Gene Therapy (2006) 13, 67–77
© 2006 Nature Publishing Group All rights reserved 0969-7128/06 $30.00
www.nature.com/gt

ORIGINAL ARTICLE

Characterization of HLA-A2-restricted HPV-16 E7-specific CD8+ T-cell immune responses induced by DNA vaccines in HLA-A2 transgenic mice

S Peng[1], C Trimble[2], L He[1], Y-C Tsai[1], C-T Lin[1,3], DAK Boyd[1], D Pardoll[1,4], C-F Hung[1] and T-C Wu[1,2,4,5]

[1]*Department of Pathology, The Johns Hopkins University School of Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD, USA;* [2]*Department of Obstetrics and Gynecology, The Johns Hopkins University School of Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD, USA;* [3]*Department of Obs/Gyn, Chang Gung Memorial Hospital, Taipei, Taiwan;* [4]*Department of Oncology, The Johns Hopkins University School of Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD, USA and* [5]*Department of Molecular Microbiology and Immunology, The Johns Hopkins Medical Institutions, Baltimore, MD, USA*

We have recently demonstrated that linkage of DNA-encoding calreticulin to DNA-encoding human papillomavirus-16 E7 antigen strongly enhances the efficacy of DNA vaccines against E7-expressing tumors in animal models. In this study, as a prelude to clinical translation, we characterized the ability of DNA-encoding calreticulin linked to DNA-encoding E7 antigen to generate HLA-A2-restricted E7-specific CD8+ T-cell responses in HLA-A2 (AAD) transgenic mice, as well as antitumor effects against an E7+ HLA-A2+ tumor cell line, TC-1/A2. Our results show that while vaccination with CRT/E7 DNA generates strong H-2D^b-restricted E7 (amino acid (aa)49–57)-specific CD8+ T-cell immune responses in both C57BL/6 and HLA-A2 (AAD) transgenic mice, no such responses were generated to HLA-A2-restricted epitopes in either type of mouse. In contrast, vaccination with DNA-encoding calreticulin linked to DNA encoding a mutant version of E7 with a deleted aa49–57 epitope leads to the generation of an HLA-A2-restricted E7 (aa11–20)-specific CTL response in HLA-A2 (AAD) transgenic mice. More importantly, vaccination with CRT/mtE7 (del aa49–57) DNA protects against a lethal challenge with TC-1/A2 tumor cells in HLA-A2 (AAD) transgenic mice. Furthermore, our in vitro *studies demonstrate that the presence of the E7 (aa49–57) epitope does not suppress presentation of the HLA-A2-restricted E7 (aa11–20) epitope through MHC class I molecules. Thus, the predominant E7 aa49–57-specific CD8+ T-cell immune response in HLA-A2 transgenic mice vaccinated with CRT/E7 is likely due to preferred expansion of E7 aa49–57-specific CD8+ T cells in vaccinated mice. These results highlight the importance of epitope immunodominance in the evaluation of immune responses in HLA-A2 (AAD) transgenic mice.*
Gene Therapy (2006) **13,** 67–77. doi:10.1038/sj.gt.3302607; published online 18 August 2005

Keywords: *HLA-A2 transgenic mice; calreticulin; human papillomavirus (HPV); E7; DNA vaccines*

Introduction

It has been established that DNA vaccine potency can be significantly enhanced by gene gun delivery of DNA plasmids into professional antigen-presenting cells *in vivo* (see, for a review, Hung and Wu[1]). We have developed several DNA vaccines against human papillomavirus-16 (HPV-16) E7 antigen using intracellular targeting strategies to enhance MHC class I and II presentation (see, for a review, Hung and Wu[1]). We chose E7 because it is consistently expressed in most HPV-associated cancer cells, and is responsible for their malignant transformation. Thus, E7 represents an ideal target antigen for the development of vaccines and immunotherapeutic strategies against HPV-associated neoplasms. We have recently performed a head-to-head comparison of E7 DNA vaccines employing various intracellular targeting strategies, and have identified the DNA vaccine encoding calreticulin fused to full-length E7 (CRT/E7) as one of the most potent in generating E7-specific immune responses and antitumor effects.[2] Thus, CRT/E7 represents a promising therapeutic vaccine for clinical translation to control HPV-associated lesions in humans.

The identification of an HLA-A2-restricted E7-specific CTL epitope will facilitate the development of quantitative human T-cell immunological assays for characterization of E7-specific CD8+ T-cell immune responses in patients receiving effective E7 DNA vaccines. Such assays include intracellular cytokine staining, ELISPOT and MHC class I tetramer staining. HLA-A2 transgenic mice have been extensively used to identify CTL epitopes that are functional in humans and to evaluate the efficacy of candidate vaccines, since these transgenic mice may have CTL repertoires similar to those of humans. Previously, Ressing *et al*.[3] have identified several E7-specific HLA-A2-restricted CTL epitopes

Correspondence: Dr T-C Wu, Department of Pathology, The Johns Hopkins University School of Medicine, Richard Ross Research Building, Room 512, 720 Rutland Avenue, Baltimore, MD 21205, USA.
E-mail: wutc@jhmi.edu
Received 21 November 2004; revised 17 May 2005; accepted 8 July 2005; published online 18 August 2005

(11–20, YMLDLQPETT; 82–90, LLMGTLGIV; 86–93, TLGIVCPI), using HLA-A2 transgenic mice. Thus, the HLA-A2 transgenic mice may potentially be useful for evaluating HLA-A2-restricted CD8 responses with specificities correlating with those observed in humans.[4] We have acquired HLA-A2.1(AAD) transgenic mice that express a chimeric HLA class I molecule, comprising the α-1 and α-2 domains from human HLA-A*0201 and the α-3 transmembrane/cytoplasmic domain from mouse H-2D^d.[5] These transgenic mice have been used by other researchers to characterize the immunogenicity and vaccine effects of several HLA-A2-restricted CTL epitopes from viral antigens.[6–8]

In this study, we characterized the HLA-A2-restricted E7-specific CD8$^+$ T-cell immune response generated by our CRT/E7 DNA vaccine in HLA-A2 (AAD) transgenic mice. We found that vaccination with CRT/E7 DNA generates strong H-2D^b-restricted E7 (amino acid (aa)49–57)-specific CD8$^+$ T-cell immune responses[9] in both C57BL/6 and HLA-A2 (AAD) transgenic mice. We also found that vaccination with CRT/mtE7 (del aa49–57) leads to the generation of an HLA-A2-restricted E7 (aa11–20)-specific CTL response in HLA-A2 (AAD) transgenic mice, suggesting that the presence of the D^b-restricted E7 immunodominant epitope (aa49–57) in E7 may influence the generation of HLA-A2-restricted E7 (aa11–20)-specific CD8$^+$ T-cell immune responses in HLA-A2 (AAD) transgenic mice that concomitantly express H-2D^b molecules. Furthermore, we observed that alterations (through deletion or mutation) of the E7 gene at the regions aa49–57 and aa11–20 were able to influence the generation of HLA-A2-restricted E7-specific T-cell-mediated immune responses in HLA-A2 (AAD) transgenic mice vaccinated with pcDNA3-CRT/E7. More importantly, we found that HLA-A2 (AAD) transgenic mice vaccinated with pcDNA3-CRT/mtE7 (del aa49–57) were capable of controlling a lethal challenge with HLA-A2-expressing, E7-expressing tumor cells (TC-1/A2). The clinical implications of the current study are discussed.

Results

Vaccination with CRT/E7 DNA generates strong H-2D^b-restricted E7 (aa49–57)-specific CD8$^+$ T-cell immune responses in both HLA-A2 (AAD) transgenic mice and C57BL/6 mice

In order to characterize the HLA-A2-restricted E7-specific CD8$^+$ T-cell immune response in HLA-A2 (AAD) transgenic mice vaccinated with pcDNA3-CRT/E7, we harvested splenocytes from vaccinated mice 1 week after the last vaccination. The splenocytes were characterized for E7 peptide-specific CD8$^+$ T-cell immune responses. As shown in Figure 1, vaccination with pcDNA3-CRT/E7 resulted in a significant number of IFN-γ-expressing E7 (aa49–57) peptide-specific CD8$^+$ T cells in both C57BL/6 (933 ± 4.24) and HLA-A2 (AAD) transgenic mice (653 ± 70.71). In comparison, very few E7

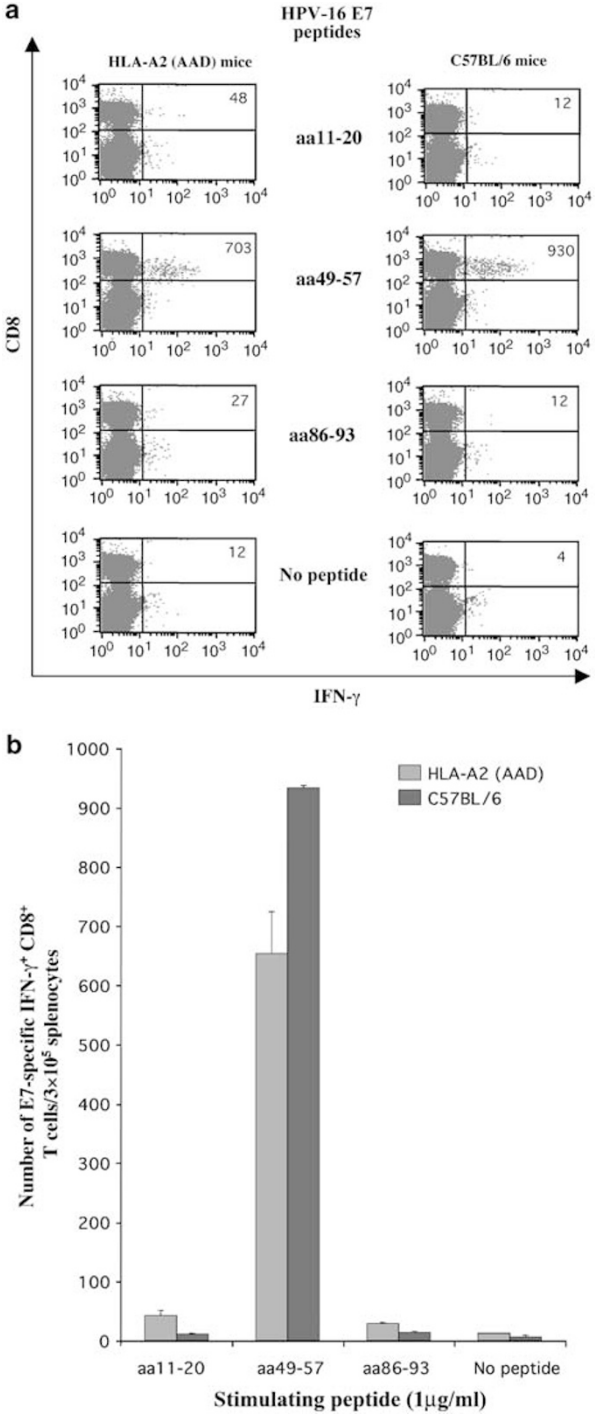

Figure 1 Intracellular cytokine staining with flow cytometry analysis to determine E7 peptide-specific CD8$^+$ T cells in pcDNA3-CRT/E7-vaccinated mice. C57BL/6 and HLA-A2 (AAD) transgenic mice were vaccinated with pcDNA3-CRT/E7. Splenocytes were harvested 1 week after the last vaccination. Pooled splenocytes from vaccinated mice (five per group) were stimulated *in vitro* with HPV-16 E7 peptides (aa11–20, aa49–57 or aa86–93) overnight. Splenocytes without stimulation with peptides were used as a negative control. Cells were stained for both CD8 and intracellular IFN-γ and followed by flow cytometry analysis to identify IFN-γ-secreting, E7-specific CD8$^+$ T-cell precursors. (**a**) Representative sample of flow cytometry analysis. The numbers in the upper right corners indicate the number of IFN-γ-secreting E7-specific CD8$^+$ T cells per 3×10^5 splenocytes. (**b**) Bar graph depicting the number of E7-specific, IFN-γ-secreting CD8$^+$ T cell precursors/3×10^5 splenocytes (mean ± s.d.). The data shown here are from one representative experiment of two performed.

peptide-specific CD8+ T cells were generated against the previously characterized HLA-A2-restricted E7 (aa11–20) (42 ± 8.49) or E7 (aa86–93) (28.5 ± 2.12) epitopes in either C57BL/6 or HLA-A2 (AAD) transgenic mice. These data indicate that vaccination of HLA-A2 (AAD) transgenic mice with pcDNA3-CRT/E7 predominantly generates H-2D^b-restricted E7 (aa49–57) peptide-specific CD8+ T-cell immune responses and that only minimal amounts of HLA-A2-restricted E7 peptide-specific CD8+ T cells were generated in the vaccinated mice.

Vaccination with the DNA vaccine encoding CRT linked to E7 with a deletion of aa49–57 leads to a significant HLA-A2-restricted E7 (aa11–20) peptide-specific CD8+ T-cell immune response in HLA-A2 (AAD) transgenic mice

The failure to observe responses to the aa11–20 or aa86–93 epitopes in HLA-A2 (AAD) transgenic mice that also expressed murine H-2 molecules suggested that the potent D^b-restricted E7 response might somehow be suppressing or masking weaker HLA-A2-restricted responses. To address this possibility, we created a mutant *E7* gene with a deletion of aa49–57 (mtE7 (del aa49–57)) and generated a chimeric CRT/mtE7 (del aa49–57) DNA vaccine. To determine if vaccination with pcDNA3-CRT/mtE7 (del aa49–57) in HLA-A2 (AAD) transgenic mice would generate an E7-specific HLA-A2 restricted CD8+ T-cell immune response, we isolated the splenocytes 1 week after the last vaccination and pulsed the splenocytes with various E7 peptides, including (aa11–20, aa49–57, aa82–90 and aa86–93). As shown in Figure 2, HLA-A2 (AAD) transgenic mice vaccinated with pcDNA3-CRT/mtE7 (del aa49–57) generated the highest number of HLA-A2-restricted E7 (aa11–20)-specific CD8+ T-cell precursors among all the E7 peptide-specific CD8+ T-cell precursors examined. In comparison, HLA-A2 (AAD) transgenic mice vaccinated with pcDNA3-CRT/E7 generated the highest number of H-2D^b-restricted E7 (aa49–57) peptide-specific CD8+ T-cell precursors. These data suggest that the presence of the E7 49–57 aa sequence within the *E7* gene of CRT/E7 DNA vaccine may influence the generation of E7 (aa11–20)-specific CD8+ T-cell immune responses in HLA-A2 (AAD) transgenic mice.

Modification of aa11–20 or aa49–57 in E7 is able to improve E7 presentation through the HLA-A2 molecule.

It has previously been demonstrated that mutation of E7 at aa57 (from Phe to Arg) decreases the binding affinity of E7 (aa49–57) to H-2D^b molecules, resulting in a reduction of the tumor protection ability of E7

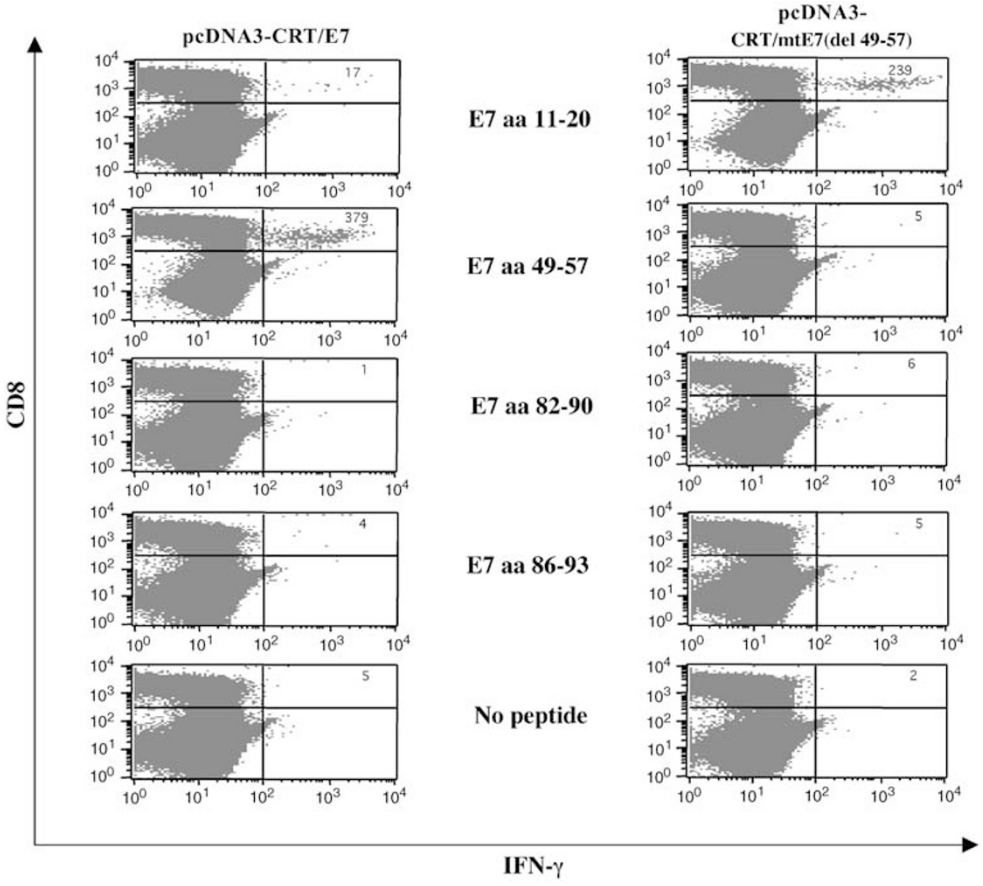

Figure 2 Intracellular cytokine staining with flow cytometry analysis to determine E7 peptide-specific CD8+ T-cell immune responses in HLA-A2 (AAD) transgenic mice vaccinated with pcDNA3-CRT/E7 or pcDNA3-CRT/mtE7 (del aa49–57). Pooled splenocytes from vaccinated HLA-A2 (AAD) mice (five per group) were cultured *in vitro* with E7 peptides aa11–20, aa49–57, aa82–90 or aa86–93 overnight and were stained for both CD8 and intracellular IFN-γ. Splenocytes without stimulation with peptides were used as a negative control. The numbers in the upper right corners indicate the number of IFN-γ-secreting E7-specific CD8+ T cells per 3×10^5 splenocytes.

(aa49–57).[10] It has also been shown that mutation of E7 at aa20 (from Thr to Val) was able to enhance the binding affinity of E7 (aa11–20) peptide to HLA-A2 molecules.[11] We therefore generated DNA constructs encoding CRT linked to E7 with a mutation at aa57 (CRT/mtE7 (F57R)) or a mutation at aa20 (CRT/mtE7 (T20V)). As shown in Figure 3, vaccination of HLA-A2 (AAD) transgenic mice with CRT/mtE7 (F57R) DNA enhanced the generation of E7 (aa11–20)-specific CD8+ T cells, but decreased the generation of E7 (aa49–57)-specific CD8+ T cells compared to vaccination with CRT/E7 DNA. Furthermore, vaccination of HLA-A2 (AAD) transgenic mice with CRT/mtE7 (T20V) DNA significantly enhanced the generation of E7 (aa11–20)-specific CD8+ T cells and slightly increased the generation of E7 aa49–57-specific CD8+ T cells compared to vaccination with CRT/E7 DNA. Taken together, these data suggest that it is possible to improve the presentation of E7 through the HLA-A2 molecule by mutations that lead to a decrease of binding affinity of E7 (aa49–57) to H-2D^b or by mutations that lead to an increase of binding affinity of E7 (aa11–20).

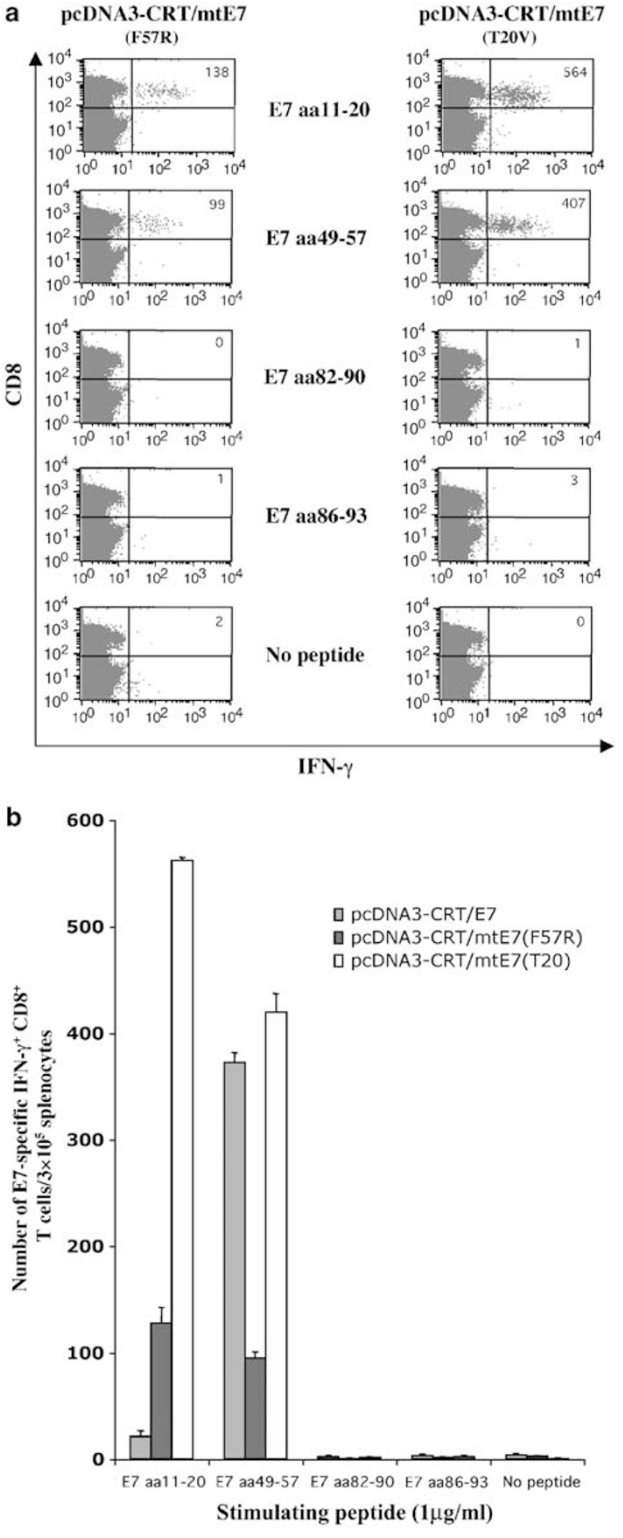

Figure 3 Intracellular cytokine staining with flow cytometry analysis to determine the E7-specific CD8+ T-cell response to vaccination with various DNA vaccines. We vaccinated HLA-A2 (AAD) transgenic mice with pcDNA3-CRT/E7, pcDNA3-CRT/mtE7 (F57R) or pcDNA3-CRT/mtE7 (T20V) constructs and harvested splenocytes from vaccinated mice 1 week after the last vaccination. Pooled splenocytes from each vaccination group (five per group) were incubated *in vitro* with various E7 peptides (aa11–20, aa49–57, aa82–90, aa86–93) or without peptide overnight. The splenocytes were stained for CD8 and IFN-γ, and subjected to flow cytometry analysis to detect IFN-γ-expressing, E7 peptide-specific CD8+ T cells. (**a**) Representative figure of flow cytometry data. The numbers in the upper right corners indicate the number of IFN-γ-secreting E7 peptide-specific CD8+ T cells per 3×10^5 splenocytes. (**b**) Bar graph showing mean number of IFN-γ-expressing, E7 peptide-specific CD8+ T cells per 3×10^5 splenocytes. Data are expressed as means $\pm$ s.d.

Tumor cells that express E7 as well as the HLA-A2 (AAD) molecule are capable of presenting E7 (aa11–20) through the HLA-A2 molecule

A syngeneic tumor cell line that is capable of presenting E7 through HLA-A2 molecules would be valuable for characterizing the antitumor responses to vaccination with an E7-expressing DNA vaccine in HLA-A2 (AAD) transgenic mice. We transduced TC-1, an established E7-expressing tumor cell line, with a retrovirus encoding the HLA-A2 (AAD) molecule. The expression of HLA-A2 in the TC-1/A2 cells was confirmed by flow cytometry analysis using an antibody against HLA-A2 (data not shown). We then confirmed that E7 (aa11–20) was presented through HLA-A2 molecules in the TC-1/A2 tumor cells using an HLA-A2-restricted E7 (aa11–20)-specific CD8+ T-cell line. As shown in Figure 4a, TC-1/A2, but not TC-1, was able to activate HLA-A2-restricted E7 (aa11–20)-specific CD8+ T cells in splenocytes from pcDNA3-CRT/mtE7 (del aa49–57)-vaccinated HLA-A2 (AAD) transgenic mice. To further confirm processing and presentation of the E7 (aa11–20) epitope in TC-1/A2 cells, we performed CTL assays using an E7 (aa11–20)-specific CD8+ T-cell line. As shown in Figure 4b, specific lysis was observed in TC-1/A2 cells but not in TC-1 cells at E:T ratios of 1:1, 10:1 and 25:1. Taken together, these data suggest that TC-1/A2 tumor cells are capable of processing and presenting the E7 (aa11–20) epitope through HLA-A2 molecules.

Vaccination of HLA-A2 (AAD) transgenic mice with pcDNA3-CRT/mtE7 (del aa49–57) protects against a lethal challenge with TC-1/A2

We performed tumor protection experiments to test whether vaccination with pcDNA3-CRT/mtE7 (del

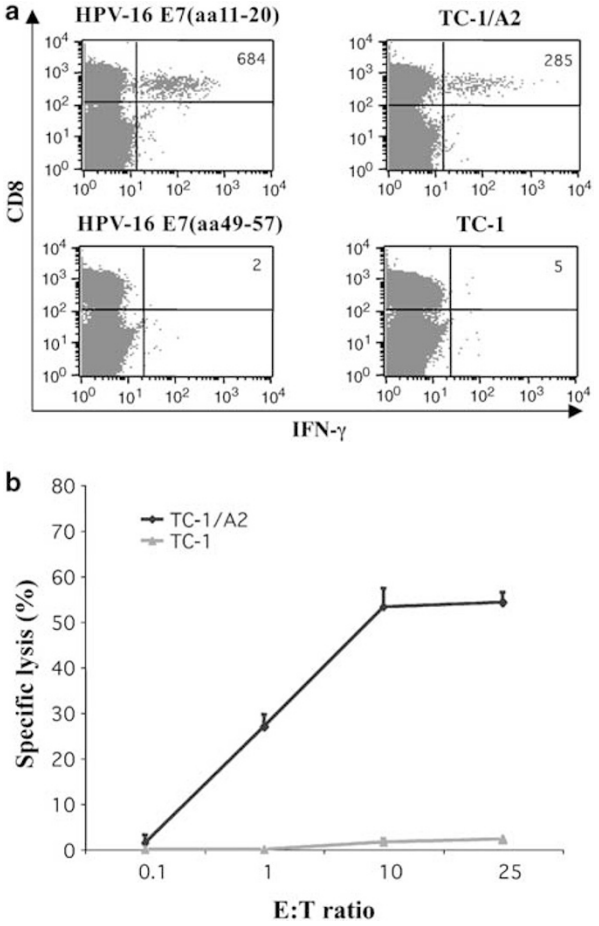

Figure 4 Characterization of HLA-A2 (AAD)-expressing TC-1 cells (TC-1/A2). (**a**) Intracellular cytokine staining followed by flow cytometry analysis to determine activation of E7 (aa11–20)-specific $CD8^+$ T cells by TC-1/A2 cells. Splenocytes from HLA-A2 (AAD) transgenic mice vaccinated with pcDNA3-CRT/mtE7 (del aa49–57) were used for this study. Data shown are representative sample of flow cytometry analysis. The numbers in the upper right corners indicate the number of activated IFN-γ-secreting E7-specific $CD8^+$ T cells per 3×10^5 splenocytes. Note: TC-1/A2 cells, but not TC-1, were able to activate E7 (aa11–20)-specific $CD8^+$ T cells. (**b**) CTL assay to demonstrate specific killing of TC-1/A2 cells by E7 (aa11–20)-specific $CD8^+$ T cells. Splenocytes from HLA-A2 (AAD) transgenic mice vaccinated with pcDNA3-CRT/mtE7 (del aa49–57) were stimulated with irradiated TC-1/A2 cells in the presence of IL-2 for 5 days to generate effector cells. Standard chromium release CTL assays were performed to assess the ability of E7 (aa11–20)-specific T cells to lyse target cells. E7 (aa11–20) peptide-pulsed T2 cells were used as positive controls (data not shown). The data shown in this figure are from one representative experiment of two performed.

aa49–57) can provide protection against tumor challenge with TC-1/A2 in HLA-A2 (AAD) transgenic mice. TC-1 cells were used as negative controls. As shown in Figure 5, 60% of mice vaccinated with pcDNA3-CRT/mtE7 (del aa49–57) remained tumor free 6 weeks after TC-1/A2 challenge. In contrast, all mice vaccinated with pcDNA3-CRT or without vaccination developed tumors within 2 weeks after tumor challenge. Furthermore, none of the mice challenged with TC-1 could be protected by vaccination with pcDNA3-CRT/mtE7 (del aa49–57). These data indicate that vaccination with pcDNA3-CRT/mtE7 (del aa49–57) was capable of generating strong HLA-A2-restricted antitumor effects.

The E7 (aa49–57) peptide epitope does not directly compete with the E7 (aa11–20) peptide epitope for HLA-A2 binding

We performed an *in vitro* peptide-binding assay to verify whether E7 (aa49–57) and E7 (aa11–20) competed for binding to HLA-A2 molecules. We added the HPV-16 E7 (aa11–20) peptide to T2 cells 3 h after incubating the cells with or without the HPV-16 E7 (aa49–57) peptide. After 1 day, the HLA-A2 expression level on the surface of T2 cells was characterized by flow cytometry analysis with HLA-A2-specific monoclonal antibody. As shown in Figure 6, the HLA-A2 expression in E7 (aa11–20) peptide-loaded T2 cells that were preincubated with E7 (aa49–57) peptide was similar to that of E7 (aa11–20) peptide-loaded T2 cells that were not preincubated with E7 (aa49–57) peptide. Furthermore, loading of T2 cells with E7 (aa49–57) peptide did not lead to upregulation of HLA-A2 expression in T2 cells. Our data suggest that the E7 (aa49–57) peptide epitope does not directly compete with the E7 (aa11–20) peptide epitope for HLA-A2 binding.

The aa49–57 region of E7 does not suppress presentation of the HLA-A2-restricted E7 aa11–20 epitope in HLA-A2 (AAD)-expressing dendritic cells

We further investigated the influence of the H-$2D^b$-restricted E7 CTL epitope (aa49–57) on the presentation of E7 through the HLA-A2 molecule using a murine dendritic cell line, DC-1. We first transfected DC-1 with pcDNA3-HLA-A2 (AAD) DNA in conjunction with CRT, CRT/E7, CRT/mtE7 (del aa49–57), CRT/mtE7 (F57R) or CRT/mtE7 (T20V) DNA. The various transfected DC-1 cells were incubated with an HLA-A2-restricted E7 (aa11–20) peptide-specific $CD8^+$ T-cell line. The activation of the HLA-A2-restricted E7 (aa11–20) peptide-specific $CD8^+$ T-cell line was determined by intracellular cytokine staining for IFN-γ secretion. As shown in Figure 7, DC-1 cotransfected with pcDNA3-HLA-A2 and CRT/mtE7 (del aa49–57) activated similar numbers of HLA-A2-restricted E7 (aa11–20) peptide-specific $CD8^+$ T cells compared to DC-1 cotransfected with pcDNA3-HLA-A2 and pcDNA3-CRT/E7. DC-1 cotransfected with pcDNA3-HLA-A2 and CRT failed to activate E7 (aa11–20)-specific $CD8^+$ T cells. Interestingly, DC-1 cells cotransfected with pcDNA3-HLA-A2 and pcDNA3-CRT/mtE7 (T20V) were capable of activating the highest number of E7 (aa11–20)-specific $CD8^+$ T cells among the various transfected DC-1 cells. These data suggest that the presence of the H-$2D^b$-restricted E7 CTL epitope (aa49–57) within E7 does not suppress the presentation of E7 (aa11–20) by the HLA-A2 molecule in dendritic cells and that modification of E7 (aa11–20) epitope (T20V) may enhance the presentation of E7 (aa11–20) through HLA-A2 molecules.

Discussion

In this study, we characterized the HLA-A2-restricted E7-specific immune response in HLA-A2 (AAD) transgenic mice. The HLA-A2 (AAD) transgenic mice used in our study express both human HLA class I heavy chains and murine H-2 class I molecules (see, for a review, Lemonnier[12]). Our data showed that vaccination with

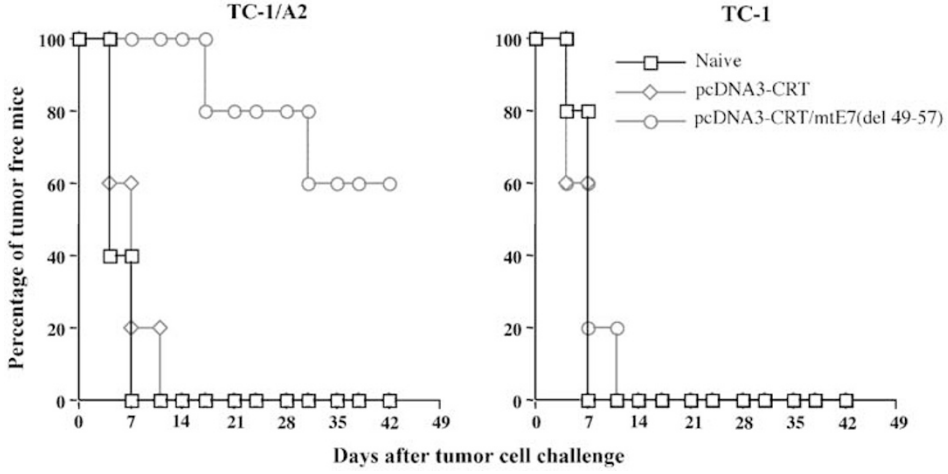

Figure 5 *In vivo* tumor protection experiments. HLA-A2 (AAD) transgenic mice (five per group) were immunized with either pcDNA3-CRT or pcDNA3-CRT/mtE7 (del aa49–57) DNA vaccines. At 1 week after the last vaccination, the vaccinated mice were challenged subcutaneously with 1×10^5 TC-1 or TC-1/A2 cells/mouse and monitored for evidence of tumor growth by inspection and palpation twice a week. The graphs show the percentage of tumor-free mice days after tumor challenge. The data shown here are from one representative experiment of two performed.

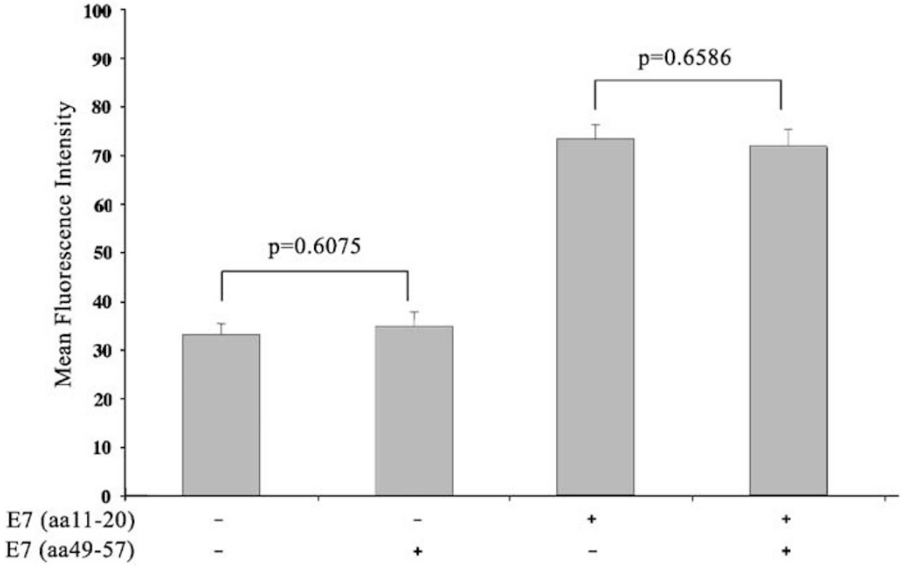

Figure 6 Characterization of HLA-A2 expression in T2 cells by flow cytometry analysis. T2 cells were pulsed with HPV-16 E7 (aa49–57) peptide or HPV-16 E7 (aa11–20) peptide for 24 h. In another set of experiments, T2 cells were pulsed with HPV-16 E7 (aa49–57) peptide 3 h before they were pulsed with HPV-16 E7 (aa11–20) peptide for 24 h. The peptide-pulsed T2 cells were then washed and characterized for HLA-A2 expression by flow cytometry analysis. Data are expressed as mean fluorescence intensity of HLA-A2 expression on T2 cells.

CRT/E7 DNA elicited a strong murine H-2D^b-restricted E7 (aa49–57)-specific CD8$^+$ T-cell immune response, but did not elicit HLA-A2-restricted E7-specific CD8$^+$ T-cell immune responses in the HLA-A2 (AAD) transgenic mice. Our results are consistent with previous studies which show that mouse CD8$^+$ T cells in these HLA-A2 (AAD) transgenic mice could preferentially, or exclusively, use murine H-2 class I molecules as restricting elements in responses against pathogens or vaccination.[13,14] We have addressed this limitation by generating a DNA vaccine that contains the *E7* gene with a deleted murine H-2D^b-restricted immunodominant epitope (aa49–57). As demonstrated in Figure 2, we showed that vaccination with CRT/mtE7 (del aa49–57) DNA was capable of generating a strong HLA-A2-restricted E7 (aa11–20)-specific CD8$^+$ T-cell response in HLA-A2 (AAD) transgenic mice.

Another approach to avoid presentation of murine H-2-restricted CTL epitope in HLA-A2 (AAD) transgenic mice is to use a new generation of HLA-A2 transgenic mice, HLA-A2.1 transgenic/H-2 class I knockout mice (also known as HHD mice). In HHD mice, the H-2 class I gene was knocked out, and a chimeric HLA-A2.1 monochain (HHD) was generated by linking the C-terminal of the human β2m covalently to the N-terminus of the chimeric HLA-A2 heavy chain (containing the α1 and α2 domains of HLA-A2.1 and the α3 domain of H-2D^b) via a peptidic arm.[12] This ensures that the HHD

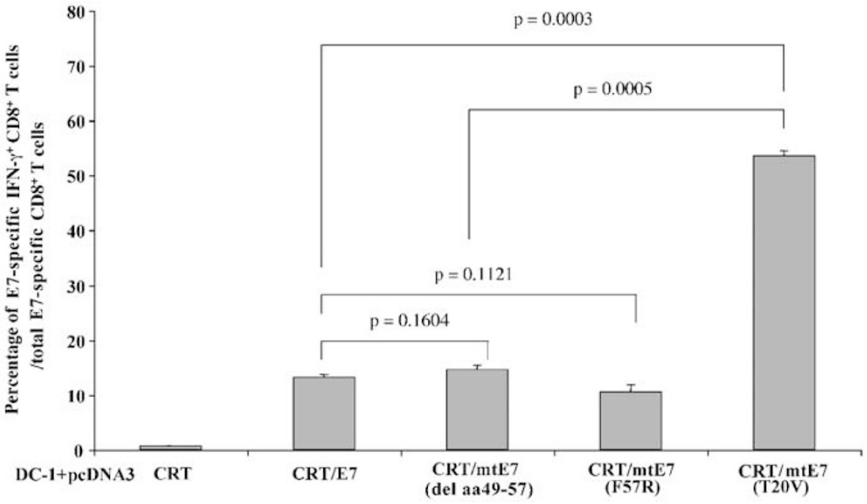

Figure 7 Intracellular cytokine staining and flow cytometry analysis to determine E7 (aa11–20)-specific CD8+ T cells activated by transfected DC-1 cells. DC-1 cells were first transfected with pcDNA3-HLA-A2 (AAD) in conjunction with CRT, CRT/E7, CRT/mtE7 (del aa49–57), CRT/mtE7 (F57R) or CRT/mtE7 (T20V) DNA. The various transfected DC-1 cells were subsequently cocultured with an HLA-A2-restricted HPV-16 E7 (aa11–20)-specific CD8+ T-cell line. IFN-γ-secreting E7 (aa11–20)-specific CD8+ T cells were detected by intracellular IFN-γ staining followed by flow cytometry analysis. Data are expressed as the mean number of IFN-γ-secreting E7-specific CD8+ T cells per 1×10^4 total E7-specific CD8+ T cells $\pm$ s.d. The data presented here are representative of two experiments performed.

molecule will be the only MHC class I molecule expressed on cell surfaces, ensuring that any identified CTL epitopes are HLA-A2 restricted. Thus, these HLA-A2 transgenic mice have been genetically engineered to promote usage of the human HLA class I molecules by the mouse CD8+ T cells. It will be of interest to test our DNA vaccine in these mice.

Our study agrees with previous studies, which showed that modification of the anchor residues of the E7 aa11–20 epitope[11] or the E7 aa49–57 epitope[10] can affect CTL responses elicited by vaccination with E7-encoding DNA vaccines. Our data suggest that a modified aa49–57 epitope can reduce presentation of the immunodominant aa49–57 epitope in mice vaccinated with CRT/E7 DNA, resulting in increased E7 (aa11–20)-specific CD8+ T-cell immune responses in vaccinated mice (see Figure 3). Furthermore, our data also suggest that the aa11–20 epitope can be modified to enhance its presentation on antigen-expressing dendritic cells (see Figure 7) as well as to enhance E7 (aa11–20)-specific CD8+ T-cell immune responses in vaccinated mice (see Figure 3). The understanding that modification of peptide anchor residues can influence immunodominance makes possible the design of more effective multiepitope vaccines.

We have developed an HLA-A2-expressing tumor model, TC-1/A2, that can naturally process and present E7 antigen via HLA-A2 molecules. This model should be useful for assays of HLA-A2-restricted E7-specific antitumor responses and antitumor effects. Eiben *et al*[15] have created a transgenic cell line, HLF16, that is tumorigenic in HLA-A*0201 mice by transfecting heart lung fibroblasts from HLA-A*0201 mice with HPV-16 E6 and E7 oncogenes and H-Ras V12. In that tumor cell line, the H-2D^b-restricted HPV-16 E7 immunodominant epitope (aa49–57) was removed from the E7 construct to ensure that all antitumor responses were mediated through the HLA-A*0201-restricted epitopes. While the HLF16 tumor cell line is potentially useful, the HLA-A2 expression level is not high and it requires injection with a large dose (2×10^6 cells/mouse) to develop stable tumor growth in challenged mice.[15] In comparison, our TC-1/A2 model has the advantage of high HLA-A2 expression and smaller tumor challenge dose for tumor growth than the HLF16 tumor cell line (1×10^4 cells versus 2×10^6 cells). Thus, we have selected the TC-1/A2 tumor cell line for our study. The TC-1/A2 tumor model could also be transfected with other tumor-associated antigens and used for testing the antitumor effect generated by other antigen-specific vaccines (e.g. MART-1 for a melanoma model or Her2/neu for an ovarian model).

Our data indicate that vaccination with CRT/mtE7 (del aa49–57) DNA vaccine generated a significant HLA-A2-restricted E7 (aa11–20)-specific CD8+ T-cell immune response, but did not generate E7 (aa82–90)- or E7 (aa86–93)-specific CD8+ T-cell immune responses in vaccinated mice (see Figure 2). Our findings agree with previous studies.[16,17] Bauer *et al*.[16] reported that HLA-A2 transgenic mice vaccinated with the E7 protein did not produce a detectable E7 (aa86–93)-specific CTL response. Similarly, Street *et al*.[17] also found that the E7 epitopes (aa82–90 and aa86–93) were not processed out of either endogenously expressed or immunization-introduced E7 in HLA-A2 transgenic mice. In comparison, Eiben *et al*.[15] have shown that HLA-A2 transgenic mice vaccinated with the Venezuelan equine encephalitis virus-based vector encoding HPV E6 and E7 or a multiepitope DNA vaccine encoding HPV-16 E6 and E7 epitopes were able to generate (aa86–93)-specific CD8+ T-cell immune responses. Such discrepancy may be related to the kinds of vectors (viral versus DNA), strains of HLA-A2 transgenic mice (A2.1K^b versus A2.1D^d) as well as the immunostimulatory molecules (with or without CRT molecule) used in the different studies.

In this study, we observed that HLA-A2 (AAD)-expressing dendritic cell lines transfected with CRT/E7 DNA were capable of activating E7 (aa11–20)-specific CD8+ T cells in a similar level compared to dendritic cells

transfected with CRT/mtE7 (del aa49–57) (see Figure 7). Thus, our data suggest that the presence of the E7 (aa49–57) epitope within the *E7* gene does not suppress the presentation of E7 (aa11–20) epitope through HLA-A2 molecules. However, we observed that the HLA-A2 (AAD) transgenic mice vaccinated with CRT/E7 generated strong E7 (aa49–57)-specific and weak E7 (aa11–20)-specific $CD8^+$ T-cell immune responses in vaccinated HLA-A2 transgenic mice (see Figures 1 and 2). Taken together, our results suggest that there is a preferred expansion of E7 (aa49–57)-specific $CD8^+$ T cells in CRT/E7 DNA-vaccinated mice.

In summary, we have demonstrated that vaccination with CRT/E7 DNA can generate a strong HLA-A2-restricted E7 (aa11–20) $CD8^+$ T-cell immune response when the H-$2D^b$-restricted E7 (aa49–57) immunodominant epitope is deleted or suppressed in the *E7* gene of the CRT/E7 DNA vaccine. Furthermore, we have shown that HLA-A2 (AAD) transgenic mice vaccinated with the CRT/E7 DNA vaccine are capable of preventing tumor growth after challenge with TC-1/A2 cells. These results are encouraging for our planned clinical trials using the pNGVL4a-CRT/E7 (detox) DNA vaccine[2] since they suggest that our CRT/E7 vaccine strategy may be capable of eliciting HLA-A2-restricted E7-specific $CD8^+$ T-cell immune responses and possible therapeutic against HPV-associated lesions in vaccinated individuals. It will be interesting to compare the results of these preclinical studies with the results of our future clinical trials.

Materials and methods

Plasmid DNA constructs and preparation

The generation of pcDNA3-CRT, pcDNA3-E7 and pcDNA3-CRT/E7 has been described previously.[18] To generate pcDNA3-CRT/mtE7 (del aa49–57), we first generated the mtE7 (del aa49–57) gene by polymerase chain reaction (PCR). The DNA fragment encoding aa1–48 of E7 was generated by PCR using a pair of primers (5′tttgaattcatgcatggagatacacctaca3′ and 5′ctggacaagcagaaccggactgttgcaagtgtgactctac3′) and pcDNA3-E7 as a template. The DNA fragment encoding aa58–98 of E7 was generated by PCR using a pair of primers (5′gtagagtcacacttgcaacagtccggttctgcttgtccag3′ and 5′gggaagcttttatggtttctgagaacagat3′) and pcDNA3-E7 as a template. The mtE7 (del aa49–57) DNA fragment was generated by PCR using primers (5′tttgaattcatgcatggagatacacctaca3′ and 5′gggaagcttttatggtttctgagaacagat3′) and E7 DNA fragments encoding aa1–48 and aa58–98 as templates. This final amplified product was further cloned into the *Eco*RI/*Hind*III sites of pcDNA3-CRT to generate pcDNA3-CRT/mtE7 (del aa49–57).

To create pcDNA3-CRT/mtE7 (T20V), we used PCR to generate the mtE7 (T20V) DNA fragment. The E7 DNA fragment of nucleotides 1–80 containing a mutation at the position 20 aa of E7 (from Thr to Val) was amplified using a pair of primers (5′tttgaattcatgcatggagatacacctaca3′ and 5′tgctcataacagtagagatctactgtctctggttgcaaatcta3′) and pcDNA3-E7 as a template. The E7 DNA fragment of nucleotides 38–297 containing the same mutation was amplified using a pair of primers (5′tagatttgcaaccagagacagtagatctctactgttatgagca3′ and 5′gggaagcttttatggtttctgagaacagat3′) and pcDNA3-E7 as a template. The final mtE7 (T20V) DNA fragment was generated by PCR using a pair of primers (5′tttgaattcatgcatggagatacacctaca3′ and 5′gggaagcttttatggtttctgagaacagat3′) and the E7 DNA fragments of nucleotides 1–80 and nucleotides 38–297 as templates. The amplified product was further cloned into the *Eco*RI/*Hind*III sites of pcDNA3-CRT to generate pcDNA3-CRT/mtE7 (T20V).

To produce pcDNA3-CRT/mtE7 (F57R), we used PCR to generate the mtE7 (F57R) DNA fragment. The E7 DNA fragment of nucleotides 1–191 containing a mutation at the position 57 aa of E7 (from Phe to Arg) was amplified using a pair of primers (5′tttgaattcatgcatggagatacacctaca3′ and 5′gtagagtcacacttgcaacaccgggttacaatattgtaatggg3′) and pcDNA3-E7 as a template. The E7 DNA fragment of 149–297 nucleotide containing the same mutation was amplified by using a pair of primers (5′cccattacaatattgtaacccggtgttgcaagtgtgactctac3′ and 5′gggaagcttttatggtttctgagaacagat3′) and pcDNA3-E7 as a template. The final E7 (F57R) DNA fragment was generated by PCR using a pair of primers (5′tttgaattcatgcatggagatacacctaca3′ and 5′gggaagcttttatggtttctgagaacagat3′) and the E7 DNA fragments of nucleotides 1–191 and nucleotides 149–297 as templates. The amplified product was further cloned into the *Eco*RI and *Hind*III sites of pcDNA3-CRT to generate pcDNA3-CRT/mtE7 (F57R).

To generate pMSCV-HLA-A2 (AAD), the DNA fragment encoding the full-length HLA-A2 (AAD) chimeric protein was amplified with a pair of primers (5′tttagatctatggccgtcatggcgccccga3′ and 5′tttgaattctcacactttacaatctgggag3′) and total RNA from the splenocytes of HLA-A2 (AAD) transgenic mice as templates using Superscript One-Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). The amplified DNA fragment was further cloned into *Bgl*II/*Eco*RI sites of pMSCVpuro retroviral vector (Clontech, Palo Alto, CA, USA). To generate pcDNA3-HLA-A2 (AAD), the amplified HLA-A2 (AAD) DNA fragment was cloned into *Bgl*II/*Eco*RI sites of pcDNA3-plus vector (Invitrogen, Carlsbad, CA, USA). The accuracy of these constructs was confirmed by DNA sequencing. DNA was amplified in *Escherichia coli* DH5α and purified as described previously.[19]

Peptides

Peptides representing defined HPV-16 E7 CTL epitopes, including H-$2D^b$-restricted aa49–57 (RAHYNIVTF),[9] and HLA-A*0201-restricted aa11–20 (YMLDLQPETT), aa86–93 (TLGIVCPI) and aa82–90 (LLMGTLGIV),[3] were synthesized by Macromolecular Resources (Denver, CO, USA) at a purity of greater than or equal to 70% (verified by HPLC).

Cells

DC-1 cells were generated from the dendritic cell line[20] provided by Dr Kenneth Rock at the University of Massachusetts. With continued passage, we have generated subclones of DCs (DC-1) that can be easily transfected.[21] T2 ($174 \times$ CEM.T2) cells deficient in the transporters for antigen processing (TAP) were obtained from American Type Culture Collection (ATCC CRL-1992, Manassas, VA, USA). TC-1 cells were generated as described previously.[22] To generate TC-1/A2, the construct pMSCV-HLA-A2 (AAD) was transfected into Phoenix packaging cell line and the retrovirus virion-containing supernatant was collected 48 h after transfec-

tion. The supernatant was filtered with 0.45-μm cellulose acetate sterile filter and used to infect TC-1 in the presence of 8 μg/ml polybrene (Sigma, St Louis, MO, USA). At 1 day after retroviral transduction, the viral supernatant was replaced with normal culture medium, and when the cells reach 70% confluency, puromycin (7.5 μg/ml) was used to select for cells with integrated HLA-A2 (AAD). The expression level of HLA-A2 (AAD) chimeric protein was verified by staining the cells with anti-HLA-A2 antibody, BB7.1 (Pharmingen, San Diego, CA, USA) and analyzing by flow cytometry. All cells were maintained in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, and 20 mM HEPES, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA, USA).

Mice

HLA-A*0201/D^d (AAD) transgenic female C57BL/6 mice, 6–8 weeks of age, were kindly provided by Dr Victor Engelhard at the University of Virginia Health Sciences Center.[5] These transgenic mice express a chimeric HLA class I molecule comprising the α-1 and α-2 domains of HLA-A*0201, and the α-3 transmembrane and cytoplasmic domain of H-$2D^d$. This allows the murine CD8 molecule on the murine $CD8^+$ T cells to interact with the syngeneic α-3 domain of the chimeric MHC class I molecule. Wild-type C57BL/6 female mice (6–8 weeks old) were also purchased from the National Cancer Institute. All animals were maintained under specific pathogen-free conditions, and all procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

DNA vaccination

DNA-coated gold particles were prepared and DNA vaccination was performed using a helium-driven gene gun (BioRad Laboratories Inc., Hercules, CA, USA) according to a protocol described previously.[19] pcDNA3-CRT, pcDNA3-CRT/E7, pcDNA3-CRT/mtE7 (del aa49–57), pcDNA3-CRT/mtE7 (F57R) or pcDNA3-CRT/mtE7 (T20V) DNA-coated gold particles were delivered to the shaved abdominal region of mice using a helium-driven gene gun (BioRad Laboratories, Hercules, CA, USA) with a discharge pressure of 400 psi. Mice were immunized with 2 μg of the various DNA vaccines via gene gun and were boosted with the same dose and regimen 1 week later.

Intracellular cytokine staining and flow cytometry analysis

Splenocytes from vaccinated mice (five per group) were harvested 1 week after the last vaccination. Pooled splenocytes from naïve or vaccinated groups of C57BL/6 mice or HLA-A2 (AAD) transgenic mice were incubated overnight with 1 μg/ml of E7 peptide, (H-$2D^b$-restricted aa49–57 (RAHYNIVTF), HLA-A*0201-restricted aa11–20 (YMLDLQPETT), aa86–93 (TLGIVCPI) or aa82–90 (LLMGTLGIV), or incubated with tumor cells TC-1 or TC-1/A2 cells. GolgiPlug (BD Pharmingen, San Diego, CA, USA) was added 6 h before harvesting the cells from the culture according to the vendor's protocol. Cells were then washed once with FACScan buffer and stained with phycoerythrin-conjugated monoclonal rat anti-mouse CD8 antibody (clone 53.6.7; BD Pharmingen). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). Intracellular IFN-γ was stained with FITC-conjugated rat anti-mouse IFN-γ. All antibodies were purchased from BD Pharmingen. Flow cytometry analysis was performed using FACSCalibur with CELLQuest software (BD Biosciences, Mountain View, CA, USA).

In vitro *generation of HLA-A*0201 restricted HPV-16 E7 (11–20)-specific CTL lines*

Splenocytes from pcDNA3-CRT/mtE7 (del aa49–57)-immunized HLA-A2 (AAD) transgenic mice were harvested 1 week after the last vaccination. These splenocytes were stimulated with irradiated TC-1/A2 tumor cells in the presence of murine IL-2 (20 IU/ml). The cells were restimulated every week for at least 2 months. The specificity of the CTL lines was tested by intracellular IFN-γ staining or chromium release CTL assay using HLA-A2-restricted HPV-16 E7 (aa11–20) peptide-pulsed T2 cells as target cells.

CTL assay

Lytic activity of T cells was assessed using a standard chromium release assay. All target cells including TC-1 and TC-1/A2 cells were labeled with 100 μCi $^{51}Cr/10^6$ cells at 37°C for 90 min. T2 target cells pulsed with HPV-16 E7 peptide (aa11–20) for 2 h at 37°C at the concentration of 1 μg/ml before chromium labeling were used as positive control cells. After extensive washing, the labeled target cells were incubated with HLA-A2-restricted E7 (aa11–20) peptide-specific $CD8^+$ T-cell lines at various effector to target cell ratios in triplicate 96-well U-bottomed plates. Negative controls included wells containing target cells but no effector cells (i.e. background). A measure of 100 μl of supernatants was harvested after a 4-h incubation, and ^{51}Cr release was quantitated using a gamma counter. Results are expressed as percent cytotoxicity $\pm$ s.d. calculated from the following formula: percent specific lysis $-$ [experimental release value $-$ spontaneous release value)/(maximum release value $-$ spontaneous release value)] $\times$ 100, where spontaneous release value represents counts in supernatants from wells containing target cells in medium only, and maximum release value represents counts in supernatant from wells containing target cells in medium supplemented with 10% SDS.

In vivo *tumor protection experiment*

For the tumor protection experiment, HLA-A2 (AAD) transgenic C57BL/6 mice (five per group) were immunized via gene gun with 2 μg of pcDNA3 without insert, pcDNA3-CRT/E7 or pcDNA3-CRT/mtE7 (del aa49–57) DNA. Mice were boosted with the same dose three times at 1-week intervals. At 1 week after the last vaccination, mice were subcutaneously challenged with 1×10^5 TC-1/A2 or TC-1 tumor cells in the right leg, and then monitored by inspection and palpation twice a week. The day of the first gene gun vaccination was considered the starting day for counting postoperative survival days. Analysis was performed using SAS version 6.12 (SAS

Institute Inc., Cary, NC, USA). Their percentage of tumor-free mice was analyzed according to Kaplan–Meier methods. Statistical significance was tested using log-rank statistics.

In vitro *T2 peptide-binding assay*

The *in vitro* T2 peptide-binding assay was performed as described previously with a minor modification.[23] Briefly, 1×10^5 T2 cells were pulsed with HPV-16 E7 (aa49–57) peptide (30 μg/ml) or HPV-16 E7 (aa11–20) peptide (30 μg/ml) in serum-free RPMI-1640 medium in a 96-well culture plate for 24 h at 37°C. In another set of experiments, T2 cells were pulsed with 30 μg/ml of HPV-16 E7 (aa49–57) peptide 3 h before they were pulsed with HPV-16 E7 (aa11–20) peptide (30 μg/ml) for 24 h at 37°C. The peptide-pulsed T2 cells were then washed once with FACS wash buffer and stained with anti-human HLA-A2 monoclonal antibody antibody (clone BB7.2, Cat. No. 551285, BD Pharmingen, San Diego, CA, USA), followed by flow cytometry analysis.

In vitro *transfection of DC-1 cells*

DC-1 cells have been shown to have a high transfection efficiency.[24] DC-1 cells were transfected with 4 μg of pcDNA3-HLA-A2 (AAD) and 4 μg of pcDNA3-CRT, CRT/E7, CRT/mtE7 (del aa49–57), CRT/mtE7 (F57R) or CRT/mtE7 (T20V) DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 24 h at 37°C. The expression of HLA-A2 was confirmed by staining with FITC-conjugated anti-HLA-A2 monoclonal antibody (clone BB7.2, Cat. No. 551285, BD Pharmingen, San Diego, CA, USA) (data not shown). The cells were collected 24 h after transfection to incubate with HLA-A2-restricted E7 (aa11–20) peptide-specific CD8+ T-cell lines for 16 h. The activation of E7 (aa11–20) peptide-specific CD8+ T cells was characterized using intracellular cytokine staining for IFN-γ secretion using methods as described above.

Acknowledgements

We thank Drs Keerti V Shah and Robert J Kurman for helpful discussions. Calreticulin cDNA was kindly provided by by Dr Marek Michalak (University of Alberta, Edmonton, Canada). We would also like to thank Drs Ralph Hruban, Ken-Yu Lin and Richard Roden for critical review of the manuscript. We would like to thank Mr Bruno Macaes for the preparation of the manuscript. This work was supported by the SPORE (P50 CA098252-02) of the National Cancer Institute.

References

1 Hung CF, Wu TC. Improving DNA vaccine potency via modification of professional antigen presenting cells. *Curr Opin Mol Ther* 2003; **5**: 20–24.
2 Kim JW, Hung CF, Juang J, He L, Kim TW, Armstrong DK *et al*. Comparison of HPV DNA vaccines employing intracellular targeting strategies. *Gene Therapy* 2004.
3 Ressing ME, Sette A, Brandt RM, Ruppert J, Wentworth PA, Hartman M *et al*. Human CTL epitopes encoded by human papillomavirus type 16 E6 and E7 identified through *in vivo* and *in vitro* immunogenicity studies of HLA-A*0201-binding peptides. *J Immunol* 1995; **154**: 5934–5943.
4 Wentworth PA, Vitiello A, Sidney J, Keogh E, Chesnut RW, Grey H *et al*. Differences and similarities in the A2.1-restricted cytotoxic T cell repertoire in humans and human leukocyte antigen-transgenic mice. *Eur J Immunol* 1996; **26**: 97–101.
5 Newberg MH, Smith DH, Haertel SB, Vining DR, Lacy E, Engelhard VH. Importance of MHC class 1 alpha2 and alpha3 domains in the recognition of self and non-self MHC molecules. *J Immunol* 1996; **156**: 2473–2480.
6 Satoi J, Murata K, Lechmann M, Manickan E, Zhang Z, Wedemeyer H *et al*. Genetic immunization of wild-type and hepatitis C virus transgenic mice reveals a hierarchy of cellular immune response and tolerance induction against hepatitis C virus structural proteins. *J Virol* 2001; **75**: 12121–12127.
7 Murata K, Lechmann M, Qiao M, Gunji T, Alter HJ, Liang TJ. Immunization with hepatitis C virus-like particles protects mice from recombinant hepatitis C virus-vaccinia infection. *Proc Natl Acad Sci USA* 2003; **100**: 6753–6758.
8 Diamond DJ, York J, Sun JY, Wright CL, Forman SJ. Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. *Blood* 1997; **90**: 1751–1767.
9 Feltkamp MC, Smits HL, Vierboom MP, Minnaar RP, de JB, Drijfhout JW *et al*. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol* 1993; **23**: 2242–2249.
10 Vierboom MP, Feltkamp MC, Neisig A, Drijfhout JW, ter Schegget J, Neefjes JJ *et al*. Peptide vaccination with an anchor-replaced CTL epitope protects against human papillomavirus type 16-induced tumors expressing the wild-type epitope. *J Immunother* 1998; **21**: 399–408.
11 Schreurs MW, Scholten KB, Kueter EW, Ruizendaal JJ, Meijer CJ, Hooijberg E. *In vitro* generation and life span extension of human papillomavirus type 16-specific, healthy donor-derived CTL clones. *J Immunol* 2003; **171**: 2912–2921.
12 Lemonnier FA. The utility of H-2 class I knockout mice. *Virus Res* 2002; **82**: 87–90.
13 Barra C, Perarnau B, Gerlinger P, Lemeur M, Gillet A, Gibier P *et al*. Analysis of the HLA-Cw3-specific cytotoxic T lymphocyte response of HLA-B7 X human beta 2m double transgenic mice. *J Immunol* 1989; **143**: 3117–3124.
14 Engelhard VH, Lacy E, Ridge JP. Influenza A-specific, HLA-A2.1-restricted cytotoxic T lymphocytes from HLA-A2.1 transgenic mice recognize fragments of the M1 protein. *J Immunol* 1991; **146**: 1226–1232.
15 Eiben GL, Velders MP, Schreiber H, Cassetti MC, Pullen JK, Smith LR *et al*. Establishment of an HLA-A*0201 human papillomavirus type 16 tumor model to determine the efficacy of vaccination strategies in HLA-A*0201 transgenic mice. *Cancer Res* 2002; **62**: 5792–5799.
16 Bauer M, Wagner H, Lipford GB. HPV type 16 protein E7 HLA-A2 binding peptides are immunogenic but not processed and presented. *Immunol Lett* 2000; **71**: 55–59.
17 Street MD, Doan T, Herd KA, Tindle RW. Limitations of HLA-transgenic mice in presentation of HLA-restricted cytotoxic T-cell epitopes from endogenously processed human papillomavirus type 16 E7 protein. *Immunology* 2002; **106**: 526–536.
18 Cheng WF, Hung CF, Chai CY, Hsu KF, He L, Ling M *et al*. Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. *J Clin Invest* 2001; **108**: 669–678.
19 Chen C-H, Wang T-L, Hung C-F, Yang Y, Young RA, Pardoll DM *et al*. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. *Cancer Res* 2000; **60**: 1035–1042.
20 Shen Z, Reznikoff G, Dranoff G, Rock KL. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J Immunol* 1997; **158**: 2723–2730.

21 Kim TW, Hung CF, Boyd DA, He L, Lin CT, Kaiserman D *et al*. Enhancement of DNA vaccine potency by coadministration of a tumor antigen gene and DNA encoding serine protease inhibitor-6. *Cancer Res* 2004; **64**: 400–405.
22 Lin K-Y, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August T, Pardoll DM *et al*. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996; **56**: 21–26.
23 Drijfhout JW, Brandt RM, D'Amaro J, Kast WM, Melief CJ. Detailed motifs for peptide binding to HLA-A*0201 derived from large random sets of peptides using a cellular binding assay. *Hum Immunol* 1995; **43**: 1–12.
24 Kim TW, Hung CF, Juang J, He L, Hardwick JM, Wu TC. Enhancement of suicidal DNA vaccine potency by delaying suicidal DNA-induced cell death. *Gene Therapy* 2004; **11**: 336–342.