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

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Summary

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

Keywords: DNA vaccine; HIV gp120; Ii suppression; MHC class II

Introduction

While DNA vaccines induce cytotoxic T lymphocyte (CTL) activity successfully and are considered to be generally safe and economic, they often stimulate a relatively poor immune response. Investigators have sought to enhance the immunogenicity of DNA vaccines using a variety of methods. These techniques include: inoculation with genes encoding costimulatory molecules,[1] enhancing the *in vivo* transfection efficiency by mixing DNA with cationic lipids,[2] coating DNA onto microparticles such as poly(lactide-coglycolide)[3] and *in vivo* electroporation.[4] Other methods that are being explored to enhance vaccine gene expression include using a gene gun to deliver DNA vaccines directly into cells,[5] the addition of CpG motifs to plamsids to generate innate immune stimuli[6,7] and the development of different prime/boost regimes using DNA/virus, DNA/protein and DNA/peptides.[8–14] In addition, cytokine genes, such as granulocyte–macrophage colony-stimulating factor (GM-CSF), have been used in DNA vaccine regimes to augment DNA vaccine efficiency.[6,15–17] Our studies add to the work of the above investigators, with a novel and potentially clinically useful method to enhance the potency of DNA vaccines.

$CD4^+$ T cell activation plays an important role in the enhancement of DNA vaccine efficacy.[18] We have developed a vaccine strategy, based on suppression of the expression of major histocompatibility complex (MHC) class II associated invariant chain protein (Ii), that augments $CD4^+$ T cell activation by endogenously synthesized antigens. The Ii protein normally binds to MHC class II molecules in the endoplasmic reticulum (ER), blocking the antigenic epitope-binding groove. Ii protein is later digested in a post-Golgi vesicle and released from the MHC class II molecule in a concerted fashion coupled to the charging of antigenic peptides.[19] One of the major functions of Ii is to protect the antigenic peptide binding site on MHC class II molecules from binding endogenously derived antigenic peptides.[20–22] Suppression of Ii leads to the induction of 'unprotected' MHC class II molecules in an antigen-presenting cell (APC), enabling it to present endogenous antigens by both MHC class I (the normal functional pathway) and 'unprotected' MHC class II molecules, simultaneously activating $CD4^+$ and $CD8^+$ T cells. In an earlier study, we generated a potent sarcoma tumour cell vaccine by inhibiting Ii with Ii anti-sense oligonucleotides.[23] Ii anti-sense oligonucleotides also effectively inhibited Ii expression in dendritic cells (DCs), leading to the presentation of endogenously expressed ovalbumin (OVA) epitopes to $CD4^+$ T cells and a potent tumour vaccine.[24] We have subsequently generated an active Ii suppression plasmid construct: Ii reverse gene construct [Ii-RGC(− 92,97), A in the AUG start codon equals 1], to suppress Ii expression in tumour cells.[25–27] Ii-RGC(− 92,97) codes for expression of an anti-sense mRNA, which hybridizes to the native mRNA for Ii

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protein, thereby leading to Ii suppression. This strategy generated MHC class $I^+/II^+/Ii^-$ phenotype tumour cell vaccines in different experimental animal models.[23,25–27]

In this study we have utilized Ii suppression technology to enhance a HIV gp120 DNA vaccine model. Our rationale was that an APC, e.g. DC, that takes up DNA plasmids containing both the gp120 gene and Ii-RGC, will generate $gp120^+$/MHC class II^+/Ii^- DC. 'Unprotected' MHC class II along with MHC class I (the normal functional pathway) molecules will be charged by endogenously produced gp120 epitopes. The DC will subsequently present MHC class II epitopes to activate $CD4^+$ T cells. The enhanced activation of gp120-specific $CD4^+$ T cells will, in turn, help to strengthen the activation of gp120-specific $CD8^+$ T cells, which are sensitized by MHC class I presentation on the same DC, thereby significantly augmenting the efficiency of HIV gp120 DNA vaccines. We report here that addition of the Ii-suppression technology to HIV gp120 DNA vaccine significantly enhances the potency of the gp120 DNA vaccine and serves as a basis for the rational design of human Ii-RNAi constructs, to be used with established DNA vaccines for enhanced antigen-specific $CD4^+$ T cell activation, all of which could potentially have a significant benefit therapeutically or as preventive vaccinations.

Materials and methods

Mice

BALB/c mice (8–12 weeks old) were purchased from Jackson Laboratory and kept in the animal facility at the University of Massachusetts Medical Center, Worcester, MA, USA. All animal procedures were performed following the University of Massachusetts Medical School animal care guidelines under an approved protocol and overseen by the University of Massachusetts IACUC Committee.

Cell lines and antibodies

Murine macrophage J774 cells cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) with 10% fetal calf serum (FCS) were obtained from Dr Gary Ostroff. Anti-murine Ii monoclonal antibody, from culture supernatant, In.1, and anti-murine MHC class II monoclonal antibody, M5/114·15·2, purified from culture supernatant, were used.[28,29]

Plasmids

Murine Ii cDNA[30] was obtained from Dr James Miller of the University of Chicago. Ii-RGC(− 92,97) (numbers represent nucleotide position in Ii cDNA gene, 1 is A in AUG start codon and − 92 represents 5′ upstream 92 nucleotides from AUG) has been described previously.[25–27] The pBudCE4·1 plasmid was purchased from Invitrogen (San Diego, CA, USA). The murine GM-CSF plasmid (pNGVL1-mGM-CSF) was from Dr Gilda G. Hillman of Wayne State University, Detroit, MI and the pCEP4/CI-ITA plasmid[31] was from Dr Laurie Glimcher of the Harvard School of Public Health (Boston, MA, USA). HIV-1 IIIB gp120 cDNA[16] from Dr Norman Letvin at the Beth Israel Deaconess Medical Center (Boston, MA, USA) was cloned into a Rous sarcoma virus (RSV.5) expression vector.[32] Expression of the HIV-1 IIIB gene was confirmed by transfecting RSV.5/gp120 into COS cells (data not shown).

Peptide synthesis

Two peptides, a 15-mer, termed p18 (RIQRGPGRAFV-TIGK), and a 10-mer, termed p18–I10 (RGPGRAFVTI), were synthesized by Commonwealth Biotechnologies, Inc., Richmond, VA, USA. Peptide p18 is presented by both $H\text{-}2D^d$ and $H\text{-}2A^d$ molecules while p18–I10 is presented only by $H\text{-}2D^d$.[33,34] That is, the shorter p18–I10 peptide contains only the MHC class I-presented epitope while the longer p18 peptide contains both a MHC class II-presented epitope and a MHC class I-presented epitope.

DNA coating of gold particles for gene gun delivery

Plasmid DNA was precipitated onto gold particles. Briefly, 15 mg of 1 μm gold microcarriers (enough for 30 cartridges) (Bio-Rad Laboratories, Inc., Richmond, CA) were resuspended by sonication in 100 μl of 0·1 M spermidine. DNA, at a concentration of 1 mg/ml in endotoxin-free water, was then added and sonicated, after which 200 μl of 2 M $CaCl_2$ was added dropwise. The gold-DNA mixture was allowed to stand for 10 min to precipitate before being washed three times with 1 ml aliquots of 100% ethanol. After the final wash, the pellet was resuspended by vortexing and sonication in 1·86 ml of 100% ethanol. After precipitating, the plasmid DNA was adsorbed onto gold beads and the gold beads were coated evenly onto the inner surface of Tefzel tubing (Bio-Rad). After coating the tubing was then cut into 0·5-inch cartridges. Different DNA loading ratios were designed for respective experiments, as described in Results. Cartridges were stored at 4° with desiccant.

DNA transfection of cells

For gene gun transfection of J774 cells, 10^6 cells in 20 μl of medium were pipetted onto a tissue culture dish in approximately 1 cm diameter circles and then subjected to gene gun shooting with one 0·5-inch cartridge (loaded with 1 μg of DNA) at a helium pressure of 300 pounds per square inch (psi). After culturing at 37° for 42 hr, cells were stained with anti-MHC class II

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antibody, M5/114·15·2 or anti-murine Ii monoclonal antibody, In.1, and then with fluorescently labelled secondary antibodies. Stained cells were analysed by fluorescence activated cell sorter (FACS) to determine transfection efficiency.

Gene gun immunization of mice

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

Enzyme-linked immunosorbent spot (ELISPOT) assay

Splenocytes were obtained from spleens of the killed mice, according to UMMC IACUC-approved procedures. Immunoaffinity-purified $CD4^+$ and $CD8^+$ splenic lymphocytes (> 95% purity) were obtained from the pooled splenocytes. ELISPOT procedures were the same for all groups. BD Pharmingen kits for murine interferon (IFN)-γ and interleukin (IL)-4 ELISPOT assays were used according to the manufacturer's instructions. Briefly, plates were coated overnight at 4° with the cytokine capture specific antibodies. The plates were then blocked with 10% fetal bovine serum (FBS) in RPMI-1640 for 2 hr at room temperature (RT) and washed four times with 1× phosphate-buffered saline (PBS) containing 0·05% Tween-20 (wash buffer). Freshly isolated single splenocyte suspensions (10^6/well) and p18 or p18–I10 peptides (5 μg/well) were added to the anti-cytokine precoated plates. After 42–66 hr of incubation, the plates were washed five times with wash buffer, biotinylated detection antibodies (2 μg/ml) were added and incubated for an additional 2 hr at RT. The plates were washed four times with wash buffer and avidin horseradish peroxidase (avidin-HRP) was added, at a 1 : 100 dilution, followed by a 1-hr incubation at RT. Avidin-HRP was removed by washing four times with wash buffer and two times with 1× PBS. Spots were developed by adding 3-amino-9-ethylcarbazole HRP substrate to the plates for 30 min at RT. Finally, the plates were washed twice with sterile water and dried for 1–2 hr at RT. Digitized images of the spots were analysed with a series 1 Immunospot Analyzer and Immunospot 1·7e software (Cellular Technology Limited, Cleveland, OH, USA).

Statistical analysis

Statistical differences were calculated by Student's *t*-test. Significance was defined as $P < 0.05$.

Results

Construction of Ii suppression plasmids

We had previously generated an Ii reverse gene construct Ii-RGC(− 92,97) that effectively inhibited Ii expression in tumour cells and created a potent tumour immunotherapy animal model.[25–27] Ii-RGC(− 92,97) was generated by cloning an Ii gene fragment (− 92,97) (1 is A in AUG) into an expression vector in reverse orientation. The anti-sense RNA produced by this Ii-RGC hybridizes with Ii mRNA to block translation of Ii mRNA and/or triggers the destruction of Ii mRNA.[35,36] In this study, we cloned three copies of the Ii-RGC(− 92,97) gene fragment into one pBudCE4·1 plasmid, generating pBudCE4·1/Ii-RGC(× 3) (Fig. 1) to increase the efficiency of Ii suppression. In pBudCE4·1/Ii-RGC(× 3), each Ii-RGC(− 92,97) gene fragment is driven by a different promoter. The first Ii-RGC(− 92,97) gene fragment was inserted into pBudCE4·1 by *Hin*dIII and *Bam*HI under the control of a cytomegalovirus (CMV) promoter to generate a one-copy pBudCE4·1/Ii-RGC(− 92,97) (pBudCE4·1/Ii-RGC). The second copy of Ii-RGC(− 92,97) was first cloned into the pUB6/V5-His plasmid (Invitrogen, San Diego, CA, USA) under control of a UbC promoter. The UbC promoter, Ii-RGC(− 92,97) fragment and poly A signal sequence were then amplified by polymerase chain reaction (PCR) and cloned into the pBudCE4·1/Ii-RGC to generate a two-copy pBudCE4·1/Ii-RGC(− 92,97) [pBudCE4·1/Ii-RGC(× 2)]. The third copy of Ii-RGC(− 92,97) was first cloned into the RSV.5 plasmid under the control of a RSV promoter.[25,27] The RSV promoter, Ii-RGC(− 92,97) fragment and poly A signal were PCR amplified and cloned into pBudCE4·1/Ii-RGC(− 92,97(× 2) to generate a three-copy pBudCE4·1/Ii-RGC(− 92,97) [pBudCE4·1/Ii-RGC(× 3)] (Fig. 1). More detailed procedures and enzyme sites for cloning are explained in the legend of Fig. 1.

Ii suppression in macrophage cells by pBudCE4·1/Ii-RGC(× 3)

The activities of the three different pBudCE4·1/Ii-RGC plasmids were tested in COS cells to define the most active pBudCE4·1/Ii-RGC plasmid. This was achieved by determining inhibition of expression of a cotransfected Ii cDNA in COS cells. The results (data not shown) indicated that pBudCE4·1/Ii-RGC(× 3) is most active and has almost completely inhibited Ii expression in transfected COS cells. DCs, macrophages and Langerhans cells play

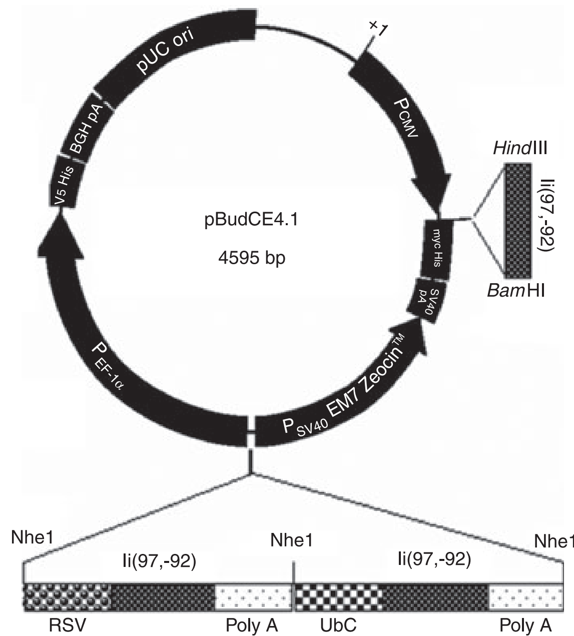

Figure 1. Generation and the map of BudCE4·1/Ii-RGC(× 3). The Ii-RGC(− 92,97) fragment was cloned into the pBudCE4·1 plasmid by *Hin*dIII (97 end, A in AUG codon is 1) and *Bam*HI (− 92 end), under control of a CMV promoter, to create the plasmid one-copy pBudCE4·1/Ii-RGC(− 92,97) (pBudCE4·1/Ii-RGC). For the two-copy Ii-RGC plasmid, the Ii-RGC(− 92,97) fragment was first cloned into pUB6/V5-His by *Hin*dIII (close to the UbC promoter from the 97 end) and *Bam*HI (− 92 end), under control of a UbC promoter, to generate pUB6/V5-His/Ii-RGC(− 92,97). The UbC promoter, Ii-RGC(− 92,97) gene fragment and poly A signal sequence were then amplified by polymerase chain reaction (PCR) and cloned into the Nhe1 site of pBudCE4·1/Ii-RGC to generate a two-copy pBudCE4·1/Ii-RGC(− 92,97) [pBudCE4·1/Ii-RGC(× 2)]. For the three-copy Ii-RGC(− 92,97), the Ii-RGC(− 92,97) gene fragment was first cloned into a RSV.5 plasmid by Sal1 (close to the RSV promoter from the 97 end) and *Bam*HI (− 92 end) under control of a Rous sarcoma virus (RSV) promoter to generate RSV.5/Ii-RGC(− 92,97).[25] The RSV promoter, Ii-RGC(− 92,97) gene fragment and poly A signal sequence were amplified by PCR and then cloned into the Nhe 1 site of pBudCE4·1/Ii-RGC(× 2) to generate a three-copy pBudCE4·1/Ii-RGC(− 92,97) [pBudCE4·1/Ii-RGC(× 3)]. The figure illustrates that each Ii-RGC(− 92,97) gene fragment was cloned in reverse orientation relative to its promoter. The '97' end of the Ii-RGC(− 92,97) gene fragment is always close to the promoter side, indicating that an anti-sense RNA will be produced. In pBudCE4/Ii-RGC(× 3), each Ii-RGC(− 92,97) gene fragment is driven by a different promoter in order to avoid possible promoter competition.

important roles in the induction of immunity against DNA vaccine antigens, especially when a gene gun is used for DNA delivery.[37,38] For this reason, we tested the activity of our Ii suppression constructs on the murine macrophage line J774. As J774 is an MHC class II-positive and Ii-positive cell line, pBudCD4·1/Ii-RGC(× 3) was used to assess inhibition of endogenously expressed Ii (Fig. 2).

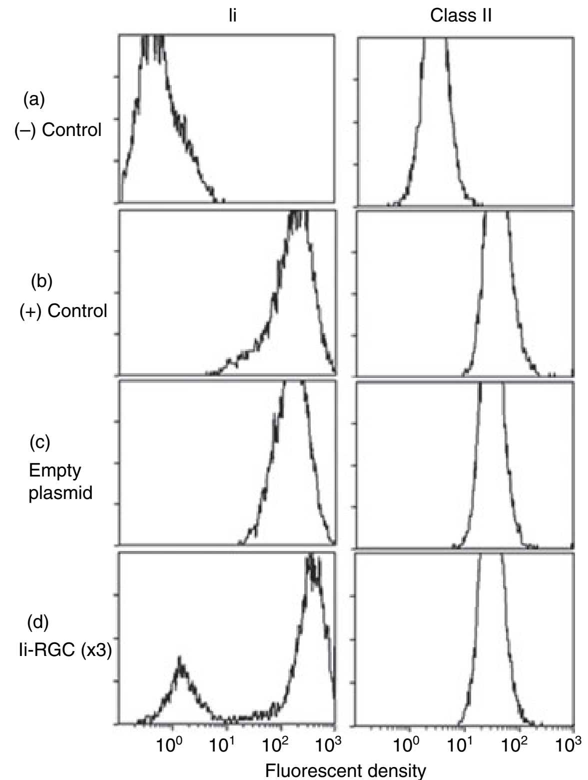

Figure 2. Ii suppression in J774 cells by pBudCD4·1/Ii-RGC(× 3); 10^6 J774 cells were transfected with 1 μg of pBudCE4·1/Ii-RGC(× 3) by gene-gun delivery as described in Materials and Methods. Three replicates of transfected J774 cells (3×10^6) were cultured together for 48 hr, harvested, and stained for both Ii and MHC class II proteins followed by fluorescence activated cell sorter (FACS) analysis. (a) J774 cells stained with only fluorescein isothiocyanate (FITC)-labelled second antibody only (negative control); (b) J774 cells stained with anti-Ii (In.1) or anti-major histocompatibility complex (MHC) class II (M5/114·15·2) antibodies followed by FITC-labelled secondary antibody (positive controls); (c) J774 cells treated with pBudCE4·1 empty plasmid and stained as in (b); (d) J774 cells treated with pBudCE4·1/Ii-RGC(× 3) and stained as in (b). Similar Ii inhibition results were obtained with several other cell lines using gene gun transfection (data not shown).

We normally obtain a 30–70% transient transfection efficiency using the gene gun, depending on the cell line used (unpublished observations). As shown in Fig. 2(d), Ii was significantly suppressed in 31% of transfected J774 cells (> 95% as measured by fluorescence intensity) without apparent change in MHC class II expression by pBudCE4·1/Ii-RGC(× 3). Under normal conditions, the Ii protein is synthesized in excess relative to MHC class II molecules in APC;[39] therefore, > 95% of Ii inhibition could lead to most MHC class II molecules in a transfected DC being unprotected by Ii molecules. These 'unprotected' MHC class II molecules should be available for charging by epitopes (including gp120 epitopes) in the ER, directed to the cell surface, followed by subsequent

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presentation to CD4$^+$ T cells. Based on these results pBudCE4·1/Ii-RGC(× 3) was used in all subsequent experiments.

Ii suppression enhances gp120 DNA vaccine efficiency

Next we tested whether Ii suppression enhanced HIV gp120 DNA vaccine efficiency. BALB/c mice were immunized with the gene for gp120, with or without pBudCE4·1/Ii-RGC(× 3). In our *in vivo* experiments, the gene for GM-CSF was included in all groups (except the naive group) as an adjuvant. In Fig. 3, one sees that both p18- and p18–I10-specific ELISPOT assays demonstrated roughly five times the enhancement of IFN-γ secreting cells in the Ii-suppressed groups (groups d and e) compared to the Ii unsuppressed group (group c). Similar reaction patterns to p18 and p18–I10 stimulation were observed, and the p18 peptide gave a greater response in most reactions. This result is consistent with previous reports which show p18–I10 is restricted only by H-2D^d, while the p18 peptide is restricted by both H-2D^d and H-2A^d.[33,34] The response to p18 reflects both CD4$^+$ and CD8$^+$ reactions and the p18I−10 response reflects only a CD8$^+$ reaction. These results indicate that most IFN-γ spots were produced by CD8$^+$ T cells in ELISPOT with total splenocytes. This is consistent with ELISPOT data using purified CD4$^+$ and CD8$^+$ T cells (next section).

In order to determine whether Ii suppression induced a Th1 or Th2 response, IL-4 secretion was also examined by ELISPOT and was found to be induced in all groups (Fig. 3), and that IL-4 production was enhanced by Ii suppression. As the enhancement pattern of IL-4 is similar to the enhancement of IFN-γ, we conclude that Ii suppression influences the magnitude of the immune response, but does not influence the Th1/Th2 pattern in our model.

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

In order to elucidate clearly whether Ii suppression enhances activation of only gp120-specific Th cells or both Th cells and CTL, we purified CD4$^+$ and CD8$^+$ T cells prior to analysis of cytokine expression. Pooled splenocytes (5×10^6/ml) from each group (five mice) were cultured with p18 peptide (25 μg/ml) for 5 days. The cells were purified with MiniMACs separation units (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated for another 48 hr prior to the IFN-γ ELISPOT assay. As indicated in Fig. 4, both CD4$^+$ and CD8$^+$ T cells were activated by gp120 DNA vaccine and Ii suppression enhanced gp120 DNA vaccine activation. This result is consistent with the working hypothesis that Ii suppression enhances the activation of CD4$^+$ Th cells.[40–42] CD4$^+$ T cells in turn augment and strengthen the activation of CD8$^+$ CTL. The frequency of generation of p18-specific CD4$^+$ T cells is consistent with previous reports, showing that the frequency of MHC class II epitope-specific CD4$^+$ Th cells resulting from DNA vaccine is much lower than that of CD8$^+$ T cells.[3,43]

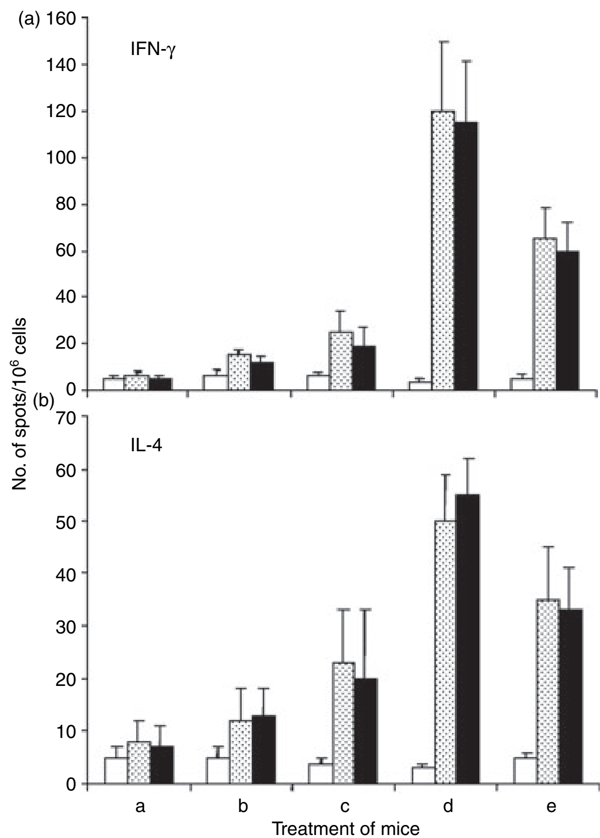

Figure 3. Interferon (IFN)-γ and interleukin (IL)-4 enzyme-linked immunosorbent spot (ELISPOT) assay with splenocytes of mice immunized with gp120 with or without Ii suppression. All groups except (a) (naive mice) were immunized using the gene-gun with 2 μg of RSV.5/gp120 plasmid and each of the following DNA plasmids, respectively: (b) empty pBudCE4·1 (1·35 μg); (c) pNGVL1/GM-CSF (0·35 μg) + empty pBudCE4·1 (1·0 μg); (d) pNGVL1/GM-CSF (0·35 μg) + pBudCE4·1/Ii-RGC(× 3) (0·325 μg) and empty pBudCE4·1 (0·675 μg); (e) pNGVL1/GM-CSF (0·35 μg) + pBudCE4·1/Ii-RGC(× 3) (1·0 μg). Compared to (c), Ii suppression (d) resulted in an approximately five-fold enhancement of IFN-γ secretion. Addition of three times more Ii-RGC(− 92,97)(× 3) (e) did not result in more IFN-γ secretion; instead, IFN-γ secretion in (e) was slightly more reduced than in (d). Splenocytes (10^6/well) from individual mice (five mice/group) were cultured in either medium alone (open bar) or p18 peptide (dotted bar), or p18–I10 peptide (solid bar) in triplicate wells within a precoated IFN-γ ELISPOT plate. Each bar represents the mean ± SD for each of five mice. Immune response enhancement in groups (d) and (e) was statistically significant (compared to group c) ($P < 0·05$). Similar results were obtained in three repeated experiments.

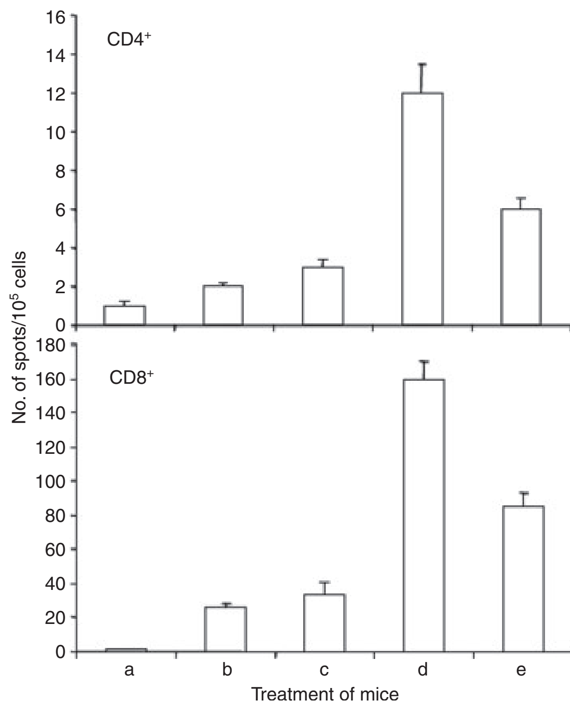

Figure 4. Interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assays of immunopurified $CD4^+$ and $CD8^+$ T cells. Splenocytes were pooled together (five mice/group) and incubated (5×10^6/ml) with p18 peptide (25 μg/ml) for 5 days. Then, $CD4^+$ and $CD8^+$ T cells were magnetically isolated using MiniMACS separation units according to the manufacturer's instructions. The purified $CD4^+$ or $CD8^+$ T cells (10^5 cells/well) were cultured along with p18 peptide in triplicate wells within an IFN-γ precoated ELISPOT plate for 48 hr. Each bar illustrates the mean ± SD for triplicate assays. Immune response enhancement in groups (d) and (e) was statistically significant (compared to group c) ($P < 0{\cdot}05$). Group design was the same as for Figure 3. Similar results were obtained in three repeated experiments.

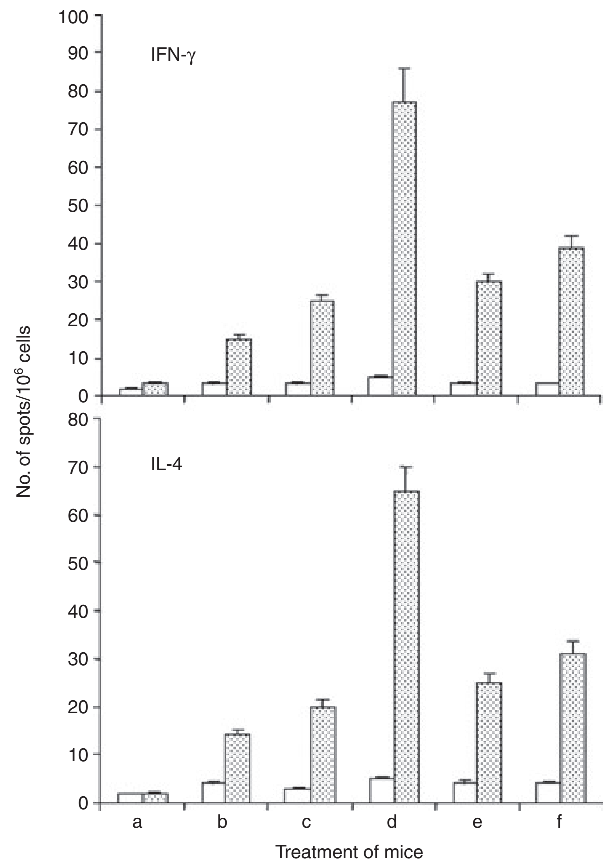

Figure 5. Interferon (IFN)-γ and interleukin (IL)-4 enzyme-linked immunosorbent spot (ELISPOT) assay with vaccine formula in the absence or presence of CIITA. All groups except (a) (naive mice) were immunized using the gene-gun with 2 μg of RSV.5/gp120 plasmid and, respectively, each of the following DNA plasmids: (b) empty plasmid (1 μg); (c) pNGVL1/GM-CSF (0·35 μg) + empty pBudCE4·1 (0·65 μg); (d) pNGVL1/GM-CSF (0·35 μg) + pBudCE4·1/Ii-RGC(× 3) (0·65 μg); (e) pNGVL1/GM-CSF (0·35 μg) + pCEP4/CIITA (50 ng) + empty pBudCE4·1 (0·65 μg); and (f) pNGVL1/GM-CSF (0·35 μg) + pCEP4/CIITA (50 ng) + pBudCE4·1/Ii-RGC(× 3) (0·65 μg). IFN-γ ELISPOT assays were performed with 10^6 cells/well in medium alone (white bar) or stimulated with p18 (dotted bar). Each bar illustrates the mean ± SD for individual mice (five mice/group) tested in groups (c) and (d). Similar results were obtained in three repeated experiments.

Addition of CIITA abolishes the enhancing activity of Ii suppression

Because keratinocytes might play a role in augmenting the magnitude of the immune response to DNA vaccines and are not normally MHC class II-positive,[44–46] the gene encoding MHC class II transactivator (CIITA)[31,47] was added to the DNA used to immunize mice. CIITA is a universal inducer of MHC class II and Ii in a variety of cells[25,47,48] and was used in these experiments to induce MHC class II expression in keratinocytes. Coupling this with Ii suppression increases the frequency of the MHC class II^+/Ii^- phenotype in keratinocytes, which might lead in turn to augmentation of potency of the gp120 DNA vaccine. From Fig. 5, one sees that addition of CIITA (group e) did not enhance the vaccine efficiency (compared to group c); instead, addition of CIITA abolished the vaccine efficiency enhanced by Ii suppression (compare group f with group c). Addition of CIITA also abolished IL-4 enhancement by Ii suppression (Fig. 5). Possible mechanisms for this effect are considered in the Discussion.

Ii suppression enhancement of gp120 DNA vaccine was more profound at the lower concentration of pBudCE4·1/Ii-RGC(× 3) group (Fig. 3, group d). In order to confirm this phenomenon, the medium concentration of pBudCE4·1/Ii-RGC(× 3) plasmid was used (Fig. 5). The three different concentrations of pBudCE4·1/Ii-RGC(× 3) used in experiments of Figs 3 and 5 were compared in Fig. 6 and one can see that the correlation between the doses of pBudCE4·1/Ii-RGC(× 3) and IFN-γ production

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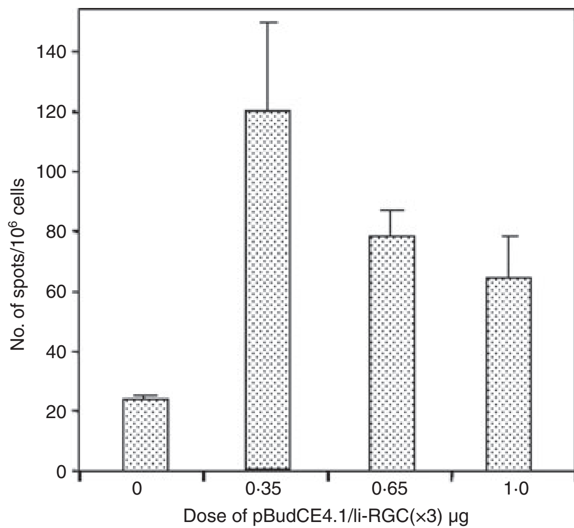

Figure 6. The correlation between concentration of pBudCE4·1/Ii-RGC(× 3) and interferon (IFN)-γ production to P18 stimulation. The IFN-γ production by p18 stimulation data of groups (c), (d) and (e) (dotted bars) in Figure 3 were pooled together with the IFN-γ production data of groups (c) and (d) in Figure 5. The data from the two groups (c) (no Ii-RGC(× 3) plasmid) were identical (25 spots in Figs 3 and 5), indicating good reproducibility of results. One can see more clearly from this figure that the IFN-γ produced by p18 stimulation is well correlated with the dose of pBudCE4·1/Ii-RGC(× 3) used.

indeed exists. The higher the dose of pBudCE4·1/Ii-RGC(× 3) used, the less IFN-γ was produced. This may reflect less promoter competition among gp120-, GM-CSF- and pBudCE4·1/Ii-RGC(× 3).

Discussion

The concept that induction of a MHC class II^+/Ii^- phenotype APC results in the presentation of endogenously derived antigenic epitopes by MHC class II molecules was first developed by Dr Ostrand Rosenberg and colleagues.[40,41,49] They showed that immunization of mice with tumour cells transfected with syngeneic MHC class II molecules (without Ii) led to a potent tumour cell vaccine and that reintroduction of the Ii protein into such tumour cells abolished the induced vaccine potency.[49] Further studies have demonstrated that MHC class II^+/Ii^- tumour cells present cytosolic or ER-retained hem egg lysozyme (HEL) through MHC class II molecules to activate $CD4^+$ T cells and that Ii limits MHC class II presentation.[40] In a recent $CD4^+$ and $CD8^+$ T cell depletion experiment it has further been shown that MHC class II^+/Ii^- tumour cells present endogenously expressed tetanus toxoid to $CD4^+$ T cells but not to $CD8^+$ T cells[42]. Zhao *et al.* have also shown that Ii inhibited DCs present endogenously expressed MHC class II epitopes to $CD4^+$ T cells.[24] Because transfection of tumour cells with a syngeneic MHC class II gene is not clinically feasible, given the great polymorphism of MHC class II genes in humans, we have developed a more practical method based on inhibition of the monomorphic Ii gene. Concomitant administration of Ii-RGC and the gene for CIITA, which is a universal inducer of both MHC class II molecules and the Ii protein,[26,27,47,48] ensures generation of the MHC class II^+/Ii^- phenotype. This method leads to potent tumour cell vaccines when MHC class I is also positive in tumour cells.[23,25–27] In the current study, we applied this strategy to develop a more potent DNA vaccine; i.e. using a construct to suppress Ii in DNA-transfected cells resulting in the presentation of endogenously synthesized vaccine antigens (gp120 epitopes) by 'unprotected' MHC class II, thereby activating $CD4^+$ T helper cells (p18-specific $CD4^+$ T cells). Our results are consistent with previous reports and support the working hypothesis that Ii suppression directly enhances the activation of $CD4^+$ T cells.[27,40,42] Activation of antigen-specific $CD4^+$ T cells is essential for the activation of antigen-specific $CD8^+$ T cells. In this manner, enhanced activation of gp120-specific $CD4^+$ T cells, and in turn enhanced activity of $CD8^+$ cells, is obtained.

Several studies have shown that endogenously expressed antigens can be processed and presented by MHC class II molecules.[50–52] Lepage *et al.*[52] have demonstrated that endogenous expressed membrane gp100 can be presented by both MHC class I and class II molecules in the absence of Ii. Deletion of the signal and transmembrane sequences decreased the class II presentation without affecting class I presentation. This result indicates that membrane antigens can be presented by MHC class II, as is the membrane protein gp120. Secondly, studies by us and others have indicated that MHC class II^+/Ii^- phenotype tumour cells are potent tumour cell vaccines,[23,25,27,40,41] while MHC class II^-/Ii^- and MHC class II^+/Ii^+ tumour cells are not, indicating that Ii limits the presentation of endogenous tumour antigens. Armstrong *et al.* have shown that Ii inhibits the ER-retained HEL epitope to be presented by MHC class II to $CD4^+$ T cells.[40] Zhao *et al.* have shown that Ii inhibition enhances DC to present endogenously expressed OVA epitopes through MHC class II and leads to enhanced activation of $CD4^+$ T cells, and subsequently to an enhanced $CD8^+$ CTL activity.[24] However, Thompson *et al.* found that Her2/neu MHC class II epitopes are not limited by Ii.[48] These discrepancies can be well explained by the direct chemical evidence of Muntasell *et al.*[53] Their mass spectroscopy study has revealed that MHC class II molecules of II^+ Ii^- cells contain epitopes presented by MHC class II^+ Ii^+ cells plus additional novel peptides which are not presented by MHC class II^+ Ii^+ cells. This direct evidence indicates that some endogenous epitopes are limited by Ii and some are not. Our experimental results indicate that p18 is limited by Ii.

Ii suppression enhancement of gp120 DNA vaccine seems to correlate with the dose of pBudCE4·1/Ii-RGC(× 3) plasmid used (Fig. 3). In order to define whether the dose of pBudCE4·1/Ii-RGC(× 3) is correlated with the enhancement of gp120 DNA vaccine efficiency, we used a middle dose of pBudCE4·1/Ii-RGC(× 3) in a further experiment (Fig. 5). Combining the data of Fig. 3 (groups c, d and e) with the data from Fig. 5 (groups c and d), we have drawn Fig. 6. One sees that the dose of pBudCE4·1/Ii-RGC(× 3) is indeed correlated with enhancement of gp120 DNA vaccine efficiency. The mechanisms underlying this phenomenon are unclear. Promoter competition could be a potential explanation.[54] The construct pBudCE4·1/Ii-RGC(× 3) contains an RSV promoter that shares with the RSV/gp120 plasmid.

Gene gun-mediated DNA immunization results in the transfection of keratinocytes and local DC.[5,38] Porgador *et al.*[55] demonstrated that after gene gun delivery of DNA, 20 000–30 000 DCs were recruited per draining lymph node and that 20–75 DCs were directly transfected with the administered DNA. Furthermore, they showed that the transfected DCs are the predominant APCs for CTL activation. Akbari *et al.* showed that DNA vaccination led to a relatively low frequency of DC transfection.[56] However, it was these transfected DCs that led to the general activation of all DCs, providing good conditions for effective Th cell activation. Our hypothesis is that gene gun-mediated vaccination of mice with pBudCE4·1/Ii-RGC(× 3) plus pcDNA(3)/gp120 led to the generation of gp120$^+$/MHC class II$^+$/Ii$^-$ DCs, that are more effective in activating gp120-specific CD4$^+$ Th cells through the presentation of endogenously synthesized gp120 epitopes by 'unprotected' MHC class II molecules.

Other studies have indicated that local cells (e.g. keratinocytes) also play a key role in the induction of the humoral and CTL activities of a DNA vaccine. Ablation of the injected skin or local inhibition of gene expression abolished the efficacy of a DNA vaccine.[44–46] We added the CIITA gene to the Ii-RGC/gp120 DNA vaccine in the hope of increasing the frequency of MHC class II$^+$/Ii$^-$ keratinocytes, further enhancing the DNA vaccine potency. However, we found that the addition of the CIITA gene did not improve efficacy of the vaccine (compare group c to group e in Fig. 5) and, instead, decreased the vaccine potency enhancement imparted by Ii suppression (compare group d to group f in Fig. 5). Our previous experiments have shown that three times more Ii-RGC plasmid is needed to suppress Ii induced by CIIT-A (unpublished observation). We conclude that the MHC class II$^+$/Ii$^-$ phenotype of keratinocytes in our *in vivo* experiment is induced by our vaccine as the concentration of CIITA plasmid was 13 times lower than the Ii-RGC plasmid (see Fig. 5 legend). Mechanisms to explain this phenomenon relate to the findings of Landmann *et al.*[57] They demonstrated that in the process of DC maturation, there is enhanced cell surface MHC class II expression followed by the *de novo* biosynthesis of MHC class II mRNA being turned off. This is due to a rapid reduction in the synthesis of CIITA, triggered by a variety of different maturation stimuli, including lipopolysaccharide (LPS), tumour necrosis factor (TNF)-α, CD40 ligand, IFN-α and infection with *Salmonella typhimurium* or Sendai virus.[57] The addition of CIITA could potentially block the *de novo* process that turns off MHC class II mRNA, disturbing the normal maturation of DCs. Our data indicate that keratinocytes are not major APCs for a DNA vaccine under our condition, because increased antigen presentation by MHC class II$^+$/Ii$^-$ keratinocytes did not enhance the efficiency of gp120 DNA vaccine.

The major advantage of using Ii suppression to augment MHC class II presentation of endogenously expressed DNA antigens is that it induces strong antigen-specific CD4$^+$ T cell activation, while at the same time the induction of CD8$^+$ T cells continues uninterrupted. Vaccine antigens are also released and phagocytosized by DCs or other APCs, through the exogenous antigen processing and presentation pathway to activate Th cells.[58] However, the availability of soluble antigen to MHC class II molecules is limited by the low levels of released antigen; while these antigens need to be taken up by APCs and DCs throughout the body, losing the advantage of colocalized stimulation of CD4$^+$ and CD8$^+$ cells. Our Ii inhibition strategy leads to a simultaneous transfection of DCs with DNA containing HIV gp120 and Ii-RGC, resulting in the expression of gp120 and 'unprotected' MHC class II molecules in a single DC. Following endogenous synthesis of gp120, processing and presentation of gp120 epitopes through MHC class I and class II molecules occurs simultaneously. This results in a stronger localized CD4$^+$ and CD8$^+$ T cell collaboration to increase efficacy of the DNA vaccine.

Our results support the feasibility of a novel strategy to augment the efficacy of DNA vaccines in a clinical setting. We have generated active human Ii-RNAi constructs for just such a purpose. Ii suppression technology does not conflict with other vaccine enhancement technologies and can be used in combination with other vaccine methods, including cytokines and adjuvants to further enhance DNA vaccines.

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