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RESEARCH ARTICLE

Generating MHC Class II+/Ii- phenotype after adenoviral delivery of both an expressible gene for MHC Class II inducer and an antisense Ii-RNA construct in tumor cells

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Tumor cells engineered by gene transduction to be MHC Class II+/Ii- are novel APCs capable of presenting endogenous tumor antigen epitopes to activate T helper cells. The MHC Class II+/Ii- tumor cell phenotype is created by transfecting genes for either CIITA or IFN-γ, and inhibiting induced Ii mRNA by an Ii reverse gene construct (Ii-RGC). Adenoviral vectors are preferred for the delivery of such genes because of high infection efficiency and ubiquity of the adenoviral receptor on many cell types and tumors. Here we show that at 5 MOI (multiplicity of infection), recombinant adenoviruses with CIITA or IFN-γ genes converted virtually all MC-38 colon adenocarcinoma cells and Renca renal carcinoma cells in culture to MHC Class II+/Ii+ cells. A single recombinant adenovirus with both genes for IFN-γ and Ii-RGC (rAV/IFN-γ/Ii-RGC) efficiently induced the MHC Class II+/Ii- phenotype. Injection of tumor nodules with rAV/Ii-RGC and rAV/CIITA/IFN-γ combined with a suboptimal dose of rAV/IL-2 induced a potent antitumor immune response. The methods are adaptable for producing enhanced genetic vaccines, attenuated virus vaccines (eg, vaccinia), and ex vivo cell-based vaccines (dendritic and tumor cells).
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Introduction

Generating strong CTL responses is required for a curative anticancer immune response. Potent activation of CD4+ T helper cells is essential to priming and maintaining that CTL response.[1,2] Dr Ostrand-Rosenberg and colleagues discovered that murine tumor cells converted to the MHC Class II+/Ii- phenotype by transfecting genes of the alpha and beta chains of MHC Class II molecules are: (1) potent stimulators of anti-tumor CD4+ T helper cells and (2) capable of providing protection against challenge with parental tumor.[3–5] Presumably, peptides processed into the ER for binding to MHC Class I molecules become bound to MHC Class II molecules in the absence of the normally peptide-binding-site-blocking Ii protein. We sought a clinically feasible approach to this novel immunotherapy, avoiding transfection of each patient's tumor cells with genes for her or his autologous MHC Class II molecules. This is carried out converting tumor cells into MHC Class II+/Ii+ cells by transfecting the genes for either the MHC Class II transactivator (CIITA) or IFN-γ, both of which induce endogenous MHC Class II molecules and the Ii protein.[6,7] The mRNA for Ii protein is then suppressed by an antisense method. We have created a potent Ii antisense oligonucleotide that inhibited Ii expression in a variety of cells and generated effective tumor cell vaccines.[8] We have also created several active Ii reverse gene constructs (Ii-RGCs) that inhibited Ii expression in A20 B lymphoma cells *in vitro* and RENCA renal adenocarcinoma tumors *in vivo* following transfection. *In vivo* injection of Ii-RGC resulted in effective intratumoral immunotherapy of renal adenocarcinoma tumors.[9]

In order to develop an efficient reagent for inducing MHC Class II+/Ii- tumor vaccine cells either *in vitro* or *in situ*, we have exploited the favorable characteristics of adenoviruses as vectors for our genetic constructs. Since their development in the early 1980s, recombinant adenoviruses (rAV) have been used to deliver a wide variety of genes into mammalian cells.[10] Numerous researchers have shown that recombinant advenovirus (rAV) is a very efficient DNA delivery vehicle.[11–13] Compared to other viral delivery methods, rAV has the advantages of high infection rate (nearly 100% cells can be infected *in vitro*), wide spectrum of cellular tropism (variety of human and nonhuman cells and tissues), robust growth in the culture, and the capacity to infect mitotically quiescent cells.[14,15] Since the adenovirus does not integrate into the cellular genome, it cannot be used to make stable cell lines.[16] This is a shortcoming when stable transfection is desired;

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however, a favorable feature when only transient expression is sought.

Adenoviruses have been widely used to deliver antisense constructs.[17–20] Here, we created a rAV containing CIITA (rAV/CIITA), IFN-γ (rAV/IFN-γ), or the combination of IFN-γ and an Ii-RGC rAV/IFN-γ/Ii-RGC. Using either the rAV/CIITA or rAV/IFN-γ to induce MHC Class II expression, plus an Ii-antisense oligonucleotide to suppress Ii, or using rAV/IFN-γ/Ii-RGC, we generated MHC Class II+/Ii- MC-38 murine adenocarcinoma cells. Injection of Renca tumor nodules with rAV/Ii-RGC and rAV/CIITA/IFN-γ combined with a suboptimal dose of rAV/IL-2 induced a potent antitumor immune response. We have relatively optimized the procedures for creating these cells, which could be potent inducers of anticancer immunity upon *in vitro* use to prepare cancer cell vaccines, or via direct injection into tumors. A major function of Ii protein is protection of the antigenic peptide binding site on MHC Class II molecules from binding to endogenously derived antigenic peptides at the time of synthesis in the ER.[22] The strategy of creating MHC Class II+/Ii- phenotype is thus of value not only for tumor cell vaccines, but also for dendritic cell and DNA vaccines.

Results

Construction of rAVs containing CIITA, IFN-γ, and Ii-RGC

We constructed rAV containing CIITA or INF-γ according to the manufacturer's instructions. In order to ensure that each infected cell receives both IFN-γ and Ii-RGC genes, we cloned IFN-γ and Ii-RGC in one adenoviral sequence-containing plasmid, pQBI/Ad/BN (Figure 1). Each class of gene is driven by a different promoter (CMV promoter for IFN-γ and RSV promoter for Ii-RGC) in order to avoid promoter competition between these classes. The pQBI/Ad/BN/IFN-γ/Ii-RGC was cotransfected with *Cla*1-digested AV DNA into 293A packaging cells. The resulting rAVs were confirmed by PCR to contain both IFN-γ and Ii-RGC along with their promoters and Poly A signals. The rAV containing CIITA or IFN-γ genes were also constructed and confirmed to contain the genes. These rAVs were expanded and stored for experiments. rAV containing other gene combinations were also constructed according to these methods.

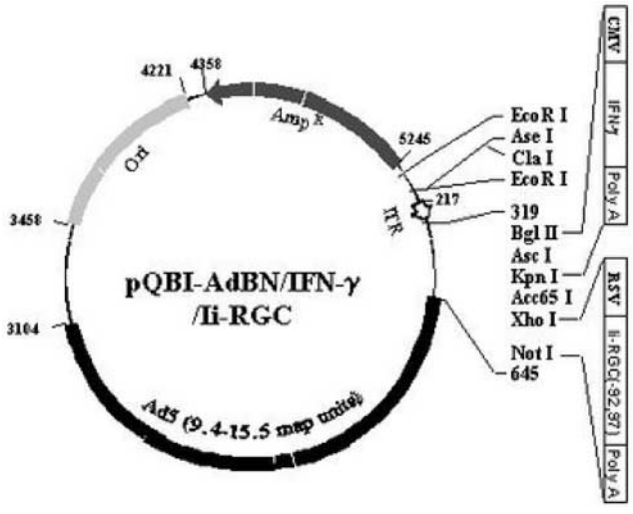

Figure 1 *Diagram of IFN-γ and Ii-RGC containing plasmid. The fragment containing RSV promoter, Ii-RGC, and poly A signal was generated from RSV.5/Ii-RGC by PCR and the fragment containing CMV promoter, IFN-γ, and poly A signal was generated from pCDNA(3)/IFN-γ by PCR. These fragments were cloned into pQBI/Ad/BN using* Not*1 and* Xho*1 for the Ii-RGC fragment, and* Kpn*1 and* Bgl*2 for IFN-γ. The resulting plasmid pQBI/Ad/BN//IFN-γ/Ii-RGC was used to cotransfect adenovirus packaging cell line 293A with* Cla*1-digested adenoviral DNA to generate rAV/IFN-γ/IiRGC.*

High efficiency of adenoviral delivery of CIITA or IFN-γ genes into cells to induce the expression of MHC Class II molecules

MC-38 or Renca cells, infected with 10 MOI of rAV/CIITA, were harvested at different times and assayed for MHC Class II expression. Figure 2 shows that at 24 h, some cells became MHC Class II-positive and at 72 h, virtually all cells were MHC Class II-positive. MHC Class II expression peaked at 96 h without affecting cell viability (>95%). In repeated experiments, more than 90% of MC-38 cells were induced to express MHC Class II molecules after infecting cells with as low as 3 MOI of rAV/IFN-γ. MHC Class II induction was used as an indicator of Ii protein expression, as well, since, in previous experiments with CIITA, Ii protein was always coinduced with MHC Class II.

Efficient inhibition of Ii protein expression by an Ii antisense oligonucleotide in MHC Class II+/Ii+ MC-38 cells infected with rAV/CIITA

The efficiency of Ii protein suppression in rAV/CIITA-infected cells by an Ii antisense oligonucleotide was

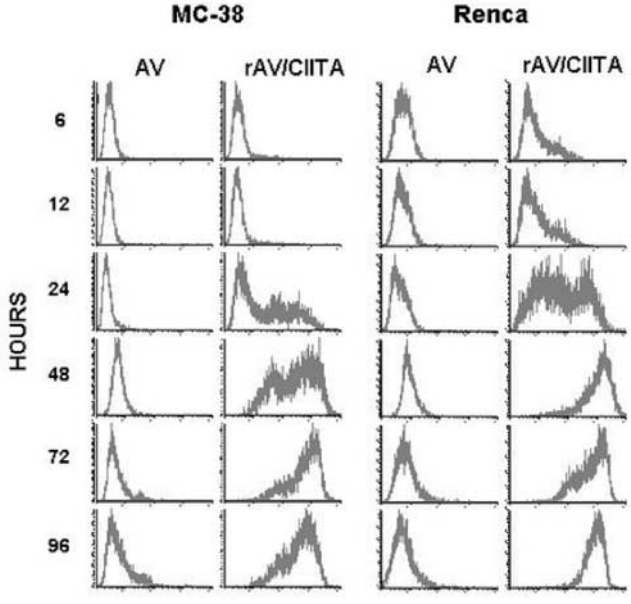

Figure 2 *Induction of MHC Class II expression in MC-38 and Renca cells by rAV/CIITA. MC-38 and Renca cells were infected with 10 MOI of recombinant adenovirus containing CIITA (rAV/CIITA) and wild-type adenovirus (AV). MHC Class II expression of transduced cells was determined by immunostaining with M5/114.15.2 mAb followed by fluorescent-labeled second antibody and FACS-analyzed at different time points. X-axis is fluorescence intensity and Y-axis is cell number.*

determined. MC-38 cells were infected with 10 MOI of rAV/CIITA for 27 h. Cells were trypsinized and subjected to electroporation in the presence of 25 μM of the Ii antisense oligonucleotide, AE54, or control oligonucleotides.[8] Ii protein expression was efficiently inhibited by the Ii antisense oligonucleotide (Figure 3e), but not by either sense or mismatch oligonucleotides (Figure 3c, d), indicating that Ii inhibition was sequence specific. MHC Class II expression was not significantly altered (Figure 3e). Consistent findings have been observed in repeated experiments. Similar results were also obtained in cells infected with rAV/IFN-γ treated with the same antisense and control oligonucleotides.

Efficient inhibition of Ii protein expression in MC-38 cells infected with RAV/IFN-γ/Ii-RGC at low concentrations. Since electroporation kills about 50% of cells *in vitro* and is poorly suited for *in vivo* use, for example, in primary and metastatic tumor nodules, we constructed several active Ii-RGCs in plasmids. These Ii-RGCs effectively inhibited Ii expression in a variety of cells after lipid-mediated or gene gun transfection.[8,9] One of the most effective Ii-RGCs was cloned into adenoviruses containing IFN-γ, generating rAV/IFN-γ/Ii-RGC construct (Figure 1). MC-38 cells were infected with different concentrations of rAV/IFN-γ/Ii-RGC for 120 h (Figure 4). MC-38 cells converted into the MHC Class II+/Ii- phenotype following infection with a low concentration of adenovector (<10 MOI). At 3 or 5 MOI, virtually all MC-38 cells were MHC Class II+/Ii-. Control MC-38 cells infected with rAV/IFN-γ were mostly MHC Class II+/Ii+ (Figure 4). These data were reproduced in repeated experiments.

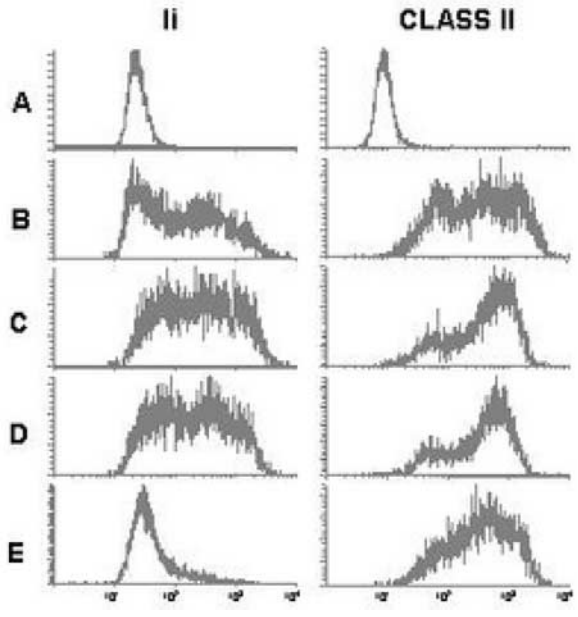

Figure 3 *Generation of MHC Class II+/Ii- phenotype MC-38 cells by rAV/CIITA and Ii antisense oligonucleotide. MC-38 cells were infected with 10 MOI rAV/CIITA for 27 h and then electroporated in the presence of an Ii antisense oligonucleotide, or a sense oligonucleotide, or a mismatch oligonucleotide. Following 24 h incubation in complete medium, cells were analyzed for MHC Class II and Ii expression by immunostaining with M5/114.15.2 and In.1 monoclonal antibodies, respectively. (a) Parental MC-38 cells; (b) rAV/CIITA infected MC-38 cells; (c) the cells of (b) treated with sense oligonucleotide (d). The cells of (b) treated with mismatch oligonucleotide; and (e) the cells of (b) treated with antisense oligonucleotide. X-axis is fluorescence intensity and Y-axis is cell number.*

Time course of Ii protein inhibition in MC-38 cells with rAV/IFN-γ/Ii-RGC

The time course for induction of the MHC Class II+/Ii- phenotype by rAV/IFN-γ/Ii-RGC was investigated. MC-38 cells were infected with 3 MOI of rAV/IFN-γ/Ii-RGC and harvested at different time points. The greatest Ii inhibition occurred at 120 h (Figure 5). Already at 96 h

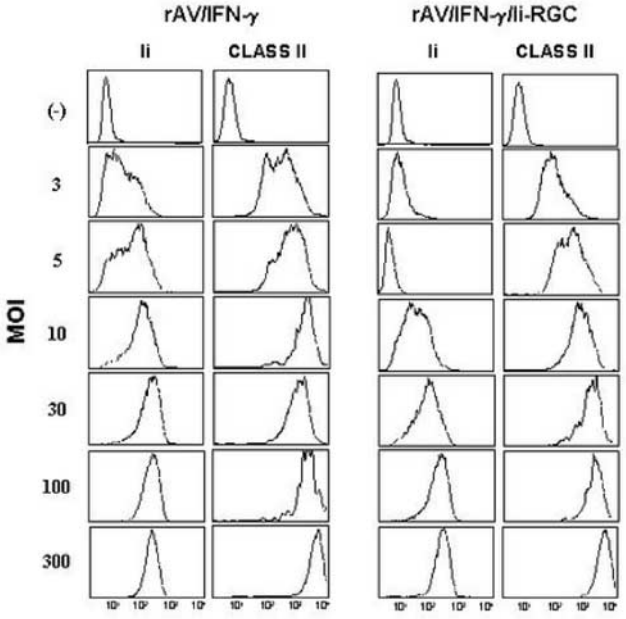

Figure 4 *Generation of MHC Class II+/Ii- phenotype MC-38 cells by different doses of rAV/IFN-γ/Ii-RGC. MC-38 cells were infected with different doses of rAV/IFN-γ/Ii-RGC or rAV/IFN-γ for 120 h and cells were then stained for MHC Class II and Ii. At a concentration of 5 MOI, 100% of MC-38 cells are MHC Class II+/Ii- phenotype. X-axis is fluorescence intensity and Y-axis is cell number.*

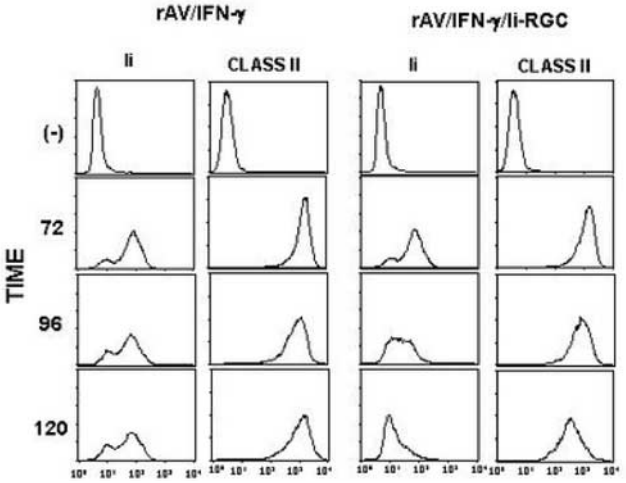

Figure 5 *Time course of generation of MHC Class II+/Ii- phenotype MC-38 by rAV/IFN-γ/Ii-RGC. MC-38 cells were infected with 3 MOI of rAV/IFN-γ/Ii-RGC or rAV/IFN-γ. Cells were harvested for MHC Class II and Ii staining at different time points and FACS-analyzed. X-axis is fluorescence intensity and Y-axis is cell number.*

after infection, cells started to become MHC Class II+/Ii- in the rAV/IFN-γ/Ii-RGC group, while most cells in the rAV/IFN-γ-infected group retained the MHC Class II+/Ii+ phenotype (Figure 5).

Intratumoral gene therapy of Renca tumors with recombinant adenovectors containing CIITA and IFN-γ and Ii-RGC

The *in vivo* function of Ii-suppression was tested in Renca cells. Prior to *in vivo* studies, to confirm the rAVs activity, Renca cells were infected with rAV/CIITA/IFN-γ and rAV/Ii-RGC *in vitro*. MHC Class I and II molecules were expressed on nearly 100% of cells and Ii was suppressed in 84% of cells. Based on these findings, a combination of these two rAV constructs was used for intratumoral treatment *in vivo*. Established s.c. Renca tumors of about 0.3 cm in diameter were treated by intratumoral injection of a mixture of rAVs (Figure 6). A subtherapeutic dose of rAV-IL-2 of 3.8×10^8 particles compared to an optimal dose of 1.5×10^{10} particles was used to enhance the immune response triggered by MHC Class II+/Ii-tumor cells. Control mice developed tumors by day 8 that grew with rapid kinetics (Figure 6a). Mice treated with rAV/CIITA/IFNγ+rAV/IL-2 + rAV(wild type) showed delayed tumor growth in three of five mice, with tumor regrowing in two of these mice, resulting in one of five mice being tumor free on day 60 (Figure 6b). Tumor regression was observed in three of four mice treated with rAV/CIITA/IFNγ+rAV/IL-2+rAV/Ii-RGC (Figure 6c). Tumor-free mice were challenged on day 63 with 1×10^5 Renca cells injected s.c. in the flank. These three mice did not develop tumor in a follow-up of 34 days postchallenge in contrast to the fact that three naïve mice injected with the same number of Renca cells developed tumors. This experiment was repeated with additional control groups and with a larger number of mice in the experimental group testing the rAV/CIITA/IFNγ+rAV/IL-2+rAV/Ii-RGC combination (Table 1). Treatment of Renca tumors with wild-type AV alone did not affect tumor growth. rAV/ IL-2 or rAV/CIITA/IFNγ alone only delayed tumor growth up to days 20–30 (Table 1). Treatment with rAV/CIITA/IFNγ+rAV/IL-2 caused complete tumor regression and rejection of Renca tumor challenge in one of five mice. The combination of rAV/CIITA/IFNγ+rAV/IL-2+rAV/Ii-RGC led to complete tumor regression in 10 of 17 mice (Table 1). When these 10 tumor-free mice were challenged on day 64 with 1×10^5 Renca cells injected s.c. in the flank, none of the mice developed tumors while three naïve mice injected with the same number of Renca cells, developed tumors, confirming the results from the previous experiment.

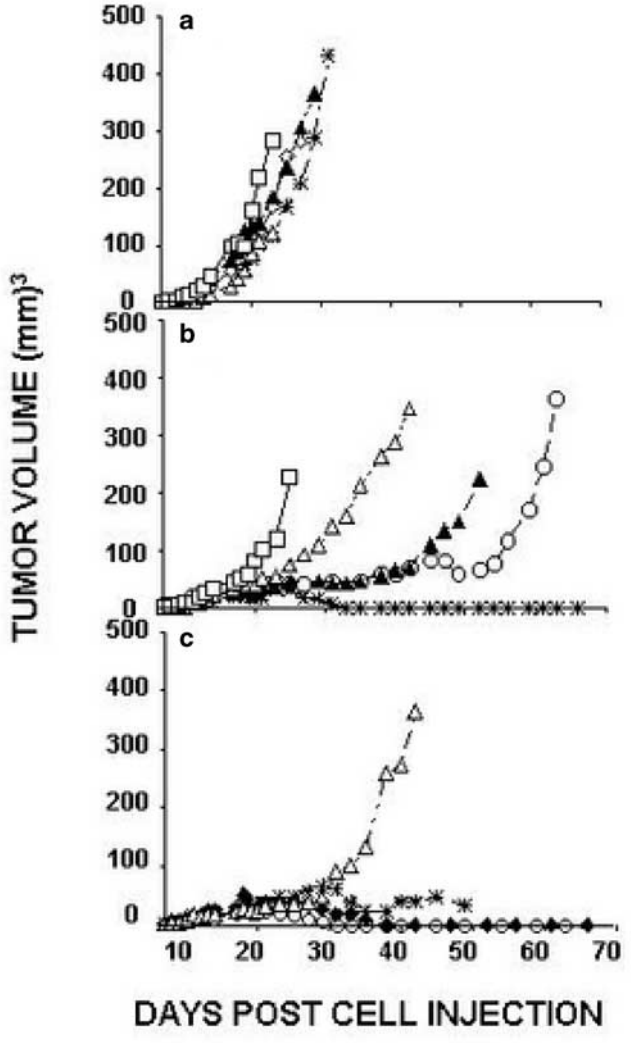

Figure 6 *Intratumoral treatment of Renca s.c. tumors with Ad-vectors. Established Renca tumors were treated with a combination of adenovirus. (a) Mice received five injections of PBS over 5 days. (b) Mice received five injections of rAV/CIITA/IFN-γ+rAV/IL-2+wild-type AV over 5 days. (c) Mice received five injections of rAV/CIITA/IFN-γ+rAV/IL-2+rAV/Ii-RGC over 5 days. The tumor volumes for individual mice are plotted.*

Discussion

The great efficiency of rAV as a gene delivery vehicle has been established in numerous studies.[11,12] rAV infects many cell types, and both dividing and nondividing cells.[13,14] Its only shortfall is that rAV does not integrate into cellular genomes and, therefore, it is not suitable for creating stably infected cells. However, in our application, transient generation of MHC Class II+/Ii- tumor

Table 1 Antitumor response of mice treated with intratumoral adenoviral vector gene therapy

	Tumor-free mice	
Treatment	*Post treatment*	*Post challenge Tumor*
Control+Ad-empty	0/7	NA
rAV-IL-2	0/4	NA
rAV-CIITA/IFN-γ	0/5	NA
rAV-CIITA/IFN-γ+Ad-IL-2	1/5	1/1
rAV-CIITA/IFN-γ+Ad-IL-2+Ad-Ii-RGC	10/17	10/10
Naïve mice	NA	0/3

Established Renca tumors were treated, on day 9, with intratumoral injections of adenoviral vectors given once a day over five consecutive days. The proportion of tumor-free mice at the end of the observation period, on day 64 postcell injection, is reported. Tumor-free mice and naïve mice were challenged with Renca cells on day 64, the proportion of challenge-tumor-free mice by day 24 postchallenge is reported.

cells is preferred both *in situ* in primary or metastatic tumors, and *ex vivo* in the preparation of cancer and dendritic cells for reinjection.

rAV gene delivery is very efficient and nontoxic at low MOI of the virus. rAV carrying the CIITA gene strongly induced MHC Class II molecules at 5 MOI in almost 100% of MC-38 and Renca tumor cells (Figure 2). Even at 3 MOI, nearly 100% of such cells were induced to express MHC Class II molecules (Figure 4). In all our experiments, we have demonstrated clearly that relatively low MOI of rAV efficiently delivers CIITA and IFN-γ genes into MC-38 and Renca tumor cells, with better cell viability than at high MOI. Efficient induction at such a low MOI is a significant advantage in our system because the high concentrations of rAV, currently used by many investigators, might produce relatively larger amounts of viral product, which can disrupt various biological functions of the infected cell including antigen processing and presentation.[21]

Our adenoviral vectors are an effective reagent to create MHC Class II+/Ii- tumor cells *in vitro*. rAVs have been widely used to deliver antisense constructs into cells and gene suppression has been observed to varying degrees both *in vitro* and *in vivo*.[17–20] We previously characterized a potent Ii antisense oligonucleotide, and now showed that it inhibited Ii expression in rAV-induced MHC Class II+/Ii+ cells after intracellular delivery by electroporation (Figure 3).[8] In order to develop better *in vivo* Ii suppression reagents, we created Ii-RGC plasmid vectors to produce an antisense RNA that dimerizes with the mRNA for Ii protein, thereby inhibiting Ii protein expression. The Ii-RGC plasmid potently inhibits Ii protein in many cell lines following lipid-mediated transient delivery or stable transfection.[8,9] In order to take advantage of the high efficiency of gene delivery by rAV, we developed rAVs to deliver Ii-RGCs into cells. MC-38 cells were infected with different concentrations of either rAV//IFN-γ/Ii-RGC or rAV//IFN-γ. At low concentrations (<10 MOI, Figure 4), the MHC Class II+/Ii- phenotype was induced in >90% cells with rAV/IFN-γ/Ii-RGC, while rAV/IFN-γ-transduced cells remained MHC Class II+/Ii+. At a concentration of 3 MOI, almost all cells are MHC Class II+/Ii-. At vector concentrations greater than 10 MOI, Ii protein was not inhibited, probably because Ii proteins are too strongly induced by the greater copy number of IFN-γ introduced by higher concentrations of rAV/IFN-γ/Ii-RGC. Our results are consistent with those of others, indicating that adenovirus-mediated delivery of antisense RNA is an efficient and clinical feasible method to specifically induce or suppress gene expression.[17–20] This is the first report, we know, of a single adenoviral vector delivering an expressible transacting gene and an antisense inhibitory gene. This indicates the feasibility of cloning two or more genes with different functions into one adenoviral vector, thereby creating an effective and convenient tool for research and therapeutic use.

A potent anticancer immune response was generated in Renca-bearing mice after intratumoral gene therapy with our new adenoviral vectors, which induced MHC Class II+/Ii- phenotype in Renca tumor cells. Injection of Renca tumor nodules with rAV/CIITA/INF-γ and rAV/Ii-RGC combined with a suboptimal dose of rAV/IL-2 induced in a much more potent antitumor immune response in 75% of the mice, compared to a 20% response in mice treated with rAV/CIITA/IFNγ+rAV/IL-2+wild-type AV. Indeed, tumor regression was seen in three of four mice treated with Ii-RGC. These data indicate that suppression of Ii protein in MHC Class II-expressing tumors augments vaccine activity. Surviving tumor-free mice rejected Renca tumor challenge, demonstrating that adenoviral gene therapy induced specific antitumor immunity. A repeated experiment with a larger number of mice demonstrated complete antitumor immune response in 60% of the mice, associated with rejection of subsequent Renca tumor challenge, confirming specific antitumor immunity (Table 1). Our findings are consistent with the observations of Ostrand-Rosenberg and our previous studies that generation of MHC Class II+/Ii- tumor cells potentiates a strong T helper cell response leading to control or rejection of the tumor.[3–5,8] It is also possible that tumor-infiltrating dendritic cells (DCs) were also transfected by rAV and modified to express MHC Class II+/Ii- phenotype. These DCs might also engulf tumor antigens released by apoptotic tumor cells and then present them to T helper cells.

The important messages from this study for future clinical applications of these novel reagents are the following. First, higher concentrations of adenovirus are not necessarily better because: (1) high concentration of adenovirus may produce viral proteins which disturb the function of the delivered therapy gene[21] and (2) in this system, low level of CIITA- or IFN-γ-induced Ii protein can easily be inhibited by the Ii-RGC. Since Ii protein is always coinduced with MHC Class II molecules by CIITA and IFN-γ, Ii-RGC is required to induce the MHC Class II+/Ii- phenotype.[22,23] The second point is that our method to induce MHC Class II and Ii expression and then to inhibit Ii protein expression through adenoviral vectors offers simplicity for clinical use for generating MHC Class II+/Ii- tumor cell vaccines or directly injecting into tumors *in situ* to induce a potent CD4+ T helper cell response to tumor antigens. One construct can be used for all patients without considering the MHC Class II allele specificity. One of the major functions of Ii is to protect the antigenic peptide binding site on MHC Class II molecules from binding to endogenously derived antigenic peptides in ER.[22] Suppression of this highly conserved, nonallele-restricted protein allows for novel epitopes to be presented on MHC Class II molecules. The final point is that our strategy for generating MHC Class II+/Ii- phenotype not only serves as a promising tumor therapy, but also as a potent adjuvant to augment the efficacy of attenuated viral vaccines (eg, vaccinia and hepatitis), genetic vaccines, and dendritic cell vaccines.

Materials and methods

Cell lines and antibodies

The MC-38 is a murine colon adenocarcinoma cell line.[24] Renca, a murine renal cell carcinoma line, of spontaneous origin in a BALB/c mouse was maintained *in vivo* by serial intraperitoneal (i.p.) passages and by *in vitro* culture.[25] Both cell lines were cultured in DMEM medium supplemented with 10% FCS. Antimurine MHC Class II monoclonal antibody M5/114.15.2 was obtained from the culture supernatant of hybridoma HB120 (ATCC, Rockville, MD, USA) by purification on a protein A affinity column (Pierce, Rockford, IL, USA).

Anti-murine Ii monoclonal antibody was used as the supernatant of a culture of hybridoma In.1, which was the gift of Dr Victor Reyes, University of Texas Medical School, Galveston, TX, USA.[26]

DNA oligonucleotides

AE54 (5-TGGTCATCCATGGCTCTA) and sense (5′-TAGAGCCATGGATGATTA) oligonucleotides were synthesized by Boston Biosystems, Inc. (Bedford, MA, USA). Mismatch oligonucleotide (5′-TGACCATTCATTGCACTA) was synthesized by Oligo Etc. (Wilsonville, OR, USA).

Plasmids and adenovirus

The pQBI/Ad/BN plasmid was purchased from Quantum Biotechnologies (Montreal, Canada). The pcDNA3.1 plasmid was purchased from Invitrogen (San Diego, CA, USA). RSV.5 plasmid was a gift of Dr Long at NIH.[27] Murine IFN-γ cDNA gene was obtained from Dr Ananthaswamy at MD Anderson Medical Center, Dallas, TX, USA.[28] The CIITA cDNA gene in pCEP4 expression plasmid was from Dr Laurie Glimcher.[29] Cla I-digested adenoviral DNA and necessary reagents for making recombinant adenoviruses were purchased from Quantum Biotechnology. rAV/IL-2 was obtained from Transgene (Strasbourg, France).[30]

Plasmid construction

The CIITA gene was already available in the pCEP4 expression vector, driven by a CMV promoter. IFN-γ was cloned into pcDNA(3) by standard molecular biological methods to generate pCDNA(3)/IFN-γ, driven by a CMV promoter. RSV.5/Ii-RGC was constructed by cloning Ii (-92,97) (A in AUG start codon is 1) into the RSV.5 vector in reverse orientation. This RSV.5/Ii-RGC effectively inhibited Ii expression in stably transfected A20 B lymphoma cells and CIITA stably transfected Sarcoma cell line, Sal 1, and in Renca cells *in vivo* by intratumoral injection. Two additional Ii-RGCs, Ii-RGC(32,136), and Ii-RGC(314,459) cloned into pcDNA(3) vector were also shown to be active in inhibiting Ii in A20 cells. In order to enhance the activity of Ii inhibition, a plasmid containing Ii-RGC(-92,97/32,136/314,459) has been constructed each with its own promoters and poly A signals. The rAV containing this Ii-RGC was used for intratumoral injections of Renca nodules *in vivo.*

Generation of recombinant adenoviruses containing CIITA, IFN-γ, or both IFN-γ and Ii-RGC

We then created recombinant adenoviruses containing one gene or gene combinations. All gene(s) (or gene combinations) alone with their promoters and poly A signals were PCR excised and cloned into pQBI/BN vector by standard molecular biology methods. These vectors were cotransfected into 293A adenoviral packaging cells with Cla1-digested adenoviral DNA (the left arm of the virus was deleted to reduce background) according to the manufacturer's instruction. At 3 weeks after cotransfection, resulting plaques were screened by PCR to ensure the presence of the CIITA gene, IFN-γ gene, and Ii-RGC genes along with their promoters. The viruses were then expanded and titrated according to the manufacturer's instructions.

Infection of MC-38 cells and Renca cells with recombinant adenoviruses

MC-38 or Renca cells were plated at 5×10^4 cells per well in 24-well plates 18–24 h before infection. The viruses were added at different concentrations, at 24 h after virus infection, 1 ml of fresh medium was added. The culture medium was replaced every 24 h with 2 ml of fresh medium. At various times after infection, the cells were washed, trypsinized, and FACS-analyzed for expression of MHC Class II molecules after staining with rat anti-mouse I-E^k monoclonal antibody M5/114.15.2.

Transfection of MC-38 cells with Ii antisense oligonucleotide with electroporation

After rAV infection, $1–2 \times 10^6$ MC-38 cells were trypsinized and washed twice with RPMI 1640 without serum and resuspended in 0.5 ml RPMI 1640 without serum. Cells were transferred into a 4-mm gap electroporation cuvette (BTX, San Diego, CA, USA) and 25 μM (final concentration) of antisense, sense, or mismatch oligonucleotides were added and mixed. The cuvette was placed in ice for 10 min and subjected to electroporation (1250 μF/250 V) with an Electro Cell Manipulator 600 (BTX). The cuvette was then kept in RT for another 10 min, and cells were recultured in six-well plate with complete DMEM medium until the cells were analyzed for the expression of MHC Class II and Ii.

Immunostaining of cultured cells

Cells were surface stained with M5/114.15.2 mAb for MHC Class II molecules and intracellularly stained with In.1 mAb for Ii protein. For cell surface staining of MHC Class II molecules, $0.5–1 \times 10^6$ of cells were washed and incubated with M5/114.15.2 antibody in a total volume of 200 μl for 45 min at RT. Cells were washed and incubated with fluorescein-conjugated anti-rat IgG (Southern Biotechnologies, Birmingham, AL, USA) for 30 min at RT. Cells were washed and fixed with 10% formaldehyde for 10 min. For intracellular staining of Ii protein, procedures of Rosenberg *et al* were followed with modifications.[4,5] Briefly, $0.5–1 \times 10^6$ cells were washed with Hanks' balanced salts solution (HBSS) and fixed with 2% formalin at RT for 10 min. Cells were then washed with 1 ml of 1 M glycine (Sigma, St Louis, MO, USA) at RT for 5 min. The cells were permeablized with 0.2% saponin (Sigma) and incubated with 200 μl of In.1 (with 0.2% saponin) for 60 min on ice. Cells were washed and labeled with fluorescein-conjugated anti-rat IgG (Southern Biotechnologies) in 0.2% saponin for 30–45 min. Cells were washed and fixed with 10% formaldehyde for 10 min. Cells were FACS-analyzed for the expression of MHC Class II and Ii proteins.

Intratumoral treatment with adenoviral vectors

Mice were injected subcutaneously (s.c.) with Renca cells at 2×10^5 cells in 0.1 ml HBSS. On day 8 postcell injection, established Renca tumors of about 0.3 cm diameter were treated with intratumoral injections of rAVs mixtures. Dissociation of a 0.3 cm tumor into single-cell suspension was found to contain $1–5 \times 10^5$ tumor cells and thus an average of about 1.25×10^5 cells. Since 3–10 MOI of rAV/CIITA or rAV/IFN-γ induced MHC Class II expression in $>95\%$ of cells *in vitro* (Figure 2), a dose of 1.25×10^6 particles for rAV/CIITA/IFNγ

was used for *in vivo* injection to result into 10 MOI per tumor cells in the tumor. To ensure the suppression of Ii induced by CIITA and IFN-γ, a 10-fold dose of 2×10^7 particles for rAV/Ii–RGC was used. A subtherapeutic dose of 3.8×10^8 particles for rAV/IL-2 was used.[30] A total of 42 μl were injected in each tumor using 0.3 ml insulin syringes. To keep a constant number of total rAVs particles between treatment groups, in groups not receiving rAV/Ii-RGC, the number of particles was supplemented with wild-type AV. Mice were injected for five consecutive days and monitored for tumor growth. Tumors were measured three times a week with a caliper. Tumor volume was calculated using the formula: 0.5236 × length × width × height.

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