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ORIGINAL ARTICLE

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Tumor immunotherapy by converting tumor cells to MHC class II–positive, Ii protein–negative phenotype

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Abstract A potent antitumor $CD4^+$ T-helper cell immune response is created by inducing tumor cells in vivo to a MHC class II^+/Ii^- phenotype. MHC class II and Ii molecules were induced in tumor cells in situ following tumor injection of a plasmid containing the gene for the MHC class II transactivator (CIITA). Ii protein was suppressed by the antisense effect of an Ii-reverse gene construct (Ii-RGC) in the same or another co-injected plasmid. The MHC class II^+/Ii^- phenotype of the tumor cells was confirmed by FACS analysis of cells transfected in vitro and by immunostaining of tumor nodules transfected by injections in vivo. Subcutaneous Renca tumors in BALB/c mice were treated by intratumoral injection with CIITA and Ii-RGC, in combination with a subtherapeutic dose of IL-2, to up-regulate the activation of T cells. Significant tumor shrinkage and decrease in rates of progression of established Renca tumors were seen in the groups injected with Ii-RGC, compared with groups in which only IL-2 plus empty plasmid controls were injected. Our method provides an effective immunotherapy warranting further development for human cancers.

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Abbreviations CIITA MHC class II transactivator · DMRIE 1,2-dimeristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide/cholesterol · FCS fetal calf serum · RGC reverse gene construct

Introduction

The critical role of $CD4^+$ T-cell stimulation by MHC class II–presented antigenic epitopes in promoting a robust immune response to tumors is widely recognized [13, 22, 24, 31]. Down-regulation of MHC class I is one way for tumors to escape immunosurveillance [9, 10]. When some tumors do express variable levels of MHC class II molecules, they often up-regulate expression of the Ii protein [14, 30, 32]. The Ii protein functions to block the binding of endogenous antigenic peptides to MHC class II molecules from time of synthesis in the endoplasmic reticulum (ER) until cleavage and release in a post-Golgi antigen-charging compartment [5, 18]. By preventing MHC class II presentation of endogenous tumor antigens, tumors escape from immunosurveillance, although MHC class I presentation is maintained [32]. Tumors expressing MHC class II molecules without the Ii protein are expected to present to $CD4^+$ T cells endogenous tumor antigenic peptides that are transported into the ER destined for MHC class I presentation. $CD4^+$ T cells specific for endogenous tumor antigens are thus activated and a robust antitumor immune response is induced.

Dr Ostrand-Rosenberg and colleagues demonstrated that transfection of genes for MHC class II molecules with or without B7, created tumor cells that were potent vaccines for stimulating SaI tumor rejection in tumor-bearing mice [3, 7, 25]. The MHC class II gene–transfected tumor cells were demonstrated to present endogenous antigen [2, 26]. Using hen egg lysozyme genetically targeted to nuclei, cytoplasm, mitochondria,

and ER, all forms were presented to specific $CD4^+$ T cells in the absence of invariant chain and H-2 M [27]. Co-expression of invariant chain restored tumorigenicity [7] and inhibited presentation of some of the lysozyme epitopes [27]. H-2 M has no effect on endogenous tumor antigen presentation [26]. In vivo studies demonstrate that MHC class II^+/Ii^- tumor cells, and not host-derived cells, were the predominant antigen-presenting cell for MHC class II–restricted nuclear antigens [27]. Induction of MHC class II by transfecting MHC class II transactivator (CIITA), which also induces Ii, into tumor cells has failed to create an effective tumor vaccine, thereby confirming that the therapeutic phenotype of converted cells is MHC class II^+/Ii^- [2, 20].

Due to allele heterogeneity, the transfection of genes for autologous MHC class II molecules is not practical clinically. We have developed an alternative approach inducing expression of MHC class II molecules with CIITA or IFN-γ, and suppressing Ii protein by antisense methods. Effective suppression of Ii protein was achieved with phosphorothioate antisense oligonucleotides, which were selected from an overlapping library through upstream and first and second exons of an Ii cDNA, using assays for RNase H protection [28]. Inoculation of mice with such MHC class II–induced, Ii-suppressed tumor vaccine cells led to much greater protection against challenge with the parental SaI sarcoma than did inoculation with parental or Ii-expressing cells [28].

We have extended these studies to intratumoral injection of lipid-delivered plasmids containing CIITA, Ii-reverse gene constructs (Ii-RGCs, express antisense RNA) to induce the MHC class II^+/Ii^- phenotype in vivo. Since the injection of CIITA plus Ii-RGC does not elicit an appreciable T-cell infiltrate into the tumor site, an IL-2 gene plasmid was co-injected to provide for local release of IL-2 to promote T-cell infiltration and activation [22] against tumor antigens. Intratumoral IL-2 plasmid therapy alone at higher, tumoricidal doses in Renca tumors was demonstrated by Horton et al. [12] and Saffran et al. [29]. Here we report that in vivo induction of the MHC class II^+/Ii^- phenotype in the context of a subtherapeutic IL-2 gene dose (2 μg/injection in contrast to 50 μg/injection) in the studies of Horton et al. [12] and Saffran et al. [29] effectively shrinks or greatly reduces the rates of progression of established Renca tumors.

Materials and methods

Plasmids

pQBI/Ad/BN plasmid was purchased from Quantum Biotechnologies (Montreal, Canada). And pcDNA3.1 plasmid was purchased from Invitrogen (San Diego, CA). Murine Ii cDNA gene in RSV.5 plasmid was from Dr Eric Long of the NIH [19]. Ii genomic DNA [25] was from Dr Suzanne Ostrand-Rosenberg at the University of Maryland. CIITA cDNA gene in pCEP4 plasmid was from Dr Laurie Glimcher [33]. IL-2 in VR1012 plasmid was provided by the Vical Corporation [12].

Cell lines and antibodies

The renal adenocarcinoma cell line Renca [8], from Dr Gilda G. Hillman of Wayne State University, Detroit, MI, was cultured in DMEM medium supplemented with 10% FCS. Antimurine MHC class II monoclonal antibody M5/114.15.2, was obtained from the culture supernatants of hybridoma HB120 (ATCC, Rockville, MD) by purification on a protein A affinity column (Pierce, Rockford, IL). Antimurine Ii monoclonal antibody was used as the supernatant of hybridoma In.1 [17], which was obtained from Dr Victor Reyes. Antibodies to MHC class II, Ii, CD4, CD3, CD8, CD19, and MAC were from BD Pharmingen (San Diego, CA) and used according to the manufacturer's instructions.

Design and construction of Ii-RGCs

Ii fragments were generated by PCR with appropriate oligonucleotide primers using either a murine genomic Ii gene [16] or a murine Ii cDNA gene [19] as templates. The primers were designed with 18–21 base pair (bp) match with the Ii segments, and appropriate restriction enzyme sites were designed on each primer for the cloning of the fragments. These Ii fragments were cloned into either RSV.5 or pcDNA3.1 plasmids in reverse orientation. All constructs were confirmed by sequencing. For cloning of the triple Ii-RGC construct, the respective promoters, Ii fragments, and poly A signals in active Ii-RGC plasmids, were PCR excised and cloned into the pQBI/Ad/BN plasmid sequentially. Ii-RGC (–92,97) was cloned first to generate the pQBI/Ad/BN/Ii-RGC (–92,97). The CIITA cDNA gene was excised from pCEP4/CIITA plasmid by PCR to delete 5′ and 3′ untranslated regions, and that product was cloned into the pcDNA3.1 plasmid and then its promoter, CIITA, and poly A signal were PCR excised and cloned into pQNI/Ad/BN plasmid (pQBI/Ad/BN/CIITA). For cloning of the CIITA/Ii-RGC (–92,97) construct, the promoter, Ii-RGC (–92,97), and poly A signals were PCR-generated and cloned into pQBI/Ad/BN/CIITA plasmid.

DNA transfection

DNA transfections were performed with lipid-mediated methods. The lipofectin was purchased from Gibco, BRL (San Diego, CA) and used according to the manufacturer's instructions. After transfection, the cells were selected with hygromycin (ICN Biomedicals, Costa Mesa, CA) for 2–3 weeks until the clones were isolated.

Immunostaining of cultured cells

The selected clones were surface-stained with M5/114.15.2 for MHC class II and intracellularly stained with In.1 for Ii. For cell-surface staining of MHC class II, 0.5×10^6 to 1×10^6 cells were washed and M5/114.15.2 antibody was added to the cells in total 200 μl volume and incubated for 45 min at room temperature (RT). Cells were then washed, and fluorescein-conjugated anti-rat IgG (Southern Biotechnologies, Birmingham, AL) was added and incubated for another 30 min at RT. Cells were then washed and fixed with 10% formaldehyde for 10 min. For intracellular staining of Ii, procedures described by Rosenberg et al were followed [2]. Briefly, 0.5×10^6 to 1×10^6 cells were washed with Hank's buffer and fixed with 2% formalin at RT for 10 min. Cells were then washed with 1 ml 1 M glycine (Sigma, St. Louis MO), at RT for 5 min. The cells were then permeabilized with 0.2% saponin (Sigma), and

200 μl of In.1 (with 0.2% saponin) was added and incubated for 60 min on ice. Cells were then washed, and fluorescein-conjugated anti-rat IgG (Southern Biotechnologies) with 0.2% saponin was added for additional 30–45-min incubation. Cells were washed and fixed with 10% formaldehyde for 10 min. Cells were analyzed for the expression of MHC class II and Ii proteins by FACS analysis.

Intratumoral DNA injections

BALB/c mice were purchased from Jackson Laboratory and were housed in the animal facility of the University of Massachusetts Medical School. Mice were injected subcutaneously in the right flank with 2×10^5 of Renca cells. Tumors were selected for comparable size (30–100 mm^3) before being injected with a total of 25 μg DNA in a plasmid DNA solution (total 40 μl) prepared as follows. One to three minutes before injection, the DNA was mixed with DMRIE/c (1,2-dimeristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide/cholesterol) (Gibco BRL) at ratio of 1:1 (w:w). For immunohistochemical staining, tumors were excised 5 days after DNA injection, immediately frozen, and subsequently processed into slides. For immunotherapy studies, mice were injected intratumorally with different plasmid combinations (see figure legends) on each of 4 consecutive days when tumor volume reached 30–100 mm^3. In order to control for nonspecific (e.g., CpG) effects, the amount of DNA (23 μg) per injection was kept constant among the groups by the addition of empty pQBI/Ad/BN plasmid. Tumor sizes were measured every 2–3 days. The tumor volumes were calculated from the formula for an ellipsoidal solid $(4/3\ \pi \times 1/2 \text{ length} \times 1/2 \text{ width} \times 1/2 \text{ height}) = 0.52 \times \text{length} \times \text{width} \times \text{height}$ [12]. Tumor-free mice were terminated at 60 days. Principles of laboratory animal care (NIH publication No. 85-23, revised 1986) were followed, as well as the current version of the United States law on the protection of animals, where applicable.

Immunohistochemical staining of the tumor sections

Consecutive frozen sections of the tumors were mounted on slides and stained for MHC class II, Ii, CD4, CD3, CD8, CD19, and MAC. The slides were fixed in 70% and then 50% acetone, each at 4°C for 30 s. Slides were then rinsed with PBS containing 1% mouse serum and blocked for endogenous peroxidase activity with 0.3% H_2O_2/1%BSA/PBS for 10 min at RT and then incubated with avidin and biotin (Vector Laboratories, Burlingame, CA) for 20 min and washed. The slides were stained with In.1 for Ii and M5/114.15.2 antibody for MHC class II at RT for 30 min. After wash, the slides were incubated with biotinylated anti-rat IgG (H + L) antibody at RT for 30 min, incubated with avidin-horseradish peroxidase for 30 min, stained with DBA kit (cat. no. SK4100, Vector Laboratories) for 3–6 min, immersed in hematoxylin solution (Vector Laboratories) for 30–60 seconds, dipped in 2% sodium borate 10 times, and finally rinsed consecutively in distilled water, 75% ethanol, 95% ethanol, 100% ethanol and xylenes. Positive staining was defined as brown cytoplasmic staining in the tumor epithelial cells, which can be observed easily at low-power magnification ($<100\times$). To estimate the effect of the transfected genes, comparable areas within consecutive serial tumors showing activity of the transfected genes were scored for percentages of MHC class II^+ and Ii^+ cells. For this observation 2–5 high-powered fields were examined, scoring about 200 nuclei per high-powered field.

Statistics

Mean, median, and standard deviations of tumor sizes were calculated for each group at each time. Average tumor sizes were plotted over time to display the trend of tumor development in each treatment group. A general linear regression model [1] was used to test the significance of different treatments on tumor growth with the adjustment for the effect of time. Statistical analyses were carried out using the SAS 8.02 program (SAS Institute, Cary, NC).

Results

Construction of murine Ii-RGCs and screening for effective Ii-RGC

14 Ii-RGCs were constructed and screened for inhibition of Ii-protein expression either in a murine lymphoma cell line, A20 (MHC class II^+/Ii^+), or in a stably CIITA-transfected murine sarcoma cell line, SaI/CIITA (MHC class II^+/Ii^+). The Ii fragments generated were designed to cover different parts of the Ii gene, including the coding region, 5′ untranslated region (5′UT), and first intron. Ii-RGC stably transfected A20 cells or SaI/CIITA cells were stained with antibodies against murine MHC class II (M5/114.15.2) or Ii (In.1) [16] and then FACS-analyzed. The Ii-RGCs that induced Ii inhibition while not altering MHC class II expression, in either A20 or SaI/CIITA cells were judged to be active Ii-RGCs (Table 1). Ii-RGC (−92,97) was chosen for subsequent in vivo experiments because it covers the AUG start codon and contains all the DNA sequences of several other Ii-RGCs that were shown to be individually active (Table 1). Ii-RGC (−92,97) inhibits Ii expression in vitro in many cell lines we have studied (data not shown), including Renca cells [11]. In addition, a construct of three active Ii-RGCs—Ii-RGC (−92,97), Ii-RGC (32,136), and Ii-RGC (314,458) (see Table 1)—each with its own promoter and poly A signal, was constructed (termed Ii-RGC [triple] below) in order to increase the efficiency of plasmid delivery of Ii-RGC. A construct containing CIITA and Ii-RGC (−92,97) (each with its own promoter and poly A signal) was constructed in order to increase the probability that individual cells were transfected with both CIITA and Ii-RGC (−92,97).

Induction of MHC class II^+/Ii^- phenotype in vivo in Renca tumors by intratumoral injection of CIITA plasmid with Ii-RGC plasmid

The MHC class II^+/Ii^- phenotype in tumor cells in situ was created by intratumoral injection of plasmids

Table 1 Ii-RGCs which generate MHC class II^+/Ii^- phenotype in A20 or SaI1/CIITA cells

Ii-RGC	Position in Ii gene	Promoter used
RSV.5/mIi (−92,97)	5′UT to 1st exon	RSV
RSV.5/mIi (−50,97)	5′UT to 1st exon	RSV
RSV.5/mIi (−26,97)	5′UT to 1st exon	RSV
pcDNA3.1/mIi (32,136)	1st exon to 2nd exon	CMV
pcDNA3.1/mIi (314,458)	4th exon to 5th exon	CMV
pcDNA3.1/mIi (−92,77)	5′UT to 1st exon	CMV
pcDNA3.1/mIi (−2, 1in81)	1st exon to 1st intron	CMV
pcDNA3.1/mIi (−26,77)	5′UT to 1st exon	CMV
pcDNA3.1/mIi (−92,15)	5′UT to 1st exon	CMV

containing either CIITA or CIITA plus Ii-RGC. Five days after the DNA injection into tumors, consecutive frozen sections of excised tumor were made and stained with antibodies against murine MHC class II molecules and Ii protein in order to assay the extent and quality of conversion of tumor cells to the class II^+/Ii^- phenotype. In tumors injected with CIITA only, comparable frequencies of MHC class II and Ii^+ cells were observed in well-induced regions of comparable areas of serial sections. In tumors injected with both CIITA and Ii-RGC (97,−92), the frequency of Ii^+ cells was significantly reduced (about 85%) compared with the frequency of MHC class II^+ cells. Likewise, in tumors injected with both CIITA and Ii-RGC (triple), the frequency of Ii^+ cells was also inhibited significantly compared with MHC class II^+ cells (about 95%). Figure 1 presents representative photographs of the well-induced regions. CD4-, CD8-, and CD3-staining showed very few positive cells, indicating that the MHC class II^+ cells in the tumor were not infiltrating T cells. Staining for CD19 and MAC showed that the MHC class II^+ cells were not B cells or macrophages. T cells, or other activated responding cells, are not expected to be found in these histological sections because no IL-2 was co-injected and the plasmids had been injected only once, and not multiple times as is the case during the therapeutic protocol. The validity of the antibodies and staining techniques was confirmed in parallel studies on spleen sections, showing strongly positive staining with all antibodies and no staining in the negative controls (data not shown).

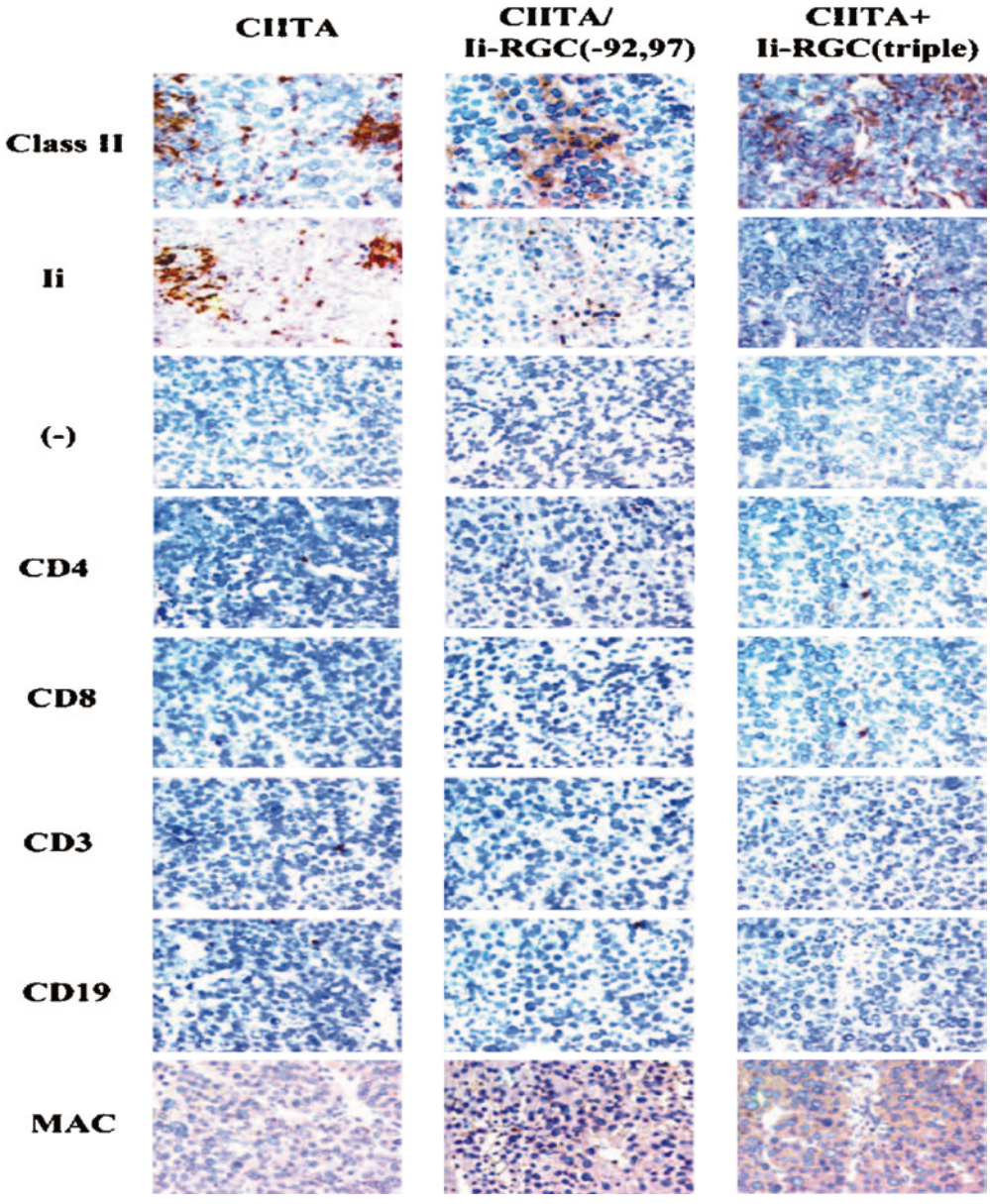

Fig. 1 Intratumoral injection of Ii-RGC and CIITA generates MHC class II^+/Ii^- phenotype. Renca tumors of 30–100 mm^3 were selected and injected with CIITA plasmid alone *(left)*, CIITA/Ii-RGC (−92,97) plasmid *(middle)*, and CIITA plus Ii-RGC (−92,97/32,136/314,459) plasmids at a ratio of CIITA to Ii-RGC (triple) 1:6 (w:w) *(right)*. Five days after DNA injection, the slides were made and stained for (from *top* to *bottom*): class II, Ii, (−), CD4, CD8, CD3, CD19, and MAC. No T or B lymphocyte infiltration was found in any slide

Therapeutic effect of Ii-RGC injected into established renal adenocarcinomas

Since staining of the frozen section 5 days after CIITA and Ii-RGC injection (Fig. 1) showed no T and B cell infiltration, we added 2 μg of IL-2 plasmid to CIITA and Ii-RGC DNA mixture to produce IL-2 to attract immune cells to the locale [15] and to promote proliferation of activated T cells [22]. The therapeutic effect of IL-2 plasmid was demonstrated previously by Horton et al. [12] who used 50 μg of IL-2 plasmid for intratumoral injections on 4 consecutive days, with a significant therapeutic effect, curing five of eight mice [12]. We hypothesized that in vivo induction of the MHC class II^+/Ii^- phenotype plus a subtherapeutic dose of IL-2 (2 μg of IL-2) plasmids might be as therapeutic as the tumoricidal dose of IL-2 given alone. Tumors were injected with plasmids containing CIITA and Ii-RGCs (see legend of Fig. 2), in combination with 2 μg of IL-2 plasmid. BALB/c mice were injected s.c. in the flank with 5×10^5 Renca cells. Upon reaching 30–100 mm^3, the tumors were injected with different plasmid combinations on each of 4 consecutive days. The size of the tumors was then measured every 2–3 days. To avoid a very high level of Ii expression being marginally inhibited by Ii-RGCs, CIITA plasmid was only injected on day 1 or on days 1 and 3, in most experiments. Intratumoral injection with CIITA + Ii-RGC (−92,97) provided the best therapeutic effect (tumors became smaller or disappeared, see Fig. 2B). Due to substantial tumor growth, control mice (2-μg IL-2 and empty plasmid) were sacrificed at day 32 (Fig. 2A). These results indicate that CIITA + Ii-RGC induced an effective therapeutic activity. CIITA + Ii-RGC (triple) provided intermediate therapeutic effects (Fig. 2C and D). Injection of IL-2 plasmid plus Ii-RGC plasmid has no therapeutic effect (Fig. 2E).

We conducted another therapeutic experiment in which the Ii-RGC plasmid and immunization schedules were slightly modified (see Fig. 3). IL-2 and CIITA/Ii-RGC (−92,97) plasmids were injected on 4 consecutive days in one group (*circle* in Fig. 3). IL-2 + CIITA + Ii-RGC (−92,97) was injected on days 1 and 3, and IL-2 + Ii-RGC (−92,97) was injected on days 2 and 4 in another group (*triangle* in Fig. 3). In this experiment, there was a significant degree of tumor inhibition or cure in both groups receiving CIITA/Ii-RGC therapy compared with the control group (*diamond* in Fig. 3) receiving IL-2 + empty plasmid for 4 consecutive days ($p < 0.001$ for *circle* and $p < 0.002$ for *triangle,* respectively, versus *diamond*). These results demonstrated a

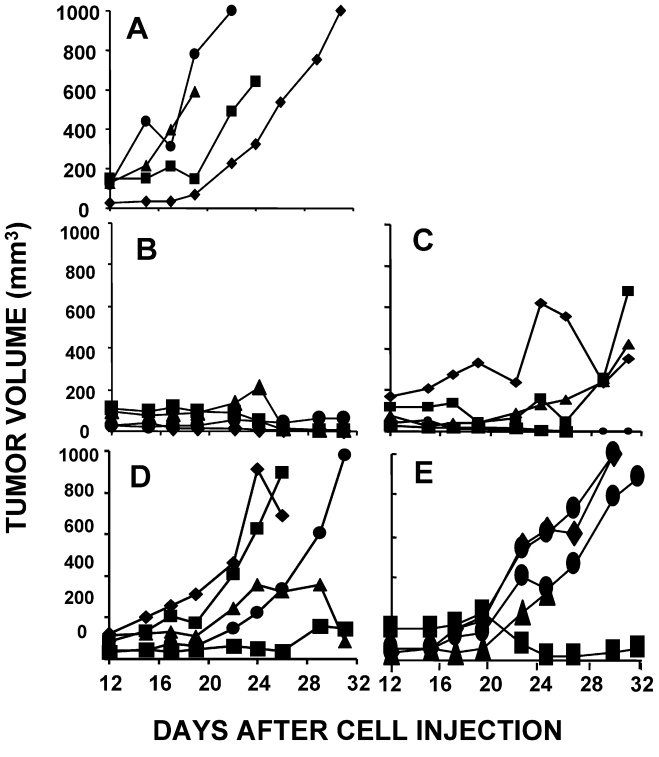

Fig. 2A–E Therapeutic synergy of Ii-RGC and IL-2 in Renca tumors of individual mice. **A** Two micrograms of IL-2 and 21-μg empty plasmid (pQBI/Ad/BN) for all four injections; **B** 2-μg IL-2, 3-μg pQBI/Ad/BN/CIITA, and 18-μg pQBI/Ad/BN/Ii-RGC (−92,97) on days 1 and 3; 2-μg IL-2, 18-μg pQBI/Ad/BN/Ii-RGC (−92,97), and 3-μg empty plasmid (pQBI/Ad/BN) (without CIITA) on days 2–4; **C** 2-μg IL-2, 3-μg pQBI/Ad/BN/CIITA, and 18-μg pQBI/Ad/BN/Ii-RGC (triple) for all four injections; **D** 2-μg IL-2, 3-μg pQBI/Ad/BN/CIITA, and 18-μg pQBI/Ad/BN/Ii-RGC (triple) for day 1, and 2-μg IL-2, 3-μg empty plasmid, and 18-μg PQBI/Ad/BN/Ii-RGC (triple) on days 2–4; **E** 2-μg IL-2, 3-μg pQBI/Ad/BN, and 18-μg pQBI/Ad/BN/Ii-RGC (−92,97) for all four injections. On day 40, all mice in groups C, D, and E had developed tumors. Two of four mice in group B had also developed tumor and the other two mice were tumor free as of day 60 when they were terminated

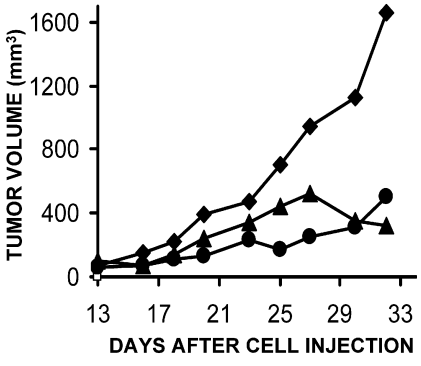

Fig. 3 Therapeutic synergy of Ii-RGC and IL-2 in Renca tumors. Seven mice were injected with IL-2 (2 μg) and empty plasmid (21 μg) on each of 4 consecutive days *(diamonds)* (all mice developed tumors between days 24–38). Five mice were injected with IL-2 (2 μg), CIITA (3 μg), and Ii-RGC (−92,97) (18 μg) on days 1 and 3; with IL-2 (2 μg), Ii-RGC (18 μg), and empty plasmid (3 μg) on days 2 and 4 *(circles)* (three of five mice were tumor free until terminated on day 60 and an other two mice developed tumor by day 40). Four mice were injected with IL-2 (2 μg), CIITA/Ii-RGC (−92,97) (5 μg), and empty plasmid (16 μg) on each of 4 consecutive days *(triangles)* (two of four mice were tumor free on day 60). The data were averaged within a group and significant tumor shrinkage was seen in two therapeutic groups compared with the control group ($p < 0.002$ for group *triangle* and $p < 0.001$ for group *circle*)

significant therapeutic effect of MHC class II^+/Ii^- in combination with a subtherapeutic dose of IL-2, over IL-2 gene therapy alone. The experiment (*triangle* group) was repeated again (seven mice/group), with similar results being obtained (data not shown).

Discussion

Creation of a potent tumor cell vaccine by transfecting tumor cells with MHC class II genes (generating the MHC class II^+/Ii^- phenotype) has been extensively studied by Ostrand-Rosenberg and colleagues [2, 3, 7, 25, 26, 27]. Specifically, this group has demonstrated that (1) MHC class II^+/Ii^- tumor cells are a potent tumor cell vaccine, generating a long-term immune response which protects mice from challenge with parental tumor cells; (2) MHC class II^+/Ii^+ tumor cells (made by co-transfecting Ii or inducing both MHC class II and Ii by CIITA) are not immunogenic; and (3) these $CD4^+$ T cells transferred specific antitumor activity to na cells [4]. The novel mechanism enhancing the immunogenecity of tumor cells with the MHC class II^+/Ii^- phenotype has been well studied by Dr Ostrand-Rosenberg et al. [2, 3, 26]. They demonstrated that MHC class II^+/Ii^- tumor cells present endogenous tumor antigens to T cells while MHC class II^+/Ii^+ tumor cells do not [2]. Since transfecting heterogeneous MHC class II alleles to each patient is not clinically practical, we developed a method to induce MHC class II and Ii by CIITA or IFN-γ, and subsequently inhibiting Ii by antisense oligonucleotides to create a potent tumor cell vaccine [28]. Here we demonstrate a clinically practical method of intratumoral induction of the MHC class II^+/Ii^- phenotype to create a potent antitumor immunotherapy in vivo.

The best therapeutic effect was observed in the group of mice receiving IL-2 + CIITA + Ii-RGC (−92,97), while mice receiving IL-2 and empty plasmid were sacrificed, as per protocol, when their tumors reached about 1,000 mm^3 (Fig. 2B and A). The therapeutic effect of Ii-RGC (−92,97) was confirmed in experiments (Fig. 3 and data not shown) in which tumors injected with CIITA/Ii-RGC (−92,97) or CIITA plus Ii-RGC (−92,97) plasmid, in combination with IL-2 plasmid, were significantly smaller (about 50% were tumor-free on day 60, see legend of Fig. 3) than tumors injected with IL-2 and empty plasmids. Mice injected with CIITA, Ii-RGC (triple), and IL-2 plasmids demonstrated a lesser therapeutic effect in two different immunization protocols (Fig. 2C and D). The Ii-RGC (triple) did provide significant Ii inhibition in vivo (Fig. 1). The comparatively lesser therapeutic effect of the Ii-RGC (triple) plasmid might result from promoter competition between the Ii-RGCs (triple) and IL-2. Ii-RGC (triple) contains two CMV promoters driving Ii-RGC (32,136) and Ii-RGC (314,459) (Table 1). When 6 times the amount of Ii-RGC (triple) was used in the experiments, the number of CMV promoters in the triple Ii-RGC (18 $\mu g = 36$ copies of CMV promoter for Ii-RGCs) competes with two copies of CMV promoter for the IL-2 gene (2 $\mu g = 2$ copies of CMV promoter), resulting in relatively reduced synthesis of IL-2, thereby decreasing therapeutic efficacy in this group. The IL-2 dose in our experiments was subtherapeutic relative to the tumoricidal doses reported by Horton et al. [12]. All experiments showed that 2 μg of IL-2 without Ii-RGC had no therapeutic effect.

MHC Class II^+/Ii^- phenotype immunotherapy significantly shrank tumors or decreased progression of the tumors injected with CIITA, Ii-RGC (−92,97), and suboptimal dose of IL-2. In parallel work we have tested the activities of our CIITA and Ii-RGC genes in adenoviral vectors, to transform subcutaneous Renca tumor and induce tumor regression or cure [11]. Those results substantiate the findings of this paper by two methods of gene delivery into established tumors. These results will be further improved by optimizing therapeutic doses/schedules and by avoiding promoter competition. New multiple copy Ii-RGCs will be constructed similar to the Ii-RGC (triple), but with each Ii-RGC being driven by a different promoter.

One advantage of intratumoral induction of the MHC class II^+/Ii^- phenotype is that one does not need to identify tumor antigens in advance. Ii–"unblocked" MHC Class II molecules survey the antigenic peptide pool in ER and present whatever tumor epitopes bound to activated $CD4^+$ T cells [23, 28]. This provides a better chance to prevent the tumor from escaping the host's immune surveillance since a broader spectrum of heterogeneous tumor antigens is expected to be surveyed by MHC class II molecules. CIITA also enhances DM and MHC class I expression, especially when class I expression becomes lower [6, 20, 21], and thus class I antigen presentation is also promoted. This is significant because deletion of MHC class I alleles is a frequent way for tumors to escape from immunosurveillance [9]. Another advantage of MHC class II^+/Ii^- phenotype induction is that it can be synergistic with other antitumor therapies such as injections with IL-12 and B7 genes [25]. A third advantage is that the monomorphic structure of the Ii gene means that one vector construct can be used in all patients, without regard to the heterogeneity of MHC class II alleles.

Finally, conversion of cancer cells into antigen-presenting cells via induction of the MHC class II^+/Ii^- phenotype in vivo by this method is simple to achieve. Transduction of a focal population of cells within a tumor mass precipitates an immune response that potentially leads to destruction of all cells within the mass as well as metastases. This is particularly poignant with respect to anticipated use clinically where local treatment to induce a potent systemic antitumor response is the goal.

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