

# 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

YU WANG,<sup>1</sup> MINZHEN XU,<sup>2</sup> MINGXIN CHE,<sup>3</sup> ERIC VON HOFE,<sup>2</sup> ASAD ABBAS,<sup>1</sup>  
NIKOLETTA L. KALLINTERIS,<sup>2</sup> XUEQING LU,<sup>2</sup> ZACHARY J. LISS,<sup>1</sup> JEFFREY D. FORMAN,<sup>1</sup>  
and GILDA G. HILLMAN<sup>1</sup>

## 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- $\gamma$  (pIFN- $\gamma$ ), 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- $\gamma$ , 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- $\gamma$  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- $\gamma$ , pCIITA, pIi-RGC, and pIL-2. We demonstrate further that both CD4<sup>+</sup> helper T cells and CD8<sup>+</sup> 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)- $\gamma$ , 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- $\gamma$ , 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<sup>+</sup> T helper cells and CD8<sup>+</sup> 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

Departments of <sup>1</sup>Radiation Oncology and <sup>3</sup>Pathology, Barbara Ann Karmanos Cancer Institute at Wayne State University School of Medicine and Harper Hospital, Detroit, MI 48201.

<sup>2</sup>Antigen Express, Inc., A Subsidiary of Genex Biotechnology, Corp., Worcester, MA 01606.

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  $\alpha$  and  $\beta$  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- $\gamma$  (pIFN- $\gamma$ ) 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- $\gamma$ , 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- $\gamma$ , 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- $\gamma$ , 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- $\gamma$ , 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- $\gamma$ , CIITA, Ii-RGC and supplemented with adjuvant cytokine plasmid IL-2. Selective *in vivo* depletion of CD4<sup>+</sup> helper T cells or CD8<sup>+</sup> 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  $\mu$ 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  $2 \times 10^5$  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- $\gamma$  (pIFN- $\gamma$ ) 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  $2 \times 10^5$  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- $\gamma$ , and IL-2 DNA plasmids were injected at a dose of 3  $\mu$ g per injection per day while Ii-RGC DNA plasmid was injected at 31  $\mu$ 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  $\mu$ 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  $1 \times 10^5$  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<sup>+</sup> or CD8<sup>+</sup> T cell subsets*

Mice were injected subcutaneously with RM-9 cells at  $2 \times 10^5$  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<sup>+</sup> T cells, 0.1 ml ascites fluid of GK 1.5 mAb was injected intraperitoneally. To deplete CD8<sup>+</sup> 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- $\gamma$  + 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<sup>+</sup> and CD8<sup>+</sup> T cells respectively (Caltag Laboratories, Burlingame, CA). Gates were set for nonspecific binding using cells labeled with the isotypes rat IgG<sub>2b</sub>-FITC and rat IgG<sub>2b</sub>-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% CO<sub>2</sub>/5% O<sub>2</sub>/90% N<sub>2</sub> 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  $\mu$ 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  $\chi^2$  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- $\gamma$ , 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  $\mu$ 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- $\gamma$  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- $\gamma$  and pIi-RGC. No complete responders were observed in eight treated mice showing that upregulation of MHC class I molecules by pIFN- $\gamma$  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 <sup>a</sup>	NA
Radiation	0/20 <sup>a</sup>	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- $\gamma$ + pIi-RGC	0/8	NA
Radiation + pIFN- $\gamma$ + 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 <sup>b</sup>	4/4
<i>IV. MHC Class I+/Class II+</i>		
Radiation + pCIITA + pIFN- $\gamma$	0/5	NA
Radiation + pCIITA + pIFN- $\gamma$ + pIi-RGC	1/7	1/1
Radiation + pCIITA + pIFN- $\gamma$ + pIL-2	3/11 <sup>b</sup>	3/3
Radiation + pCIITA + pIFN- $\gamma$ + pIi-RGC + pIL-2	11/21 <sup>b</sup>	11/11

<sup>a</sup>In 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.

<sup>b</sup>In 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.

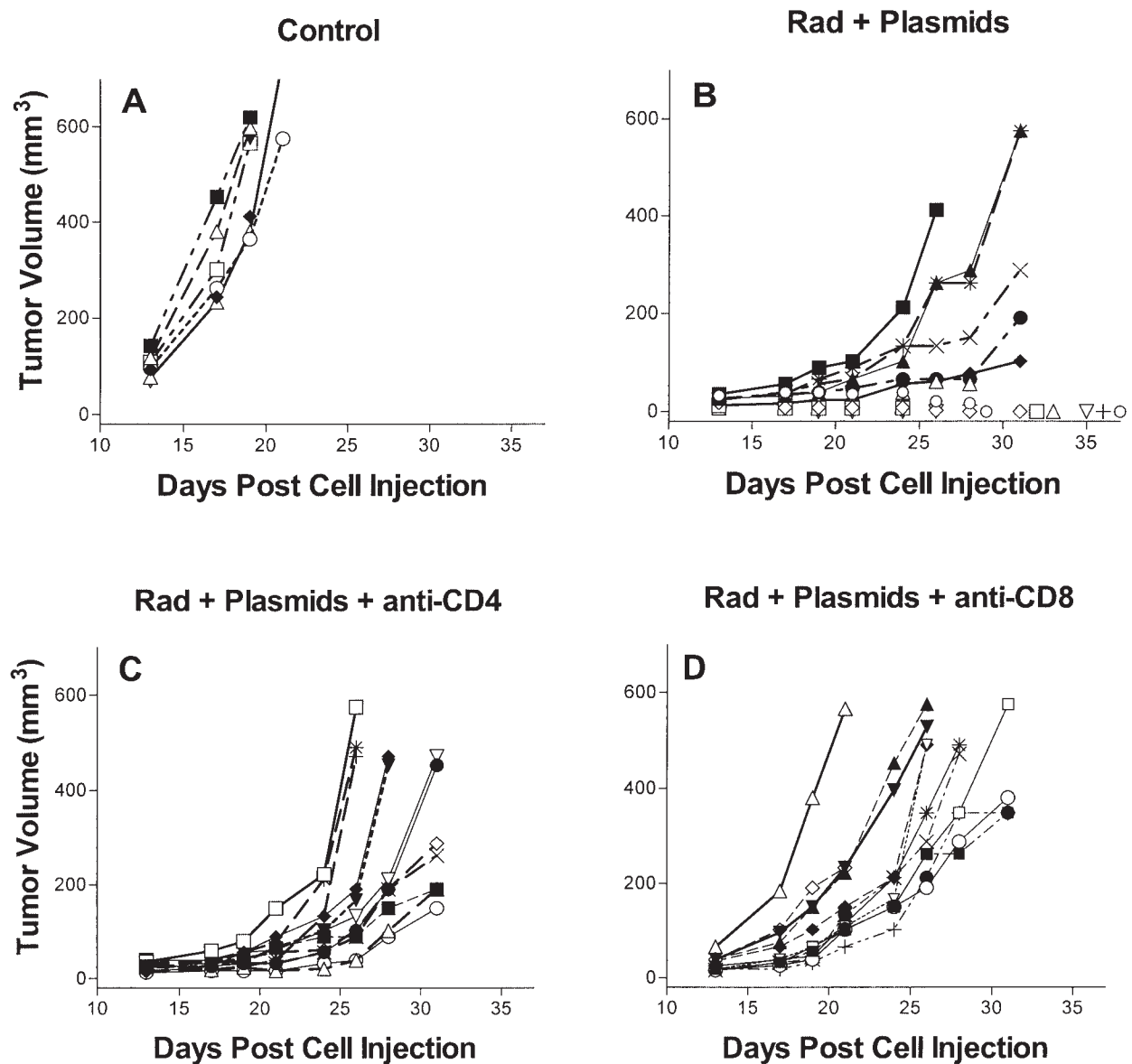
firmed by rejection of RM-9 challenge in the four complete responders of 13 treated mice (Table 1, *III*).

Treatment of mice with tumor irradiation followed by pIFN- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\gamma$  was significant ( $p < 0.005$ ) and addition of pCIITA to pIFN- $\gamma$  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<sup>+</sup> or CD8<sup>+</sup> T cells on the antitumor response induced by radiation and gene therapy in RM-9 tumors*

To assess the role of CD4<sup>+</sup> helper T cells and CD8<sup>+</sup> cytotoxic T cells, mice were injected with mAb specific to these subpopulations before and after treatment with tumor irradiation and pCIITA plus pIFN- $\gamma$  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- $\gamma$  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<sup>+</sup> T cells (**C**) or CD8<sup>+</sup> 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<sup>+</sup> helper T cells or CD8<sup>+</sup> 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<sup>+</sup> T cell subsets or CD8<sup>+</sup> T cell subsets on days 7, 13, and 27 by immunofluorescent staining of mouse splenocytes, confirmed the depletion of these populations. CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells and CD8<sup>+</sup> T cells started to regenerate. The percent of CD4<sup>+</sup> T cells and CD8<sup>+</sup> 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<sup>+</sup> T cells and 9–12% for CD8<sup>+</sup> 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- $\gamma$  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

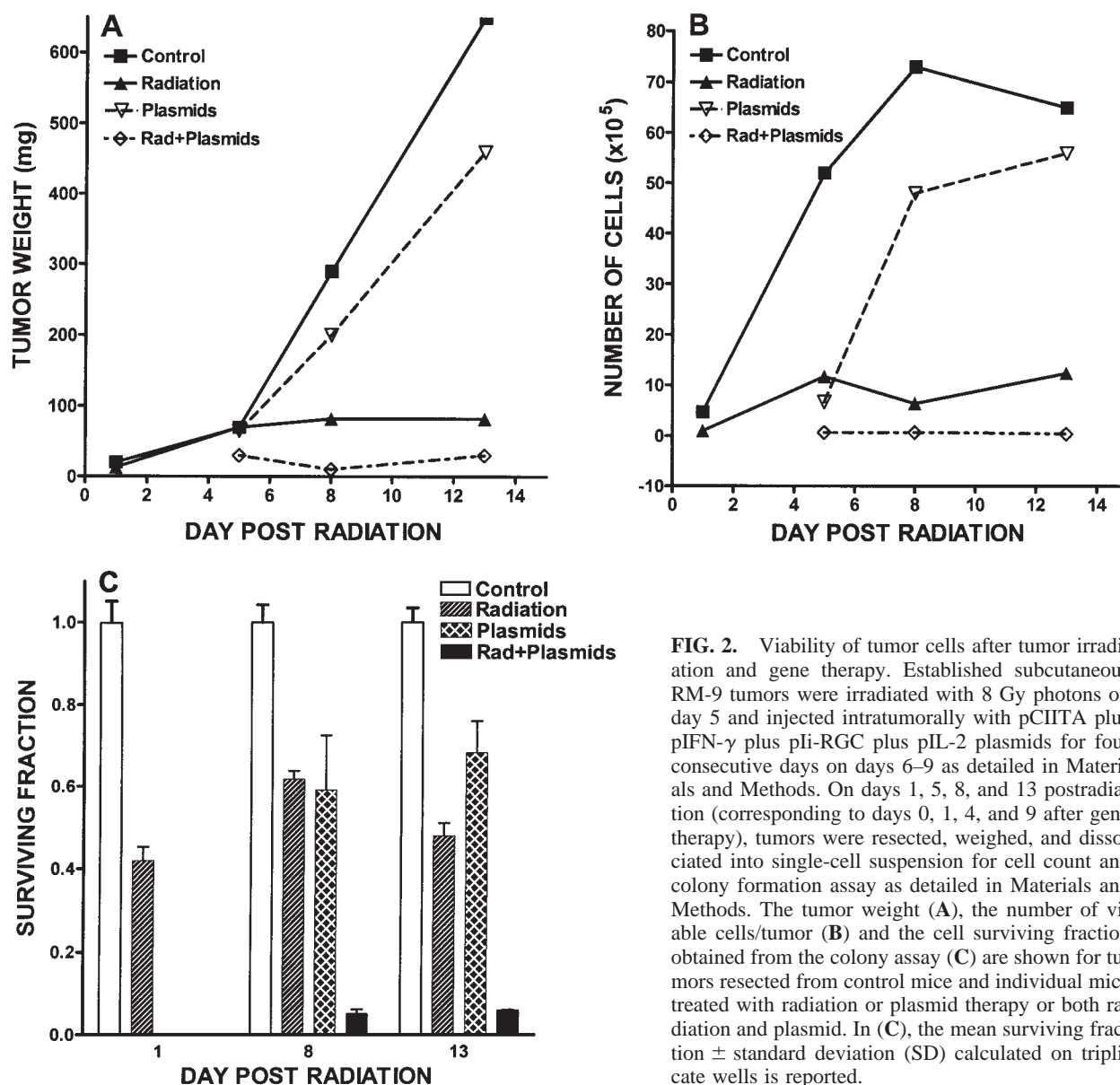
of 30–40% colony formation was observed relative to control (Fig. 2C). Treatment with radiation and plasmids almost completely abrogated the ability of tumor cells to divide, corroborating the low tumor weight and the low number of cells recovered from these tumors (Fig. 2C). These data were consistently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

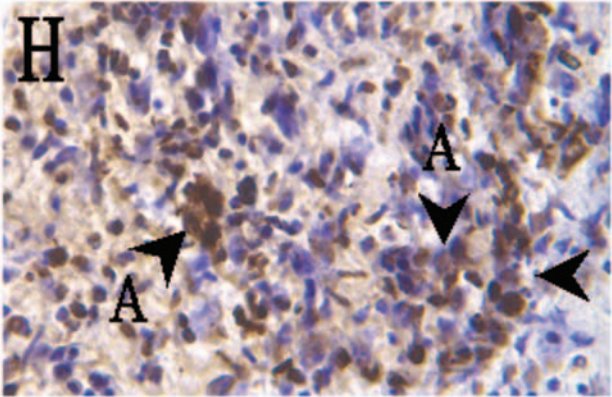
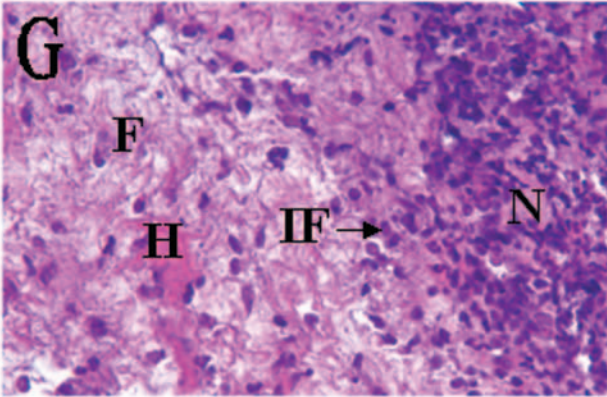
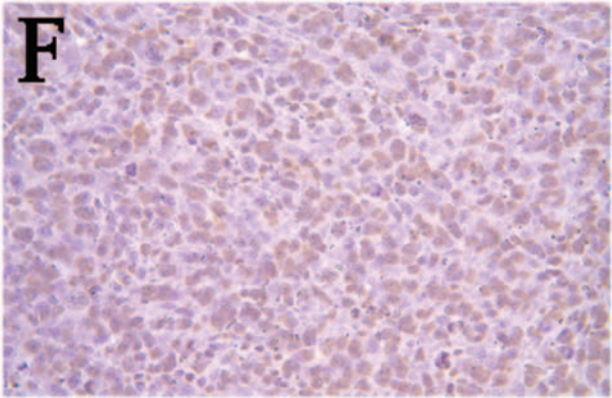
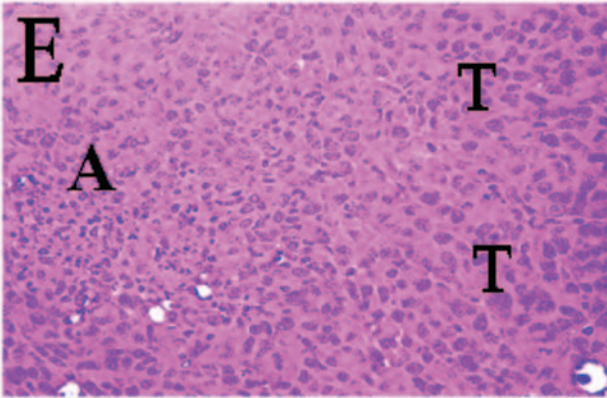
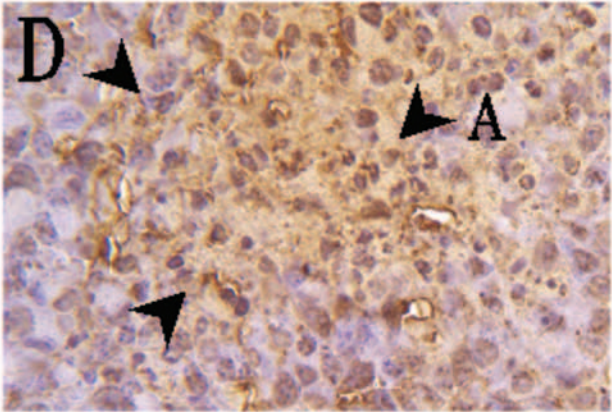
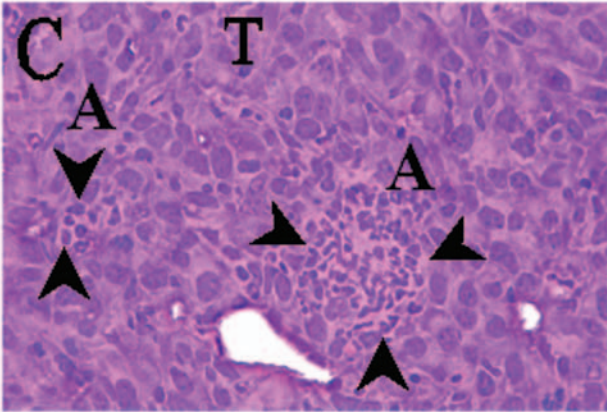
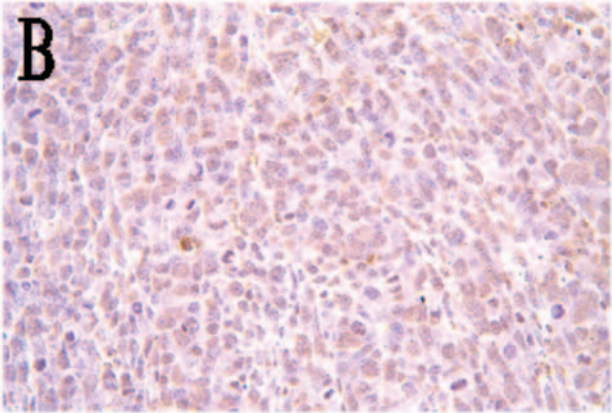
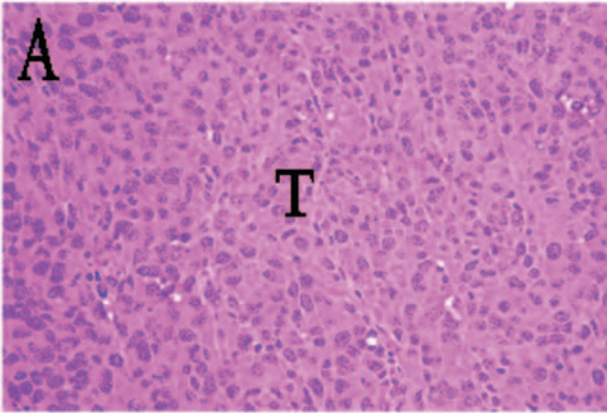
We have developed a novel approach combining selective tumor irradiation with gene-mediated immunotherapy that converts tumor cells, *in situ*, into a curative cancer vaccine in the murine RM-9 prostate tumor model. We showed that intratumoral gene therapy of established RM-9 subcutaneous tumor nodules with plasmid cDNAs coding for the MHC class I inducer IFN- $\gamma$ , 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- $\gamma$  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- $\gamma$  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 $\gamma$ , 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  $\mu$ g used in these studies in contrast to the 50- $\mu$ 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- $\gamma$  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<sup>+</sup> 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<sup>+</sup> 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- $\gamma$  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- $\gamma$ , 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- $\gamma$ , 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cytotoxic T cells also resulted in the elimination of complete responders. Immune monitoring of CD4<sup>+</sup> T cells and CD8<sup>+</sup> 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<sup>+</sup> helper and CD8<sup>+</sup> 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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Address reprint requests to:

*Gilda G. Hillman, Ph.D.*

*Department of Radiation Oncology*

*Wayne State University School of Medicine*

*Karmanos Cancer Institute*

*Hudson-Webber Building, Room 515, 4100 John R*

*Detroit, MI 48201*

*E-mail: hillmang@karmanos.org.*

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