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14. ABSTRACT A large amount of effort has been focused on the optimization of human li-RNAi constructs during the past year. We have optimized the use of a combination of human li-RNAi constructs, which target different sites of li mRNA, to obtain optimal li suppression. More importantly, we have defined the influence of the promoter on the activity of li-RNAi. Our results indicate that two elements are important for the activity of an li-RNAi construct: a) selection of the correct li-RNAi sequence that targets a specific location on li mRNA; and, b) selection of the best promoter that is active in that cell line. We have defined three active li-RNAi constructs and tested their activity of three different promoters, U6, CMV, and EF-1a. Each is active in different types of cells. Lastly, we have tested the activity of our li-RNAi constructs in fresh tumor cells. Concurrently, the task of optimization of the doses of IL-2 and IFN- γ is being addressed and optimized with our collaborator, Dr. Hillman. Substantial progress has been made in the optimization of li-RNAi constructs. We will continue our effort to pick the most active human li-RNAi constructs for prostate cancer clinical trial.						
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INTRODUCTION

Antigen Express, Inc. has 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 to activate CD8+ T cells and eradicate residual tumor and micrometastases. Tumor cells are converted into APC by inducing MHC Class II molecules and then suppressing the immunoregulatory Ii protein. The Ii protein 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 I+/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 (normal pathway) and “unprotected” MHC class II molecules to activate both CD4+ and CD8+ T cells, generating a very potent tumor cell vaccine. Prior to this grant we had demonstrated the principle that *in situ* intratumor generation of MHC class II+/Ii- tumor cell phenotype was a potent therapeutic.

Our first annual report detailed the optimization of a more potent Ii suppression reverse gene construct containing 3 reverse Ii gene fragments and the generation of potent RNA interference constructs (Ii-RNAi) to inhibit Ii protein expression. The field of RNAi is moving very swiftly and we conceived of adapting this method to our objective in the original proposal. The initial results were excellent, leading to potent synthetic oligonucleotide RNAi dimers, and genetic constructs suitable for Ii suppression in murine and human cells. In the past year we have optimized the dose of Ii-RNAi constructs and the combined use of multiple RNAi constructs. We have also tested the influence of promoters in the activity and function of Ii-RNAi constructs. We have generated Ii-RNAi constructs that are suitable not only for prostate cancer but also for other tumor cells. Our results have justified the possible clinical trials for prostate cancer immunotherapy as well as other human tumors.

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*.

- a. Construction of a plasmid containing triple Ii-RGC and CIITA. We previously constructed a three-copy Ii-RGC(-92,97)₃ which is an effective Ii-suppressor. We will clone the 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 cell 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, followed by immunohistostaining with antibodies to MHC Class II and Ii.

1. Construction of Ii suppressing genetic constructs: Ii-RNAi

(This paragraph (as well as Figure 1) was showed in the last year report. For better introduction to this year's report, this paragraph and Figure 1 are included here).

We previously constructed effective Ii-RGCs (Ii reverse gene constructs), which effectively inhibited Ii expression in many murine tumor cells. 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 cancer cells. Since the DU145 human prostate cancer cell line is MHC class II-negative and Ii negative, we did the initial testing of the activity of Ii-RNAi constructs in Raji MHC class II+/Ii+ 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 in Raji cells. **Figure 1** 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 method. 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 were used for the Ii suppression in the experiments planned in DU145 prostate cells (see last year's report).

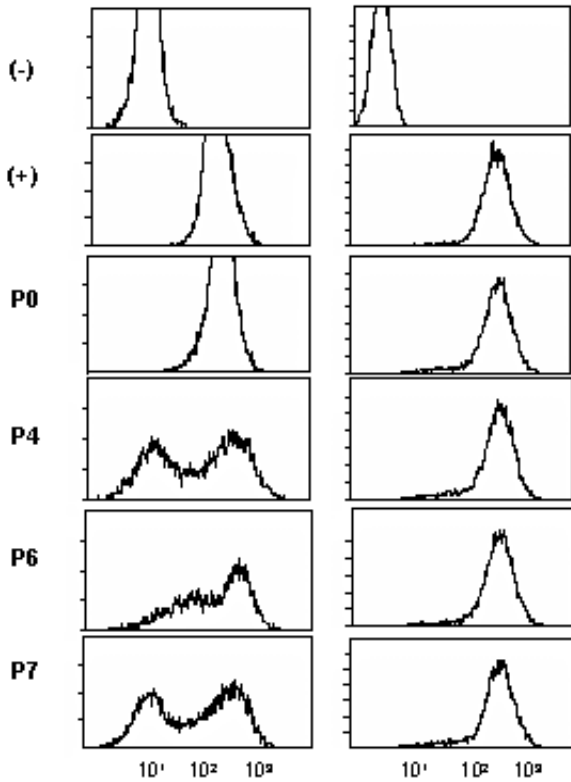


Figure 1. Ii inhibition in Raji cells. Cells were stained with anti-human Ii (left) and HLA-DR antibodies (right). P0 is empty plasmid control

Task 2: Optimize therapeutic protocols including the doses of IL-2 and IFN- γ . The dose of radiation will also be optimized.

a. We have 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 that have received one dose of radiation. The optimum dose of IL-2 will be optimized first and IFN- γ secondly. We expect a high rate 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.

b. 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.

2. Optimization of combination use of human Ii-RNAi constructs.

Since we have changed our focus of building Ii-RGC to building more potent Ii-RNAi constructs for Ii suppression, our effort has largely been switched to the optimization of the doses, and the use of combinations of Ii-RNAi constructs to obtain more efficient inhibition of Ii expression. The optimization of IL-2 and IFN- γ was with our collaborator, Dr. Hillman at the same time.

A large body of the literature has shown that the combined use of RNAi constructs that target different sites of mRNA yields better inhibition of gene expression. Thus we have put our effort into optimizing the combined use of Ii-RNAi constructs. The active Ii-RNAi constructs, P4, P6, and P7, target different sites of Ii mRNA. We have performed experiments in which all combinations of P4, P6, and P7 were used, respectively, to inhibit the Ii expression in Raji cells. From **Figure 2**, one can see that all combinations of the active human Ii-RNAi constructs showed better Ii suppression than single use of any of the three active Ii-RNAi constructs. Among these combinations, the P4/P7 combination gave the most profound Ii inhibition (the negative peak is much larger than other negative peaks of other combinations under same experiment condition). This result indicates that the simultaneous use of P4 and P7 Ii-RNAi constructs is the best combination for Ii inhibition and could be use as our final formula for potential clinical trials.

3. Test the influence of promoter on the activity of Ii-RNAi constructs.

In the early stage of constructing our Ii-RNAi constructs, we first cloned all ten Ii-RNAi fragments into the plasmid under the control of a U6 promoter. Transfection of Raji cells with

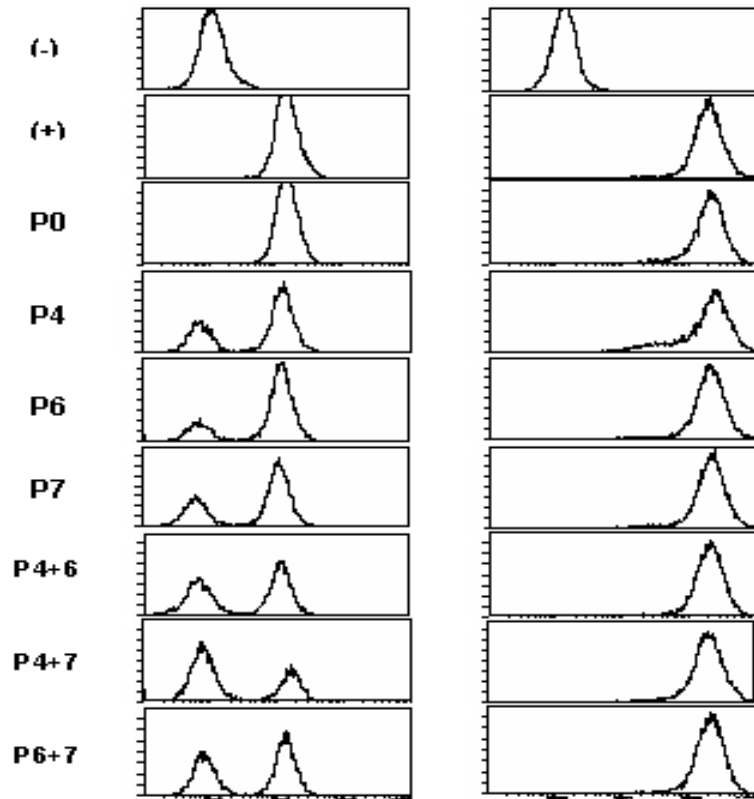


Figure 2. Combination use of Ii-RNAi in Raji cells. Raji cells were gene gun transfected with equal amount of Ii-RNAi constructs (For single use, 1 ug of DNA was used. For combination use, 0.5 ug of each plasmid was used). Cells were then incubated for another 48 hours and then stained for HLA-DR and Ii. P0 is empty plasmid control.

these U6/Ii-RNAi constructs showed that all U6/RNAi constructs were inactive (data not shown). We then changed the promoter and cloned all ten Ii-RNAi fragments into plasmids under the control of a CMV promoter. Transfection of Raji cells defined the three active Ii-RNAi constructs (**Figure 1**, *last years work and showed in the last year report.*). These results indicated that a promoter may play an important role in the activity of an Ii-RNAi construct in a given cell line. In an attempt to further confirm this idea, we chose an EF-1a promoter for our further experiments. P4 and P7 Ii-RNAi fragments were cloned into a plasmid under the control of an EF-1a promoter which is active in most mammalian cells. Transfection of an acute myeloid leukemia (AML) cell line, KG-1, with EF-1a/P4 and EF-1a/P7 constructs indicated that these two constructs were active in KG-1 cells, while the transfection of KG-1 cells with CMV/P4 and CMV/P7 were relatively inactive (**Figure 3**). Our result indicates that the activity of a promoter is important for the RNAi activity in a given cell line or a given type of cell, paving the way for using our human Ii-RNAi constructs in different tumor models.

4. Activity of human Ii-RNAi constructs in fresh tumor cells.

In order to pursue a clinical trial with our Ii-RNAi constructs, we must be able to transfect them into fresh tumor cells and demonstrate activity. We have used fresh AML cells as samples to test this idea. These experiments were performed with our collaborator, Dr. Daopei Lu. Fresh acute myeloid leukemia (AML) cells were collected from newly diagnosed AML patients and immediately frozen. For each experiment, AML cells were thawed and incubated for 24 hours before gene gun-mediated DNA transfection. Cells were transfected with EF-1a/P4 and EF-1a/P7 constructs and then incubated for another 48 hours, collected and stained with anti-HLA-DR and anti-human Ii monoclonal antibodies and FACS analyzed. The experiments indicate that the EF-1a/P4 and EF-1a/P7 Ii-RNAi constructs were active in fresh AML cell samples (**Figure 4**). The solid area represents the untransfected cells and the area under the green line represents cells that have been transfected with the human Ii-RNAi constructs. From **Figure 4**, one can see that Ii has been strongly suppressed without influencing HLA-DR expression. Six samples have been tested and similar results were obtained (data not shown). Our results indicate that the activity of a promoter is important for the activity of Ii-RNAi in tumor cells and that our results demonstrate

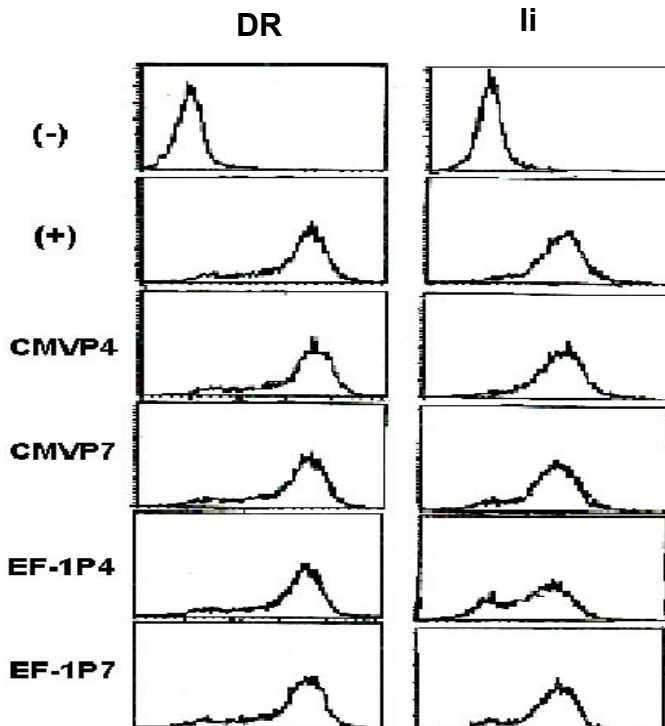


Figure 3. Influence of EF-1a promoter and CMV promoter in the activity of Ii-RNAi activity. KG-1 cells were gene gun transfected with CMV/P4, CMV/P7, EF-1a/P4, and EF-1a/P7, respectively. Cells were then stained with anti-HLA-DR and Ii antibodies and FACS analyzed.

that we have active Ii-RNAi constructs for use in clinical trials with tumor models including prostate cancer.

5. Optimize therapeutic protocols including the doses of IL-2 and IFN- γ is our second task.

In order to obtain optimized doses of IL-2 and IFN- γ in combination with Ii-RNAi constructs, we determined these parameters under the condition that Ii suppression is mediated by Ii-RNAi constructs rather than Ii-RGCs. We have developed murine Ii-RNAi constructs and will optimize the doses of IL-2, IFN- γ , and irradiation with our collaborator, Dr. Hilman. The concrete procedures are: 1) *In vitro* transfection of murine RM-9 prostate tumor cells with murine Ii-RNAi constructs to induce MHC class I+/class II+/Ii-phenotype RM-9 cells; 2) To obtain therapeutic efficacy of MHC class I+/class II+/Ii- phenotype RM-9 cells induced by murine Ii-RNAi constructs in a syngeneic mouse tumor model; 3) *In vitro* transfection of human tumor cell lines with human Ii-RNAi constructs, mentioned previously, to induce MHC class I+/class II+/Ii- phenotype human prostate cell line and other tumor cell lines including, PC-3 prostate carcinoma, KCI-18 renal cell carcinoma (established in the Hilman laboratory), BR231 breast cancer cell line, and HN4 head and neck squamous cell carcinoma; and 4) Modification of mouse xenografts of human tumor cells *in vivo* by Ii suppression gene therapy and radiation: To test for *in situ* genetic modification of human tumor cells, s.c. xenografts of PC-3 tumor cells will be injected with hIi-RNAi (p4 and P7) + pCIITA + pIFN- γ + pIL-2 in immunodeficient nude mice. Induction of MHC class I+/class II+/Ii- phenotype by this gene therapy will be monitored by immunostaining of tumor sections and analyzed by LSC.

6. Define the enhancement of Ii suppression in DNA vaccine.

Meanwhile, we have also tested Ii suppression for the enhancement of the potency of DNA vaccines. The rationale for enhancement of DNA vaccines by Ii suppression is the same as for enhancing the potency of tumor cell vaccines. Specifically, suppression of Ii allows MHC class II molecules to acquire endogenously synthesized antigenic epitopes, whether they are endogenous genes aberrantly expressed by a tumor cell or exogenous DNA vaccine genes acquired by an APC. When an APC simultaneously acquires DNA for an antigen and for an Ii-RGC plasmid, the APC becomes antigen+/class I+/class II+/Ii-. In that APC, antigen will be

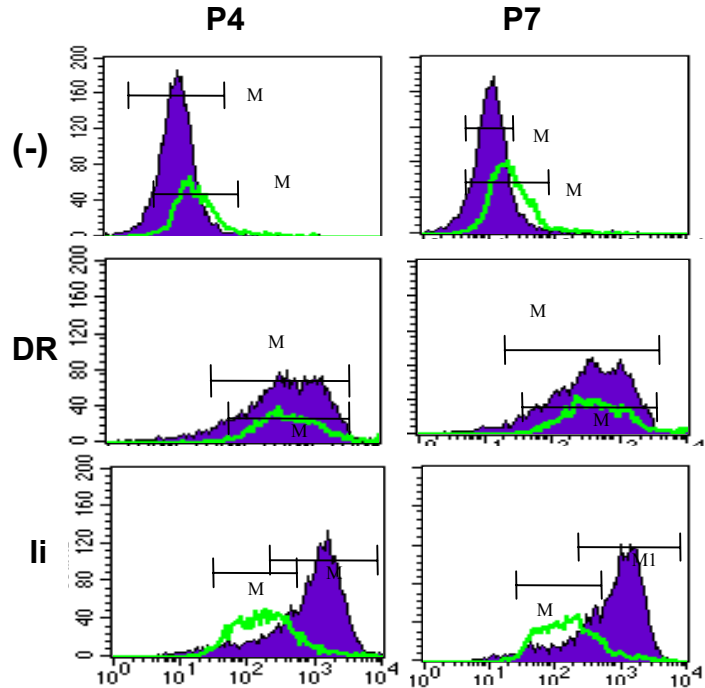


Figure 4. One example of Ii suppression in fresh AML cells by EF-1a/P4 and EF-1a/P7. The solid blue are represents the untransfected cells and the green line area represents the cells that have been transfeted with human Ii-RNAi constructs. Cells were stained 48 hour after gene transfection.

synthesized and processed as an endogenous antigen and presented through both MHC class I and class II molecules. Gp120 cDNA was used as our experimental model. GM-CSF DNA was used as a DNA adjuvant and a triple murine Ii-RGC was used as the Ii suppression reagent. All DNAs were mixed in a specific ratio (see Figure 5 legend), coated onto gold beads and delivered by gene gun to the skin cells. The ability of Ii suppression to enhance HIV gp120 DNA vaccine efficiency was tested in BALB/c mice. Mice were immunized with the gene for gp120 and for GM-CSF, with or without the Ii suppression construct. Two epitopes (p18, restricted by H-2D^d and H-2A^d, and p18-I10, restricted by H-2D^d) were used to measure the immune response to gp120 antigen. In Figure 5, one sees that both p18- and p18-I10-specific ELISPOT assays demonstrated roughly 5 times the enhancement of IFN- γ secreting cells in the Ii-suppressed groups (groups D and E) compared to the Ii un-suppressed group (group C). The enhancement was related to the Ii suppression and not related to the use of GM-CSF. The enhancement was more profound at the lower concentration of pBudCE4.1/Ii-RGC(x3) (compare group D to group E). This phenomenon may reflect less promoter competition among gp120-, GM-CSF-, and Ii-RGC-containing plasmids. Similar reaction patterns to p18 and p18-I10 stimulation were observed, the p18 peptide gave a greater response in most reactions. This result is consistent with previous reports which show that p18-I10 is restricted only by H-2D^d while the p18 peptide is restricted by both H-2D^d and H-2A^d. The p18 reaction reflects both CD4⁺ and CD8⁺ reactions and p18-I10 reaction reflects only the CD8⁺ reaction. In order to determine whether Ii suppression induced a Th1 or Th2 response, IL-4 secretion was also examined in the ELISPOT assay. IL-4 was induced in all groups (Figure 5). Compared to IFN- γ production, IL-4 production was lower, indicating that the addition of the GM-CSF gene induced a Th1-biased immune response.

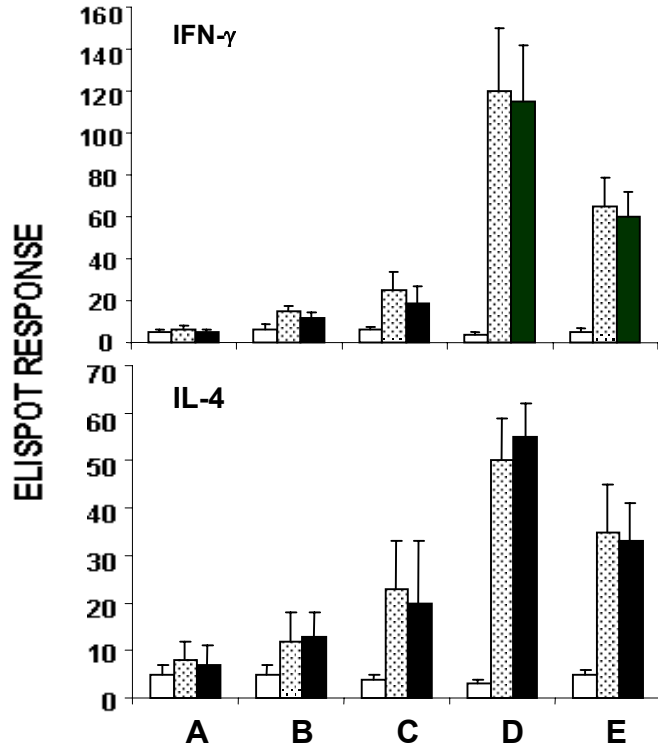


Figure 5. IFN- γ and IL-4 ELISPOT assays with splenocytes of mice immunized with gp120 with or without Ii suppression. All groups except A) (naïve mice) were immunized using the gene-gun with 2 μ g of RSV.5/gp120 plasmid and each of the following DNA plasmids, respectively: B) Empty pBudCE4.1 (1.35 μ g); C) pNGVL1/GM-CSF (0.35 μ g) + empty pBudCE4.1 (1.0 μ g); D) pNGVL1/GM-CSF (0.35 μ g) + pBudCE4.1/Ii-RGC(x3) (0.325 μ g) and empty pBudCE4.1 (0.675 μ g); E) pNGVL1/GM-CSF (0.35 μ g) + pBudCE4.1/Ii-RGC(x3) (1.0 μ g). Medium only is represented by open bar. p18 peptide is represented by dotted bar, or p18-I10 peptide is represented by black bar.

KEY RESEARCH ACCOMPLISHMENTS

1. We have optimized the combined use of human Ii-RNAi constructs that target different sites of Ii mRNA. Such combinations enhanced the potency of Ii suppression when the total quantity of plasmids is as same as in single plasmid use. This achievement enables us to construct more active double Ii-RNAi construct, e.g. put EF-1a/P4 and P7 into one plasmid. This achievement also enables us to construct the Ii-RNAi constructs into viral vector for clinical trials.
2. Defined the influence of the promoter on the activity of Ii-RNAi. Our results indicate that two elements are important for the activity of an Ii-RNAi construct: a) Define the right Ii-RNAi sequence to target a specific region of Ii mRNA; and, b) Select the promoter that is most active in that cell line. We have defined three active Ii-RNAi segments and tested the activity of three different promoters, U6, CMV, and EF-1a.
3. Tested the activity of our Ii-RNAi constructs in fresh AML cells. It is important for a clinical trial that human Ii-RNAi constructs be active in fresh tumor cell samples. It cannot be concluded that human Ii-RNAi constructs that are active in stable cells line are active in fresh samples.
4. The doses of IL-2 and IFN- γ are being addressed and optimized with our collaborator, Dr. Hillman.
5. Ii suppression and its role in the enhancement of DNA vaccines has been tested and confirmed by using a gp120 DNA vaccine model. We have obtained a five-fold enhancement of a gp120 DNA vaccine by Ii suppression. This result further confirms that Ii suppression enhances the endogenous antigen presentation by MHC class II molecules without interrupting MHC class I antigen presentation, thus enhancing the potency of tumor cell and DNA vaccines. Demonstration of the enhancement of a DNA vaccine presents another potential avenue for treatment of prostate cancer.

REPORTABLE OUTCOMES

1. Papers.
 - 1) Our data is sufficient to write a paper on the construction and defining the activity of human Ii-RNAi constructs.
 - 2) Construction of human Ii-RNAi active in both cell lines and fresh tumor cells. Manuscript in preparation.
 - 3) Suppression of MHC class II-associated invariant chain enhances the potency of an HIVgp120 DNA vaccine Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu.

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 Kallinteris, Shuzhen Wu, Robert Humphreys, Eric Von Hofe, Minzhen Xu. Antigen Express, Worcester, MA 01606
- 2) "Tumor irradiation potentiates gene-mediated immunotherapy for induction of a curative cancer vaccine." Hillman GG, Xu M, Che M, Von Hofe E, Lu X, Forman JD and Wang Y. ICTR presentation.

CONCLUSIONS

Importance and implications. The generation of potent Ii-RNAi constructs, optimization of the combined use of human Ii-RNAi constructs, and defining the most active promoter to drive the expression of an Ii-RNAi in a given cell line or fresh tumor cells are the necessary steps to ensure the generation of MHC class I+/II+/Ii- phenotype in prostate cancer cells. These cells induce potent anti-prostate cancer immunity by presenting their own tumor antigens to the immune system. This is the only technology that can force living tumor cells to actively present tumor antigens to the immune system. These achievements continue to pave the way to achieve our major long term goal – curative immunotherapy of prostate cancer. We expect this therapy to far exceed the efficacy of other DNA vaccines, dendritic cell vaccines, dendritic/tumor fusions, or dendritic/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. In particular, the identification of potent Ii-RNAi constructs to suppress Ii protein is likely to offer more effective reagents relative to those we developed previously.

Changes in future work to better address the problem. At this point in time, we do not foresee significant changes in the long-term goal: prostate cancer immunotherapy. However, we expended large amounts of effort on the construction of the most active human Ii-RNAi constructs. The human Ii-RNAi constructs are now ready for clinical trials. The determination of immunizing doses and schedules and the doses of IL-2 and IFN- γ will be optimized with our collaborator, Dr. Hillman in mice using murine Ii-RNAi constructs. Her work will reveal the most suitable parameters for prostate cancer immunotherapy. We will further perform the experiments to establish the most active promoter/Ii-RNAi constructs, EF-1a/P4 and EF-1a/P7 vs CMV/P4 and CMV/P4 and P7 in human prostate cancer cell lines for use in a prostate cancer clinical trial.

Evaluation of the knowledge as a scientific or medical product. The experiments of the past year demonstrate the activity of Ii-RNAi constructs in both cell lines and fresh tumor cells, paving the way for the clinical trials for tumor immunotherapies including prostate cancer. This immunotherapy converts tumor cells into the MHC class II molecules-positive and Ii protein-negative phenotype, initiating a potent immune response to cure animals of established tumor.

REFERENCES -None

APPENDICS

Abstracts.

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 Kallinteris, Shuzhen Wu, Robert Humphreys, Eric Von Hofe, Minzhen Xu. Antigen Express, Worcester, MA 01606
2. TUMOR IRRADIATION POTENTIATES GENE-MEDIATED IMMUNOTHERAPY FOR INDUCTION OF A CURATIVE CANCER VACCINE. Hillman GG¹, Xu M², Che M¹, Von Hofe E², Lu X², Forman JD¹ and Wang Y¹.¹Department of Radiation Oncology and Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, United States and ²Antigen Express, Inc., GenereX Biotechnology Corp., Worcester, MA 01606, United States.

Papers.

1. Xueqing Lu, Nikoletta L. Kallinteris, Shuzhen Wu, Robert E. Humphreys, Eric von Hofe, and Minzhen Xu. Suppression of MHC class II-associated invariant chain enhances the potency of an HIVgp120 DNA vaccine. Manuscript submitted.
2. Wang Y, Xu M, Che M, Hofe EV, Abbas A, Kallinteris NL, Lu X, Liss ZJ, Forman JD, Hillman GG. 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. Hum Gene Ther. 2005;16:187-99

ABSTRACTS

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 Kallinteris, Shuzhen Wu, Robert Humphreys, Eric Von Hofe, Minzhen Xu. Antigen Express, Worcester, MA 01606

RNAi is a potent method to inhibit specific gene expression. This method has been evaluated as a potential tool to treat cancer, for example, to specifically inhibit oncogene expression. The biggest challenge for using RNAi to inhibit oncogene expression 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 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 class II molecules pick up endogenous antigenic peptides (including tumor antigens) in the ER. Simultaneous presentation of these tumor antigens by both MHC class I and class II molecules to both CD4+ and CD8+ T cells generates 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. Our novel method forces tumor cells to actively present their tumor antigens and thus has the potential to lead to a feasible and potent tumor cell immunotherapy. We have now generated human Ii-RNAi constructs that effectively inhibit Ii-expression in Raji lymphoma cells and 293 kidney cells. Ii inhibition by active Ii-RNAi constructs reached 95% in Raji cells while a combination of different Ii-RNAi constructs targeting different positions of the ii gene has a synergistic effect on Ii inhibition, reaching about 99% Ii suppression. Because Ii is monomorphic, one Ii-RNAi construct(s) may be sufficient for all patients regardless of their HLA-DR allele. The generation of these active Ii-RNAi constructs provides suitable reagents paving the way for human cancer clinical trials.

TUMOR IRRADIATION POTENTIATES GENE-MEDIATED IMMUNOTHERAPY FOR INDUCTION OF A CURATIVE CANCER VACCINE. Hillman GG¹, Xu M², Che M¹, Von Hofe E², Lu X², Forman JD¹ and Wang Y¹.¹Department of Radiation Oncology and Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201, United States and ²Antigen Express, Inc., Genex Biotechnology Corp., Worcester, MA 01606, United States.

Objective: We have shown that tumor irradiation preceding the transfection of 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 in murine RM-9 prostate carcinoma syngeneic to C57BL/6 mice. Such cancer cells become antigen-presenting cells (APCs) that present both class I and class II endogenous tumor antigens, triggering a potent T-helper response essential for robust cytotoxic T cell activity (CTL). Inhibition of Ii protein increases presentation of endogenous tumor peptides by class II molecules to helper T cells. The mechanism by which tumor irradiation enhances the efficacy of gene therapy for induction of cancer vaccine was investigated.

Materials and Methods: To induce, *in situ*, the MHC class I+/class II+/Ii- phenotype, we used cDNA plasmids containing genes for interferon gamma (pIFN-g) to upregulate MHC class I, MHC class II transactivator (pCIITA) to upregulate MHC class II, an Ii reverse gene construct (pIi-RGC) to suppress Ii, and a subtherapeutic dose of adjuvant IL-2 (pIL-2). Established RM-9 tumors were treated with 8 Gy photon radiation followed by 4 days of intratumoral injections with a mixture of pCIITA + pIFN-g + pIi-RGC + pIL-2 plasmids. Viability of cells isolated from treated tumors at different time points was assessed by colony formation assay. Tumor destruction was assessed on tumor sections by histology and TUNEL assay.

Results: An optimal and specific anti-tumor response is achieved in more than 50% of the mice when, following radiation, tumor nodules are treated with the four pIFN-g, pCIITA, pIi-RGC and pIL-2 plasmids. Mice responding with complete tumor regression rejected tumor rechallenge and demonstrated tumor-specific CTLs. Such therapeutic effect was achieved only when tumor irradiation preceded gene therapy and when the combination of the four plasmids were injected intratumorally to convert tumors to MHC class I+/class II+/Ii- phenotype. Omission of radiation or either one of the plasmids decreased the tumor response and giving gene therapy prior to radiation was not as effective. We demonstrated 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 abrogated the response. Apoptosis was documented in tumor sections by TUNEL assay as early as one day after radiation, at the time gene therapy was initiated. 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. Complete tumor destruction by combined radiation and gene therapy was determined by lack of colony formation of cells isolated from these tumors and by histological observation. Histological analysis of tumor sections shows that tumor irradiation combined with plasmids causes extensive destruction of tumor cells, large areas of apoptosis and necrosis associated with a massive infiltration of lymphocytes and PMN. This effect is seen 1 day after gene therapy and persists for several days while tumor regrowth follows the initial focal apoptosis and necrosis observed after radiation or plasmids alone. We further showed that radiation potentiates the genetic modification of tumor cells by increasing both the level and duration of expression of transfected genes.

Conclusions: Our findings suggest that radiation potentiates gene therapy by causing tumor debulking, increasing gene transfection and the permeability of tumors to infiltration of inflammatory cells. These data emphasize the efficacy of tumor irradiation preceding gene therapy to modify tumor cells *in situ* into a MHC class I+/class II+/Ii- phenotype converting these cells into a potent therapeutic cancer vaccine.

Suppression of MHC class II-associated invariant chain enhances the potency of an HIV gp120 DNA vaccine

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Summary

One function of the MHC class II-associated invariant chain (Ii) is to prevent MHC class II molecules from binding endogenous antigenic epitopes in the endoplasmic reticulum. Ii inhibition leads to MHC class II presentation of endogenous antigens by APC without interrupting MHC class I presentation. We present data that *in vivo* immunization of BALB/c mice with HIV gp120 cDNA plus an Ii suppressive construct significantly enhances the activation of both gp120-specific Th cells and CTL. Our results support the concept that MHC class II-positive/Ii-negative (class II⁺/Ii⁻) APC present endogenously synthesized vaccine antigens simultaneously by MHC class II and class I molecules to activate both CD4⁺ and CD8⁺ T cells. Activated CD4⁺ T cells strengthen locally the activation of CD8⁺ CTL, enhancing the potency of a DNA vaccine.

Introduction

While DNA vaccines induce CTL activity successfully and are considered to be generally safe and economic, various investigators have sought to enhance the immune system's relatively poor response to these vaccines. These methods include the following: Inoculation with genes encoding co-stimulatory molecules;¹ enhancing the *in vivo* transfection efficiency by mixing DNA with cationic lipids;² coating DNA onto microparticles such as poly(lactide-co-glycolide);³ and *in vivo* electroporation.⁴ Use of a gene gun is another effective method to enhance vaccine gene expression by delivering vaccine DNA directly into cells.⁵ The addition of CpG motifs to a plasmid also generates an innate immune stimulus for DNA vaccines.^{6, 7} Different prime/boost regimes using DNA/virus, DNA/protein, and DNA/peptide have been developed.⁸⁻¹⁴ Cytokine genes, especially the GM-CSF gene, have been used in DNA vaccine regimes to augment DNA vaccine efficiency.^{6, 15-17} Our studies add to the work of the above investigators, with a novel and potentially clinically useful to enhance the potency of DNA vaccines.

CD4+ T cell activation plays an important role in the enhancement of DNA vaccine efficacy.¹⁸ We have developed a vaccine strategy based on suppression of expression of the Ii protein, to augment CD4+ T cell activation by endogenously synthesized antigens. The Ii protein normally binds to MHC class II molecules in the ER, blocking the antigenic epitope-binding groove. Ii protein is later digested in a post-Golgi vesicle and released from the MHC class II molecule in a concerted fashion coupled to the charging of antigenic peptides.¹⁹ One of the major functions of Ii is to protect the antigenic peptide binding site on MHC class II molecules from binding endogenously derived antigenic peptides in the ER.^{20, 21} Suppression of Ii leads to the induction of "unprotected" MHC class II molecules in an antigen presenting cell (APC) enabling it to present endogenous antigens by MHC class I molecules (as would normally be done) and class II molecules to simultaneously activate CD4+ and CD8+ T cells. We have previously

generated active Ii suppression plasmid construct: Ii reverse gene construct (Ii-RGC(-92,97), A in AUG is 1), to effectively suppress the Ii expression in tumor cells.²²⁻²⁴ The Ii-RGC(-92,97) codes for expression of an antisense mRNA, which hybridizes to the native mRNA for Ii protein, thereby leading to Ii suppression. This strategy of enabling presentation by both MHC class I and class II molecules on a single APC generates a potent tumor cell vaccine in experimental animal models.²²⁻²⁵

In this study we utilized Ii suppression technology to enhance an HIV gp120 DNA vaccine model. The rationale is that APC, *e.g.* dendritic cells (DC) that take up DNA plasmids containing both the gp120 gene and Ii-RGC will allow for both MHC class I and “un-protected MHC class II” charging by endogenously produced gp120 epitopes in the ER. The DC will subsequently present these epitopes to activate CD4⁺ and CD8⁺ T cells, respectively. The enhanced activation of gp120-specific CD4⁺ T cells in turn helps to strengthen the activation of gp120-specific CD8⁺ T cells, thereby significantly augmenting the efficiency of HIV gp120 DNA vaccines. We report here that addition of the Ii-suppression technology to an HIV gp120 DNA vaccine greatly enhances the potency of the gp120 DNA vaccine. The studies reported here serve as a basis for the rational design of human Ii-RNAi constructs, to be used with established DNA vaccines for enhanced antigen-specific CD4⁺ activation, which could be of potentially significant benefit in therapeutic or preventive vaccinations.

Materials and Methods

Mice

BALB/c mice (8-12 weeks old) were purchased from Jackson Laboratory and kept in the animal facility of the University of Massachusetts Medical Center, Worcester, MA. All animal

procedures were performed following University of Massachusetts Medical School animal care guidelines under an approved protocol.

Cell lines and antibodies

Green monkey kidney COS cells (#CRL-1650), cultured in RPMI-1640 medium with 10% FCS, and murine macrophage J774 cells (#TIB-67), cultured in DMEM with 10% FCS, were from the ATCC. Anti-murine Ii monoclonal antibody, In.1, and anti-murine MHC class II monoclonal antibody, M5/114.15.2, were used.^{26, 27}

Plasmids

Murine Ii cDNA²⁸ was from Dr. James Miller of the University of Chicago. Ii-RGC(-92,97) was described previously.²²⁻²⁴ The pBudCE4.1 plasmid was from Invitrogen (San Diego, CA). The murine GM-CSF plasmid (pNGVL1-mGM-CSF) was from Dr. Gilda G. Hillman of Wayne State University, Detroit, MI and the pCEP4/CIITA plasmid²⁹ was from Dr. Laurie Glimcher of the Harvard School of Public Health (Boston, MA). HIV-1 IIIB gp120 cDNA¹⁶ from Dr. Norman Letvin at the Beth Israel Deaconess Medical Center (Boston, MA) was cloned into a RSV.5 expression vector.³⁰ Expression of the HIV-1 IIIB gene was confirmed by transfecting RSV.5/gp120 into COS cells, staining with anti-HIV-1 IIIB gp120 polyclonal antibody, and analysis by flow cytometry (data not shown).

Peptide synthesis

Two peptides, a 15-mer termed p18 (RIQRGPGRAFVTIGK) and a 10-mer, termed p18-I10 (RGPGRAFVTI) were synthesized by Commonwealth Biotechnologies, Inc., Richmond, VA.

The p18 is presented by both H-2D^d and H-2A^d molecules while the p18-I10 is presented only by H-2D^d.^{31, 32} That is, the shorter p18-I10 peptide contains only the MHC class I-presented epitope while the longer p18 peptide contains both a MHC class II-presented epitope and a MHC class I-presented epitope.

DNA coating of gold particles for gene gun delivery

Plasmid DNA was precipitated onto gold particles. Briefly, 15 µg of 1 µm gold microcarriers (for 30 cartridges) (Bio Rad) were resuspended by sonication in 100 µl of 0.1 M spermidine. The indicated amount of DNA at a concentration of 1 mg/ml in endotoxin-free water was then added and sonicated; 200 µl of 2 M CaCl₂ was then added dropwise. This gold-DNA mixture was allowed to stand for 10 min before being washed 3 times with 1 ml aliquots of 100% ethanol. After the final wash, the pellet was re-suspended by vortexing and sonication in 1.86 ml of 100% ethanol. After precipitating, the plasmid DNA was adsorbed onto gold beads and the gold beads were coated evenly onto the inner surface of Tefzel tubing (Bio Rad). The tubing was then cut into 0.5-inch cartridges after coating. Different DNA loading ratios were designed for respective experiments, as described in results. Cartridges were stored at 4 °C with desiccant.

DNA transfection of cells

For gene gun transfection of J774 cells, 10⁶ cells in 20 µl medium were smeared onto a tissue culture dish in approximately 1 cm diameter circles and then subjected to gene gun shooting with one 0.5-inch cartridge (loaded with 1 µg of DNA) at a helium pressure of 300 psi. After culturing at 37 °C for 18-42 h, cells were stained with anti-MHC class II or anti-Ii monoclonal antibodies and analyzed by flow cytometry.

For electroporation, 2×10^6 COS cells were resuspended in 0.5 ml of RPMI 1640 medium without serum, followed by the addition and gentle mixing of 18 μg plasmid DNA (total). The suspension was transferred to electroporation cuvettes and placed on ice for 10 min. Directly after incubation on ice the cuvetts were subjected to electroporation at 250v/1200 μF with an electroporator (BTX, Holliston, MA). They were placed at RT for 10 min, and cultured overnight at 37 °C in tissue culture flasks. COS cells were harvested and stained with anti-Ii monoclonal antibody In.1, and analyzed by flow cytometry.

Gene gun immunization of mice

Prior to vaccinating mice by the gene gun delivery of DNA, each mouse was anesthetized *i. p.* with a 50 μl of solution comprising 13 μl ketamine solution (100 mg/ml), 17 μl xylazine solution (20 mg/ml), and 20 μl saline. After anesthesia, mice were shaved on the abdomen with an electric shaver. The barrel of the gene gun was held directly against the abdominal skin and a single microcarrier shot was delivered using a helium-activated Gene Gun System at 400 psi (PowderJect). For all *in vivo* experiments in this report, each mouse received three consecutive gene gun inoculations. Two weeks later, mice were boosted with the same amount DNA by the same method. One week after boost, mice were sacrificed for assays.

ELISPOT assay

Total splenocytes were obtained from the individual spleens of sacrificed mice according to UMMC IUCAC-approved procedures. Immunoaffinity-purified CD4⁺ and CD8⁺ splenic lymphocytes were obtained from pooled splenocytes from each group of five mice, the ELISPOT procedures were the same as for total splenocytes. BD Pharmingen kits for murine IFN- γ and IL-

4 ELISPOT assays were used according to the manufacturer's instructions. Briefly, plates were coated overnight at 4 °C with the cytokine capture specific antibodies. The plates were blocked with 10% FBS in RPMI-1640 for 2 h at RT and washed four times with 1X PBS containing 0.05% Tween-20 (wash buffer). Freshly isolated single splenocyte suspensions (10^6 /well) and p18 or p18-I10 peptides (5 µg/well) were added to the anti-cytokine pre-coated plates. After 42-66 h of incubation, the plates were washed five times with wash buffer, biotinylated detection Abs (2 µg/ml) were added and incubated for an additional 2 h at RT. The plates were washed four times with wash buffer and avidin horseradish peroxidase (Avidin-HRP) added at a 1:100 dilution followed by one hr incubation at RT. Avidin-HRP was removed by washing four times with wash buffer and two times with 1X PBS. The spots were developed by adding 3-amino-9-ethylcarbazole HRP substrate to the plates for 30 min at RT. Finally, the plates were washed twice with sterile water and dried for 1 to 2 hrs at RT. Digitized images of spots were analyzed with a series 1 Immunospot Analyzer and Immunospot 1.7e software (Cellular Technology Limited, Cleveland, OH).

Results

Construction of Ii suppression plasmids

We have previously generated an Ii reverse gene construct: Ii-RGC(-92,97) that effectively inhibites Ii expression in tumor cells and created potent tumor immunotherapy in animal models.²²⁻²⁴ Ii-RGC(-92,97) was generated by cloning an Ii gene fragment (-92,97) into an expression vector in a reverse orientation. The antisense RNA produced by this Ii-RGC hybridizes with Ii mRNA to block translation of Ii mRNA and/or trigger the destruction of Ii mRNA.^{33, 34} In this study, We cloned three copies of the Ii-RGC(-92,97) gene fragment into one pBudCE4.1

plasmid, generating pBudCE4.1/Ii-RGC(x3) (Figure 1) to increase the efficiency of Ii suppression. In pBudCE4.1/Ii-RGC(x3), each Ii-RGC(-92,97) gene fragment is driven by a different promoter in order to avoid possible promoter competition. The first Ii-RGC(-92,97) gene fragment was inserted into pBudCE4.1 by Hind 3 and Bam H1 under the control of CMV promoter to generate one-copy pBudCE4.1/Ii-RGC(-92,97) [pBudCE4.1/Ii-RGC]. The second copy of Ii-RGC(-92,97) was first cloned into the pUB6/V5-His plasmid (Invitrogen, San Diego, CA) under control of the UbC promoter. The UbC promoter, Ii-RGC(-92,97) fragment, and poly A signal sequence were then amplified by PCR and cloned into the pBudCE4.1/Ii-RGC to generate two-copy pBudCE4.1/Ii-RGC(-92,97) [pBudCE4.1/Ii-RGC(x2)]. The third copy of Ii-RGC(-92,97) was first cloned into the RSV.5 plasmid under the control of an RSV promoter^{22, 24}. The RSV promoter, Ii-RGC(-92,97) fragment, and poly A signal were PCR amplified and cloned into pBudCE4.1/Ii-RGC(-92,97(x2)) to generate a three-copy pBudCE4.1/Ii-RGC(-92,97) [pBudCE4.1/Ii-RGC(x3)] (Figure 1). More detailed procedures and enzyme sites for cloning are explained in the legend of Figure 1.

***In vitro* activity of one-, two-, and three- copy pBudCE4/Ii-RGCs**

The activities of the three different pBudCE4.1/Ii-RGC plasmids were tested in COS cells to define the most active pBudCE4.1/Ii-RGC plasmid. This was achieved by determining inhibition of expression of a co-transfected Ii cDNA in COS cells. The COS cells (2×10^6) were mixed with murine Ii cDNA (3 μ g) plus 15 μ g of empty pBudCE4.1 plasmid (Ii-positive control cells), pBudCE4.1/Ii-RGC, pBudCE4.1/Ii-RGC(x2), or pBudCE4.1/Ii-RGC(x3), respectively, and subjected to electroporation (250V/1250 μ F). After 20 h in culture, cells were stained with anti-Ii antibody (In.1) and analyzed by flow cytometry. In Figure 2, one sees that pBudCE4.1/Ii-

RGC(x3) is the most active and has almost completely inhibited Ii expression in transfected cells (D); see legend of Figure 1 for a more detailed description. The pBudCE4.1/Ii-RGC and pBudCE4.1/Ii-RGC(x2) were also active (B and C) compared to empty plasmid control (A) but they are less active than pBudCE4.1/Ii-RGC(x3) (compare the main peaks of B, C, and D). The Ii-positive cells in (D) are those cells that transfected with Ii cDNA without receiving pBudCE4.1/Ii-RGC(x3) under co-transfection condition with two plasmids. The pBudCE4.1/Ii-RGC(x3) plasmid was used in all subsequent experiments.

Ii suppression in J774 macrophage cells by pBudCE4.1/Ii-RGC(x3)

Dendritic cells (DC), macrophages, and Langerhans cells play important roles in the induction of immunity against DNA vaccine antigens, especially when a gene gun is used for DNA delivery.^{35,36} For this reason, we tested the activity of our Ii suppression constructs on the murine macrophage line J774. As J774 is an MHC class II-positive and Ii-positive cell line (Figure 3B), pBudCD4.1/Ii-RGC(x3) was used to assess inhibition of endogenously expressed Ii. We usually obtained 30-70% transient transfection efficacy using the gene gun depending on the cell line used (unpublished observations). As shown in Figure 3D, Ii was significantly suppressed in transfected J774 cells (>95% as measured by fluorescence intensity) without apparent change in MHC class II expression by pBudCE4.1/Ii-RGC(x3). Under normal conditions, the Ii protein is synthesized in excess relative to MHC class II molecules in APC;³⁷ therefore, >95% of Ii inhibition could lead to most MHC class II molecules in a transfected DC being unprotected by Ii molecules. These “unprotected” MHC class II molecules should be available for charging by epitopes present in the ER (including gp120 epitopes) followed by subsequent presentation to CD4+ T cells. COS and J774 cell transfection experiments demonstrated that the pBudCD4.1/Ii-

RGC(x3) plasmid is the most active Ii-RGC plasmid for inhibiting the expression of either co-transfected Ii c DNA or endogenous Ii and was used for *in vivo* experiments in this study.

Ii suppression enhances gp120 DNA vaccine efficiency

Next we tested whether Ii suppression enhanced HIV gp120 DNA vaccine efficiency. BALB/c mice were immunized with the gene for gp120, with or without pBudCE4.1/Ii-RGC(x3). In our *in vivo* experiments, the gene for GM-CSF was included as an adjuvant. GM-CSF is frequently used to enhance the potency of a DNA vaccine.¹⁶ In Figure 4, one sees that both p18- and p18-I10-specific ELISPOT assays demonstrated roughly 5 times enhancement of IFN- γ secreting cells in the Ii-suppressed groups (groups D and E) compared to the Ii un-suppressed group (group C). The enhancement was related to the Ii suppression and not related to the use of GM-CSF. The enhancement was more profound at the lower concentration of pBudCE4.1/Ii-RGC(x3) group (group D). This phenomenon may reflect less promoter competition among gp120-, GM-CSF-, and pBudCE4.1/Ii-RGC(x3). Similar reaction patterns to p18 and p18-I10 stimulation were observed, the p18 peptide gave a greater response in most reactions. This result is consistent with previous reports which show p18-I10 is restricted only by H-2D^d while the p18 peptide is restricted by both H-2D^d and H-2A^d.^{31, 32} The p18 reaction reflects both CD4+ and CD8+ reactions and p18I-10 reaction reflects only CD8+ reaction. This result indicated that most IFN- γ spots were secreted by CD8+ T cells in ELISPOT with total splenocytes. This is consistent with ELISPOT data with purified CD4+ and CD8+ T cells (next section).

In order to determine whether Ii suppression induced a Th1 or Th2 response, IL-4 secretion was also examined in the ELISPOT assay. IL-4 was induced in all groups (Figure 4b). Compared to IFN- γ production (Figure 4a), IL-4 production was relatively lower, indicating that

the addition of the GM-CSF gene induced a Th1-biased immune response, which finding is consistent with prior studies.¹⁶ Nevertheless, IL-4 production was also enhanced by Ii suppression. Since the enhancement pattern of IL-4 is similar to the enhancement of IFN- γ , we conclude that Ii suppression influences the magnitude of immune response, but does not influence the Th1/Th2 pattern in our model.

Ii suppression enhances the activation of both gp120-specific CD4+ and CD8+ T cells

In order to elucidate clearly whether Ii suppression enhances activation of only gp120-specific Th cells or both Th cells and CTL, we purified CD4+ and CD8+ T cells prior to analysis of cytokine expression. Pooled splenocytes (5×10^6 /ml) from each group (5 mice) were cultured with p18 peptide (25 μ g/ml) for 5 days. The cells were purified with MiniMACs separation units (Miltenyi Biotec, Germany) and incubated for another 48 h prior to an IFN- γ ELISPOT assay. As indicated in Figures 5a and 5b, both CD4+ and CD8+ T cells were activated by gp120 DNA vaccine and Ii suppression enhanced activation of both CD4+ and CD8+ T cells. This result is consistent with our working hypothesis that Ii suppression enhances the activation of CD4+ Th cells, which in turn augments and strengthens the activation of CD8+ CTL. The frequency of generation of p18-specific CD4+ T cells is consistent with previous reports showing that the frequency of MHC class II epitope-specific CD4+ Th cells resulting from DNA vaccine is much lower than that of CD8+ T cells.³⁸ Ii suppression enhances the frequency of activated CD4+ T cells, which in turn enhance CD8+ T cell activation.

Addition of CIITA abolishes the enhancement activity by Ii suppression

Since keratinocytes might play a role in augmenting the magnitude of the immune response to DNA vaccines,³⁹ the gene encoding MHC class II transactivator (CIITA)⁴⁰ was added to the DNA used to immunize mice. CIITA is a strong inducer of MHC class II and Ii and was used in these experiments to induce MHC class II expression in keratinocytes. Coupling this with Ii suppression increases the frequency of the MHC class II⁺/Ii⁻ phenotype in keratinocytes, which in turn might lead to augmentation of potency of the gp120 DNA vaccine. From Figure 6a, one sees that addition of CIITA (group E) did not enhance the vaccine efficiency (compared to groups C); instead, addition of CIITA abolished the vaccine efficiency enhanced by Ii suppression (compared group F with group C). Addition of CIITA also abolished IL-4 enhancement by Ii suppression (Figure 6b). Possible mechanisms for this effect are considered in the Discussion.

Discussion

The concept that induction of MHC class II⁺/Ii⁻ phenotype APC results in the presentation of endogenously-derived antigenic epitopes by MHC class II molecules was first developed by Dr. Ostrand Rosenberg and colleagues.⁴¹⁻⁴³ They showed that immunization of mice with tumor cells transfected with syngeneic MHC class II molecules (without Ii) led to a potent tumor cell vaccine and that reintroduction of the Ii protein into such tumor cells abolished the induced vaccine potency.⁴² They also showed that MHC class II⁺/Ii⁻ tumor cells presented ER-retained antigens through MHC class II molecules to activate T cells. they have further showed that Ii limited MHC class II presentation of ER-retained antigens^{41, 43} but not membrane-bound antigen.⁴⁴ Because transfection of tumor cells with a syngeneic MHC class II gene is not clinically feasible given the great polymorphism of MHC class II genes in humans, we have developed a more practical method based on inhibition of the monomorphic Ii gene.

Concomitant administration of Ii-RGC and a gene for the transcription factor CIITA, which induces both MHC class II molecules and the Ii protein, ensures generation of the MHC class II+/Ii- phenotype.²²⁻²⁵ This method leads to a potent tumor cell vaccine, which is effective in animal models of renal and prostate cancer.^{22, 24, 25} In the current study, we applied the same strategy to develop a more potent DNA vaccine; *i.e.*, using a construct to suppress Ii in each DNA-transfected cell (*e.g.*, DC) resulting in the simultaneous presentation of endogenously synthesized vaccine antigens (gp120 epitopes) by “unprotected” MHC class II as well as MHC class I molecules. In this manner, activation of gp120-specific CD4+ T cells, and in turn enhanced activity of CD8+ cells, is greatly increased. Our data clearly show that activation of both CD4+ T cells and CD8+ T cells is enhanced by Ii suppression, supporting the working hypothesis and consistent with our previous tumor immunotherapy results.^{22, 24, 25}

Gene gun-mediated DNA immunization results in the transfection of keratinocytes and local DC.^{5, 36} Several studies have shown that direct transfection of DC after gene gun DNA immunization is a key factor for the induction of immunity. Porgador et al.⁴⁵ demonstrated that after gene gun delivery of DNA, 20,000 – 30,000 DC were recruited per draining lymph node and that 20-75 DC were directly transfected with the gene. Further, they showed in CTL activation assays that transfected DC are the predominant APC for CTL activation. Akbari et al.⁴⁶ showed that DNA vaccination led to a relatively low frequency of DC transfection. However, it was these transfected DC that led to the general activation of all DC, providing good conditions for effective Th cell activation. Our hypothesis is that gene gun-mediated vaccination of mice with pBudCE4.1/Ii-RGC(x3) plus pcDNA(3)/gp120 led to the generation of gp120+/MHC class II+/Ii- DC, that are more effective in activating both types of gp120-specific CD4+ Th cells. Presumably, this enhancement is obtained through the acquisition and

presentation of endogenously synthesized gp120 epitopes by “unprotected” MHC class II molecules.

The CIITA gene was added to the Ii-RGC/gp120 DNA vaccine in the hope of increasing the frequency of MHC class II+/Ii- keratinocytes for the further enhancement of DNA vaccine potency. However, addition of the CIITA gene did not enhance efficacy of the vaccine and, instead, abolished the vaccine potency enhanced by Ii suppression (Figure 6). Our previous experiments showed that 3 times more Ii-RGC plasmid is needed to suppress Ii induced by CIITA (unpublished observation). We conclude that Ii is well inhibited in our *in vivo* experiment since the concentration of CIITA plasmid was 7 times lower than the Ii-RGC plasmid (see Figure 6 legend). Mechanisms to explain this phenomenon relate to findings of Landmann *et al.*⁴⁷ They demonstrated that in the process of DC maturation, there is enhanced cell surface MHC class II expression followed by a turn off of *de novo* biosynthesis of MHC class II mRNA. This is due to a rapid reduction in the synthesis of CIITA which is triggered by a variety of different maturation stimuli, including LPS, TNF-alpha, CD40 ligand, IFN-alpha, and infection with *Salmonella typhimurium* or Sendai virus.⁴⁷ The addition of CIITA might block this *de novo* CIITA turn off process, which disturbs normal maturation of DC.

The major advantage of using Ii suppression to augment MHC class II presentation of endogenously expressed DNA antigens is that it induces strong antigen-specific CD4+ T cell activation, while the induction of CD8+ T cells is not interrupted. Vaccine antigens are also released and phagocytosed by DC or other APC, through the exogenous antigen processing and presentation pathway to activate Th cells.⁴⁸ However, the availability of soluble antigen to MHC class II molecules is limited by the low levels of released antigen. Released vaccine antigens can be taken up by APC and DC throughout the body, losing the advantage of co-localized stimulation of CD4+

and CD8+ cells. Our Ii inhibition strategy leads to a simultaneous transfection of DC with DNA containing HIV gp120 and Ii-RGC to result in the expression of gp120 and “unprotected” MHC class II molecules in a single DC. Following endogenous synthesis of gp120, processing and presentation of gp120 epitopes through MHC class I and Class II molecules occurs simultaneously. This results in a strong CD4+ and CD8+ T cell collaboration to increase efficacy of the DNA vaccine.

Our results support the feasibility of a novel strategy to augment the efficacy of DNA vaccines in a clinical setting. The Ii suppression technology does not conflict with other vaccine enhancement technologies and can be used in combination with other vaccine methods including cytokines and adjuvants to further enhance DNA vaccines.

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Figure Legends:

Figure 1. Generation and the map of BudCE4.1/Ii-RGC(x3). The active Ii-RGC(-92,97) fragment was cloned into pBudCE4.1 plasmid by Hind 3 (97 end, A in AUG codon is 1) and BamH 1 (-92 end) under control of a CMV promoter to create the plasmid one-copy pBudCE4.1/Ii-RGC(-92,97) [pBudCE4.1/Ii-RGC]. For two-copy Ii-RGC plasmids, the Ii-RGC(-92,97) fragment was first cloned into pUB6/V5-His by Hind 3 (close to UbC promoter for 97 end) and BamH 1 (-92 end) under control of a UbC promoter to generate pUB6/V5-His/Ii-RGC(-92,97). The UbC promoter, Ii-RGC(-92,97) gene fragment, and poly A signal sequence were then amplified by PCR and cloned into the Nhe1 site of pBudCE4.1/Ii-RGC to generate two-copy pBudCE4.1/Ii-RGC(-92,97) [pBudCE4.1/Ii-RGC(x2)]. For three-copy Ii-RGC(-92,97), the Ii-RGC(-92,97) gene fragment was first cloned into a RSV.5 plasmid by Sal1 (close to RSV promoter for 97 end) and Bam H1 (-92 end) under control of a RSV promoter to generate RSV.5/Ii-RGC(-92,97).²² The RSV promoter, Ii-RGC(-92,97) gene fragment, and poly A signal sequence were amplified by PCR and then cloned into the Nhe 1 site of pBudCE4.1/Ii-RGC(x2) to generate three-copy pBudCE4.1/Ii-RGC(-92,97) [pBudCE4.1/Ii-RGC(x3)]. Figure 1 illustrates that each Ii-RGC(-92,97) gene fragment was cloned in reverse orientation relative to its promoter. The '97' end of the Ii-RGC(-92,97) gene fragment is always close to the promoter side, indicating that an antisense RNA will be produced. In pBudCE4/Ii-RGC(x3), each Ii (-92,97) gene fragment is driven by a different promoter in order to avoid possible promoter competition.

Figure 2. Ii suppression in COS cells by one-, two-, and three-copy pBudCE4.1/Ii-RGC(-92,97). The activity of pBudCE4/Ii-RGCs was determined in Ii-negative COS cells by determining inhibition of expression of co-transfected murine Ii cDNA. COS cells (2×10^6) were transfected by electroporation with murine Ii cDNA (3 μ g) plus 15 μ g of: A) Empty pBudCE4.1 plasmid; B) pBudCD4.1/Ii-RGC; C) pBudCD4.1/Ii-RGC(x2); and, D) pBudCD4.1/Ii-RGC(x3). Cells were then cultured for 18 h and stained with anti-Ii protein antibody In.1 and analyzed by flow cytometry. The dotted line represents COS cells that were mock-electroporated (negative control). COS cells transfected with Ii cDNA plus empty plasmid (A) resulted 53% of Ii-positive cells, plus Ii-RGC(x1) (B) resulted 49% of Ii-positive cells, plus Ii-RGC(x2) resulted 42% Ii-positive cells, and plus Ii-RGC(x3) resulted 29% of Ii-positive cells. The main peak in D almost completely overlaps the Ii-negative peak, indicating almost complete inhibition of Ii expression by pBudCD4.1/Ii-RGC(x3) in 24% cells (compare to A). The remaining 29% of Ii-positive cells in D are mostly those that received the Ii cDNA gene without the Ii-RGC(x3) plasmid under two plasmid co-transfection condition.

Figure 3. Ii suppression in J774 cells by pBudCD4.1/Ii-RGC(x3). 10^6 J774 cells were subjected to gene-gun delivery of 1 μ g of pBudCE4.1/Ii-RGC(x3) as described in Materials and Methods. Three replicates of transfected J774 cells (3×10^6) were cultured for 48 h, harvested and stained for both Ii and MHC class II proteins followed by flow cytometry analysis. A) J774 cells stained with only FITC-labeled second antibody (negative control); B) J774 cells stained with anti-Ii (In.1) and anti-MHC class II (M5/114.15.2) antibodies (positive controls); C) J774 cells treated with pBudCE4.1 empty plasmid and stained as in B); D) J774 cells treated with pBudCE4.1/Ii-RGC(x3) and stained as in B). The figure shows that Ii was significantly

suppressed (>95 % suppression as determined by fluorescent density) in 31% of J774 cells (see D) while empty plasmid had no inhibition of Ii (see C). We obtain Ii inhibition in 30% to 70% using gene gun transfection and electroporation in many comparable experiments in several cell lines (unpublished observations).

Figure 4. IFN- γ and IL-4 ELISPOT assays with splenocytes of mice immunized with gp120 with or without Ii suppression. All groups except A) (naïve mice) were immunized using the gene-gun with 2 μg of RSV.5/gp120 plasmid and each of the following DNA plasmids, respectively: B) Empty pBudCE4.1 (1.35 μg); C) pNGVL1/GM-CSF (0.35 μg) + empty pBudCE4.1 (1.0 μg); D) pNGVL1/GM-CSF (0.35 μg) + pBudCE4.1/Ii-RGC(x3) (0.325 μg) and empty pBudCE4.1 (0.675 μg); E) pNGVL1/GM-CSF (0.35 μg) + pBudCE4.1/Ii-RGC(x3) (1.0 μg). Compare to C), Ii suppression (D) resulted about 5-fold enhancement of IFN- γ secretion. Addition of 3-times more Ii-RGC(-92,97)(x3) (E) did not result more IFN- γ secretion, instead, IFN- γ secretion in (E) was slightly reduced than in (D). Possible mechanisms are explained in Results and Discussion. ELISPOT assays were performed with medium only (open bar), p18 peptide (dotted bar), or p18-I10 peptide (dashed bar). A triplet-well assay was performed with 10^6 splenocytes/well for each of 5 mice. Each bar is the SD for triplet assays for each of 5 individual mice. Comparable results were obtained in 3 experiments.

Figure 5. IFN- γ ELISPOT response of immunopurified CD4+ and CD8+ T cells. Splenocytes from the mice of Figure 4 were pooled within each group of 5 mice and incubated ($5 \times 10^6/\text{ml}$) with p18 peptide (25 $\mu\text{g}/\text{ml}$) for 5 days. The cells were then purified for CD4+ and CD8+ T cells using MiniMACS separation units according to the manufacturer's instruction. The

purified CD4⁺ or CD8⁺ T cells were cultured in capture antibody-coated, 96-well plates for another 48 h in the presence of p18 peptide. CD8⁺ T cells is more frequent than CD4⁺ T cells and this is consistent with the result in Figure 4 (slightly higher ELISPOT activity were obtained with P18 than P18-I10). The result is further discussed in Discussion. The plates were ELISPOT assayed in triplet wells each with 10⁵ cells. Each bar is the SD for triplet assays. Group design was the same as for Figure 4. Comparable results were obtained in 3 experiments.

Figure 6. IFN- γ and IL-4 ELISPOT assays when CIITA was added in DNA vaccine formula. IFN- γ and IL-4 ELISPOT responses of splenocytes of mice immunized with, a) the gp120 cDNA gene, b) CIITA gene, and c) with or without Ii suppression. All groups except A) (naïve mice) were immunized using the gene-gun with 2 μ g of RSV.5/gp120 plasmid and respectively each of the following DNA plasmids: B) Empty plasmid (1 μ g); C) pNGVL1/GM-CSF (0.35 μ g) + empty pBudCE4.1 (0.65 μ g); D) pNGVL1/GM-CSF (0.35 μ g) + pBudCE4.1/Ii-RGC(x3) (0.65 μ g); E) pNGVL1/GM-CSF (0.35 μ g) + pCEP4/CIITA (50 ng) + empty pBudCE4.1 (0.65 μ g); and F) pNGVL1/GM-CSF (0.35 μ g) + pCEP4/CIITA (50 ng) + pBudCE4.1/Ii-RGC(x3) (0.65 μ g). ELISPOT assays with medium (open bar) and p18 (dashed bar) were performed in triplet with 10⁶ cells per well for each mouse. Each bar is the SD for triplet assays on each of 5 individual mice. Comparable results were obtained in 2 experiments. The possible mechanisms are considered in the Discussion.

Figure 1.

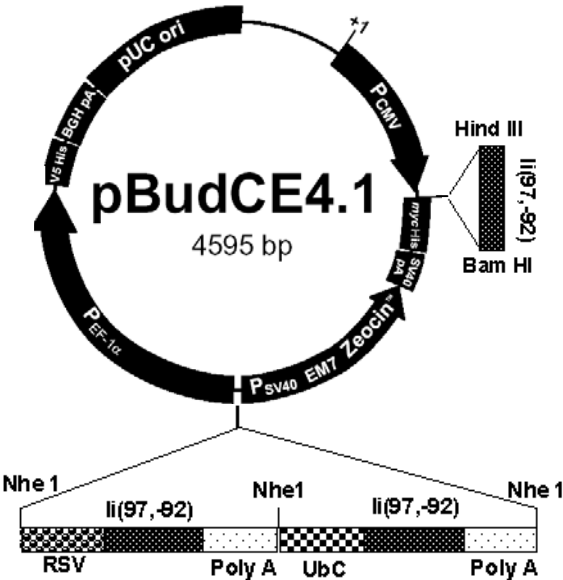


Figure 2.

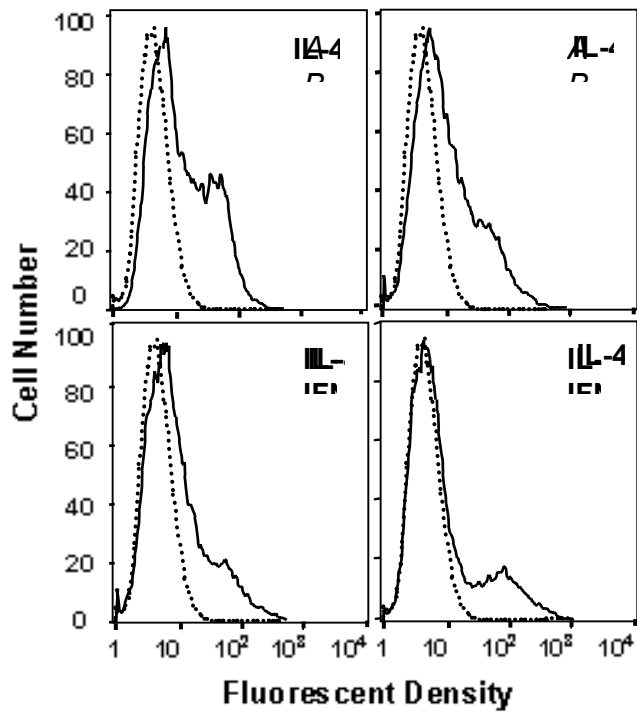


Figure 3.

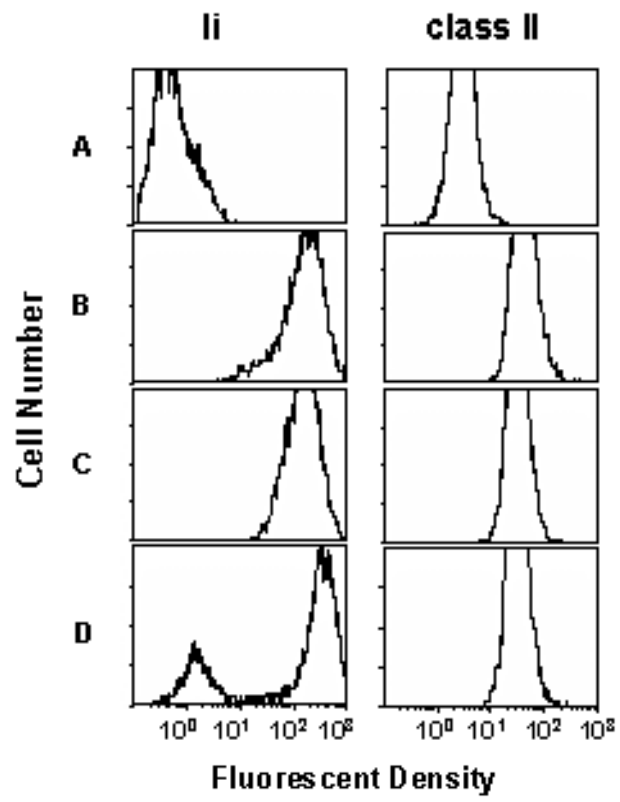


Figure 4.

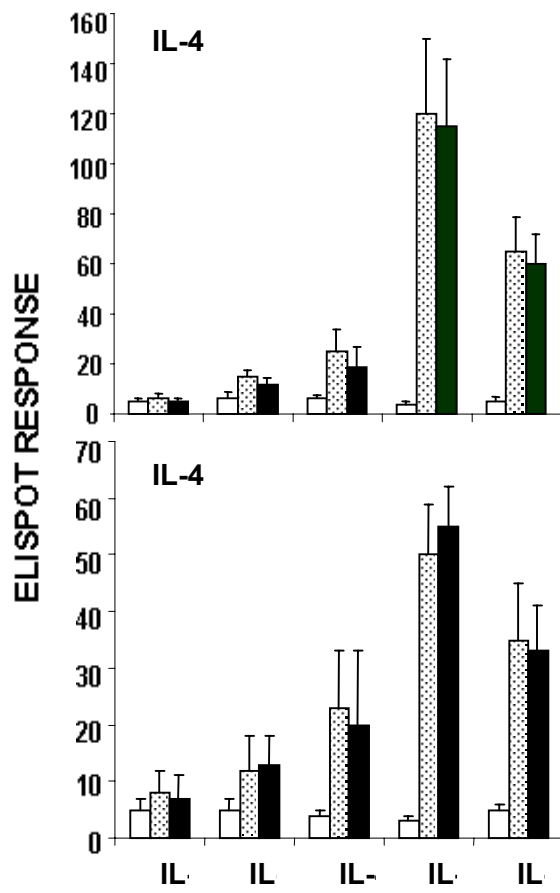


Figure 5.

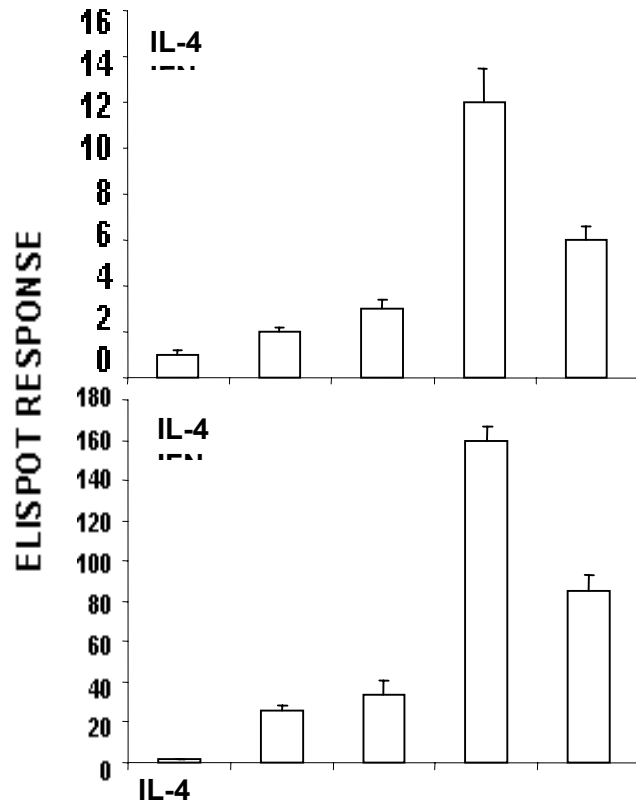
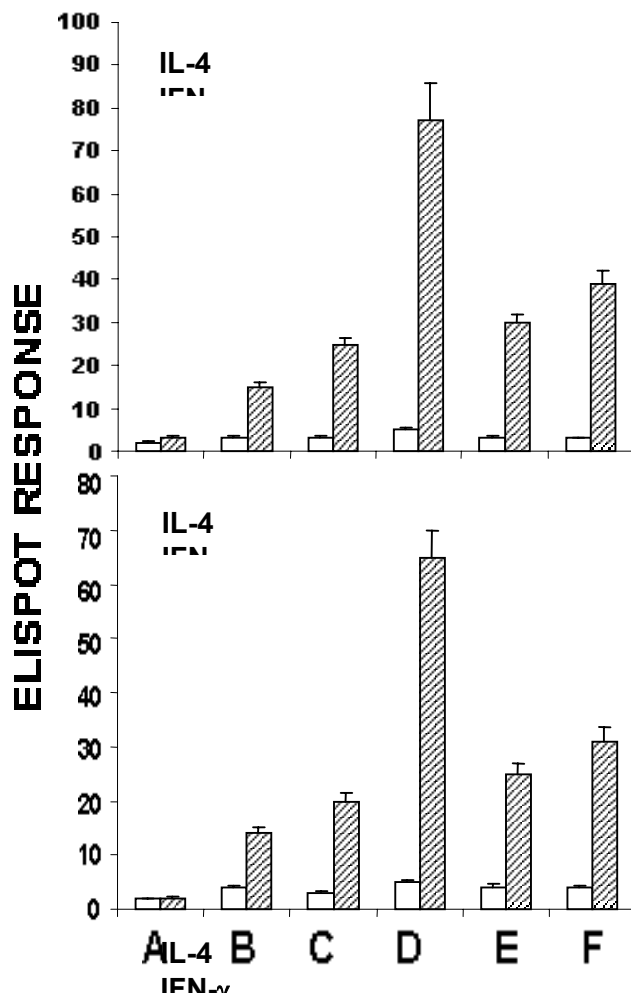


Figure 6.



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). Suprascripting 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, pIi-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, pIi-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 pIi-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 Tompson (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 Ii-RGC (pIi-RGC) was constructed by cloning an Ii 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 Ii-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 Ii-RGC DNA plasmid was injected at 31 μ g per injection per day. We used approximately 10 times more Ii-RGC than CIITA in order to ensure that each cell transfected with a CIITA gene was also transfected with Ii-RGC, and to ensure that there would be sufficient suppression of the Ii protein in light of Ii 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-dimethylhydroxyethyl 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 naive 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 + pIi-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 , pIi-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 pIi-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, 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. Combining radiation with pIi-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 pIi-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 pIi-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 pIi-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 pIi-RGC. Upregulating MHC class II molecules by pCIITA and decreasing Ii protein by pIi-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-

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-

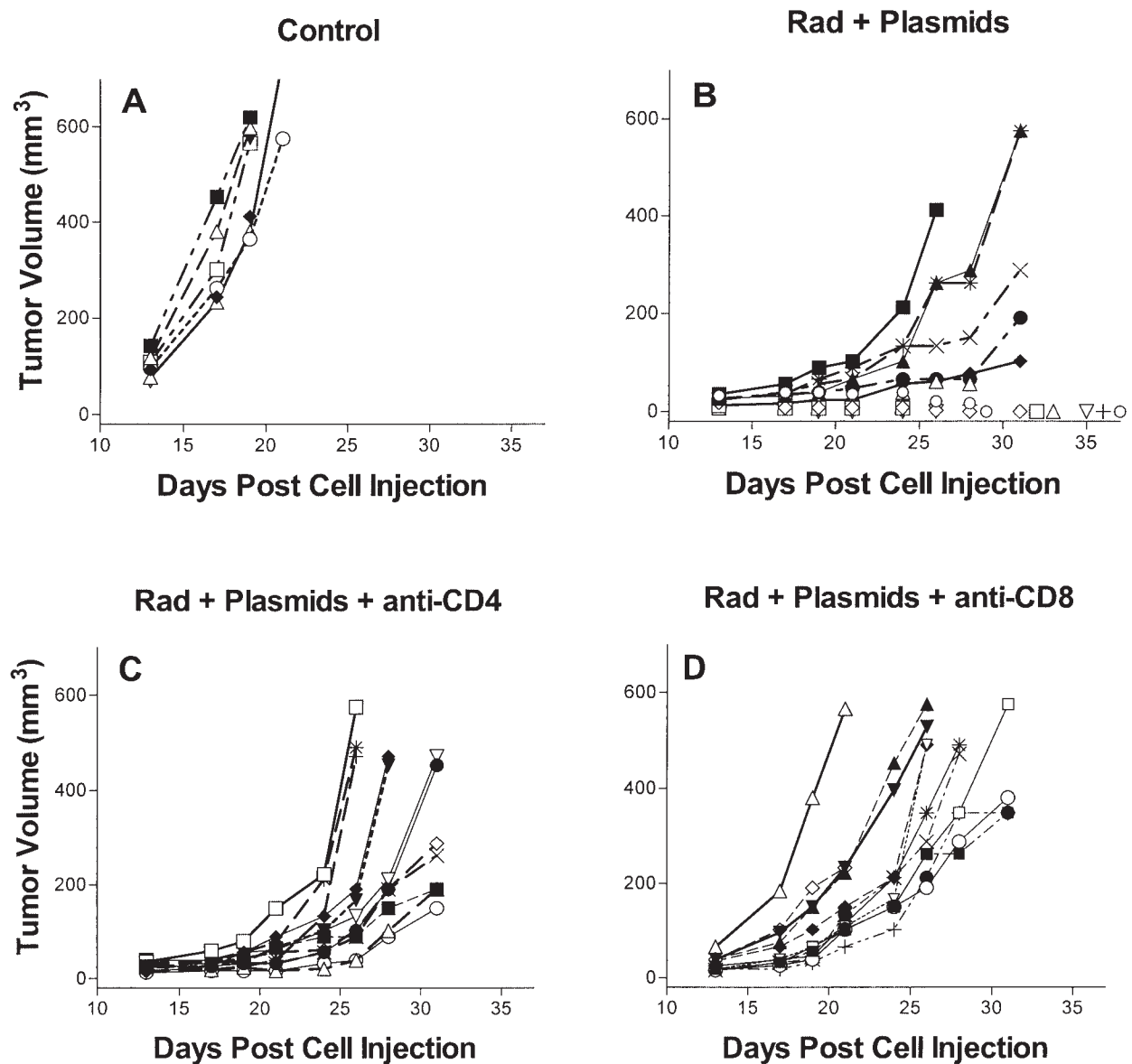


FIG. 1. Growth of RM-9 tumors in mice depleted of T cells and treated with irradiation and gene therapy. Mice were injected subcutaneously with RM-9 cells and treated with intraperitoneal injections of GK 1.5 anti-CD4 monoclonal antibody (mAb) or Ly-2 anti-CD8 mAb before and after gene therapy on day 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 pI-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 Ii suppressor gene, to upregulate MHC class I and class II molecules and suppress the Ii invariant chain, transiently inhibited tumor growth (Hillman *et al.*, 2003b). This effect suggested that this

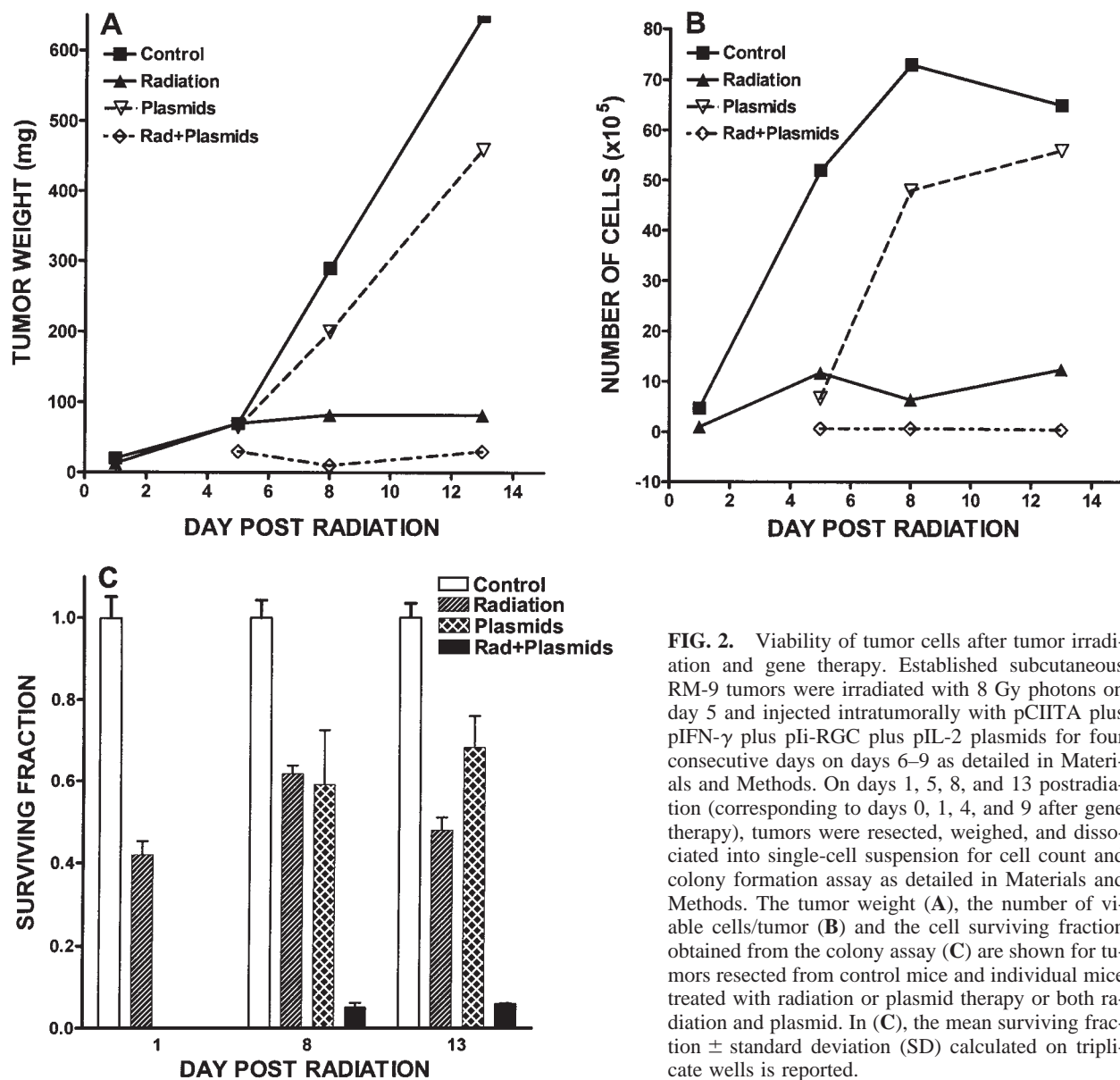
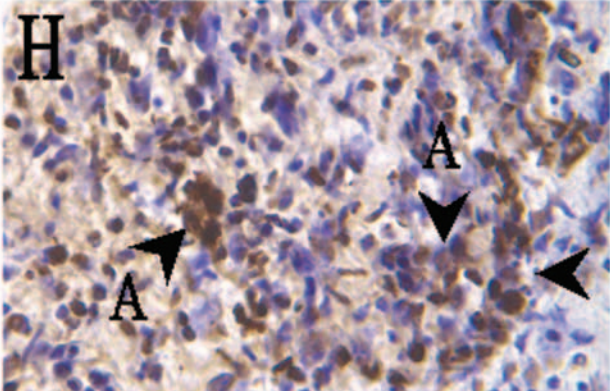
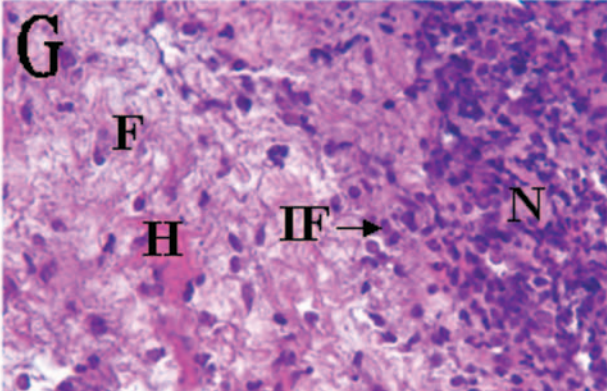
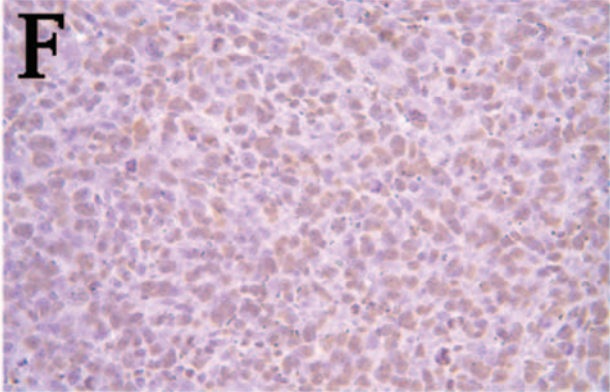
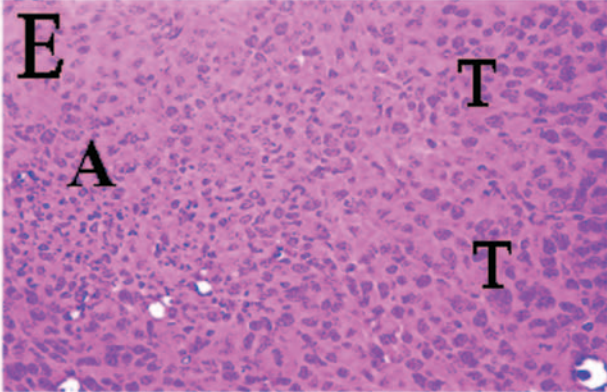
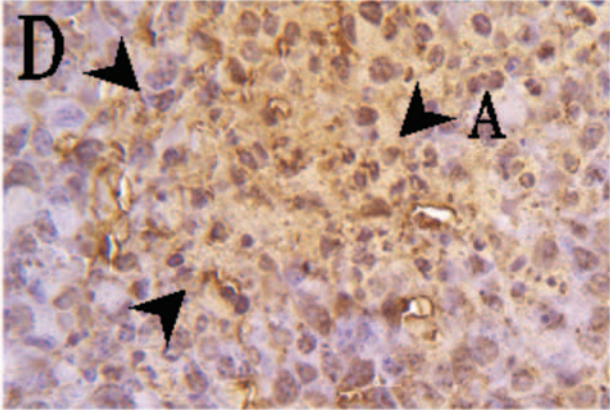
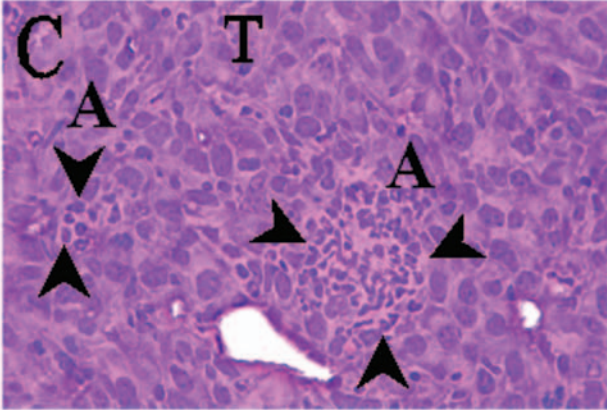
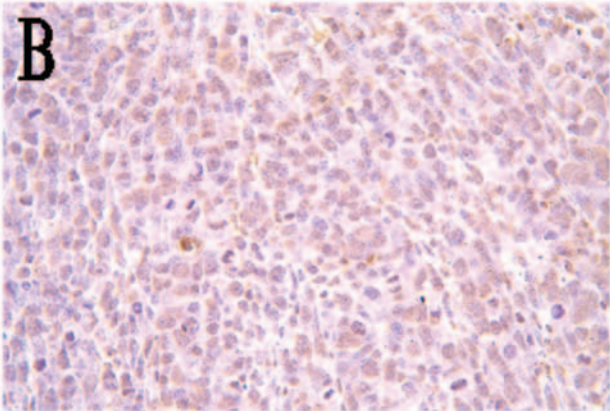
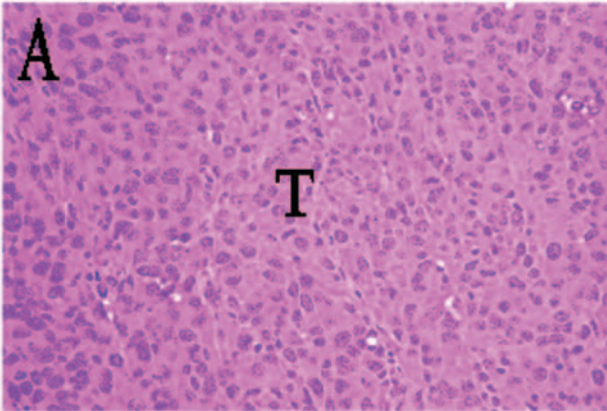


FIG. 2. Viability of tumor cells after tumor irradiation and gene therapy. Established subcutaneous RM-9 tumors were irradiated with 8 Gy photons on day 5 and injected intratumorally with pCIITA plus pIFN- γ plus pIi-RGC plus pIL-2 plasmids for four consecutive days on days 6–9 as detailed in Materials and Methods. On days 1, 5, 8, and 13 postirradiation (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 postirradiation, 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. G: 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. H: 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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