been pursued first in dendritic cells for two reasons. 1) They are already MHC class II-positive and Ii protein-positive, i.e., they do not need to be induced with CIITA, 2)

Suppression of Ii in DCs leads to additional ways to vaccinate against prostate cancer antigens, e.g., gene transduction for PSA or PSMA.

In an effort to decrease murine Ii expression in antigen presenting cells, we synthesized siRNA molecules specific for murine Ii mRNA. Two siRNA molecules specific for murine Ii mRNA were introduced into murine dendritic cells and the J774 promyelocytic leukemia cell line using

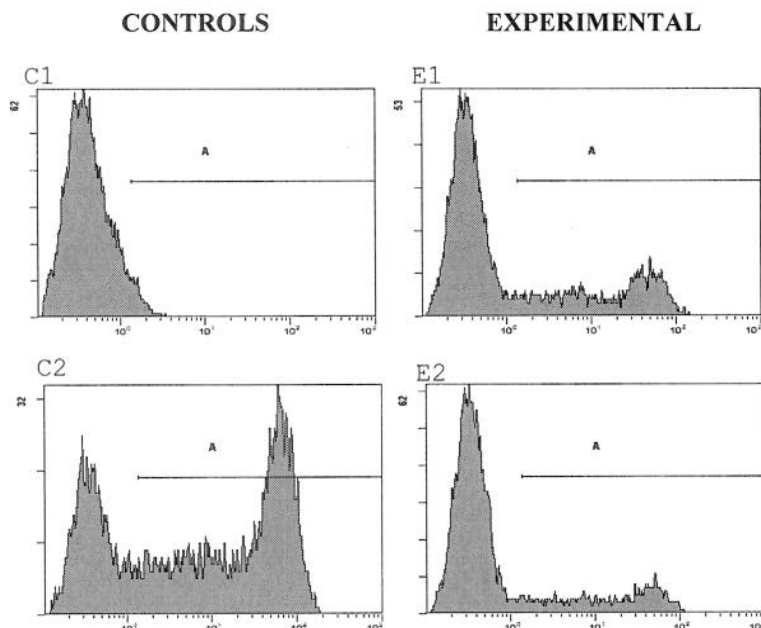


Figure 3 (C1) represents flow cytometric analysis of DC stained with only a secondary goat anti-mouse FITC antibody. (C2) DC fed with YGP were stained with a primary murine Ii-specific antibody and a secondary goat anti-mouse FITC-conjugated antibody. (E1) DC fed with YGP particles loaded with the Ii RGCx3 plasmid; stained with a primary murine Ii specific antibody and a secondary goat anti mouse FITC antibody. (E2) DC fed YGP loaded with synthetic siRNA specific for murine Ii were stained with a primary murine Ii-specific antibody and a secondary goat anti-mouse FITC-conjugated antibody.

TABLE 1

Percent Positivity of Murine HLA-DR Invariant Chain in Murine Dendritic Cells of Figure 4.

	YGP	Construct	Ii Antibody	% Ii Positive
Control 1	-		-	6
Control 2	+		+	66
Experimental 1	+	Ii RGCx3	+	36
Experimental 2	+	Ii siRNA 2	+	28

chemically modified yeast glycan particles (YGP). The YGP particles are taken up by DCs in a receptor mediated process that results in efficient expression of plasmid DNA and delivery of synthetic siRNA. We have previously used a plasmid pli RGCx3 that results in decreased expression of Ii protein when transfected into Ii positive cells. This plasmid was constructed

using a portion of the Ii coding sequence inserted into an expression plasmid in the reverse orientation. Using an antibody specific for Ii and flow cytometry, the expression of Ii is detected on 66% of murine DCs as shown in figure 1, C2. Ii expression was not affected by empty YGP particles. Transfection of pIi RGCx3 resulted in a decrease in Ii expression, as shown in **Figure 3 and Table 1**). Transfection of synthetic siRNA also resulted in a decrease in Ii expression as shown in **Figure 4 and Table 2**).

Figure 4 (C1) represents flow cytometric analysis of J774 stained with a secondary goat anti mouse FITC antibody. (C2) J774 stained with a primary murine Ii specific antibody and a secondary goat anti mouse FITC antibody. (C3) J774 transfected with empty YGP particles. (E1) J774 transfected with YGP particles loaded with the Ii RGCx3 plasmid. (E2) J774 transfected with YGP particles loaded with synthetic siRNA specific for murine Ii. (E3) J774 transfected with YGP particles loaded with an expression plasmid psiRNA1-2 encoding an siRNA specific for murine Ii. (E4) J774 transfected with YGP particles loaded with an expression plasmid psiRNA 2-11 encoding an siRNA specific for murine Ii.

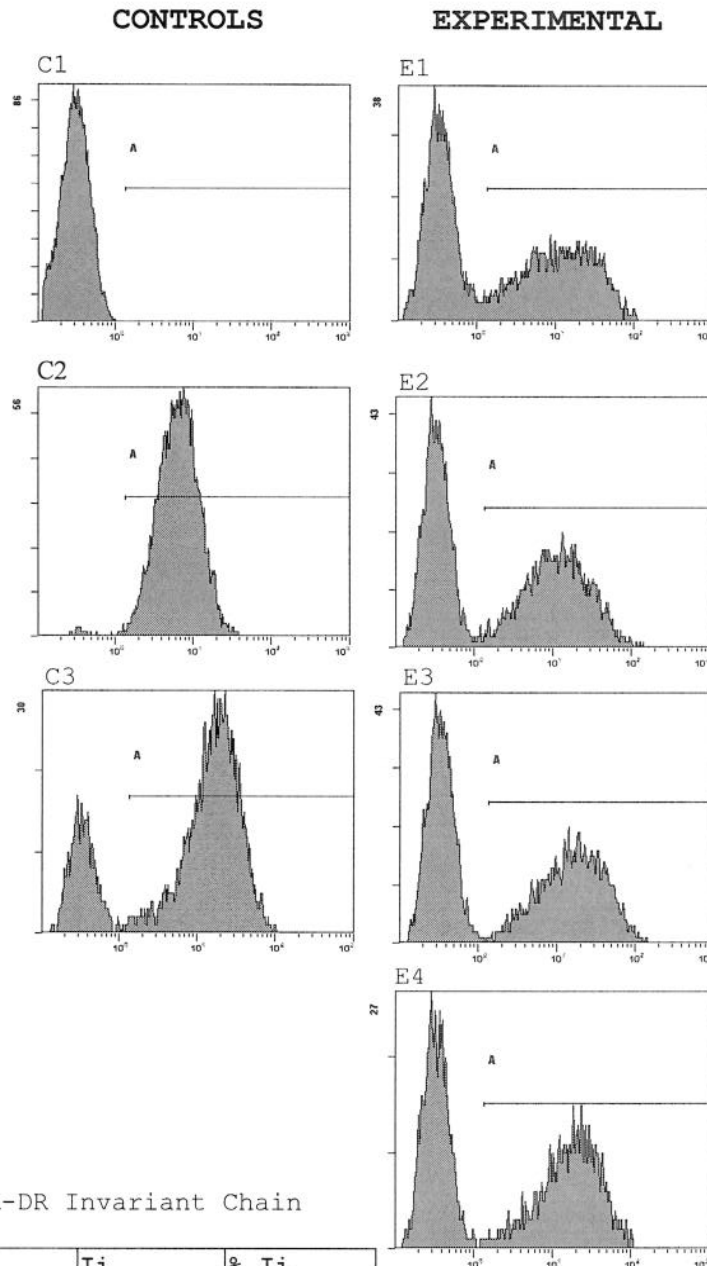


TABLE 2

Percent Positivity of Murine HLA-DR Invariant Chain In J774 Cells

	YGMP	Construct	Ii Antibody	% Ii Positive
Control 1	-		-	0
Control 2	-		+	96
Control 3	+		+	75
Experimental 1	+	Ii RGCx3	+	51
Experimental 2	+	Ii siRNA 2	+	53
Experimental 3	+	Ii psiRNA 1-2	+	52
Experimental 4	+	Ii psiRNA 2-11	+	54

For these experiments, bone marrow cells were extracted from the femurs of BALB/c mice. Cells were plated at 4×10^6 cells in 100 mm dishes, in RPMI 1640-medium supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), L-glutamine (2 mM), 2-BME (50 ug/ml), and 10% fetal calf serum. The bone marrow cells were treated for 6 days with 20 ng/ml murine GM-CSF. For the remaining 4 days of incubation, the cells were given fresh medium with 10 ng/ml murine GM-CSF. At day 10, the immature dendritic were washed with PBS, trypsinized, plated in 6 well plates 5×10^5 per 2 ml medium. The cells were fed chemically modified yeast cells, YGP particles. These particles bear a chemical group readily recognized by a receptor uniquely on DC, and leading to the uptake of the particle into the DC, with expression of DNA encapsulated in the particle. The YGP particles are taken up by DCs in a receptor-mediated process that results in efficient expression of plasmid DNA and delivery of synthetic siRNA. YGP particles are loaded with Ii RGCx3 plasmid DNA or siRNA. The Ii RGCx3 plasmid was constructed using a portion of the Ii coding sequence inserted into an expression plasmid in the reverse orientation. The RNAi plasmid results in expression of a biosynthetic siRNA which decreases expression of Ii protein, by a RNAi mechanism. Cells were harvested 48 hr after addition of the YGP particles. The cells were washed with PBS, formalin-fixed, quenched with glycine, and permeabilized with saponin in preparation for staining with Ii monoclonal antibodies.

5. Inhibition of murine HLA-DR invariant chain in J774 promyelocytic leukemia cell line.

Inhibition of Ii expression was also seen in the J774 murine promyelocytic leukemia cell line, which is considered to be of dendritic cell/macrophage lineage (**Figure 4**). This cell line constitutively expresses Ii (**Figure 4, C1**) and is transfectable with the YGP particles. Transfection of J774 with empty YGP had little effect on the expression of Ii as seen in **Figure 3, C3** whereas transfection of the pIiRGCx3 plasmid greatly decreased the expression of Ii-positive cells and increased the number of Ii-negative cells as seen in **figure 2, E1**. J774 transfected with YGP particles loaded with synthetic siRNA specific for murine Ii demonstrated a similar decrease in Ii expression, **Figure 4, E2**. siRNA expression plasmids psiRNA1-2, psiRNA 2-11 were constructed that express siRNA corresponding to synthetic siRNA specific for murine Ii that effectively suppressed Ii expression in this as well as in previous experiments. J774 transfected with YGP particles loaded with plasmids psiRNA1-2, psiRNA 2-11 demonstrated a similar decrease in Ii expression (**Figure 4, E3 and E4**). J774 were plated at 5×10^5 per 6 well plates in RPMI-1640 medium containing penicillin (100 U/ml), streptomycin (100 ug/ml), L-glutamine (2 mM), and 10% fetal calf serum. YGMP particles were added to the J774 cells and incubated for 3+ hr and fed spent:new medium. 48 hours later the cells were harvested for Ii monoclonal antibody staining.

Task 2: Optimize therapeutic protocols including the doses of IL-2 and IFN- γ . (Months 6 through 24). The dose of radiation will also be optimized.

- a. Dr. Hillman has demonstrated that radiation plus MHC Class II+/Ii- phenotype cures 60% of mice. We will optimize the doses of IL-2 and IFN- γ to obtain the best cure rate of mice with one dose of radiation. Dose of IL-2 will be optimized first and IFN- γ secondly. We expect high rates of cure will be achieved with optimized doses of IL-2 and IFN- γ since in our previous studies, only low doses of IL-2 and IFN- γ were used. We used only one dose of radiation (8 Gy on day 6) in our previous studies. With optimized doses of IL-2 and IFN- γ ,

we will optimize the frequency of radiation. Radiation will be done on day 6, on days 6 and 11, and on days 6, 11, and 16. We believe close to 100% cure will be obtained with optimized doses of IL-2, IFN- γ , and optimized frequency of radiation.

We have previously demonstrated that transfecting genes into tumors, to up-regulate MHC class I and class II molecules and inhibit invariant chain (Ii), induces a potent anti-tumor immune response when preceded by tumor irradiation, in murine RM-9 prostate carcinoma. Here we want to optimize dose of pIL-2. The transfected genes are cDNA plasmids for interferon gamma (pIFN- γ), MHC class II transactivator (pCIITA), an Ii reverse gene construct (pIi-RGC), and a subtherapeutic dose of adjuvant IL-2 (pIL-2). Responding mice rejected challenge with parental tumor and demonstrated tumor-specific CTLs. In fulfillment of the objective of Task 2, we determined the relative roles of each one of the four plasmids pIFN- γ , pCIITA, pIi-RGC, and pIL-2 in conjunction with radiation for the induction of a curative immune response. Up-regulation of MHC class I with pIFN- γ or class II with pCIITA, separately, did not lead to a complete response even if supplemented with pIL-2 or pIi-RGC. An optimal and specific anti-tumor response is achieved in more than 50% of the mice when, following tumor irradiation, tumor cells are converted *in situ* to a MHC class I+/class II+/Ii- phenotype with pIFN- γ , pCIITA, pIi-RGC and pIL-2. We demonstrate further that both CD4+ helper T cells and CD8+ cytotoxic T cells are essential for induction of an anti-tumor response because *in vivo* depletion of either subset abrogates the response. We conclude that the radiation contributes to the gene therapy by causing tumor debulking and increasing the permeability of tumors to infiltration of inflammatory cells. It may also increase the efficiency of transfection.

KEY RESEARCH ACCOMPLISHMENTS

The following accomplishments, in order of importance to the goals of this project were the following.

1. Discovery of the principle of vastly improved potency of siRNAs. Genetic constructs expressing these highly potent smaller siRNAs enable the design and molecular genetic construction of plasmid or genes in other vectors, constructs, which are important when siRNA and CIITA must be given together in one construct. In the current experiments we can use gold beads coated with fixed ratios of genes for CIITA and siRNA to deliver such fixed ratios into each cell which received a bead (shot by the gene gun) However, in applications, more adapted to a clinical setting with certain other vectors, e.g., adenovirus, canarypox virus, lipids, etc. is imperative to put both gene functions within one plasmid, usually under different promoters to avoid promoter competition. The potency and compactness of siRNA-coding genes is a very significant advance.
2. Design, synthesis and validation of human siRNA
3. Design, synthesis and validation of murine siRNA. Even though the murine reagents are needed first in the Task Schedule, we prioritize the human reagent in this list, because it might actually become the final therapeutic and the more experience we have with it early, the better.
4. Advances by our collaborator, Dr. Gali Hillman in understanding the interplay of radiation and enhancement of the activity of our genetic constructs in tumors, in terms of a) enhancing gene expression, b) enhancing immunogenicity by radiation effects on tumor suppressing T cells.

5. Developing interest of major players (oncology big pharmaceutical companies) in hearing about our efforts..

REPORTABLE OUTCOMES

1. Papers. Two papers, which we submitted in 2004 including work under this grant, have been accepted (attached in the appendix). In addition, a third manuscript is being prepared by our collaborator Dr. Gali Hillman reporting the use of using our reagents in curing RM-9 murine prostate tumors in mice. This is a very important animal model of human prostate cancer, in that the tumor cells grow in the prostate of normal mice, with local extension, much as does the human disease. This manuscript will be submitted in 6+ weeks. There is no current draft for review. Any data?

2. Presentations and abstracts. This work has been presented at 3 conferences (Abstracts attached in Appendix)

3. Development of siRNAs. We have had excellent success in development of siRNAs specific for this project. The field of siRNAs in general is moving so fast today, that although we had conceptualized abstractly they might become applied here, at the time of writing the original grant proposal, we did not at that time expect to use them. However, we tried and with rapid and lucky advances, we have discovered they are reliable and give a stronger degree of Ii suppression than we had before with our best reverse gene constructs, particularly in human cells. We therefore focused sharply to obtain the best murine and human constructs, and have validated them. We are now back on track, according to the original Task Schedule of combining the genetic constructs, which lead to biosynthetic expression of a siRNA to suppress Ii protein along with a genetic construct for CITTA to induce MHC class II molecules and the Ii protein in tumor cells.

4. Transfer of genes to adenoviral vector specialists. The RGCs and siRNA for mice have been transferred to Transgene, SA in Strasbourg France under a no-money MTA for them to evaluate after reconstructing into their adenoviral vectors. These vectors have mutations or deletions in two or three genes required for viral replication, and therefore after being used as a vehicle to transport our genetic constructs into tumor cells for transcription and translation to the molecules which mediate the biological effects of our methods. [For your information, a “no money” MTA is the customary method for initial study of another company’s reagents. We consider this to be a very promising step in the judgment of this method by another, sophisticated group of scientists in this field.]

5. Discussions with Novartis-Oncology and with Sanofi-Aventis Oncology. We presented our technology to Aventis Infectious Diseases group in Lyon, France, for the development of vaccines against the H5N1 pandemic flu originating in South East Asia, vaccinia (smallpox), and HIV. They referred us to the Aventis Oncology in Toronto Canada. A meeting is being scheduled for the last two weeks of March. About two years ago, we presented our programs to Novartis Oncology in northern New Jersey. After a recent update, we have been referred to Novartis Oncology in Boston. After a recent phone conference, we expect to present there in April. Again, while there is no “close” on a collaboration or funds to develop into the clinic, the very fact that we are having such conversations indicates support for the value of this program supported under a novel idea grant by the DOD.

Presentations

1. "Generation of the MHC class II+/Ii- phenotype on tumor cells by Ii-RGC or Ii-RNAi leads to a potent tumor cell immunotherapy." Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu. American Association for Cancer Research. Anaheim, CA, April 16-20, 2005
2. "Forcing tumor cells to actively present MHC class II-restricted endogenous tumor antigens by inhibiting MHC class II-associated invariant chain expression by Ii-RGC and Ii-RNAi." Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu. Keystone Symposium "Basic Aspects of Tumor Immunology". Keystone CO, March 19, 2005
3. "Potent Therapeutic Cancer Vaccine Generated by Tumor Irradiation and Genetic Induction of MHC class I+/class II+/Ii- Tumor Phenotype." Gilda Hillman¹, Minzhen Xu², Mingxin Che¹, Eric Von Hofe², Asad Abbas¹ and Yu Wang¹. Keystone Tumor Immunology (C3) Mar 19 - Mar 24, 2005.

Papers

1. Xu M, Lu X, Kallinteris NL, Wang Y, Wu S, von Hofe E, Gulfo J, Humphreys RE, Hillman GG. Immunotherapy of cancer by antisense inhibition of Ii protein, an immunoregulator of antigen selection by MHC class II molecules. *Curr Opin Mol Ther.* 2004; 6:160-5.
2. Wang Y, Xu M, Che M, von Hofe E, Abbas A, Kallinteris NL, Lu X, Liss ZJ, Forman JD, Hillman GG. Curative Anti-Tumor Immune Response Is Optimal with Tumor Irradiation Followed by Genetic Induction of MHC Class I and Class II Molecules and Suppression of Ii protein. *Hum Gene Ther.* 2004; In Press.

CONCLUSIONS

Importance and implications. We consider the discovery of the principle that siRNAs to suppress Ii protein expression, with the demonstration of the design and validation of both murine and human reagents to be of greatest importance to the achievement of our major long term goal – curative immunotherapy of prostate cancer. We expect this route to therapy to far exceed the efficacy of other DNA vaccines, or dendritic cell vaccines with either antigens, tumor fusions, or tumor extracts. This advance would not have been accomplished if this DOD grant had not been in place (or equivalent exploratory funding had been available). This advance will not only speed the pursuit of the remaining Tasks under this grant, but improve the chance we will actually develop clinically useful reagents. That is, the potency of siRNA to suppress Ii protein might enable the immunotherapy, relative to the even very useful reagents we had developed prior to this grant.

Changes in future work to better address the problem. At this point in time, we do not foresee significant changes in the work plan, either in objectives or time course. There was a very useful accomplishment in the past year, to develop the siRNA constructs for experiments with both murine and human cells and in mice. Fortunately, this effort was successful and rapidly accomplished. We now can proceed with the additional experiments with better tools. In the case of the human reagents it is very possible that one of the human siRNAs for Ii suppression, which we have developed, will actually be the reagent, which goes all the way into clinical trials.

Evaluation of the knowledge as a scientific or medical product. The experiments of the past year, demonstrate the robust nature of the fundamental mechanism that by suppressing Ii expression in a tumor cell, which has been converted to the MHC class II molecules-positive and Ii protein-positive phenotype, we can initiate a potent immune response to cure animals of established tumor.

REFERENCES

-None-

APPENDIX

Abstracts

Forcing tumor cells to actively present MHC class II-restricted endogenous tumor antigens by inhibiting MHC class II-associated invariant chain expression by Ii-RGC and Ii-RNAi

Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu

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Immunological cure of tumors depends on initiating both CTL and T helper cell responses to endogenous tumor antigens. In contrast to other tumor immunotherapies, we have developed a novel approach that forces tumor cells actively present endogenous tumor antigens to stimulate CD4⁺ T helper cells by converting tumor cells into MHC class II⁺/Ii⁻ phenotype. Using antisense methods previously, we suppressed expression of the invariant chain (Ii protein) that normally blocks the antigenic peptide binding site of MHC class II molecules during synthesis in the endoplasmic reticulum (ER). In such genetically engineered tumor cells, the MHC class II molecules pick up endogenous antigenic peptides (including tumor antigens), which have been transported into the ER for binding to MHC class I molecules. The simultaneous presentation of these tumor antigens by both MHC class I and II molecules generates a robust and long-lasting anti-tumor immune response. Injecting murine tumors with genes to induce MHC class II and suppress Ii protein expression, results in the cure of a significant number of animals with renal and prostate tumors. An advantage of this strategy is that we do not need to transfect all tumor cells permanently. Transfecting only a fraction of the total tumor cells transiently is sufficient to induce a complete anti-tumor immune response. We have now developed human Ii-RNAi constructs that efficiently inhibit Ii expression in human tumor cell lines including Raji lymphoma cells and 293 kidney cells. Since Ii is monomorphic, one Ii-RNAi construct is suitable for all patients with different HLA-DR alleles. The generation of active human Ii-RNA paves the way to generate MHC class II⁺/Ii⁻ human tumor cells for clinical trial.

Potent Therapeutic Cancer Vaccine Generated by Tumor Irradiation and Genetic Induction of MHC class I+/class II+/Ii- Tumor Phenotype.

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We showed that *in situ* genetic modification of murine RM-9 prostate tumor cells, to express MHC class I and class II molecules and suppress MHC class II associated invariant chain Ii, converts those cells into a cancer vaccine. Gene therapy was delivered intratumorally using plasmids coding for IFN- γ , CIITA, an Ii reverse gene construct (Ii-RGC), and a low IL-2 plasmid dose. Complete tumor regressions, and induction of specific anti-tumor immune response, were obtained only when gene therapy was preceded, one day before, by 8Gy tumor irradiation. By selective *in vivo* depletion of T cell subsets, we demonstrate further that both CD4+ helper T cells and CD8+ cytotoxic T cells are essential for induction of a potent anti-tumor response. *In vitro* colony assays of cells isolated from tumors, 1 day after radiation, show 60% inhibition in division ability, thus radiation causes tumor debulking and increases the probability of cell transfection. Histology of tumors treated with radiation and gene therapy shows complete tumor destruction and that radiation increases the permeability of tumors to infiltration of inflammatory cells. Radiation enhances gene therapy by causing tumor debulking and increasing tumor permeability.

Generation of the MHC class II+/Ii- phenotype on tumor cells by Ii-RGC or Ii-RNAi leads to a potent tumor cell immunotherapy.

Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu

RNAi is a potent method to inhibit specific gene expression. This method has been evaluated as a potential method to treat cancer by, for example, specifically inhibiting oncogene expression. The biggest challenge here is the requirement for in vivo transfection of all tumor cells permanently by RNAi constructs. We have developed Ii-RGC and Ii-RNAi methods to effectively suppress in tumor cells the expression of invariant chain (Ii protein) that normally blocks the antigenic peptide binding site of MHC class II molecules during synthesis in the endoplasmic reticulum (ER). In such genetically engineered tumor cells, both MHC class I and II molecules pick up endogenous antigenic (tumor) peptides in the ER. Simultaneous presentation of these tumor antigens by both MHC class I and II to both CD4+ and CD8+ T cells, generating a robust and long-lasting anti-tumor immune response in mice. An advantage of this strategy is that we do not need to transfect all tumor cells permanently. Transfecting only a fraction of the total tumor cells transiently is sufficient to induce an anti-tumor immune response. We have now generated a human Ii-RNAi construct that effectively inhibits Ii expression in Raji lymphoma cells and 293 kidney cells. Since Ii is monomorphic, one Ii-RNAi construct is sufficient for all patients. Our novel method forces tumor cells to actively present their tumor antigens and thus could lead to a very potent tumor cell immunotherapy.

Papers

1. Xu M, Lu X, Kallinteris NL, Wang Y, Wu S, von Hofe E, Gulfo J, Humphreys RE, Hillman GG. Immunotherapy of cancer by antisense inhibition of Ii protein, an immunoregulator of antigen selection by MHC class II molecules. *Curr Opin Mol Ther.* 2004; 6:160-5.
2. Wang Y, Xu M, Che M, von Hofe E, Abbas A, Kallinteris NL, Lu X, Liss ZJ, Forman JD, Hillman GG. Curative Anti-Tumor Immune Response Is Optimal with Tumor Irradiation Followed by Genetic Induction of MHC Class I and Class II Molecules and Suppression of Ii protein. *Hum Gene Ther.* 2004; In Press.

Immunotherapy of cancer by antisense inhibition of Ii protein, an immunoregulator of antigen selection by MHC class II molecules

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Ii protein suppression is a promising antisense drug-based therapy that dramatically enhances the immunogenicity of tumor cell major histocompatibility complex class II-presented antigenic epitopes. The strength of this approach is that the antisense only needs to be transiently effective in a fraction of the tumor cells. The systemic antitumor immune response generated subsequently eradicates both directly treated cells and distant tumor deposits. The drugs and mechanisms of this therapy are considered, in addition to practical developmental questions.

Keywords Cancer vaccine, Ii antisense, immunotherapy, major histocompatibility complex class II, T-helper cells

Introduction

Thwarting a tumor's escape from immune surveillance

Tumors escape the host immune surveillance, which normally prevents evolution of a malignancy by blocking presentation of immunogenic tumor-associated antigenic epitopes. Additionally, tumors suppress the antitumor immune response by developing active immunosuppressive mechanisms against dominant T-cell-recognized antigenic epitopes of the tumor.

Protein Ii can be inhibited in two ways against these tumor growth-favoring mechanisms. Presentation of tumor-associated antigenic epitopes is enhanced by rearranging the antigen-processing pathway to allow major histocompatibility complex (MHC) class II presentation of tumor self-proteins to T-helper (Th) cells. This method also favors presentation of cryptic and subdominant epitopes to which immunosuppression had never developed previously. The approach can even cure mice of an aggressive prostate tumor that is poorly immunogenic. The biological mechanism of this therapy and the steps taken to bring it to the clinic are reviewed.

Overview of mechanism and therapeutic potential

Normally, antigenic epitopes of cellular self-proteins are transported from the cytoplasm into the endoplasmic

reticulum for binding to newly synthesized MHC class I molecules, but not to MHC class II molecules, which are blocked by the Ii protein. The Ii protein is effectively suppressed with antisense oligonucleotides or reverse gene constructs. This process can be 'rearranged' in the endoplasmic reticulum of such Ii-suppressed tumor cells, to permit MHC class II molecules to bind tumor-associated antigenic epitopes for subsequent presentation to self-surveying T-cells. New epitopes presented by MHC class II molecules stimulate Th cells to enhance the activity of tumor-specific cytotoxic T-lymphocytes (CTLs) and create long-lasting antitumor immunological memory. The repertoire of MHC class II epitopes is also expanded to include 'cryptic epitopes', to which a cancer patient's immune system has never been exposed. The presentation of such epitopes by MHC class II molecules can reverse immunological tolerance to the tumor and cure an established tumor, at least in mice. While this antisense drug is directly therapeutic when injected into tumors that are either naturally MHC class II-positive or made such by co-transfection of genes for MHC class II transactivator (CIITA) or interferon (IFN) γ , it also enables many additional therapies. Ii suppression can enhance various DNA vaccines for tumor or infectious disease antigens. In addition, novel peptide therapeutics can be mined from the repertoire of induced MHC class II epitopes found in MHC class II+/Ii-cultured cells of tumors or antigen-presenting cells (APCs) loaded with antigens relevant to autoimmune disease. In short, antisense-induced Ii suppression enables a wide range of antigenic epitope diagnostics and therapeutics.

Evidence for mechanisms

Antigen processing and presentation pathways potentially blocked during tumorigenesis

All nucleated cells express MHC class I molecules. At the time of their synthesis, these molecules bind antigenic peptides derived from cytoplasmic proteins, which are processed into peptides by proteasomes and transported by the transporter of antigenic peptides (TAP) into the endoplasmic reticulum [1,2]. β_2 -Microglobulin binds to MHC class I molecules, locking them into a conformation that tightly holds the antigenic peptide for the duration of its presentation at the cell surface [3].

MHC class II molecules are normally expressed on professional APCs, such as dendritic cells (DCs), macrophages and B-cells, to induce a CD4⁺ Th cell response. Such Th cells induce DCs to a stage of activation defined as 'licensing', which stimulates and activates specific CTLs [4,5,6].

Normally, MHC class II molecules are blocked by the Ii protein at the time of their synthesis, and receive exogenous antigen selected by the APCs in a post-Golgi compartment. There, certain proteases that cleave the foreign antigen also

cleave and release Ii fragments from the MHC class II molecules in a concerted exchange process, during which antigenic peptide is inserted into the binding site of class II molecules [7,8]. This pathway prevents expression of endogenous peptides by MHC class II molecules in tumor cells. Interestingly, an increase in Ii in hairy leukemic cells and the inverse correlation of Ii expression and tumor-infiltrating lymphocytes in human colon carcinomas suggest an immunosuppressive role for Ii in such malignancies [9,10]. MHC class II molecules appear to influence the antitumor response [11-13,14]. The absence of, or defects in these antigen processing and presentation pathways have been individually reported to promote non-recognition of tumors.

Tumor defense by immunosuppression

Dominant epitopes within a population of tumor-associated antigenic epitopes often induce suppression, which shuts off all antitumor immune responses. This immunosuppression is sometimes associated with the T-cell subset Th3 CD4⁺/CD25⁺ immunoregulatory cells [15•,16,17•,18], which secrete interleukin (IL)-10 and transforming growth factor (TGF)β. This is in contrast to the IFNγ-secreting Th1 cells, a Th subset that promotes CTL responses [19•,20-22].

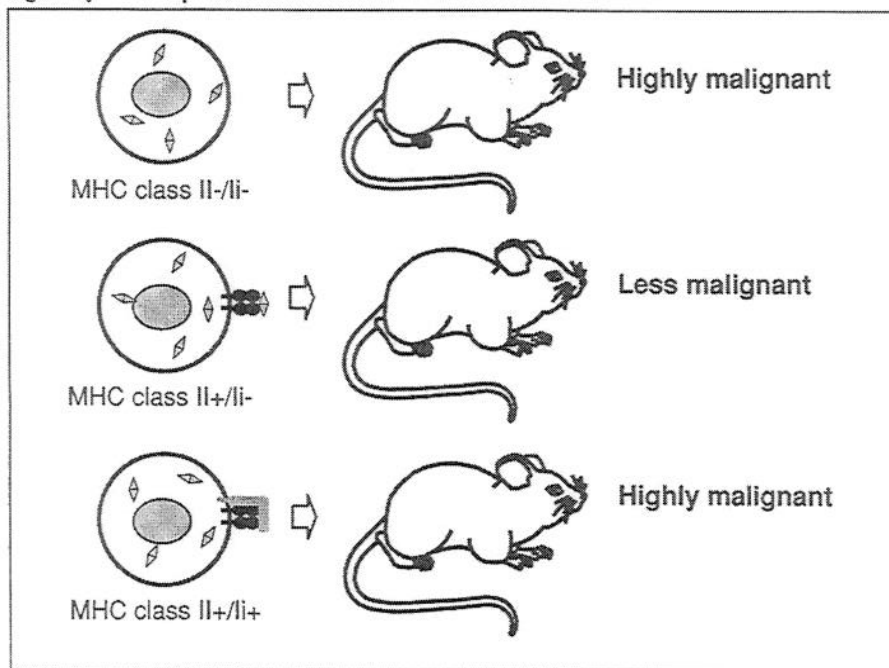
One can hypothesize that by downregulating expression of the Ii protein, a much larger repertoire of MHC class II-restricted epitopes is presented, including cryptic epitopes, which have never been seen before by the immune system. The immune system also recognizes newly exposed, lower-affinity Th cell-recognized epitopes, to which tolerance has never been developed. The response to both types of such non-immunosuppressed epitopes leads to a robust Th1

response, stimulating tumor-specific CTLs and providing long-term immunological memory.

Pioneering experiments

Suzanne Ostrand-Rosenberg and colleagues discovered the principle that MHC class II⁺/Ii⁻ tumor cells, made MHC class II-positive by transfection of genes for syngeneic MHC class II α and β chains, and not expressing Ii protein, are potent anticancer vaccines [23-25]. These researchers characterized the immunological mechanisms in detail (Figure 1). Mice vaccinated with MHC class II gene-transfected Sal 1 sarcoma cells rejected subsequent challenges with the parental MHC class I⁺/MHC class II⁻ Sal 1 cells [23]. However, supratransfecting the engineered MHC class II⁺ tumor cells with the Ii gene abrogated the vaccine potential of the modified cells. The destruction of tumor cell immunogenicity following re-introduction of the Ii gene presumably resulted from its ability to block the binding of endogenous tumor-associated peptides in the endoplasmic reticulum and consequent development of a Th cell response to epitopes from those peptides. Both CD4⁺ Th cells and CD8⁺ CTLs were found to be essential, as deletion of either T-cell subpopulation in adoptive transfer of immunity experiments abrogated the protective effect [25]. This study is consistent with the established principle that the activation of CD4⁺ Th cells by MHC class II⁺/Ii⁻ tumor cells is required for optimal activation and expansion of CD8⁺ CTLs. Finally, the importance of Th cells activated by MHC class II⁺/Ii⁻ tumor cells in prolonging immunological memory was demonstrated by the protection of mice against tumor challenge for extended periods of time after vaccination [25].

Figure 1. Tumor malignancy and Ii expression.



When tumor cells are MHC class II⁺ and Ii⁺, the tumor is highly malignant, as Ii blocks presentation of tumor antigen through MHC class II. The same situation applies to tumor cells that are MHC class II⁻ and Ii⁻. Only when they are MHC class II⁺ and Ii⁻ do tumor cells present the tumor antigen by MHC class II to activate CD4⁺ T-cells, which in turn activate CD8⁺ CTLs.

Endogenous proteins from different intracellular compartments of a tumor cell can be presented by MHC class II+/Ii- tumor cells [26,27]. This is demonstrated by the finding that cells engineered to express the gene for hen egg lysozyme (HEL) with a leader sequence targeting the endoplasmic reticulum, presented MHC class II HEL epitopes to HEL-specific CD4+ T-cells. Similar to the studies described above, co-expression of the Ii protein in these cells inhibited presentation of the HEL epitopes [27].

Antisense suppression of Ii in tumors induced for MHC class II and Ii expression

Given the numerous different MHC class II alleles in humans, even only at the human leukocyte antigen (HLA)-DR locus, generating the MHC class II+/Ii- phenotype by transfecting a patient's tumor with genes matching MHC class II α and β chain types is not a practical clinical approach. We developed an alternative approach using a non-polymorphic gene construct active in all humans. Expression of endogenous MHC class II molecules can be induced using *CIITA* or *IFN γ* genes, while the co-induced Ii protein is suppressed with a reverse gene construct (*Ii*-RGC) targeting Ii mRNA. *Ii*-RGC acts at the mRNA level to prevent translation of the Ii protein. Initially, Ii antisense oligonucleotides were used to suppress Ii expression in MHC class II+/Ii+ tumor cells [28]. In these studies, using the Sal 1 sarcoma model, MHC class II+ cells treated with Ii antisense oligonucleotides demonstrated good vaccine potency against challenge by parental tumor.

Subsequently, we created expressible Ii antisense constructs (*Ii*-RGC) for inclusion into DNA vaccine vectors. These constructs were cloned into expressible plasmids and/or engineered into adenoviruses to evaluate different methods of tumor gene transfection [29,30]. The *Ii*-RGCs were evaluated by stable or transient DNA transfections using several murine tumor cell lines; the most active, *Ii*-RGC(-92,97) (A in the AUG start codon is position 1), was selected for *in vivo* studies.

While some tumor cell lines were MHC class II+/Ii+, many of the lines we tested were MHC class II-/Ii-. In these cell lines, the *CIITA* or *IFN γ* gene, or both, were co-transfected *in vitro* with *Ii*-RGC(-92,97) to create the MHC class II+/Ii- phenotype, as detected by immunostaining [29-31]. *In vivo* induction of this phenotype in established tumors was also generated by intratumoral injection of *Ii*-RGC and *CIITA* plasmids delivered in liposomes [29,31], or using recombinant adenoviral vectors containing *Ii*-RGC(-92,97), *CIITA* and *IFN γ* [30]. While the *CIITA* gene used in the mice studies is human, its gene product fortuitously functions well on the murine promoters for MHC class II and *Ii* genes [30].

Ii suppression therapy of animal tumors

We tested our therapeutic strategy for both tumor prevention (vaccination to protect against tumor challenge) and tumor cure (therapy of established tumors). In a prevention model using Sal 1, tumor cells treated with antisense oligonucleotide-suppressed Ii protein were much more potent than Sal 1 cells treated with sense and mismatch antisense oligonucleotides [28]. In cure models, the *in vivo* activities of these therapeutic constructs were tested by intratumoral injection of plasmids or adenoviral

vectors in established subcutaneous tumors of both the Renca renal adenocarcinoma and RM-9 prostate carcinoma murine models [29,31].

In tumor cure models, complete regression of established tumors was achieved. Renca tumor regression was observed in approximately 50% of mice following four intratumoral injections of *CIITA* and *Ii*-RGC plasmid constructs over 4 days administered with a subtherapeutic dose of IL-2 plasmid [29]. In these tumor nodules, *in situ* induction of the MHC class II+/Ii- phenotype was confirmed by immunohistochemical staining of tumor sections [29]. The injection of established Renca tumors with recombinant adenovirus, containing *CIITA*, *IFN γ* and *Ii*-RGC, combined with a low suboptimal dose of IL-2 adenovector, induced complete tumor regression in approximately 60 to 70% of mice and complete protection against Renca tumor rechallenge [30]. These studies using the weakly immunogenic Renca MHC class I+/class II- model confirm that induction of the MHC class II+/Ii- phenotype triggers an antitumor immune response with long-lasting systemic immunity.

In the aggressive, poorly immunogenic MHC class I-/class II- RM-9 prostate tumor model, *in situ* induction of the MHC class I+/class II+/Ii- phenotype by intratumoral injection of the plasmids *pCIITA*, *pIFN γ* and *pIi*-RGC caused a significant but transient inhibition of tumor growth, even when suboptimal doses of pIL-2 were added to the tumor nodule treatment (Table 1) [31]. Complete responders were observed only when tumor nodules were first irradiated prior to gene therapy [31]. In a subsequent experiment, established RM-9 subcutaneous tumors were selectively irradiated and treated 1 day later with intratumoral plasmid gene therapy using a mixture of the plasmids *pCIITA*, *pIFN γ* and *pIi*-RGC combined with a subtherapeutic dose of pIL-2 for 4 consecutive days. Table 1 demonstrates that intratumoral treatment with all four plasmids induced complete tumor regression in more than 50% of the mice only when tumor irradiation was administered 1 day prior to gene therapy. Mice rendered tumor free by radiation and intratumoral gene therapy and re-challenged on day 64 were protected against RM-9 challenge but not against syngeneic EL-4 tumor challenge (Table 1) [31]. These findings demonstrate that in the RM-9 model, radiation enhanced the therapeutic efficacy of intratumoral gene therapy for *in situ* induction of tumor-specific immunogenicity.

IL-2 at subtherapeutic doses is probably acting as an adjuvant to strengthen and sustain the activation of T-cells. Our recent studies demonstrate that both CD4+ Th cells and CD8+ CTLs are essential for the induction of a complete antitumor response in the RM-9 model, specifically, the *in vivo* depletion of either T-cell subset abrogates this response [GG Hillman *et al*, unpublished data]. These studies are consistent with induction of both a Th response and a CTL response by our gene therapy approach, resulting in long-lasting tumor immunity. Furthermore, in both the Renca and RM-9 model, omission of the *Ii*-RGC vector in the intratumoral gene therapy protocol led to a significantly lower incidence of complete tumor regressions, emphasizing the essential role of Ii suppression in the induction of a complete and systemic antitumor immune response [30,31].

Table 1. Antitumor response of RM-9 tumor-bearing mice treated with radiation and plasmid gene therapy, and response of cured animals to re-challenge.

Treatment group	Proportion of tumor-free mice		
	Post-treatment	Post-challenge	
		RM-9	EL-4
Control	0/10	-	-
<i>pCIITA</i> + <i>pIFNγ</i> + <i>pIL-2</i> + <i>pIi-RGC</i>	0/7	-	-
Radiation	0/11	-	-
Radiation + <i>pCIITA</i> + <i>pIFNγ</i> + <i>pIL-2</i> + <i>pIi-RGC</i>	7/13	7/7	-
Radiation + <i>pCIITA</i> + <i>pIFNγ</i> + <i>pIL-2</i> + <i>pIi-RGC</i>	3/6	-	0/3
Naive mice	N/A	0/5	0/5

Established RM-9 tumors of 0.3 to 0.4 cm were irradiated with 8 Gy photons on day 6 after cell injection. From day 7, tumors were injected with the plasmids *pCIITA* + *pIFN γ* + *pIL-2* + *pIi-RGC* for 4 consecutive days. The proportion of tumor-free mice is presented at the end of the observation period, on day 64, following radiation and plasmid therapy. Tumor-free mice and naive mice were re-challenged with RM-9 cells or genetically identical EL-4 cells on day 64, the proportion of tumor-free mice by day 30 after challenge is reported [31]. N/A not applicable. (Reproduced with permission from Mary Ann Liebert and Hillman GG, Xu M, Wang Y, Wright JL, Lu X, Kallinteris NL, Tekyi-Mensah S, Thompson TC, Mitchell MS, Forman JD: *Radiation improves intratumoral gene therapy for induction of cancer vaccine in murine prostate carcinoma*. *Human Gene Therapy* (2003) 14(8):763-775. © 2003 Mary Ann Liebert).

Role of radiation

The role of radiation in enhancing intratumoral gene therapy for the induction of cancer immunity is particularly intriguing. Possible mechanisms for radiation enhancement of gene therapy include the following: (i) the DNA damaging and debulking effect slows tumor growth to allow time for the immune response to be effective [31,32]; (ii) radiation-induced tissue damage mobilizes inflammatory cells in the tumor vicinity that can be readily activated by *in situ* gene therapy [32]; (iii) radiation is hypothesized to limit suppressive immunoregulatory T-cells; and (iv) radiation increases gene transduction efficiency and duration of expression of surviving tumor cells, thus improving the efficiency of *in situ* genetic modification, leading to an immune response that eradicated remaining tumor cells. Stevens and colleagues demonstrated that radiation improves the transfection efficiency of plasmid DNA in normal and malignant cells *in vitro* resulting from radiation-induced DNA breaks and DNA repair mechanisms [33,34]. They demonstrated that radiation followed by plasmid or adenoviral transfection caused enhanced integration of the transgene. Preliminary studies in the Renca model using intratumoral injections of the *IL-2* adenovector demonstrated that radiation potentiates the genetic modification of the tumor cells by increasing both the level and duration of expression of transfected genes [GG Hillman, unpublished data].

Unique features of li antisense therapy

Making antisense therapy effective clinically for other cancer molecular targets has two daunting obstacles. One is the requirement for delivering antisense reagents to all tumor cells, and the other is the requirement for suppressing the target gene continuously [35,36]. Comparatively, li suppression for tumor immunotherapy has three advantages. Firstly, it stimulates tumor antigen-specific CD4⁺ Th cell activation without interrupting MHC class I presentation for CD8⁺ CTL activation. Simultaneous activation of both CD4⁺ and CD8⁺ T-cells creates a more robust tumor cell immunotherapy. Secondly, inhibition of li protein expression does not need to occur in all tumor cells, unlike antisense targeting other tumor genes. Thirdly, the li inhibition does not need to be continuous, as a transient inhibition of li protein (3 to 5 days) in a portion of tumor cells is

sufficient to generate a strong antitumor immune response [30,31]. After a specific antitumor immune response has been generated, the immune system will eradicate the residual tumor cells until all tumor cells sharing the same tumor antigens have been killed.

Future directions

Issues to address in the design of clinical trials

The biological mechanism of this therapy leads to special considerations for the design and evaluation of clinical trials. For initial trials, patients with metastatic disease poorly responsive to other means of therapy will be targeted. Although chemotherapy in such patients suppresses the anticancer immune response, once leukocyte counts have rebounded, this mode of immunotherapy eliciting an immune response within injected tumor masses, is possible. Readily accessible, ie, subcutaneous axillary masses in breast carcinoma might be preferred because they can be approached with ease. However, many radiologists feel that any tumor they can visualize is a candidate for intratumor injections, for example, colon adenocarcinoma. While melanoma has been a classic target for tumor immunotherapy procedures, the frequency of accessible masses, frequency of patients with metastatic disease, and a variable and sometimes long clinical course, indicates against melanoma for initial trials.

Injection of a tumor with *Ii*-RGC is expected to lead to presentation of normal tissue antigens, as well as tumor determinates. While we have not observed histological signs of autoimmunity in mice with cancers treated by this method, such reactions against self-tissues probably occur. Those reactions on balance might promote tumor cure. Vitiligo is observed in some melanoma patients vaccinated against melanoma-associated tumor antigens. Signs of autoimmunity to normal tissue antigens of the tumor will be monitored in trials of this immunotherapy.

Additional therapeutic uses for li suppression

This technology can also be used to identify novel MHC class II epitopes in tumor and autoimmune disease-related antigens. Specifically, tumor- and autoimmune disease-related antigenic epitopes can be identified by high performance liquid chromatography (HPLC) tandem mass

spectrometry of acid-eluted peptides from immune purified MHC class II molecules. The eluted-peptide HPLC patterns can then be compared with those of MHC class II+/Ii+ cells to identify the putative Ii suppression of specific peaks [37•]. The molecular weight of a peptide in such a peak can be precisely determined by tandem mass spectrometry and a sequence assigned from the weight.

Ii suppression can also enhance the efficiencies of DNA vaccines and of gene-transfected DC vaccines. The biological effect of Ii suppression might enhance the immune response to a co-delivered DNA vaccine containing a gene for a malignant or infectious antigen [38-40]. When a cell (eg, a professional APC) is transfected with a gene encoding an antigen and an Ii suppression construct, this cell expresses the antigen endogenously, while Ii is suppressed to produce the MHC class II+/Ii- phenotype. Consequently, the transfected cell can now present antigenic epitopes through MHC class II and I to activate both CD4+ Th cells and CD8+ CTLs, respectively. The result is a stronger DC or DNA vaccine.

Conclusion

We have developed a novel antisense approach to convert tumor cells into MHC class II+ and Ii- APCs. Suppression of Ii gene expression in the endoplasmic reticulum leads to the simultaneous presentation of endogenous tumor antigens by both MHC class I and II molecules and generates a robust and long-lasting antitumor immune response. Injecting murine tumors with genes that induce the MHC class II+/Ii- phenotype in tumor cells causes complete tumor regression (ie, cure) in a significant number of animals with renal and prostate tumors.

Compared with other antisense applications, our Ii antisense method has two major advantages; Ii only needs to be suppressed temporarily and it does not need to be suppressed in all tumor cells. Analogous human Ii antisense gene constructs that are suitable for most patients and cancers have been developed.

Conversion of cancer cells into APCs via induction of the MHC class II+/Ii- phenotype *in vivo* by this method is simple to achieve. The induction of MHC class II molecules and Ii by *CIITA* and suppression of Ii by *Ii*-RGC antisense is a clinically practical strategy, as both *CIITA* and *Ii* genes are monomorphic. Transduction of a focal population of cells within a tumor mass stimulates an immune response that potentially leads to destruction of all tumor cells within that mass as well as in distant metastases. This is particularly relevant to the anticipated clinical use where local treatment to induce a potent systemic antitumor immune response is the goal.

References

- of outstanding interest
- of special interest
- 1. Karttunen JT, Lehner PJ, Gupta SS, Hewitt EW, Cresswell P: Distinct functions and cooperative interaction of the subunits of the transporter associated with antigen processing (TAP). *Proc Natl Acad Sci USA* (2001) 98(13):7431-7436.
- 2. Bryant PW, Lennon-Dumenil AM, Fiebigler E, Lagaudriere-Gesbert C, Ploegh HL: Proteolysis and antigen presentation by MHC class II molecules. *Adv Immunol* (2002) 80:71-114.
- 3. Kessler BM, Glas R, Ploegh HL: MHC class I antigen processing regulated by cytosolic proteolysis-short cuts that alter peptide generation. *Mol Immunol* (2002) 39(3-4):171-179.
- 4. Ridge JP, Di Rosa F, Matzinger P: A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* (1998) 393(6684):474-478.
- This paper provides critical evidence for DC licensing and CTL activation being a two-step process.
- 5. Germain RN: Ligand-dependent regulation of T cell development and activation. *Immunol Res* (2003) 27(2-3):277-286.
- A good review of the effects of ligands on T-cell biology.
- 6. Dorfman JR, Stefanova II, Yasutomo K, Germain RN: Response to 'Class II essential for CD4 survival'. *Nat Immunol* (2001) 2(2):136-137.
- 7. Xu M, Capraro GA, Daibata M, Reyes VE, Humphreys RE: Cathepsin B cleavage and release of invariant chain from MHC class II molecules follow a staged pattern. *Mol Immunol* (1994) 31(10):723-731.
- 8. Daibata M, Xu M, Humphreys RE, Reyes VE: More efficient peptide binding to MHC class II molecules during cathepsin B digestion of Ii than after Ii release. *Mol Immunol* (1994) 31(4):255-260.
- 9. Spiro RC, Sairenji T, Humphreys RE: Identification of hairy cell leukemia subset defining p35 as the human homologue of Ii. *Leukemia Res* (1984) 8(1):55-62.
- 10. Jiang Z, Xu M, Savas L, LeClair P, Banner BF: Invariant chain expression in colon neoplasms. *Virchows Arch* (1999) 435(1):32-36.
- 11. Georgiannos SN, Renaut A, Goode AW, Sheaff M: The immunophenotype and activation status of the lymphocytic infiltrate in human breast cancers, the role of the major histocompatibility complex in cell-mediated immune mechanisms, and their association with prognostic indicators. *Surgery* (2003) 134(5):827-834.
- 12. Casares N, Ambillaga L, Sarobe P, Dotor J, Lopez-Diaz De Cerio A, Melero I, Prieto J, Borras-Cuesta F, Lasarte JJ: CD4+/CD25+ regulatory cells inhibit activation of tumor-primed CD4+ T cells with IFN- γ -dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J Immunol* (2003) 171(11):5931-5939.
- 13. Chil A, Sikorski M, Bobek M, Jakiel G, Marcinkiewicz J: Alterations in the expression of selected MHC antigens in premalignant lesions and squamous carcinomas of the uterine cervix. *Acta Obstet Gynecol Scand* (2003) 82(12):1146-1152.
- 14. Schroers R, Shen L, Rollins L, Xiao Z, Sonderstrup G, Slawin K, Huang XF, Chen SY: Identification of MHC class II-restricted T-cell epitopes in prostate-specific membrane antigen. *Clin Cancer Res* (2003) 9(9):3260-3271.
- This paper provides an example of searching for MHC class II tumor epitopes.
- 15. Antony PA, Restifo NP: Do CD4+ CD25+ immunoregulatory T cells hinder tumor immunotherapy? *J Immunother* (2002) 25(3):202-206.
- This paper provides evidence for T-suppressor cells in cancer.
- 16. Thornton AM, Shevach EM: Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *Immunology* (2000) 164(1):183-190.
- 17. Weiner HL: Induction and mechanism of action of transforming growth factor- β -secreting Th3 regulatory cells. *Immunol Rev* (2001) 182:207-214.
- A review of experiments demonstrating the properties of Th3 cells.
- 18. Woo EY, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, Kaiser LR, June CH: Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* (2002) 168(9):4272-4276.
- 19. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, Delleman TA, Antony PA, Spiess PJ, Palmer DC, Hellmann DM et al: Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med* (2003) 198(4):569-580.
- This paper provides evidence for T-cell suppression in cancer.
- 20. Krug A, Veeraswamy R, Pekosz A, Kanagawa O, Onanue ER, Colonna M, Cella M: Interferon-producing cells fail to induce proliferation of naive T cells but can promote expansion and T helper 1 differentiation of antigen-experienced unpolarized T cells. *J Exp Med* (2003) 197(7):899-906.

21. Chamoto K, Kosaka A, Tsuji T, Matsuzaki J, Sato T, Takeshima T, Iwakabe K, Togashi Y, Koda T, Nishimura T: Critical role of the Th1/Tc1 circuit for the generation of tumor-specific CTL during tumor eradication *in vivo* by Th1-cell therapy. *Cancer Sci* (2003) 94(10):924-928.
22. Ho WY, Yee C, Greenberg PD: Adoptive therapy with CD8+ T cells: It may get by with a little help from its friends. *J Clin Invest* (2002) 110(10):1415-1417.
23. Clements VK, Baskar S, Armstrong TD, Ostrand-Rosenberg S: Invariant chain alters the malignant phenotype of MHC class II+ tumor cells. *J Immunol* (1992) 149(7):2391-2396.
24. Baskar S, Glimcher L, Nabavi N, Jones RT, Ostrand-Rosenberg S: Major histocompatibility complex class II+B7-1+ tumor cells are potent vaccines for stimulating tumor rejection in tumor-bearing mice. *J Exp Med* (1995) 181(2):619-629.
25. Armstrong TD, Clements VK, Martin BK, Ting JP, Ostrand-Rosenberg S: Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. *Proc Natl Acad Sci USA* (1997) 94(13):6886-6891.
26. Qi L, Ostrand-Rosenberg S: MHC class II presentation of endogenous tumor antigen by cellular vaccines depends on the endocytic pathway but not H2-M. *Traffic* (2000) 1(2):152-160.
27. Qi L, Rojas JM, Ostrand-Rosenberg S: Tumor cells present MHC class II restricted nuclear and mitochondrial antigens and are the predominant antigen presenting cells *in vivo*. *J Immunol* (2000) 165(10):5451-5461.
28. Qiu G, Goodchild J, Humphreys RE, Xu M: Cancer immunotherapy by antisense suppression of li protein in MHC-class II-positive tumor cells. *Cancer Immunol Immunother* (1999) 48(9):499-506.
29. Lu X, Kallinteris NL, Li J, Wu S, Li Y, Jiang Z, Hillman GG, Gulfo JV, Humphreys RE, Xu M: Tumor immunotherapy by converting tumor cells to MHC class II-positive, li protein-negative phenotype. *Cancer Immunol Immunother* (2003) 52(10):592-598.
30. Hillman GG, Kallinteris NL, Li J, Wang Y, Lu X, Li Y, Wu S, Wright JL, Slos P, Gulfo JV, Humphreys RE, Xu M: Generating MHC class II+/li-phenotype after adenoviral delivery of both an expressible gene for MHC class II inducer and an antisense li-RNA construct in tumor cells. *Gene Ther* (2003) 10(17):1512-1518.
31. Hillman GG, Xu M, Wang Y, Wright JL, Lu X, Kallinteris NL, Tekyi-Mensah S, Thompson TC, Mitchell MS, Forman JD: Radiation improves intratumoral gene therapy for induction of cancer vaccine in murine prostate carcinoma. *Hum Gene Ther* (2003) 14(8):763-775.
32. Dezso B, Haas GP, Hamzavi F, Kim S, Montecillo EJ, Benson PD, Pontes JE, Maughan RL, Hillman GG: The mechanism of local tumor irradiation combined with interleukin 2 therapy in murine renal carcinoma: Histological evaluation of pulmonary metastases. *Clin Cancer Res* (1996) 2(9):1543-1552.
33. Zeng M, Cerniglia GJ, Eck SL, Stevens CW: High-efficiency stable gene transfer of adenovirus into mammalian cells using ionizing radiation. *Hum Gene Ther* (1997) 8(9):1025-1032.
34. Stevens CW, Zeng M, Cerniglia GJ: Ionizing radiation greatly improves gene transfer efficiency in mammalian cells. *Hum Gene Ther* (1996) 7(14):1727-1734.
35. Thierry AR, Vives E, Richard JP, Prevot P, Martinand-Mari C, Robbins I, Lebleu B: Cellular uptake and intracellular fate of antisense oligonucleotides. *Curr Opin Mol Ther* (2003) 5(2):133-138.
- This paper outlines the role of antisense oligonucleotide therapeutics.
36. Sazani P, Vacek MM, Kole R: Short-term and long-term modulation of gene expression by antisense therapeutics. *Curr Opin Biotechnol* (2002) 13(5):468-472.
37. Peakman M, Stevens EJ, Lohmann T, Narendran P, Dromey J, Alexander A, Tomlinson AJ, Trucco M, Gorga JC, Chiciz RM: Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4. *J Clin Invest* (1999) 104(10):1449-1457.
- This paper details provocative methodology to discover biologically relevant MHC class II epitopes.
38. Wolchok JD, Gregor PD, Nordquist LT, Slovin SF, Scher HI: DNA vaccines: An active immunization strategy for prostate cancer. *Semin Oncol* (2003) 30(5):659-666.
39. Ponsaerts P, Van Tendeloo VF, Berneman ZN: Cancer immunotherapy using RNA-loaded dendritic cells. *Clin Exp Immunol* (2003) 134(3):378-384.
40. Engleman EG: Dendritic cell-based cancer immunotherapy. *Semin Oncol* (2003) 30(3 Suppl 8):23-29.

Curative Antitumor Immune Response is Optimal with Tumor Irradiation Followed by Genetic Induction of Major Histocompatibility Complex Class I and Class II Molecules and Suppression of Ii Protein

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ABSTRACT

Transfecting genes into tumors, to upregulate major histocompatibility complex (MHC) class I and class II molecules and inhibit MHC class II associated invariant chain (Ii), induces a potent anti-tumor immune response when preceded by tumor irradiation, in murine RM-9 prostate carcinoma. The transfected genes are cDNA plasmids for interferon- γ (pIFN- γ), MHC class II transactivator (pCIITA), an Ii reverse gene construct (pIi-RGC), and a subtherapeutic dose of adjuvant IL-2 (pIL-2). Responding mice rejected challenge with parental tumor and demonstrated tumor-specific cytotoxic T lymphocytes (CTLs). We have extended our investigation to determine the relative roles of each one of the four plasmids pIFN- γ , pCIITA, pIi-RGC, and pIL-2 in conjunction with radiation for the induction of a curative immune response. Upregulation of MHC class I with pIFN- γ or class II with pCIITA, separately, does not lead to a complete response even if supplemented with pIL-2 or pIi-RGC. An optimal and specific antitumor response is achieved in more than 50% of the mice when, after tumor irradiation, tumor cells are converted *in situ* to a MHC class I+/class II+/Ii- phenotype with pIFN- γ , pCIITA, pIi-RGC, and pIL-2. We demonstrate further that both CD4⁺ helper T cells and CD8⁺ cytotoxic T cells are essential for induction of an antitumor response because *in vivo* depletion of either subset abrogates the response. The radiation contributes to the gene therapy by causing tumor debulking and increasing the permeability of tumors to infiltration of inflammatory cells.

OVERVIEW SUMMARY

We showed that genetic modification of murine RM-9 prostate tumor cells, *in situ*, to express major histocompatibility complex (MHC) class I and class II molecules and suppress MHC class II associated invariant chain Ii, converts those cells into a cancer vaccine. Gene therapy was delivered intratumorally using plasmids coding for interferon (IFN)- γ , CIITA, and an Ii reverse gene construct (Ii-RGC), and a subtherapeutic adjuvant dose of interleukin (IL)-2 plasmid. Complete tumor regressions, associated with the induction of a specific antitumor immune response, were obtained only when gene therapy was preceded by tumor irradiation. We

now demonstrate that each of the four plasmids IFN- γ , CIITA, Ii-RGC, and IL-2, combined with tumor irradiation, are required for optimal antitumor activity. This approach causes the induction of a strong antitumor immune response, in which CD4⁺ T helper cells and CD8⁺ cytotoxic T cells play an essential role. Radiation enhances gene therapy by causing tumor debulking and permeability.

INTRODUCTION

SEVERAL METHODS to induce an immune response against prostate cancer, including cytokines or peptides delivered

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via expression constructs, dendritic cells or *ex vivo* vaccination with cytokine gene-modified cells, induced an immune response but with only limited clinical results (Hillman *et al.*, 1999; Simons *et al.*, 1999; Steiner and Gingrich, 2000; Beldegrun *et al.*, 2001; Harrington *et al.*, 2001; Trudel *et al.*, 2003). Several clinical trials based on immunotherapy, cancer vaccines, or gene therapy to induce an antitumor immune response did not cure advanced metastatic and bulky disease, but might be effective when combined with surgery, chemotherapy, or radiation to decrease the tumor burden (Teh *et al.*, 2001). While radiation using megavoltage photons (x rays) is conventional therapy for localized prostate carcinoma, residual disease resulting in disease progression occurs in a significant number of patients (Powell *et al.*, 1997; Gray *et al.*, 2001). A high percentage (40–50%) of patients with newly diagnosed prostate cancer have intermediate- to high-risk localized prostate cancer and are at high risk of recurrence after radiotherapy, probably as a result of residual radioresistant tumor cells and occult micrometastases (Forman *et al.*, 1998; Gray *et al.*, 2001). Combining radiation with an effective cancer vaccine has the potential to eradicate tumor deposits and micrometastases, both locally and at distant sites. We have developed a novel therapeutic approach for the treatment of locally advanced prostate cancer that consists of administering local tumor irradiation with the genetic induction of cancer vaccine in tumor nodules, *in situ*, using the murine RM-9 prostate carcinoma preclinical model (Hillman *et al.*, 2003b).

To create a cancer vaccine that triggers a specific and systemic antitumor immune response, tumor-associated antigens (TAA) on tumor cells must be presented to helper T cells and cytotoxic T cells in the context of major histocompatibility complex (MHC) molecules via antigen presenting cells (APC) (Hillman *et al.*, 2004a). We have designed a strategy to convert RM-9 murine prostate carcinoma cells *in vivo* into APCs by simultaneously upregulating MHC molecules and suppressing the invariant chain (Ii). At the time of their synthesis in the endoplasmic reticulum (ER), unlike MHC class I molecules, MHC class II molecules cannot bind endogenous antigenic peptides (Xu *et al.*, 2004). The MHC class II molecule binding site initially is blocked by Ii, a membrane glycoprotein that acts as a transport-chaperone and inhibitor of binding of endogenous antigens to newly synthesized MHC class II molecules (Koch *et al.*, 1982; Stockinger *et al.*, 1989; Guagliardi *et al.*, 1990). This mechanism allows only exogenous peptide binding to MHC class II molecules and limits the endogenous repertoire of peptides presented by MHC Class II molecules (Clements *et al.*, 1992; Qi *et al.*, 2000; Hillman *et al.*, 2004a; Xu *et al.*, 2004). Inhibition or absence of Ii protein increases presentation of endogenous tumor peptides by class II molecules to helper T cells, the activation of which is essential for induction of antitumor immunity (Xu *et al.*, 2000, 2004; Hillman *et al.*, 2004a). These concepts are based on pioneering work by Ostrand-Rosenberg and colleagues demonstrating that transfecting syngeneic genes for MHC class II α and β chains into a MHC class II-negative tumor creates a tumor cell vaccine, which protects against challenge with the parental tumor (Ostrand-Rosenberg *et al.*, 1990; Clements *et al.*, 1992; Armstrong *et al.*, 1997, 1998b,a; Qi *et al.*, 2000). Supratransfecting these engineered MHC class II-positive tumor cells with a gene for the Ii protein abrogated the

vaccine potential of the modified cells (Clements *et al.*, 1992; Armstrong *et al.*, 1997).

We have shown that suppression of Ii protein synthesis by antisense methods enables MHC class II molecules to present TAA epitopes to helper T cells (Hillman *et al.*, 2003a,b; Lu *et al.*, 2003). Expressible Ii antisense reverse gene constructs (Ii-RGC) were engineered for inclusion into DNA vaccine vectors. These constructs were cloned into plasmids and/or engineered into adenoviruses to evaluate different methods of tumor gene transfection (Hillman *et al.*, 2003a,b; Lu *et al.*, 2003). The transfection of MHC class I and class II negative RM-9 cells, *in vitro*, using DNA plasmids encoding the genes for interferon- γ (pIFN- γ) and the MHC class II transactivator (pCIITA) caused upregulation of MHC class I molecules and MHC class II molecules, respectively (Hillman *et al.*, 2003b). The Ii protein, coinduced by pCIITA transfection, was suppressed by an adenovirus encoding for an antisense reverse gene construct (Ii-RGC) (Hillman *et al.*, 2003b). *In vivo*, the genes were delivered intratumorally in established RM-9 tumors using the plasmids pIFN- γ , pCIITA, pli-RGC, and a subtherapeutic dose of a DNA plasmid encoding the interleukin-2 gene (pIL-2) used as an adjuvant cytokine. This treatment led to significant tumor growth inhibition but not to complete tumor regression (Hillman *et al.*, 2003b). We showed that radiation of established tumors followed, a day later, by intratumoral injection of pIFN- γ , pCIITA, pli-RGC, and pIL-2, resulted in complete tumor regression in more than 50% of the mice (Hillman *et al.*, 2003b). Complete responders are defined by tumor regression and disappearance, and remaining tumor-free for more than 60–90 days of follow-up. Moreover, these complete responders were immune to rechallenge with parental tumor cells and demonstrated tumor-specific cytotoxic T cell activity (Hillman *et al.*, 2003b). These data demonstrated that radiation enhanced the therapeutic effect of intratumoral gene therapy for *in situ* induction of a long-lasting tumor-specific immune response.

We have now investigated the requirement for each one of the four gene vectors, IFN- γ , CIITA, Ii-RGC, and IL-2, for the induction of the cancer vaccine when combined with prior tumor irradiation. We found that radiation and gene therapy using only the adjuvant plasmids IL-2, Ii-RGC, or both together did not cause complete tumor regression. Upregulation of MHC class I molecules with pIFN- γ , or class II molecules with pCIITA, respectively, was not sufficient to lead to a complete response even if supplemented with pIL-2 or pli-RGC. An optimal and specific antitumor response was achieved in more than 50% of mice when, after tumor irradiation, tumor cells are converted *in situ* to the MHC class I+/class II+/Ii- phenotype by gene therapy with IFN- γ , CIITA, Ii-RGC and supplemented with adjuvant cytokine plasmid IL-2. Selective *in vivo* depletion of CD4⁺ helper T cells or CD8⁺ cytotoxic T cells abrogated the response to radiation and gene therapy confirming that these two T cell subsets play an essential role in the induction of complete antitumor immune response. Radiation caused significant debulking of the tumors *in situ* as demonstrated by significant colony formation inhibition of cells isolated from tumors at early time points between days 1–13 after radiation treatment. Apoptosis was documented histologically in these tumors as early as 1 day after radiation, at the time gene therapy was initiated. Complete tumor destruction by combined gene therapy was deter-

mined by lack of colony formation of cells isolated from these tumors and by histologic observation.

MATERIALS AND METHODS

Tumor model

The RM-9 murine prostate cancer cell line, provided by Dr. Timothy Thompson (Baylor College of Medicine, Houston, TX), was derived from independent primary prostate tumors induced in the Zipras/myc-9-infected mouse prostate reconstitution (MPR) model system using C57BL/6 mice as previously described (Thompson *et al.*, 1989). Cells were maintained *in vivo* by serial subcutaneous passages and were also cultured *in vitro* in complete medium (CM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100U/ml penicillin G, 100 μ g/ml streptomycin, and 10 mM HEPES buffer (Gibco BRL, Life Technologies, Grand Island, NY) (Hall *et al.*, 1997; Nasu *et al.*, 1999). Cells were passaged, *in vitro*, by trypsinization using 0.25% trypsin. For *in vivo* implantation, RM-9 cells were washed in Hanks' balanced salt solution (HBSS) and injected subcutaneously at 2×10^5 cells in 0.1 ml HBSS, in 4–6 week old C57BL/6 mice (Harlan Sprague Dawley Inc, Indianapolis, IN). For proper alignment in the radiation apparatus, cells were injected in the middle of the back, 1.5 cm from the tail (Hillman *et al.*, 2003b). Mice were shaved prior to injection for accurate location of the injection site and for monitoring tumor growth. Mice were housed and handled in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. The animal protocol was approved by the Wayne State University Animal Investigation Committee.

Gene expression vectors:

The plasmids pEF/Bsd/CIITA (pCIITA) and pcDNA (3)/IFN- γ (pIFN- γ) were constructed with cytomegalovirus (CMV) promoters based on constructs from Invitrogen (Carlsbad, CA) by standard molecular biology techniques. The plasmid li-RGC (pli-RGC) was constructed by cloning an li gene fragment of base pairs from -92 to 97 (where A in the AUG start codon is position 1) into the RSV.5 vector in a reverse orientation, being driven by a RSV promoter to avoid promoter competition when large amounts of li-RGC were used (Hillman *et al.*, 2003b). This construct was selected for our studies because it was more effective than the same construct driven by a CMV promoter (data not shown). The IL-2-containing plasmid (pIL-2), pNGVL-hIL-2 plasmid (CMV promoter/enhancer/intron A), was obtained from the National Gene Vector Laboratory (University of Michigan, Ann Arbor, MI).

Radiation

An apparatus developed for radiotherapy of mouse prostate tumors (Hillman *et al.*, 2001) was adapted for the radiation of subcutaneous tumors located in the middle of the back, 1.5 cm from the tail. Acrylic jigs were designed to place anesthetized mice in the supine position with their fore and hind limbs restrained by posts for reproducible and accurate positioning of

the subcutaneous tumor on the back as described previously (Hillman *et al.*, 2003b). Three jigs were positioned on an aluminum frame mounted on the x ray machine to irradiate three mice at a time. Lead shields of 6.4-mm thickness were designed with three cutouts for the three mice to expose the area of the tumor to photon irradiation while shielding the rest of the mouse body (Hillman *et al.*, 2003b). The radiation dose to the tumor and the scattered dose to areas of the mouse outside of the radiation field were carefully monitored. Photon irradiation was performed with a Siemens Stabilipan X ray set (Siemens Medical Systems, Inc., Malvern, PA) operated at 250 kV, 15 mA with 1-mm copper filtration at a distance of 47.5 cm from the target.

Combination of radiation and intratumoral gene therapy with DNA plasmid vectors

Mice were injected subcutaneously with RM-9 cells at 2×10^5 cells in 0.1 ml HBSS. Mice with established tumors were treated on day 6 with selective tumor irradiation administered at a single dose of 8 Gy photons. One day later, on day 7, intratumoral injections of DNA plasmid vectors were initiated and continued on days 8, 9, and 10 as previously described (Hillman *et al.*, 2003b). CIITA, IFN- γ , and IL-2 DNA plasmids were injected at a dose of 3 μ g per injection per day while li-RGC DNA plasmid was injected at 31 μ g per injection per day. We used approximately 10 times more li-RGC than CIITA in order to ensure that each cell transfected with a CIITA gene was also transfected with li-RGC, and to ensure that there would be sufficient suppression of the li protein in light of li induction caused by CIITA. A total of 40 μ g of plasmid were injected per mouse, and the total amount of plasmid DNA was adjusted when needed using empty plasmid DNA to result in the same total DNA for all groups. Plasmids vectors were mixed with a liposome formulation of cationic lipid DMRIE [1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide/cholesterol] (DMRIE-C, Gibco, Life Technologies) 2–4 min prior to injection at a ratio of 1:5 w/w, DMRIE/DNA. Experimental groups were treated either with intratumoral PBS or tumor irradiation and intratumoral PBS, or tumor irradiation and various combinations of plasmids. Mice were monitored for tumor growth and survival. Tumors were measured in three dimensions, three times per week, with a caliper. Tumor volume was calculated using the equation: $0.5236 \times \text{length} \times \text{width} \times \text{height}$. In all experiments, when tumors reached 1.5 cm in greatest diameter or 1 cm with ulceration, mice were sacrificed in accordance with animal facilities regulations. Mice with no evidence of tumor by day 64–70 underwent rechallenge with 1×10^5 parental RM-9 tumor cells injected subcutaneously in the opposite flank; as a control, three naïve mice also underwent challenge in this manner.

In vivo depletion of CD4⁺ or CD8⁺ T cell subsets

Mice were injected subcutaneously with RM-9 cells at 2×10^5 cells in 0.1 ml HBSS. On days 1, 4, 6, and 12, mice were injected with either anti-CD4 monoclonal antibody (mAb) or anti-CD8 mAb. To deplete CD4⁺ T cells, 0.1 ml ascites fluid of GK 1.5 mAb was injected intraperitoneally. To deplete CD8⁺ T cells, mice were injected intraperitoneally with 0.5 ml hy-

bridoma culture supernatant of Ly-2 mAb that was purified using the Montage Antibody Purification Kit with PROSEP-A (Millipore, Billerica, MA). On day 6, tumor irradiation was administered at 8 Gy photons followed on days 7–10 by daily intratumoral injections of pCIITA + pIFN- γ + pli-RGC + pIL-2 plasmid combinations as described above. Depletion of T cells was monitored on days 7, 13, and 27 post-cell injection, by immunofluorescent staining of mouse splenocytes with specific antibodies as previously described (Younes *et al.*, 1995). Splenocytes (10^6) were washed in phosphate-buffered saline (PBS) containing 0.1% sodium azide and 0.1% fetal calf serum (FCS) and then labeled with antibodies for 30 min at 4°C. The mAbs anti-L3T4 conjugated to phycoerythrin (PE) and anti-Lyt-2 conjugated to fluorescein isothiocyanate (FITC) were used for CD4⁺ and CD8⁺ T cells respectively (Caltag Laboratories, Burlingame, CA). Gates were set for nonspecific binding using cells labeled with the isotypes rat IgG_{2b}-FITC and rat IgG_{2b}-PE (Caltag Laboratories). Cells were analyzed on a FACScan flow cytometer.

Tumor processing for cell viability and colony formation assay

Tumors were resected at different time points, weighed, and processed into a single cell suspension. Tumors were minced into small pieces and dissociated by enzymatic digestion with 0.4 mg/ml collagenase type IV (Sigma Chemical Co., St. Louis, MO) in RPMI-1640 medium supplemented with 2 mM glutamine and 100 U/ml penicillin/streptomycin. Tumor digestion was done at 37°C for 2 hr with stirring, and then cells were filtered through a wire mesh. The cell suspension was washed twice in medium. The number of viable cells was determined by trypan blue exclusion. Cells were plated for colony assay in triplicates in 6-well plates at a concentration of 3000 cells per well for cells from control tumors, radiation-, or plasmid-treated tumors, and 1000 cells per well for radiation- plus plasmid-treated tumors in 2 ml CM. After 8 days incubation at 37°C in a 5% CO₂/5% O₂/90% N₂ incubator, colonies were fixed and stained in 2% crystal violet in absolute ethanol, then counted. The plating efficiency was calculated for each well by dividing the number of colonies by the original number of cells plated. The surviving fraction was normalized to the cell plating efficiency of control cells by dividing the plating efficiency of treated cells by that of control cells.

Histology

Tumors were resected at different time points and processed for histology studies. Tumors were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin (H&E). To detect apoptotic cells, paraffin-embedded sections were pretreated with proteinase K (20 μ g/ml) for 15 min and stained using an In Situ Cell Death Detection Kit peroxidase POD (TUNEL) according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Slides were counterstained with Mayer's hematoxylin.

Statistical analysis

To compare the proportion of mice with complete tumor regression, the χ^2 test was used at the statistical significance level of 0.05.

RESULTS

Radiation and induction of the MHC class I+/class II+/Ii- phenotype for optimal antitumor response in RM-9 tumors

We previously demonstrated that an optimal antitumor response induced by intratumoral gene therapy was obtained only when radiation was given to the tumor selectively 1 day prior to gene therapy (Hillman *et al.*, 2003b). The gene therapy, which was used to convert the tumor cells *in situ* into a potent cancer vaccine, consisted of a mixture of the four DNA plasmid vectors pCIITA, pIFN- γ , pli-RGC, and pIL-2. In order to dissect the relative roles of each plasmid in inducing the cancer vaccine response, we have now treated established RM-9 tumors of 0.3–0.4 cm with 8 Gy radiation followed a day later by intratumoral injection of various combinations of plasmids given once per day for 4 consecutive days. In repeated experiments, treatment of tumors with PBS, or with radiation and PBS, did not lead to complete tumor regression (Table 1), as shown previously (Hillman *et al.*, 2003b). Treating tumors with radiation followed by empty plasmid injections also did not cause complete tumor regression (Table 1, I). Single-plasmid gene therapy using pIL-2 or pli-RGC combined with tumor irradiation also did not result in a complete antitumor immune response (Table 1, I). These data confirm that pIL-2, *per se*, is not therapeutic at the low dose of 3 μ g used in these studies. As expected, pli-RGC by itself is not sufficient to cause an effect on tumor growth in RM-9 cells negative for Ii and MHC class II molecules. Combining radiation with pli-RGC and pIL-2 led to one of six mice having complete tumor regression; however, this mouse was not immune to RM-9 rechallenge, ruling out induction of immune response with specific tumor immunity by this treatment (Table 1, I).

We have shown that pIFN- γ transfection of RM-9 cells induces cell surface expression of MHC class I molecules (Hillman *et al.*, 2003b). In order to address whether induction of MHC class I molecules is sufficient to get a therapeutic effect, tumors were treated with radiation followed by a mixture of pIFN- γ and pli-RGC. No complete responders were observed in eight treated mice showing that upregulation of MHC class I molecules by pIFN- γ was not sufficient to induce a complete tumor response and that pli-RGC also did not affect this response as could be expected (Table 1, II). The addition of pIL-2 led to one responder out of eight, this finding might be incidental as found with radiation plus pli-RGC plus pIL-2 (Table 1, II).

We have shown that pCIITA transfection of RM-9 cells causes upregulation of MHC class II cell surface molecules and intracellular Ii protein (Hillman *et al.*, 2003b). To test whether induction of MHC class II molecules and suppression of Ii are sufficient to get a therapeutic effect, tumors were treated with radiation followed by a mixture of pCIITA and pli-RGC. Upregulating MHC class II molecules by pCIITA and decreasing Ii protein by pli-RGC were not sufficient to induce a complete tumor response (Table 1, III). However, addition of an adjuvant dose of pIL-2 cytokine induced a complete and significant antitumor response in 30% of the mice compared to the same treatment with pIL-2 alone ($p < 0.001$). This antitumor response was the result of a specific immune response as con-

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TABLE 1. RADIATION AND INDUCTION OF THE MHC CLASS I+/MHC CLASS II+/Ii- PHENOTYPE PROVIDE OPTIMAL ANTITUMOR RESPONSE TO RM-9 TUMORS

Treatment	Tumor-free mice	
	Post-treatment	Post-RM-9 challenge
PBS control	0/20 ^a	NA
Radiation	0/20 ^a	NA
<i>I. Adjuvant plasmids</i>		
Radiation + empty plasmid	0/5	NA
Radiation + pIL-2	0/5	NA
Radiation + pIi-RGC	0/7	NA
Radiation + pIi-RGC + pIL-2	1/6	0/1
<i>II. MHC Class I+</i>		
Radiation + pIFN- γ + pIi-RGC	0/8	NA
Radiation + pIFN- γ + pIi-RGC + pIL-2	1/8	NT
<i>III. MHC Class II+</i>		
Radiation + pCIITA + pIi-RGC	0/8	NA
Radiation + pCIITA + pIi-RGC + pIL-2	4/13 ^b	4/4
<i>IV. MHC Class I+/Class II+</i>		
Radiation + pCIITA + pIFN- γ	0/5	NA
Radiation + pCIITA + pIFN- γ + pIi-RGC	1/7	1/1
Radiation + pCIITA + pIFN- γ + pIL-2	3/11 ^b	3/3
Radiation + pCIITA + pIFN- γ + pIi-RGC + pIL-2	11/21 ^b	11/11

^aIn control PBS and radiation groups, 5 mice per group were used in each of the 4 experiments resulting in no antitumor response in a total of 20 mice.

^bIn these radiation + plasmids group, data from 2–3 repeated experiments were compiled.

Established subcutaneous RM-9 tumors were irradiated with 8 Gy photons on day 6. On day 7, intratumoral plasmid therapy with various plasmid combinations was initiated for 4 consecutive days as detailed in Materials and Methods. The proportion of tumor-free mice at the end of the observation period, by day 64–70 after radiation and plasmid therapy is presented. Tumor-free mice and naïve mice were challenged with RM-9 cells at that time. The proportion of challenge-tumor free mice after 3–4 weeks post-tumor challenge is reported. These data are compiled from four separate experiments.

MHC, major histocompatibility complex; PBS, phosphate-buffered saline.

firmed by rejection of RM-9 challenge in the four complete responders of 13 treated mice (Table 1, *III*).

Treatment of mice with tumor irradiation followed by pIFN- γ and pCIITA to upregulate MHC class I molecules and class II molecules associated with Ii synthesis was not sufficient to cause a complete response (Table 1, *IV*). Addition of pIi-RGC to decrease Ii synthesis induced a complete specific antitumor response in one of seven mice (14% response). Addition of pIL-2 to pCIITA and pIFN- γ caused a complete antitumor response in 27% of the mice confirming a role for IL-2 to act as an adjuvant to enhance an immune response triggered by tumor cells expressing MHC class I and class II molecules (Table 1, *IV*). However, when pIi-RGC was added to the mixture of pCIITA plus pIFN- γ plus pIL-2, to decrease Ii synthesis, the number of mice responding with complete tumor regression was consistently increased resulting in a complete and lasting response over 60 days in more than 50% of the mice (Table 1, *IV*). Comparisons between treatment groups showed that addition of pIi-RGC and pIL-2 to pCIITA and pIFN- γ was significant ($p < 0.005$) and addition of pCIITA to pIFN- γ plus pIi-RGC plus pIL-2 was significant ($p < 0.05$). The complete tumor responses observed in series *IV* of *in situ* induction of MHC class

I+/class II+ combined with adjuvant plasmids were caused by a specific antitumor immune response because all responding mice rejected RM-9 tumor cell rechallenge administered on day 64 (Table 1, *IV*). Mice rejecting challenge tumors were clear of tumors during a 3–4 week period. In contrast, all naïve mice developed RM-9 tumors by 7–10 days after challenge with RM-9 cells.

Effect of in vivo depletion of CD4⁺ or CD8⁺ T cells on the antitumor response induced by radiation and gene therapy in RM-9 tumors

To assess the role of CD4⁺ helper T cells and CD8⁺ cytotoxic T cells, mice were injected with mAb specific to these subpopulations before and after treatment with tumor irradiation and pCIITA plus pIFN- γ plus pIi-RGC plus pIL-2 intratumoral gene therapy (as detailed in Materials and Methods). Tumor growth was inhibited by radiation and gene therapy by more than approximately 20 days compared to control tumors (Fig. 1A and 1B), as previously described (Hillman *et al.*, 2003b). Tumor progression was observed in 6 of 12 mice by day 30 while the remaining 6 of 12 mice showed tumor re-

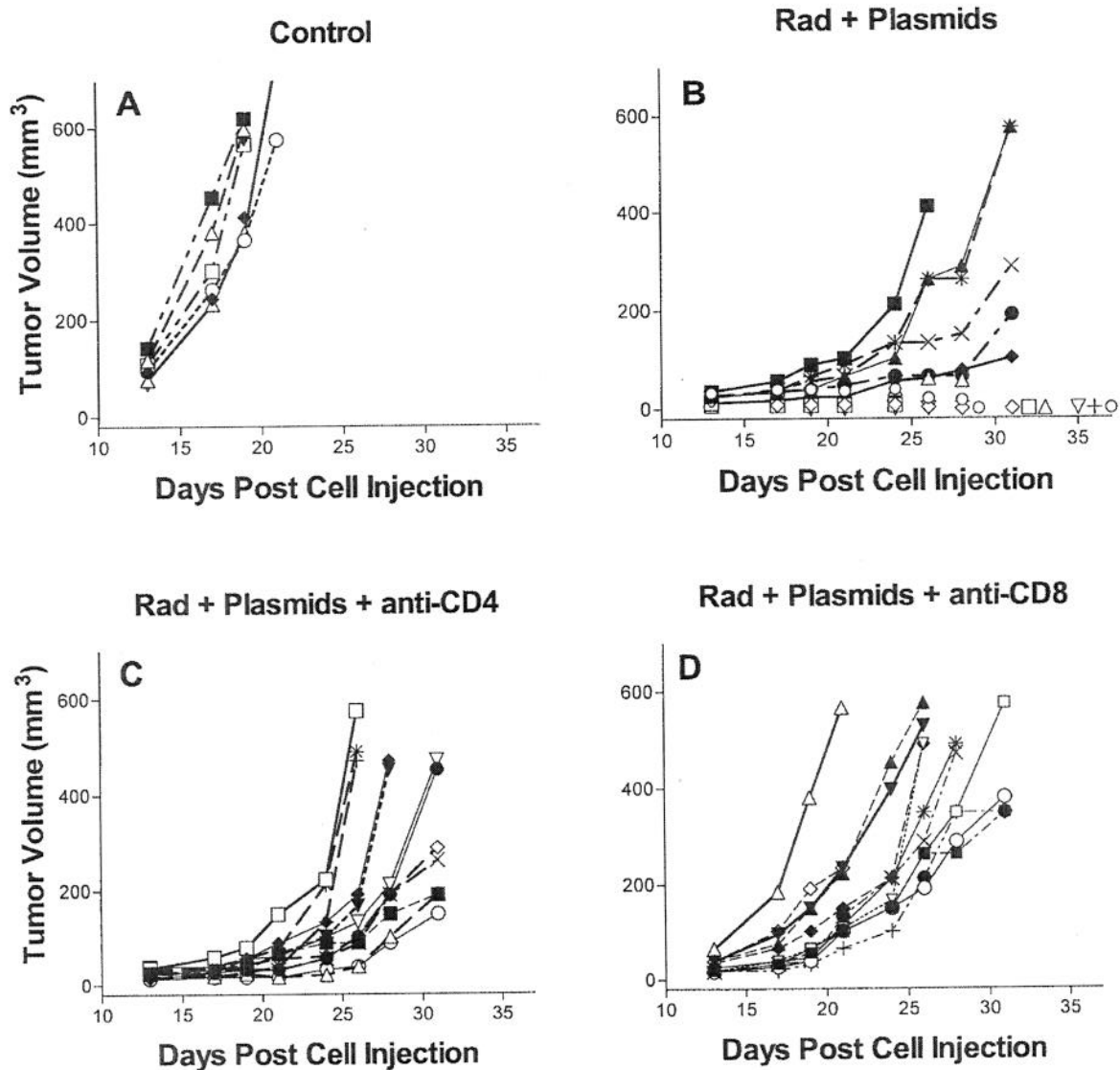


FIG. 1. Growth of RM-9 tumors in mice depleted of T cells and treated with irradiation and gene therapy. Mice were injected subcutaneously with RM-9 cells and treated with intraperitoneal injections of GK 1.5 anti-CD4 monoclonal antibody (mAb) or Ly-2 anti-CD8 mAb before and after gene therapy on day 1, 4, 6, and 12. On day 6, mice were treated with 8 Gy tumor irradiation followed on days 7–10 by daily intratumoral injections of pCIITA plus pIFN- γ plus pLi-RGC plus pIL-2 plasmids. **A:** Control mice treated with phosphate buffered saline (PBS). **B:** Mice treated with tumor irradiation plus gene therapy. **C:** Mice pretreated with anti-CD4 mAb then with radiation plus gene therapy. **D:** Mice pretreated with anti-CD8 mAb then with radiation plus gene therapy. In panels (**B**), (**C**), and (**D**), the tumor volume of 12 individual mice is represented each by a different symbol. Complete tumor regressions were observed in 6 of 12 mice treated with radiation and gene therapy (**B**) compared to 0 of 12 in mice depleted of either CD4⁺ T cells (**C**) or CD8⁺ T cells (**D**).

gression that was consistent with our previous findings of approximately 50% response (Fig. 1B, Table 1; Hillman *et al.*, 2003b). In treatment groups receiving either anti-CD4 mAb or anti-CD8 mAb, tumor growth was inhibited initially probably due to the radiation effect, but after day 20, all tumors progressed rapidly to large sizes (Figure 1 C, D). Tumor regression was observed in 0 of 12 mice treated with anti-CD4 mAb and in 0 of 12 mice treated with anti-CD8 mAb compared to 6

of 12 mice treated with radiation plus gene therapy but not depleted of T cells. Therefore, the antitumor response mediated by tumor irradiation and gene therapy was abrogated by depletion of CD4⁺ helper T cells or CD8⁺ cytotoxic T cells prior and after therapy. A second identical experiment showed reproducibility of our findings in which 4 of 10 mice had complete tumor regression after tumor irradiation and gene therapy while 0 of 10 and 1 of 8 had tumor regression in groups treated

with anti-CD4 and anti-CD8 mAbs, respectively. In both experiments, immune monitoring of CD4⁺ T cell subsets or CD8⁺ T cell subsets on days 7, 13, and 27 by immunofluorescent staining of mouse splenocytes, confirmed the depletion of these populations. CD4⁺ T cells were completely depleted *in vivo* during and after treatment with plasmids for at least 4 weeks (data not shown). Similarly, depletion of CD8⁺ T cells was also complete for several days during and after treatment with plasmids and lasted for 4 weeks (data not shown). We found that by day 42, CD4⁺ T cells and CD8⁺ T cells started to regenerate. The percent of CD4⁺ T cells and CD8⁺ T cells was comparable in naïve mice, RM-9-bearing mice and mice treated with tumor irradiation and gene therapy without T cell depletion and was in the range of 16–20% for CD4⁺ T cells and 9–12% for CD8⁺ T cells.

Viability and division ability of cells isolated from RM-9 tumors treated with radiation and gene therapy

To investigate the contribution of radiation to the extent of cell killing prior to and after gene therapy, established tumors were treated with radiation and pCIITA plus pIFN- γ plus pIL-RGC plus pIL-2 intratumoral gene therapy or each therapy alone (as detailed in Materials and Methods). On days 1, 5, 8, and 13 postradiation (corresponding to days 0, 1, 4, and 9 after gene therapy), tumors were resected and weighed. One tumor from each group was fixed in formalin for histology studies described below and one tumor was dissociated into single-cell suspension for cell count and colony formation assay as detailed in Materials and Methods. These kinetic studies showed that tumors grew rapidly in control nonirradiated tumors while radiation inhibited the growth of the tumor up to 13 days after tumor irradiation (Fig. 2A). After plasmid therapy, growth of the tumors resumed 4 days after the end of gene therapy while radiation combined with plasmid therapy decreased the tumor burden by 4 days after gene therapy with minimal measurable nodules, a lasting effect seen by day 9 after gene therapy or day 13 after radiation in contrast to tumors treated with plasmids alone (Fig. 2A). The number and viability of the tumor cells isolated from these tumors followed the same pattern with rapid increase in the number of viable cells in control tumors and relatively lower number of cells in radiation-treated tumors for up to 13 days after radiation (Fig. 2B). Already by 1 day after radiation, the recovery of viable tumor cells was five times less than in control tumor. An increase in the number of viable cells was observed 4 days after plasmid therapy while the number of viable cells isolated from radiation plus plasmid-treated tumors remained low ($< 0.4\text{--}0.6 \times 10^5$ per tumor) (Fig. 2B).

To determine the division ability of the cells isolated from treated tumors, cells were plated in an 8-day colony formation assay. The surviving fraction showed that cells isolated from radiation treated tumors, 1 day after radiation, had approximately 60% inhibition in their ability to form colonies relative to control tumors (Fig. 2C). This inhibition remained in the range of 40–50% over 13 days after radiation. These data corroborate the findings of the kinetics of tumor growth and viability of the cells over 13 days remaining at a low level after radiation. On days 4 and 9 after plasmid therapy an inhibition

of 30–40% colony formation was observed relative to control (Fig. 2C). Treatment with radiation and plasmids almost completely abrogated the ability of tumor cells to divide, corroborating the low tumor weight and the low number of cells recovered from these tumors (Fig. 2C). These data were consistently reproduced in a second experiment.

Histologic evaluation of RM-9 tumors treated with radiation and gene therapy

To determine the *in situ* alterations induced by radiation and gene therapy and the extent of tumor destruction compared to each treatment alone, separate tumors resected from the experiment described above and depicted in Figure 2 were processed for histologic studies. Tumor sections were stained with H&E and others were stained using the TUNEL assay as detailed in Materials and Methods. Untreated RM-9 tumors presented as sheets of pleomorphic epithelial cells, with large nuclei and prominent nucleoli (Fig. 3A), and few apoptotic cells (Fig. 3B). Already 1 day after radiation, areas of focal necrosis and apoptotic cells were scattered in the tumor nodules as seen by H&E staining (Fig. 3C) and confirmed by TUNEL staining (Fig. 3D). An increase in fibrosis, inflammatory infiltrates, including polymorphonuclear cells (PMN) and lymphocytes, and focal hemorrhages were observed at 5–13 days postradiation, however, approximately 50–70% of the tumor cells looked viable. A larger number of giant cells tumors were seen that are characteristic of radiation induced cell alterations. After plasmid therapy, areas of tumor destruction at the periphery of the tumor nodules were observed with apoptotic cells, infiltration of inflammatory cells and vascular damage whereas 60–70% of viable tumor was seen in the center of the tumor (Fig. 3E). By day 9 after the end of gene therapy, most of the tumor showed little apoptosis (Fig. 3F). In contrast, treatment with radiation and plasmid therapy resulted in small tumor nodules, showing significant changes already at 1 day after the end of gene therapy that became prominent at 4 and 9 days after therapy. Tumor presented with large areas of necrosis associated with cell debris, apoptotic bodies, fibrosis, and focal hemorrhages (Fig. 3H). Few or no viable tumor cells were observed as confirmed by the large number of stained apoptotic cells in TUNEL (Fig. 3G). A heavy infiltration of inflammatory cells in the periphery and inside the tumor nodule consisted of lymphocytes, histiocytes and neutrophils. These data were consistently reproduced in a second experiment.

DISCUSSION

We have developed a novel approach combining selective tumor irradiation with gene-mediated immunotherapy that converts tumor cells, *in situ*, into a curative cancer vaccine in the murine RM-9 prostate tumor model. We showed that intratumoral gene therapy of established RM-9 subcutaneous tumor nodules with plasmid cDNAs coding for the MHC class I inducer IFN- γ , the MHC class II inducer CIITA and an I α suppressor gene, to upregulate MHC class I and class II molecules and suppress the I α invariant chain, transiently inhibited tumor growth (Hillman *et al.*, 2003b). This effect suggested that this

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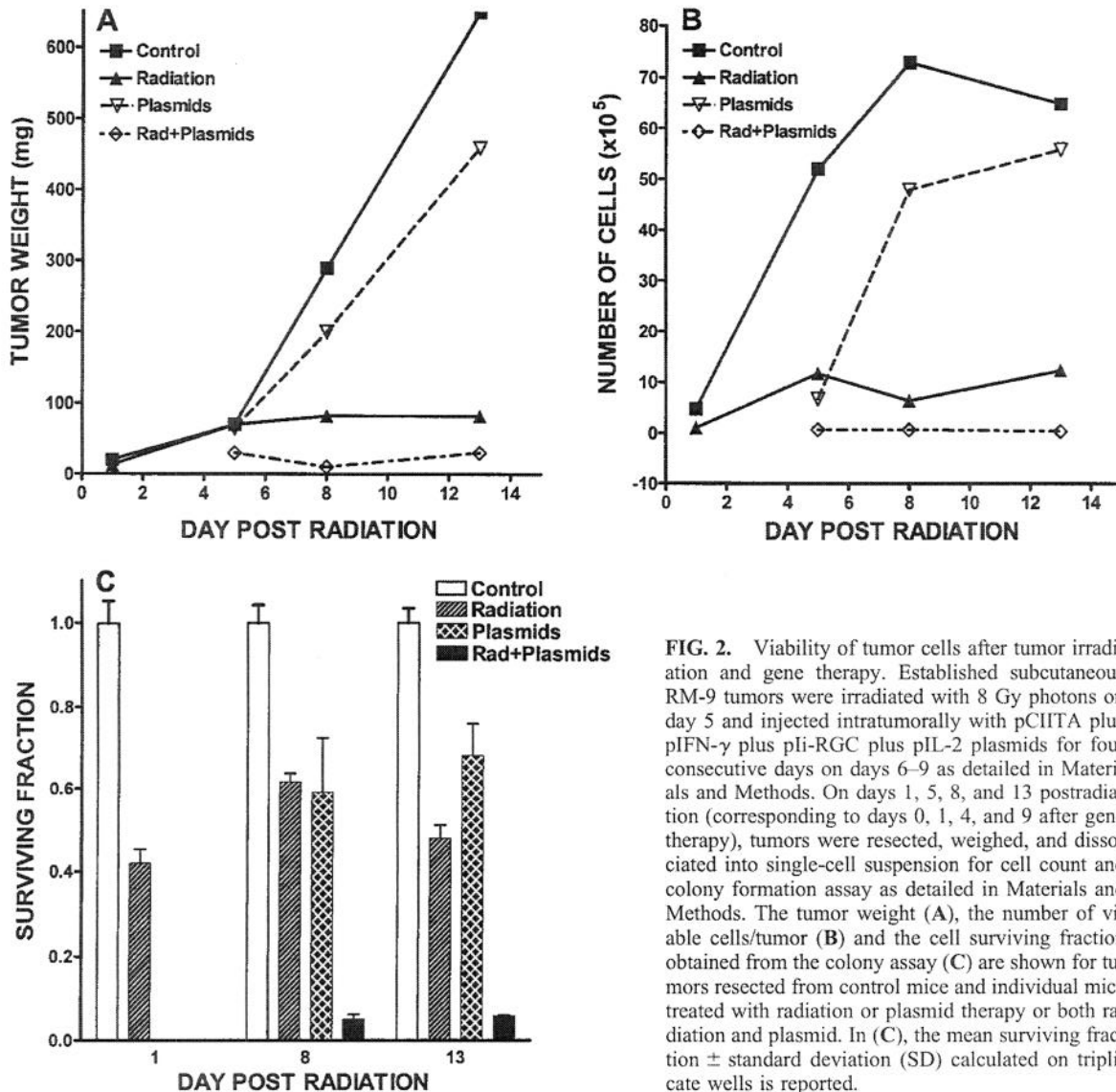
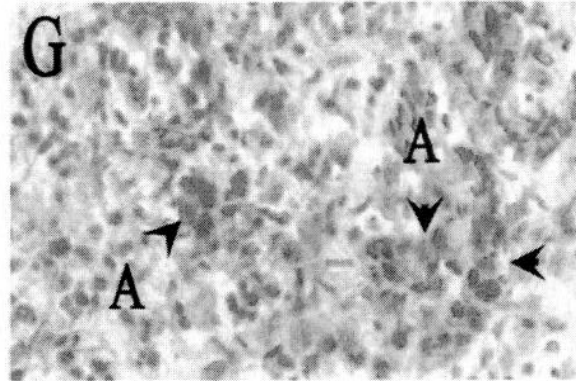
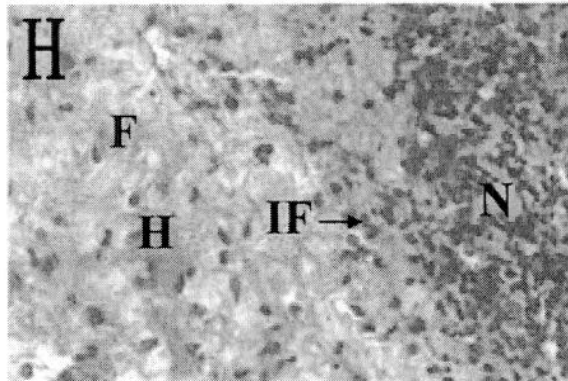
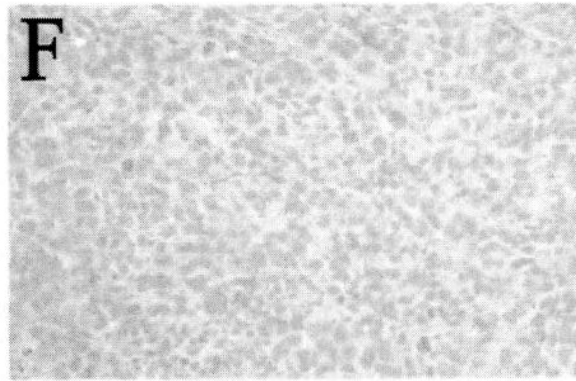
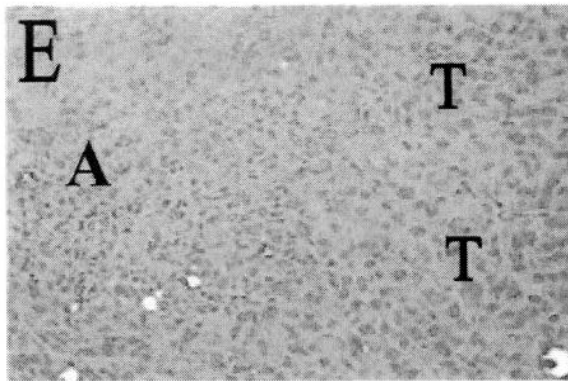
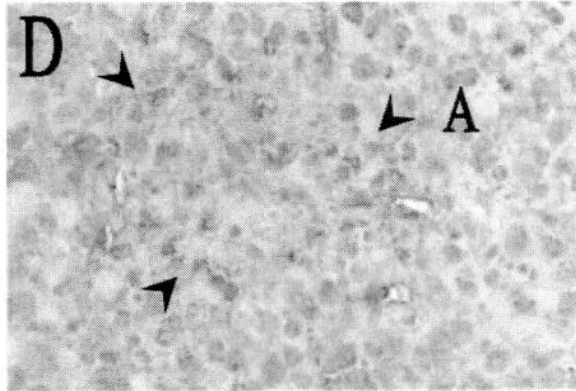
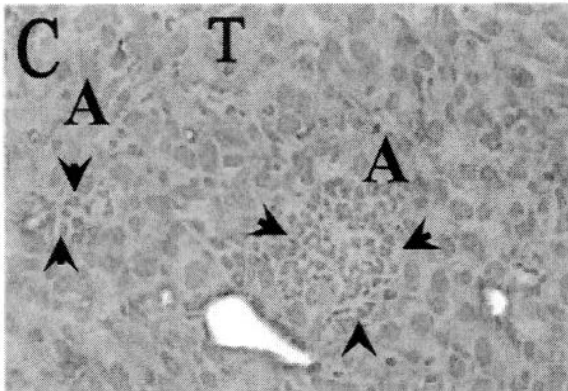
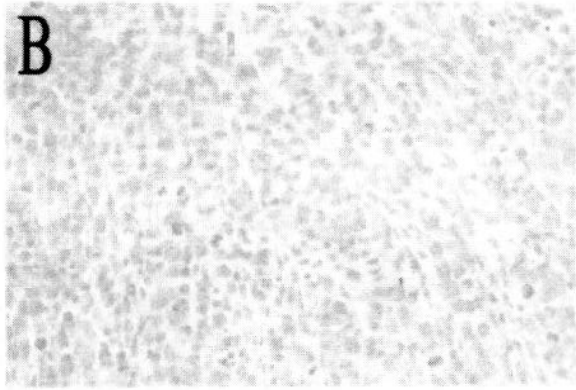
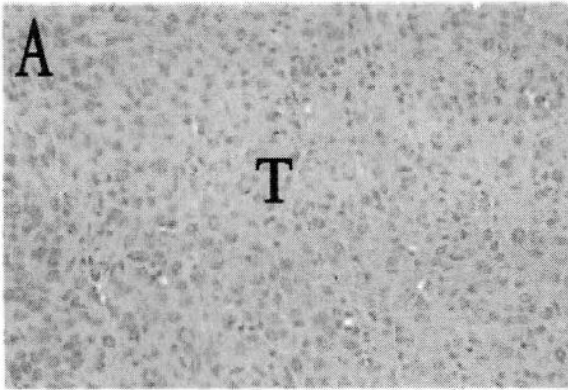


FIG. 2. Viability of tumor cells after tumor irradiation and gene therapy. Established subcutaneous RM-9 tumors were irradiated with 8 Gy photons on day 5 and injected intratumorally with pCIITA plus pIFN- γ plus pIi-RGC plus pIL-2 plasmids for four consecutive days on days 6–9 as detailed in Materials and Methods. On days 1, 5, 8, and 13 postradiation (corresponding to days 0, 1, 4, and 9 after gene therapy), tumors were resected, weighed, and dissociated into single-cell suspension for cell count and colony formation assay as detailed in Materials and Methods. The tumor weight (A), the number of viable cells/tumor (B) and the cell surviving fraction obtained from the colony assay (C) are shown for tumors resected from control mice and individual mice treated with radiation or plasmid therapy or both radiation and plasmid. In (C), the mean surviving fraction $\pm$ standard deviation (SD) calculated on triplicate wells is reported.

FIG. 3. Histology of RM-9 tumors treated with radiation and plasmid gene therapy. Established subcutaneous RM-9 tumors were irradiated with 8 Gy photons on day 5 and injected intratumorally with pCIITA plus pIFN- γ plus pIi-RGC plus pIL-2 plasmids for 4 consecutive days on days 6–9 as detailed in Materials and Methods. Tumors were resected at different time points and tumor sections were stained with hematoxylin and eosin (H&E; A, C, E, H) or for apoptosis with TUNEL In Situ Cell Death Detection Kit peroxidase POD (B, D, F, G) as described in Materials and Methods. The main findings were labeled on the prints with T for tumor, A for apoptosis, H for hemorrhages, N for necrosis, F for fibrosis, and IF for inflammatory cells. A: Untreated tumor, sheets of pleomorphic epithelial cells with frequent mitosis. B: Untreated tumor stained with TUNEL showing few stained cells. C: Radiation treated tumor on day 1 postradiation, note focal areas of apoptotic cells as confirmed by TUNEL staining in (D). E: Tumor treated with plasmids at 4 days after the end of gene therapy showing areas of tumor destruction and areas of viable tumor. F: Tumor treated with plasmids at 9 days after the end of gene therapy stained with TUNEL confirming viable tumor and minimal apoptosis. H: Radiation- plus plasmid-treated tumor at 4 days after the end of gene therapy showing large areas of necrosis with extensive apoptosis, focal hemorrhages, fibrosis, and inflammatory cells. G: Radiation- plus plasmid-treated tumor at 9 days after the end of gene therapy stained with TUNEL exhibiting large numbers of apoptotic cells. All magnifications, $\times 50$.



gene therapy approach induced an immune response, but that this response was not sufficient to eradicate the poorly immunogenic and rapidly growing tumors in the RM-9 model. However, when radiation was applied to RM-9 tumors 1 day prior to intratumoral gene therapy, complete tumor regressions occurred in approximately 50% of the mice (Hillman *et al.*, 2003b). These complete responders, rendered tumor-free by the combined therapy, were immune to rechallenge with parental tumor and demonstrated specific cytotoxic T cell activity (Hillman *et al.*, 2003b). These data confirm that tumor irradiation in conjunction with gene-mediated immunotherapy induced a significantly stronger antitumor immune response resulting in eradication of the tumor nodule and long-lasting tumor immunity. This effect was obtained when gene therapy was administered by injections of a mixture of the four individual plasmid vectors, CIITA, IFN γ , Ii-RGC, and IL-2 in liposome formulation.

To determine the role of each plasmid in induction of the antitumor immune response, we have treated established RM-9 subcutaneous tumors with radiation followed a day later by intratumoral plasmid injections using various combinations of the four plasmids. We found that radiation and gene therapy using adjuvant plasmids IL-2 or Ii-RGC or both were ineffective at causing complete tumor regression. These data confirm that pIL-2 is not therapeutic at the low dose of 3 μ g used in these studies in contrast to the 50- μ g tumoricidal dose used in other studies (Saffran *et al.*, 1998). As expected, pIi-RGC by itself is not sufficient to cause an effect on tumor growth in RM-9 cells negative for Ii and MHC class II molecules. Similarly, upregulation of MHC class I molecules by IFN- γ plasmid was not sufficient to lead to a complete response even when IL-2 plasmid was added. These data indicate that tumor cells expressing only MHC class I molecules presenting TAA and not class II molecules cannot act as APCs to mediate a strong antitumor immune response via stimulation of CD8 $^{+}$ cytotoxic T cells. However, upregulation of MHC class II molecules by the CIITA plasmid and inhibition of Ii synthesis by Ii-RGC caused complete tumor regression associated with specific immunity in 30% of the mice but only when supplemented with low doses of IL-2 plasmid. These data suggest the importance of stimulation of CD4 $^{+}$ T cells by novel endogenous TAA presented by MHC molecules (Hillman *et al.*, 2004a; Xu *et al.*, 2004). IL-2 may play a role in regulating the T cell activation. Induction of MHC class I+/class II+ by mixed CIITA and IFN- γ plasmids was not effective but addition of Ii-RGC or IL-2 plasmids led to 14–27% complete responders.

The combination of the four IFN- γ , CIITA, Ii-RGC, and IL-2 plasmids with tumor irradiation consistently led to a specific antitumor immune response associated with long-lasting complete tumor regression and immunity to tumor rechallenge in more than 50% of the mice. These data demonstrate that an optimal and specific antitumor immune response is achieved in mice treated with tumor irradiation followed by gene therapy, with a combination of the four plasmids pCIITA, pIFN- γ , pIi-RGC, and pIL-2, converting the tumor cells *in situ* to the MHC class I+/class II+/Ii- phenotype. Such a phenotype helped by the adjuvant cytokine IL-2, probably acting as the second signal for T cell stimulation in addition to MHC presenting tumor peptides to the T cell receptor, converts the cells into a cancer vaccine. IL-2 may also act to sustain and enhance the T cell ac-

tivation triggered by modified tumor cells as previously shown in other studies (Kim *et al.*, 2001).

Modified MHC class I+/class II+/Ii- cells allow for presentation of endogenous tumor antigens by MHC class II molecules to CD4 $^{+}$ T helper cells. We have now demonstrated that these helper T cells play an essential role in the induction of a complete antitumor immune response triggered by our combined radiation and gene therapy approach. Depletion of CD4 $^{+}$ T helper cells *in vivo* prior to and during radiation/gene therapy treatment abrogated the complete antitumor response induced by radiation and plasmid therapy. Depletion of CD8 $^{+}$ cytotoxic T cells also resulted in the elimination of complete responders. Immune monitoring of CD4 $^{+}$ T cells and CD8 $^{+}$ T cells confirmed that these cells were depleted before therapy and for at least 4 weeks after therapy, a crucial time for the antitumor immune response to develop. These data demonstrate that the antitumor effect observed after tumor irradiation and genetic modification of tumor cells to the MHC class I+/class II+/Ii- phenotype is mediated by induction of a robust antitumor immune response dependent on both CD4 $^{+}$ helper and CD8 $^{+}$ cytotoxic T cell subsets.

These studies provide a direct confirmation that creation of the MHC class I+/II+/Ii- phenotype to allow tumor cells simultaneously present both MHC class I- and class II-restricted TAA epitopes has the potential to trigger a robust and specific antitumor immune response able to eradicate the tumor. Induction of MHC class II molecules and Ii by CIITA together with suppression of Ii by Ii-RGC, is a clinically practical method because both CIITA and Ii genes are monoallelic (Hillman *et al.*, 2004a; Xu *et al.*, 2004). Transfecting the tumors of each patient with genes for his or her own MHC class II alleles is not clinically practical in large numbers of patients.

The mechanisms by which tumor irradiation enhances the therapeutic efficacy of intratumoral gene therapy, for *in situ* conversion of tumor cells into a cancer vaccine, is a major focus of our work. Two possible mechanisms for radiation enhancement of gene therapy are the DNA-damaging and tissue-debulking effects that slow tumor growth and give time for the immune response to become effective (Dezso *et al.*, 1996; Hillman *et al.*, 2003b). We have now shown that as early as 1 day after tumor irradiation, at the time of initiation of plasmid injections, there are already five times fewer viable cells isolated from irradiated tumors compared to control tumors. A 60% inhibition in the division ability of these *in situ* irradiated tumor cells, relative to cells from control tumors, was measured in a colony formation assay. These data confirm that at the time gene therapy is initiated in the irradiated tumor nodules, there is a significantly lower number of functional cells, increasing the probability of tumor cell transfection and consistent with the debulking effect of radiation. Moreover, this effect persists for almost 2 weeks after radiation as seen in inhibition of tumor growth, lower number of viable cells, and decrease in division ability. These findings were confirmed by the histologic observation of irradiated tumors presenting with focal areas of apoptotic cells as soon as 1 day postirradiation. By 2 weeks after radiation, remaining viable tumor was observed, consistent with subsequent tumor regrowth. As shown in our previous studies, inhibition of growth of irradiated tumors was transient and growth resumed after 2 weeks after radiation corroborating the present findings (Hillman *et al.*, 2003b). Monitoring of

cells isolated from plasmid treated tumors also showed inhibition of 30–40% of the ability to form colonies, consistent with the transient inhibition observed in tumor growth (Hillman *et al.*, 2003b). In contrast, the effect of gene therapy combined with prior tumor irradiation was more drastic and observed already just at one day after the end of gene therapy with a decrease in tumor size, recovery of few viable cells with limited or no ability to divide in the colony assay. This dramatic inhibition of tumor growth persisted and was confirmed by the histologic observation of complete destruction of tumor cells. Tumor nodules showed extensive necrosis, apoptosis, and fibrosis.

Another possibility for mechanism of interaction between the two modalities is that radiation-induced tissue damage mobilizes inflammatory cells in the tumor vicinity that can be readily activated by *in situ* gene therapy (Dezso *et al.*, 1996). In this study, we showed that radiation caused vascular damage and infiltration of PMN and lymphocytes in RM-9 tumors confirming mobilization of inflammatory cells. A large influx of inflammatory cells consisting of lymphocytes, neutrophils, and histiocytes was observed in tumors treated with radiation and plasmid therapy localized both at the periphery and inside the nodules in areas of fibrosis and necrosis. This is consistent with our findings of induction of antitumor immune response associated with T cell activity as shown in the T cell depletion experiments (Fig. 1) and cytotoxic T cell activity previously demonstrated (Hillman *et al.*, 2003b). Interestingly, an influx of inflammatory cells associated with tumor destruction was also seen in nonirradiated plasmid-treated tumors, but it was localized only at the periphery of the tumor while tumor in the center of the nodule looked viable and resulted in tumor regrowth. Radiation might enhance the permeability of the tumor allowing a greater influx of activated immune cells inside the nodules.

Radiation could increase gene transduction efficiency and duration of expression of surviving tumor cells, thus improving efficiency of *in situ* genetic modification leading to an immune response that eradicated remaining tumor cells. Radiation improved the transfection efficiency of plasmid DNA in normal and malignant cells, *in vitro*, resulting from radiation-induced DNA breaks and DNA repair mechanisms (Zeng *et al.*, 1997). These studies showed that radiation followed by plasmid or adenoviral transfection enhanced integration of the transgene (Stevens *et al.*, 1996; Zeng *et al.*, 1997). Other recent studies also showed that ionizing radiation increased adenoviral vector uptake and improved transgene expression in tumor xenografts (Zhang *et al.*, 2003). We found that tumor irradiation also enhanced the anti-tumor response mediated by intratumoral injections of the IL-2 adenovector (Ad-IL-2) in the Renca murine renal adenocarcinoma (Hillman *et al.*, 2004b). Our preliminary studies in the RM-9 and Renca models, using intratumoral injections of Ad-IL-2, show that radiation potentiates the genetic modification of the tumor cells by increasing both the level and duration of expression of transfected genes (unpublished observations). Our studies and others indicate that radiation improves gene transfection efficiency. Radiation might also limit suppressive immunoregulatory T cells; previous studies in the RM model have shown evidence that RM tumors are immunosuppressive and induce tumor-specific CD4+ regulatory T cells (Griffith *et al.*, 2001).

We are pursuing additional studies to clarify further the mechanisms by which radiation improves the efficacy of gene therapy, to optimize the conditions of radiation/plasmid combination to increase therapeutic efficacy, and to test this novel approach in orthotopic transplants for both local tumor eradication and control of spontaneous metastases. In addition, we are addressing the question as to why 100% of the mice are not cured. Possibly, we are already at nearly optimal conditions for our therapy and failure to cure lies in issues of T cell immunoregulatory function, tumor cell sequestration, and protective fibrosis. One might be able to anticipate in which mice cures will not occur by polymerase chain reaction (PCR) analysis of cytokine transcripts of defined subsets of tumor-infiltrating lymphocytes.

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REFERENCES

- ARMSTRONG, T.D., CLEMENTS, V.K., MARTIN, B.K., TING, J., and OSTRAND-ROSENBERG, S. (1997). Major histocompatibility complex class II-transfected tumor cells present endogenous antigen and are potent inducers of tumor-specific immunity. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6886–6891.
- ARMSTRONG, T.D., CLEMENTS, V.K., and OSTRAND-ROSENBERG, S. (1998a). MHC Class II-transfected tumor cells directly present antigen to tumor-specific CD4+ T lymphocytes. *J. Immunol.* **160**, 661–666.
- ARMSTRONG, T.D., CLEMENTS, V.K., and OSTRAND-ROSENBERG, S. (1998b). Class II-transfected tumor cells directly present endogenous antigen to CD4+ T cells in vitro and are APCs for tumor-encoded antigens in vivo. *J. Immunother.* **21**, 218–224.
- BELLDEGRUN, A., TSO, C.L., ZISMAN, A., NAITOH, J., SAID, J., PANTUCK, A.J., HINKEL, A., DEKERNION, J., and FIGLIN, R. (2001). Interleukin 2 gene therapy for prostate cancer: Phase I clinical trial and basic biology. *Hum. Gene Ther.* **12**, 883–892.
- CLEMENTS, V.K., BASKAR, S., ARMSTRONG, T.D., and OSTRAND-ROSENBERG, S. (1992). Invariant chain alters the malignant phenotype of MHC class II tumor cells. *J. Immunol.* **149**, 2391–2396.
- DEZSO, B., HAAS, G.P., HAMZAVI, F., KIM, S., MONTECILLO, E.J., BENSON, P.D., PONTES, J.E., MAUGHAN, R.L., and HILLMAN, G.G. (1996). Insights into the mechanism of local tumor irradiation combined with IL-2 therapy in murine renal carcinoma: Histological evaluation of pulmonary metastases. *Clin. Cancer Res.* **2**, 1543–1552.
- FORMAN, J.D., TEKZI-MENSAH, S., CAUDRELIER, J.M., FALQUEZ, R., VELASCO, J., PORTER, A.T., and MAUGHAN, R.L. (1998). Neutron radiation in the management of localized and locally

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- advanced prostate cancer. In *Advances in the Radiotherapeutic Management of Carcinoma of the Prostate*. (Chapman and Hall, New York, NY).
- GRAY, C.L., POWELL, C.R., RIFFENBURGH, R.H., and JOHNSTONE, P.A. (2001). 20-year outcome of patients with T1-3N0 surgically staged prostate cancer treated with external beam radiation therapy. *J. Urol.* **166**, 116–118.
- GRIFFITH, T.S., KAWAKITA, M., TIAN, J., RITCHEY, J., TARTAGLIA, J., SEHGAL, I., THOMPSON, T.C., ZHAO, W., and RATLIFF, T.L. (2001). Inhibition of murine prostate tumor growth and activation of immunoregulatory cells with recombinant canarypox viruses. *J. Natl. Cancer Inst.* **93**, 998–1007.
- GUAGLIARDI, L.E., KOPPELMAN, B., BLUM, J.S., MARKS, M.S., CRESSWELL, P., and BRODSKY, F.M. (1990). Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. *Nature* **343**, 133–139.
- HALL, S.J., MUTCHNIK, S.E., CHEN, S., WOO, S., and THOMPSON, T.C. (1997). Adenovirus-mediated herpes simplex virus thymidine kinase gene and ganciclovir therapy leads to systemic activity against spontaneous and induced metastasis in an orthotopic mouse model of prostate cancer. *Int. J. Cancer* **70**, 183–187.
- HARRINGTON, K.J., SPITZWEG, C., BATEMAN, A.R., MORRIS, J.C., and VILE, R.G. (2001). Gene therapy for prostate cancer: Current status and future prospects. *J. Urol.* **166**, 1220–1233.
- HILLMAN, G.G., TRIEST, J.A., CHER, M.L., KOCHERIL, S.V., and TALATI, B.R. (1999). Prospects of immunotherapy for the treatment of prostate carcinoma—A review. *Cancer Detect. Prev.* **23**, 333–342.
- HILLMAN, G.G., MAUGHAN, R.L., GRIGNON, D.J., YUDELEV, M., RUBIO, J., TEKYI-MENSAH, S., LAYER, A., CHE, M., and FORMAN, J.D. (2001). Neutron or photon irradiation for prostate tumors: Enhancement of cytokine therapy in a metastatic tumor model. *Clin. Cancer Res.* **7**, 136–144.
- HILLMAN, G.G., KALLINTERIS, N.L., LI, J., WANG, Y., LU, X., LI, Y., WU, S., WRIGHT, J.L., SLOS, P., GULFO, J.V., HUMPHREYS, R.E., and XU, M. (2003a). Generating MHC Class II+/Ii- phenotype after adenoviral delivery of both an expressible gene for MHC Class II inducer and an antisense Ii-RNA construct in tumor cells. *Gene Ther.* **10**, 1512–1518.
- HILLMAN, G.G., XU, M., WANG, Y., WRIGHT, J.L., LU, X., KALLINTERIS, N.L., TEKYI-MENSAH, S., THOMPSON, T.C., MITCHELL, M.S., and FORMAN, J.D. (2003b). Radiation improves intratumoral gene therapy for induction of cancer vaccine in murine prostate carcinoma. *Hum. Gene Ther.* **14**, 763–775.
- HILLMAN, G.G., KALLINTERIS, N.L., LU, X., WANG, Y., WRIGHT, J.L., LI, Y., WU, S., FORMAN, J.D., GULFO, J.V., HUMPHREYS, R.E., and XU, M. (2004a). Turning tumor cells in situ into T-helper cell-stimulating, MHC class II tumor epitope-presenters: Immuno-curing and immuno-consolidation. *Cancer Treat. Rev.* **30**, 281–290.
- HILLMAN, G.G., SLOS, P., WANG, Y., WRIGHT, J.L., LAYER, A., DE MEYER, M., YUDELEV, M., CHE, M., and FORMAN, J.D. (2004b). Tumor irradiation followed by intratumoral cytokine gene therapy for murine renal adenocarcinoma. *Cancer Gene Ther.* **11**, 61–72.
- KIM, J.J., YANG, J.S., DANG, K., MANSON, K.H., and WEINER, D.B. (2001). Engineering enhancement of immune responses to DNA-based vaccines in a prostate cancer model in rhesus macaques through the use of cytokine gene adjuvants. *Clin. Cancer Res.* **7**, 882s–889s.
- KOCH, N., KOCH, S., and HAMMERLING, G.J. (1982). Ia invariant chain detected on lymphocyte surfaces by monoclonal antibody. *Nature* **299**, 644–645.
- LU, X., KALLINTERIS, N.L., LI, J., WU, S., LI, Y., JIANG, Z., HILLMAN, G.G., GULFO, J.V., HUMPHREYS, R.E., and XU, M. (2003). Tumor immunotherapy by converting tumor cells to MHC class II-positive, Ii protein-negative phenotype. *Cancer Immunol. Immunother.* **52**, 592–598.
- NASU, Y., BANGMA, C.H., HULL, G.W., LEE, H.M., HU, J., WANG, J., MCCURDY, M.A., SHIMURA, S., YANG, G., TIMME, T.L., and THOMPSON, T.C. (1999). Adenovirus-mediated interleukin-12 gene therapy for prostate cancer: suppression of orthotopic tumor growth and pre-established lung metastases in an orthotopic model. *Gene Ther.* **6**, 338–345.
- OSTRAND-ROSENBERG, S., THAKUR, A., and CLEMENTS, V. (1990). Rejection of mouse sarcoma cells after transfection in MHC class II genes. *J. Immunol.* **144**, 4068–4071.
- POWELL, C.R., HUISMAN, T.K., RIFFENBURGH, R.H., SAUNDERS, E.L., BETHEL, K.J., and JOHNSTONE, P.A. (1997). Outcome for surgically staged localized prostate cancer treated with external beam radiation therapy. *J. Urol.* **157**, 1754–1759.
- QI, L., ROJAS, J.M., and OSTRAND-ROSENBERG, S. (2000). Tumor cells present MHC class II-restricted nuclear and mitochondrial antigens and are the predominant antigen presenting cells in vivo. *J. Immunol.* **165**, 5451–5461.
- SAFFRAN, D.C., HORTON, H.M., YANKAUCKAS, M.A., ANDERSON, D., BARNHART, K.M., ABAL, A.M., HOBART, P., MANTHORPE, M., NORMAN, J.A., and PARKER, S.E. (1998). Immunotherapy of established tumors in mice by intratumoral injection of interleukin-2 plasmid DNA: Induction of CD8+ T-cell immunity. *Cancer Gene Ther.* **5**, 321–330.
- SIMONS, J.W., MIKHAK, B., CHANG, J.F., DEMARZO, A.M., CARDUCCI, M.A., LIM, M., WEBER, C.E., BACCALA, A.A., GOEMANN, M.A., CLIFT, S.M., ANDO, D.G., LEVITSKY, H.I., COHEN, L.K., SANDA, M.G., MULLIGAN, R.C., PARTIN, A.W., CARTER, H.B., PIANTADOSI, S., MARSHALL, F.F., and NELSON, W.G. (1999). Induction of immunity to prostate cancer antigens: Results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. *Cancer Res.* **59**, 5160–5168.
- STEINER, M.S., and GINGRICH, J.R. (2000). Gene therapy for prostate cancer: Where are we now? *J. Urol.* **164**, 1121–1136.
- STEVENS, C.W., ZENG, M., and CERNIGLIA, G.J. (1996). Ionizing radiation greatly improves gene transfer efficiency in mammalian cells. *Hum. Gene Ther.* **7**, 1727–1734.
- STOCKINGER, B., PESSARA, U., LIN, R., HABICHT, J., GREZ, M., and KOCH, N. (1989). A role of Ia-associated invariant chains in antigen processing and presentation. *Cell* **56**, 683.
- TEH, B.S., AGUILAR-CORDOVA, E., KERNEN, K., CHOU, C.C., SHALEV, M., VLACHAKI, M.T., MILES, B., KADMON, D., MAI, W.Y., CAILLOUET, J., DAVIS, M., AYALA, G., WHEELER, T., BRADY, J., CARPENTER, L.S., LU, H.H., CHIU, J.K., WOO, S.Y., THOMPSON, T., and BUTLER, E.B. (2001). Phase I/II trial evaluating combined radiotherapy and in situ gene therapy with or without hormonal therapy in the treatment of prostate cancer—A preliminary report. *Int. J. Radiat. Oncol. Biol. Phys.* **51**, 605–613.
- THOMPSON, T.C., SOUTHGATE, J., KITCHENER, G., and LAND, H. (1989). Multi-stage carcinogenesis induced by ras and myc oncogenes in a reconstituted model. *Cell* **56**, 917–930.
- TRUDEL, S., TRACHTENBERG, J., TOI, A., SWEET, J., LI, Z.H., JEWETT, M., TSHILIAS, J., ZHUANG, L.H., HITT, M., WAN, Y., GAULDIE, J., GRAHAM, F.L., DANCEY, J., and STEWART, A.K. (2003). A phase I trial of adenovector-mediated delivery of interleukin-2 (AdIL-2) in high-risk localized prostate cancer. *Cancer Gene Ther.* **10**, 755–763.
- XU, M., QIA, G., VON HOF, E., and HUMPHREYS, R.E. (2000). Genetic modulation of tumor antigen presentation. *Trends Biotechnol.* **18**, 167–172.
- XU, M., LU, X., KALLINTERIS, N.L., WANG, Y., WU, S., VON HOF, E., GULFO, J.V., HUMPHREYS, R.E., and HILLMAN, G.G.

- (2004). Immunotherapy of cancer by antisense inhibition of Ii protein, an immunoregulator of antigen selection by MHC class II molecules. *Curr. Opin. Mol. Ther.* **6**, 160–165.
- YOUNES, E., HAAS, G.P., DEZSO, B., ALI, E., MAUGHAN, R.L., KUKURUGA, M.A., MONTECILLO, E.J., PONTES, J.E., and HILLMAN, G.G. (1995). Local tumor irradiation augments the response to IL-2 therapy in a murine renal adenocarcinoma. *Cell. Immunol.* **165**, 243–251.
- ZENG, M., CERNIGLIA, G.J., ECK, S.L., and STEVENS, C.W. (1997). High-efficiency stable gene transfer of adenovirus into mammalian cells using ionizing radiation. *Hum. Gene Ther.* **8**, 1025–1032.
- ZHANG, M., LI, S., LI, J., ENSMINGER, W.D., and LAWRENCE, T.S. (2003). Ionizing radiation increases adenovirus uptake and improves transgene expression in intrahepatic colon cancer xenografts. *Mol. Ther.* **8**, 21–28.

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