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Enhancement of DNA Vaccine Potency through Coadministration of CIITA DNA with DNA Vaccines via Gene Gun

Daejin Kim,† Talia Hoory,* Archana Monie,* Jenny Pan-Yun Ting,† Chien-Fu Hung,*§ and T.-C. Wu*†‡§

Administration of DNA vaccines via gene gun has emerged as an important form of Ag-specific immunotherapy. The MHC CIITA is a master regulator of MHC class II expression and also induces expression of class I molecules. We reasoned that the gene gun administration of CIITA DNA with DNA vaccines employing different strategies to improve MHC I and II processing could enhance DNA vaccine potency. We observed that DC-1 cells transfected with CIITA DNA lead to higher expression of MHC I and II molecules, leading to enhanced Ag presentation through the MHC I/II pathways. Furthermore, our data suggested that coadministration of DNA-encoding calreticulin (CRT) linked to human papillomavirus (HPV) 16 E6 Ag (CRT/E6) with CIITA DNA leads to enhanced E6-specific CD8+ T cell immune responses in vaccinated mice. In addition, coadministration of the combination of CRT/E6 DNA with CIITA DNA and DNA encoding the invariant chain (Ii) linked to the pan HLA-DR-reactive epitope (Ii-PADRE) further enhanced E6-specific CD8+ T cell immune responses in vaccinated mice. Treatment with the combination vaccine was also shown to enhance the antitumor effects and to prolong survival in TC-1 tumor-bearing mice. Vaccination with the combination vaccine also led to enhanced E6-specific CD8+ memory T cells and to long-term protection against TC-1 tumors and prolonged survival in vaccinated mice. Thus, our findings suggest that the combination of CIITA DNA with CRT/E6 and Ii-PADRE DNA vaccines represents a potentially effective means to combat tumors in the clinical setting. The Journal of Immunology, 2008, 180: 7019–7027.

Deoxyribonucleic acid vaccines have emerged as an interesting approach for Ag-specific immunotherapy because they are safe, stable, and easy to produce. Gene gun administration of DNA vaccines represents an effective means of directly delivering antigenic DNA into dendritic cells (DCs), the most potent of the professional APCs. The Ag-expressing DCs mature and migrate to the draining lymph nodes, where they activate naive T lymphocytes in vivo to differentiate into activated, Ag-specific T cells (1, 2). Gene gun administration enables us to test the strategies that require direct delivery of the DNA vaccines into DCs to improve the potency of these DNA vaccines. We have previously used the gene gun delivery system for the development of several innovative strategies to enhance DNA vaccine potency (for review, see Refs. 3 and 4).

One of these strategies involves intracellular targeting of the encoded Ag to subcellular compartments to enhance MHC class I and class II Ag presentation in DCs. For example, DNA vaccines encoding Ag linked to calreticulin (CRT) are able to target the linked Ag to the endoplasmic reticulum, resulting in enhanced MHC class I presentation of the linked Ag (5). CRT is an abundant Ca2+-binding protein that resides in the endoplasmic reticulum (for review, see Ref. 6) and has been shown to aid in Ag presentation by associating with peptides delivered to the endoplasmic reticulum by TAP molecules (7) and with MHC class I molecules (8). We have demonstrated that DNA vaccines encoding CRT linked to model Ag HPV-16 E6 and E7 generated increased HPV Ag-specific CD8+ T cell responses and antitumor effects (5, 9). Therefore, DNA vaccines encoding CRT linked to a tumor-specific Ag present the opportunity to enhance vaccine potency via enhancing MHC class I processing and presentation.

Another strategy to enhance DNA vaccine potency involves the induction of CD4+ T cell help. The activation of CD8+ T cells can be significantly enhanced by CD4+ Th cells (for review, see Ref. 10). Thus, strategies to induce CD4+ Th cells at sites of CD8+ T cell priming can potentially enhance CTL immune responses. In previous studies, it has been shown that DNA vaccines encoding the invariant (Ii) chain in which the CLIP region is replaced with a high-affinity and “promiscuous” CD4+ T cell epitope such as the pan HLA-DR-reactive epitope (PADRE) (11) leads to the stable, accelerated presentation of PADRE through MHC class II molecules. More recently, we have shown that immunization with DNA vaccines encoding Ii-PADRE DNA leads to the generation of high numbers of PADRE-specific CD4+ T cell immune responses in mice (12). Furthermore, coadministration of E7 DNA vaccines with Ii-PADRE DNA has led to enhanced E7-specific CD8+ T cell
immune responses and antitumor effects in vaccinated mice (12, 13). Therefore, DNA vaccination encoding Ag of interest with II-PADRE DNA serves as a potentially useful means to improve DNA vaccine potency through the induction of CD4+ T cells.

We reasoned that a strategy that is capable of enhancing the MHC class I and II expression on DCs may further enhance DNA vaccine potency. The MHC CIITA is known as a master control factor for the genes required for MHC class II Ag presentation (14, 15). It has also been shown that CIITA induces the surface expression of MHC class I molecules (16, 17). Thus, we reasoned that coadministration of DNA vaccines with CIITA DNA would increase the levels of MHC class I/Ii molecules and lead to enhanced presentation of the Ag via the MHC class I and II processing pathways, resulting in enhanced DNA vaccine potency. Therefore, the employment of CIITA DNA in DNA vaccines represents a potential strategy to improve vaccine potency through enhancing Ag presentation via the MHC class I and II processing pathways.

In the current study, we used a combination of DNA vaccines encoding CRT/E6, II-PADRE DNA, and CIITA DNA to further improve DNA vaccine potency. We showed that DC-1 cells transfected with CIITA DNA exhibited increased MHC class I/Ii expression, resulting in enhanced Ag presentation through the MHC class I/Ii pathways. Furthermore, we found that coadministration of DNA vaccines with CIITA DNA and II-PADRE further enhanced E6-specific CD8+ T cell immune responses and improved the antitumor effects against E7-expressing tumors. Thus, the combination of CIITA DNA with CRT/E6 and II-PADRE DNA vaccines represents a potentially effective means to enhance the potency of DNA vaccines. The clinical implications of the study are discussed.

Materials and Methods

Abs, peptides, cell lines, and mice
The HPV-16 E6 (YDFAFRDL) and PADRE (AKFVAAWTLKAAA) peptides were synthesized by Macromolecular Resources at a purity of ≥70%. Abs against mouse CD4 (PE-conjugated, clone L3T4), IFN-γ (FITC-conjugated, clone XMG1.2), CD8a (PE-conjugated, clone Ly-1), I-Ab (PE-conjugated, clone AF6-120.1), H-2Kb (PE-conjugated, clone KH95), and H-2Db (PE-conjugated, clone AF6-88.5) were purchased from BD Pharmingen.

The immortalized DC line was provided by Dr. K. Rock (University of Massachusetts, Worcester, MA) (18). With continued passage, we have generated subclones of DCs (DC-1) that are easily transfected using Lipofectamine 2000 (Invitrogen) (19). The production and maintenance of TC-1 have been described previously (20).

Six- to 8-wk-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and housed in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Table I. Vaccinations of mice with various DNA combinations of pcDNA3-CIITA

<table>
<thead>
<tr>
<th>Mouse Group</th>
<th>Names of the DNA Constructs Used in the Mixtures</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pcDNA3-CRT/E6 pcDNA3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>pcDNA3-CRT/E6 pcDNA3-CIITA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>pcDNA3-CRT/E6 pcDNA3-Ii-PADRE</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>pcDNA3-CRT/E6 pcDNA3-mtCIITA</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>pcDNA3-CRT/E6 pcDNA3-II-PADRE</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>pcDNA3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>pcDNA3-Ii-PADRE</td>
<td></td>
</tr>
</tbody>
</table>

Amount of DNA in one bullet (μg)

<table>
<thead>
<tr>
<th></th>
<th>Mouse Group</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Total DNA ~1.0 μg/bullet

*C57BL/6 mice (five per group) were administered two bullets of the DNA mixtures twice with a 1-wk interval.

**a** pcDNA3 vector backbone for all plasmids.

Plasmid DNA constructs and DNA preparation

The generation of pcDNA3-Ii and pcDNA3-II-PADRE has been described previously (12). pcDNA3-CRT/E6 (9) was generated as described previously. The generation of the pcDNA3/F-CIITA and pcDNA3/F-CIITA59-94 (pcDNA3-mtCIITA) expression vectors have been previously described (21). The DNA were amplified and purified as described previously (22).

**Generation of PADRE-specific CD4+ T cell line and E6-specific CD8+ T cell line**

Six-week-old female C57BL/6 mice were immunized with pcDNA3-Ii-PADRE by gene gun. After prime and booster vaccination at 1-wk intervals, splenocytes were harvested 1 wk after the last vaccination. For initial in vitro stimulation, 5 × 10^6 splenocytes were pulsed with IL-2 (10 U/ml) and PADRE peptide (1 μg/ml) in RPMI 1640 medium containing 10% FBS for 6 days. Propagation of the PADRE-specific CD4+ T cell line was performed in 24-well plates by mixing 1 × 10^6 splenocytes containing...
PADRE-specific CD4<sup>+</sup>T cells with 1 × 10<sup>6</sup> irradiated DCs that were pulsed with PADRE peptide and then cultured in RPMI 1640 medium containing IL-2 (10 U/ml) for 6 days. This procedure was repeated weekly. Flow cytometry was performed to demonstrate the expression of the CD4 marker. The preparation of E6-specific CD8<sup>+</sup>T cell line was performed similarly.

Transfection and detection of MHC class molecules

DC-1 cells (1.0 × 10<sup>6</sup>) were transiently transfected with pcDNA3, pcDNA3-mtCIITA, or pcDNA3-CIITA plasmid constructs using Lipofectamine 2000 according to the vendor’s manual. Cells were grown at 37°C and 5% CO<sub>2</sub>. At 24 h after transfection, the effects of CIITA on the expression of MHC class I and II molecules were characterized by flow cytometry. Naive DC-1 cells or DC-1 cells cotransfected with the same plasmid constructs (pcDNA3, pcDNA3-mtCIITA, or pcDNA3-CIITA) and pcDNA-I<sub>i</sub> or pcDNA-I<sub>i</sub>-PADRE (5 × 10<sup>4</sup>/well) were mixed with PADRE-specific CD4<sup>+</sup>T cells (5 × 10<sup>5</sup>/well) in 24-well plates.

Naive DC-1 cells or DC-1 cells transiently cotransfected with the same plasmid constructs pcDNA3, pcDNA3-mtCIITA, or pcDNA3-CIITA and pcDNA3 or pcDNA3-CRT/E6 (5 × 10<sup>4</sup>/well) were mixed with E6-specific CD8<sup>+</sup>T cells (5 × 10<sup>5</sup>/well) in 24-well plates. Cells were cultured at 37°C and 5% CO<sub>2</sub> for 24 h. After mixed culture, the cells were subjected to intracellular cytokine staining with fluorescein-conjugated anti-mouse IFN-γ.

DNA vaccination using a gene gun

DNA-coated gold particles were prepared according to a previously described protocol (22). DNA-coated gold particles were delivered to the shaved abdominal region of mice using a helium-driven gene gun (Bio-Rad) with a discharge pressure of 400 lb/in<sup>2</sup>. C57BL/6 mice were immunized with various combinations of the DNA constructs illustrated in Table I. Each cartridge contained 1 μg of plasmid DNA mixture and mice received two shots of the DNA mixtures per mouse by gene gun bombardment for a total of 2 μg/mouse. Each mouse received a booster of the same regimen 1 wk later.

Intracellular cytokine staining and flow cytometry analysis

Splenocytes were harvested from mice (five per group) 1 wk or 60 days after the last vaccination. Before intracellular cytokine staining, 5 × 10<sup>7</sup>
mouse of pooled splenocytes from each vaccination group were incubated for 16 h with 1 μl/ml E6 peptide (YDFAFRDL) containing a MHC class I (H-2Kb or Db) epitope (aa 50–57) for detecting Ag-specific CD8+ T cell precursors or MHC class II (I-Aβ) PADRE peptide (AKFVAAWTLKAAA) for detecting Ag-specific CD4+ T cell precursors in the presence of GolgiPlug (BD Pharmingen). Intracellular IFN-γ staining and flow cytometry analysis were performed as described previously (22). Analysis was performed on a BD Biosciences FACScan with CellQuest software (BD Immunocytometry Systems).

In vivo tumor treatment experiment

For the tumor treatment experiment, C57BL/6 mice (five per group) were challenged with 5 × 104 of TC-1 tumor cells/mouse by s.c. injection in the right leg. At 3 days after challenge with TC-1 tumor cells, mice were administered via gene gun 2 μg of each designated plasmid DNA mixture/mouse three times at 4-day intervals. Tumor growth was monitored by visual inspection and palpation twice weekly as described previously (20).

Long-term in vivo tumor protection experiment

For long-term tumor protection experiments, mice (five per group) were vaccinated via gene gun with 2 μg/mouse twice with a 1-wk interval of the DNA combinations listed in Table I. Splenocytes from vaccinated mice were harvested 1 wk after the last vaccination and characterized for E6-specific CD8+ T cells using intracellular IFN-γ staining followed by flow cytometry analysis. A, Representative flow cytometry data. The numbers in the upper right-hand corner represent the number of E6-specific IFN-γ-secreting CD8+ T cells per 5 × 106 pooled splenocytes. B, Bar graphs depicting the numbers of E6-specific IFN-γ-secreting CD8+ T cells per 5 × 106 pooled splenocytes (means ± SD). The data presented in this figure are from one representative experiment of two performed.

Tumor measurement and conditional survival

Three-dimensional tumor sizes were measured two or three times per week with Vernier calipers. Tumor sizes were approximated by multiplying the measured lengths. From day 25 after challenging tumor cells, tumors were measured every other day, and mice with tumor sizes >19 mm in diameter or projected tumor volumes >10% body weight or >2700 mm3 were considered moribund and were sacrificed. Tumor volume was calculated using the following formula:

\[ V = \frac{4}{3} \pi r^3 \]

where V is the volume and r is the radius of the tumor cross-section.

FIGURE 4. Characterization of the E6-specific CD8+ T cells in mice vaccinated with CRT/E6, CIITA DNA, and Ii-PADRE DNA vaccines. C57BL/6 mice (five per group) were immunized with 2 μg/mouse twice with a 1-wk interval of the DNA combinations listed in Table I. Splenocytes from vaccinated mice were harvested 1 wk after the last vaccination and characterized for E6-specific CD8+ T cells using intracellular IFN-γ staining followed by flow cytometry analysis. A, Representative flow cytometry data. The numbers in the upper right-hand corner represent the number of E6-specific IFN-γ-secreting CD8+ T cells per 5 × 106 pooled splenocytes. B, Bar graphs depicting the numbers of E6-specific IFN-γ-secreting CD8+ T cells per 5 × 106 pooled splenocytes (means ± SD). The data presented in this figure are from one representative experiment of two performed.
the following formula: $V = (L \times W \times D)$, where $V$ is tumor volume, $L$ is length, $W$ is width, and $D$ is depth. All of the animal studies were approved by the Institutional Animal Care and Use Committee at Johns Hopkins Hospital.

**Statistical analysis**

All data expressed as mean $\pm$ SD are representative of at least two different experiments. Comparisons between individual data points were made using Student’s $t$ test. Kaplan-Meier survival curves for tumor treatment and protection experiments were applied; for differences between curves, $p$ values were calculated using the log-rank test. The value of $p < 0.05$ was considered significant.

**Results**

**DC-1 cells transfected with CIITA DNA lead to higher expression of MHC class I and II molecules**

We have previously developed a DC line, DC-1, that can be transfected with DNA with high efficiency (19). To characterize the expression of MHC class I and II molecules in DC-1 cells transfected with CIITA DNA, we performed flow cytometry analysis using Abs specific for MHC class I H-2 K$^b$, D$^a$, or MHC class II I-A$^b$. DC-1 cells transfected with mutant CIITA, vector backbone DNA, or untransfected were used as controls. The expression of MHC class I and II molecules on transfected
DC-1 cells was characterized by flow cytometry 24 h after transfection. As shown in Fig. 1, DC-1 cells transfected with CIITA DNA expressed higher levels of MHC class I and II molecules compared with DC-1 cells transfected with the control constructs or nontransfected cells. Thus, our data indicate that transfection of DC-1 cells with CIITA DNA leads to increased expression of MHC class I and II molecules.

**DCs transfected with CIITA DNA lead to enhanced Ag presentation through the MHC class I and II pathways**

We then characterized the Ag presentation through MHC class I pathways in DCs cotransfected with CRT/E6 DNA and CIITA DNA. The transfected DCs were then incubated with E6-specific CD8$^+$ T cells overnight. The degree of MHC class I presentation of E6 would correlate with the number of IFN-γ-secreting activated E6-specific T cells. The activation of E6-specific CD8$^+$ T cells was characterized by intracellular cytokine staining followed by flow cytometry analysis. As shown in Fig. 2A, DC-1 cells cotransfected with CRT/E6 DNA and CIITA DNA generated significantly higher numbers of activated E6-specific CD8$^+$ T cells compared with DC-1 cells transfected with CRT/E6 DNA and mutant CIITA. A graphic representation of the number of activated E6-specific CD8$^+$ T cells is depicted in Fig. 2B. Taken together, our data indicate that the increased MHC class I expression mediated by CIITA has led to enhanced MHC class I presentation of E6 in DC-1 cells transfected with CRT/E6 DNA.

We also characterized the Ag presentation through the MHC class II pathway in DCs cotransfected with Ii-PADRE DNA and CIITA DNA. The transfected DCs were incubated with PADRE-specific CD4$^+$ T cells overnight. The increase in MHC class II presentation of PADRE would lead to an increased number of IFN-γ-secreting activated PADRE-specific T cells. We characterized the activation of PADRE-specific CD4$^+$ T cells using intracellular cytokine staining followed by flow cytometry analysis. As shown in Fig. 2C, DC-1 cells cotransfected with CIITA DNA and Ii-PADRE DNA generated significantly higher numbers of activated PADRE-specific CD4$^+$ T cells compared with DC-1 cells transfected with Ii-PADRE DNA and CIITA DNA. A graphic representation of the number of PADRE-specific CD4$^+$ T cells is depicted in Fig. 2D. Thus, our data similarly suggest that the increased MHC class II expression mediated by CIITA led to enhanced MHC class II presentation of PADRE in DC-1 cells transfected with Ii-PADRE DNA.

**Coadministration of CRT/E6 DNA with CIITA DNA leads to enhanced E6-specific CD8$^+$ T cell immune responses in vaccinated mice**

We further characterized the Ag-specific CD8$^+$ T cell immune responses in C57BL/6 mice vaccinated with CRT/E6 DNA in conjunction with CIITA DNA or pcDNA3. Mice vaccinated with pcDNA3 and CIITA DNA were used as a negative control. One week after the last vaccination, we measured the E6-specific CD8$^+$ T cell immune responses in vaccinated mice using intracellular IFN-γ staining followed by flow cytometry analysis. As shown in Fig. 3, mice vaccinated with both CRT/E6 DNA and CIITA DNA generated the highest numbers of E6-specific CD8$^+$ T cells among all of the vaccinated groups. We also characterized the Ag-specific CD4$^+$ T cell immune responses in mice vaccinated with Ii-PADRE DNA in conjunction with CIITA DNA or pcDNA3. Mice vaccinated with the Ii-chain DNA and CIITA DNA were used as a negative control. We observed that mice vaccinated with Ii-PADRE DNA and CIITA DNA generated the highest numbers of PADRE-specific CD4$^+$ T cells among all of the vaccinated groups (data not shown). Thus, our data indicate that the increased MHC class I and II presentation by CIITA is capable of enhancing both
the Ag-specific CD8+ and CD4+ T cell immune responses in vaccinated mice.

**Coadministration of CRT/E6 DNA with CIITA DNA and Ii-PADRE further enhances E6-specific CD8+ T cell immune responses in vaccinated mice**

We recently demonstrated that DNA vaccines encoding HPV-16 E6 and/or E7 Ags coadministered with Ii-PADRE could improve the HPV Ag-specific CD8+ T cell immune responses in vaccinated mice (12). Because the strategy to enhance CD4+ T cell help represents a different strategy to enhance DNA vaccine potency, we explored whether this strategy (Ii-PADRE) can be combined with the strategies to enhance MHC class I/II presentation (CIITA) to further enhance the Ag-specific T cell immune responses in vaccinated mice. Thus, we vaccinated C57BL/6 mice with the combinations of DNA constructs illustrated in Table I. One week after the last vaccination, we measured the E6-specific CD8+ T cell immune responses in vaccinated mice using intracellular IFN-γ staining followed by flow cytometry analysis. As shown in Fig. 4, mice vaccinated with CRT/E6, Ii-PADRE, and CIITA DNA generated significantly higher numbers of E6-specific CD8+ T cells compared with mice vaccinated with CRT/E6, Ii-PADRE, and pcDNA3 DNA (p = 0.00343) or mutant CIITA DNA (p = 0.00201). We also characterized the PADRE-specific CD4+ T cell immune responses in vaccinated mice. We found that mice vaccinated with CRT/E6, Ii-PADRE, and CIITA DNA generated a significantly higher number of PADRE-specific CD4+ T cell immune responses in vaccinated mice. Thus, we vaccinated C57BL/6 mice with the combinations of DNA constructs illustrated in Table I. One week after the last vaccination, we measured the E6-specific CD8+ T cell immune responses in vaccinated mice using intracellular IFN-γ staining followed by flow cytometry analysis. As shown in Fig. 4, mice vaccinated with CRT/E6, Ii-PADRE, and CIITA DNA generated significantly higher numbers of E6-specific CD8+ T cells compared with mice vaccinated with CRT/E6, Ii-PADRE, and pcDNA3 DNA (p = 0.00343) or mutant CIITA DNA (p = 0.00201). We also characterized the PADRE-specific CD4+ T cell immune responses in vaccinated mice. We found that mice vaccinated with CRT/E6, Ii-PADRE, and CIITA DNA generated a significantly higher number of PADRE-specific CD4+ T cell immune responses in vaccinated mice. Thus, we vaccinated C57BL/6 mice with the combinations of DNA constructs illustrated in Table I. One week after the last vaccination, we measured the E6-specific CD8+ T cell immune responses in vaccinated mice using intracellular IFN-γ staining followed by flow cytometry analysis. As shown in Fig. 4, mice vaccinated with CRT/E6, Ii-PADRE, and CIITA DNA generated significantly higher numbers of E6-specific CD8+ T cells compared with mice vaccinated with CRT/E6, Ii-PADRE, and pcDNA3 DNA (p = 0.00343) or mutant CIITA DNA (p = 0.00201). We also characterized the PADRE-specific CD4+ T cell immune responses in vaccinated mice. We found that mice vaccinated with CRT/E6, Ii-PADRE, and CIITA DNA generated a significantly higher number of PADRE-specific CD4+ T cell immune responses in vaccinated mice.

**FIGURE 7.** Long-term in vivo tumor protection experiments. C57BL/6 mice (five per group) were immunized with 2 μg of DNA/mouse twice with a 1-wk interval of the various DNA vaccine mixtures listed in Table I. Two months after the last vaccination, the mice were challenged by s.c. injection of 1 × 10⁵ of TC-1 cells/mouse. The mice were monitored for evidence of tumor growth by inspection and palpation twice a week. Tumor volumes were measured starting from day 7 after tumor challenge. A, Line graph depicting tumor volume in mice challenged with TC-1 cells (means ± SD). B, Kaplan-Meier survival analysis in mice challenged with TC-1 cells. The data shown here are from one representative experiment of two performed.
cells compared with vaccination with CRT/E6, Ii-PADRE, and mutant (mt) CIITA DNA (data not shown). Thus, our results suggest that coadministration of CRT/E6 DNA with CIITA DNA and Ii-PADRE DNA further enhances E6-specific CD8+ T cell immune responses in vaccinated mice.

**Treatment with a combination of CIITA DNA, CRT/E6, and Ii-PADRE DNA leads to enhanced antitumor effects and prolonged survival in TC-1 tumor-bearing mice**

To determine whether the enhanced E6-specific T cell response generated by coadministration of the combination of CIITA, CRT/E6, and Ii-PADRE DNA translates into therapeutic antitumor effects, we performed in vivo tumor treatment experiments using a HPV-16 E6/E7-expressing tumor model, TC-1. C57BL/6 mice were first challenged s.c. with TC-1 tumor cells and then, 3 days later, treated with the combinations of DNA constructs illustrated in Table I. The treated mice were monitored for tumor growth. As shown in Fig. 5A, tumor-bearing mice treated with the combination of CRT/E6, CIITA DNA, and Ii-PADRE DNA exhibited significantly decreased tumor growth compared with the tumor-bearing mice treated with CRT/E6, Ii-PADRE DNA, and the mutant CIITA DNA (p = 0.00136). We also performed Kaplan-Meier survival analysis of the treated mice. As shown in Fig. 5B, tumor-challenged mice treated with the combination of CRT/E6, CIITA DNA, and Ii-PADRE DNA also exhibited significantly prolonged survival compared with the other treatment groups. Thus, our data indicate that treatment with a combination of CIITA DNA, CRT/E6, and Ii-PADRE DNA leads to enhanced antitumor effects and prolonged survival in TC-1 tumor-bearing mice.

**Coadministration of CRT/E6 DNA with CIITA DNA and Ii-PADRE leads to enhanced E6-specific CD8+ memory T cells in vaccinated mice**

We also characterized the long-term Ag-specific immune responses to vaccination with the combination of CIITA, CRT/E6, and Ii-PADRE DNA. C57BL/6 mice were vaccinated with the combinations of DNA constructs illustrated in Table I. Sixty days later, we measured the E6-specific CD8+ cell immune responses in vaccinated mice using intracellular IFN-γ staining followed by flow cytometry analysis. As shown in Fig. 6, vaccination with CRT/E6, Ii-PADRE, and CIITA DNA generated higher numbers of E6-specific CD8+ memory T cells compared with vaccination with CRT/E6 and Ii-PADRE with mutant CIITA DNA (p = 0.00756). We also determined the PADRE-specific CD4+ T cell long-term immune responses in mice vaccinated with CRT/E6, Ii-PADRE, and CIITA DNA compared with the other DNA construct combinations. Mice vaccinated with CRT/E6, Ii-PADRE, and CIITA DNA generated a significantly higher number of PADRE-specific CD4+ T memory cells compared with vaccination with CRT/E6, Ii-PADRE, and CIITA DNA and Ii-PADRE DNA compared with the other DNA constructs illustrated in Table I. Two months after the last vaccination, immunized mice were s.c. challenged with TC-1 tumor cells and then monitored for tumor growth. As shown in Fig. 7A, mice vaccinated with CIITA DNA, CRT/E6 DNA, and Ii-PADRE DNA demonstrated almost complete inhibition of tumor growth compared with mice vaccinated with CRT/E6 DNA, Ii-PADRE DNA, and the mutant CIITA DNA. We also performed Kaplan-Meier survival analysis. As shown in Fig. 7B, we observed significantly prolonged survival in these mice. These data suggest that the coadministration of the combination of CIITA DNA and Ii-PADRE DNA can further enhance the ability of CRT/E6 DNA to generate long-term protective antitumor effects against TC-1 tumors in vaccinated mice.

**Discussion**

In the current study, we used a combination of DNA vaccines encoding CRT/E6, Ii-PADRE DNA, and CIITA DNA to further improve DNA vaccine potency. We showed that DC-1 cells transfected with CIITA DNA exhibited enhanced MHC class I/II expression leading to improved Ag presentation through the MHC class I/II pathways. Coadministration of the combination of CRT/E6 DNA with CIITA DNA and Ii-PADRE further enhanced E6-specific CD8+ T cell immune responses and the antitumor effects in TC-1 tumor-bearing mice. Vaccination with the combination vaccine also led to enhanced E6-specific CD8+ memory T cell response, long-term protection against TC-1 tumors, and prolonged survival in vaccinated mice. Thus, the combination of CIITA DNA with CRT/E6 and Ii-PADRE DNA vaccines represents a promising approach to further enhance the potency of DNA vaccines.

The use of gene gun administration is important for the success of the current strategy. All of these strategies most likely require the direct delivery of DNA into the DCs to effectively influence the priming of the T cells. For example, intracellular targeting strategies using CRT require the linked Ag to be directly targeted to the endoplasmic reticulum in order to enhance the Ag processing. Furthermore, the Ii-PADRE DNA strategy requires the induction of CD4+ Th cells in the vicinity of Ag-specific CD8+ T cells to enhance T cell activation. Finally, the strategy using CIITA DNA is required to be delivered directly to the DCs so that it can increase expression of MHC class I/II molecules on its surface, leading to enhanced MHC class I/II Ag processing and presentation. Thus, all of the strategies used in the current study rely heavily on the intradermal delivery of Ag via gene gun.

In our study, we have successfully used DNA vaccines encoding the CIITA DNA to enhance DNA vaccine potency. Previously, the CIITA has been used in other vaccine systems, particularly tumor cell-based vaccines, to successfully improve vaccine potency (23–26). Furthermore, tumor cells transfected with CIITA and/or CD80 have been shown to activate tumor-specific CD4+ T cells (25, 26). Thus, the use CIITA has been shown to be promising in the enhancement of cancer vaccine potency.

It is important to identify the best vaccine for future clinical translation. DNA vaccines using different strategies to improve vaccine potency modify the properties of DCs through different, complementary mechanisms. In our study, we showed that the intradermal administration of the combination of CRT/E6, Ii-PADRE, and CIITA DNA vaccines generated the best E6-specific CD8+ T cell immune responses and antitumor effects against TC-1 tumors. Each of the DNA constructs employs strategies to modify the properties of DCs through different mechanisms. It is likely that the potency of this combination of DNA vaccines can be further enhanced by the addition of a DNA vaccine using a strategy that operates through another mechanism. For example, one potential strategy is to modify the properties of DCs using coadministration of DNA-encoding antiapoptotic proteins. We
have previously shown that coadministration of DNA vaccines with DNA-encoding antiapoptotic proteins such as Bcl-xL has led to the prolonged life of DCs, resulting in significant enhancement of Ag-specific CD8+ T cell immune responses (27). It would be of interest to see whether this antiapoptotic strategy can be combined with the aforementioned DNA vaccine strategies to further enhance DNA vaccine potency for eventual clinical translation.

In summary, we have identified an innovative strategy to increase the expression of MHC class I/II molecules on DCs to enhance DNA vaccine potency. This strategy can potentially be used in other antigenic systems for the control of infectious diseases and cancer.

Disclosures

The authors have no financial conflict of interest.

References