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Ii-Key/HPV16 E7 hybrid peptide immunotherapy for HPV16+ cancers

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ABSTRACT

Activation of antigen-specific CD4+ T cells is critical for vaccine design. We have advanced a novel technology for enhancing activation of antigen-specific CD4+ T helper cells whereby a fragment of the MHC class II-associated invariant chain (Ii-Key) is linked to an MHC class II epitope. An HLA-DR4-restricted HPV16 E7 epitope, HPV16 E7(8–22), was used to create a homologous series of Ii-Key/HPV16 E7 hybrids testing the influence of spacer length on *in vivo* enhancement of HPV16 E7(8–22)-specific CD4+ T lymphocyte responses. HLA-DR4-tg mice were immunized with Ii-Key/HPV16 E7(8–22) hybrids or the epitope-only peptide HPV16 E7(8–22). As measured by IFN-γ ELISPOT assay of splenocytes from immunized mice, one of the Ii-Key/HPV16 E7(8–22) hybrids enhanced epitope-specific CD4+ T cell activation 5-fold compared to the HPV16 E7(8–22) epitope-only peptide. We further demonstrated that enhanced CD4+ T cell activation augments the CTL activity of a H-2D^b-restricted HPV16 E7(49–57) epitope in HLA-DR4+ mice using an *in vivo* CTL assay. Binding assays indicated that the Ii-Key/HPV16 hybrid has increased affinity to HLA-DR4+ cells relative to the epitope-only peptide, which may explain its increased potency. In summary, Ii-Key hybrid modification of the HLA-DR4-restricted HPV16 E7(8–22) MHC class II epitope generates a potent immunotherapeutic peptide vaccine that may have potential for treating HPV16+ cancers in HLA-DR4+ patients.

1. Introduction

Nearly 100% of cervical cancers contain HPV of the high-risk type, HPV 16 or 18 (90% of other ano-genital cancers also contain HPV) [1–3]. Persistent infection of the cervix with these viruses is considered to be the most common cause of cervical cancer. Oral cancer and some neck cancers may be related to HPV infection as well. There are no good therapies when metastasis of cervical cancer occurs and thus effective new therapies are needed urgently. Prophylactic vaccines for HPV infection are approved by the FDA. Gardasil, a virus like particle that targets the L1 protein of HPV types 6, 11, 16 and 18 protects women from infection by HPV close to 100% [4,5]. However, this kind of vaccine is ineffective for therapeutic treatment of HPV+ cancers as it is insufficient to induce both CD4+ and CD8+ T cell immunity which is critical to eradicate HPV+ cancers [6,7].

There is accumulating data suggesting that it is possible to combat cervical and other HPV+ cancers using immunotherapy. Expression of the HPV E6 and E7 genes in HPV+ cancers strongly implicates the oncogenic E6 and E7 proteins in the induction and maintenance of cellular transformation and suggests that they should be good targets for immunotherapy [1–3,6–9]. Several types of therapeutic HPV vaccines are being studied, including: (1) viral vector-based DNA vaccines such as a recombinant adenovirus containing the E6 and/or E7 genes [10]; (2) plasmid-based DNA vaccines containing the E6 and E7 genes [11,12], which induce both a cellular (CTL) and humoral immune response; and, (3) dendritic cell (DC)-based vaccines such as autologous DCs pulsed with E7 or E6 proteins [13]. While potent, the high cost and labor-intensive methods required for every DC vaccination hampers their easy transitioned into the clinic.

Peptide vaccines represent a safe, economical, and convenient form of immunotherapy with the potential for treating HPV+ cancers [14–16]. Past research has focused on CTL epitopes derived from HPV oncogene products, but their inherently weak immunogenicity has hindered their development in the clinic [14–17]. One of the most important steps for improving the efficacy of CTL activity is the inclusion of both MHC class II and class I epitope(s) in one vaccine formulation, as has been demonstrated in both animal studies and human clinical trials [16–18]. It has been clearly established that CD4+ T helper cell lymphocytes play an important role in the activation and maintenance of CD8+ CTL [19–21]. By combining MHC class I epitopes with potent MHC class II epitopes, a more effective vaccine can be developed. Through linking the Ii-Key functional moiety, LRMK, either directly or through a polymethylene linker, to an MHC class II epitope we have developed a novel technology to enhance the activity of MHC class II epitope vaccines [22–26]. Here

Abbreviations: Ii, invariant chain protein; Ii-Key, a peptide from Ii protein; Tg, transgenic.

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we report the identification of a potent Ii-Key/HPV16 E7(8–22) hybrid vaccine in HLA-DR4-tg mice. The Ii-Key/HPV16 E7(8–22) hybrid vaccine is five times more potent in the activation of HPV16 E7(8–22)-specific CD4+ T cells than is the HPV16 E7(8–22) epitope-only peptide in immunized mice. We further show that Ii-Key/HPV hybrid enhanced CD4+ T cell activation in-turn augments the activity of H-2D^b-restricted HPV16 E7(49–57) CTL epitopes as measured by CTL epitope-specific cell killing in HLA-DR4-tg mice. These studies provide evidence that Ii-Key modification of an HPV MHC class II epitope may generate a safe yet potent immunotherapeutic peptide with the potential for treating HPV16+ cancers.

2. Materials and methods

2.1. Mice

DR4-IE-tg mice express HLA-DRA-IE alpha and HLA-DRB1*0401-IE beta chimeric genes consisting of the α1 and β1 binding domains from the human HLA-DRA and HLA-DRB1*0401-IE molecules, respectively, and the remaining domains from the murine I-E^d-α2 and I-E^d-β2 chains, respectively [27]. Female HLA-DR4-IE-tg mice were purchased from Taconic Labs, while C57BL/6 mice were obtained from The Jackson Labs (Bar Harbor, ME). All mice were kept at University of Massachusetts animal facility supervised by IACUC.

2.2. Cell line and antibodies

The HLA-DR4+ human lymphoma cell line, H9 [28], was purchased from ATCC (HIB176). Cells were cultured in RPMI1640 with 10% FBS. Mouse anti-HA tag monoclonal antibody, 12CA5 [29], was purchased from Roche Diagnostic GmbH (Germany). FITC-conjugated anti-mouse IgG monoclonal antibody was purchased from BD Pharmingen.

2.3. Peptides

All peptides, including Ii-Key/epitope hybrids, were synthesized and purified to >95% purity by 21st Century Biochemicals, Inc. (Marlborough, MA). All peptides except the CTL epitope were N-acetylated and C-amidated to inhibit exopeptidases. The peptides were dissolved in sterile water with 20% DMSO and stored at −20 °C.

2.4. CpG ODN

Phosphorothioate-modified CpG ODN 1826, (5′ TCC ATGACGTTC CTG ACGTT 3′, CpG motif underlined) [30] immunostimulatory nucleotide was synthesized and purified by Oligos Etc. (Wilsonville, OR). CpG ODN was dissolved in distilled water at 5 mg/ml as stock solution before use.

2.5. Immunization of mice

HLA-DR4-IE-tg mice were immunized subcutaneously at the base of the tail with the indicated dose of Ii-Key/HPV16 E7(8–22) hybrid, epitope-only peptide, and/or CTL epitope peptides using 28 G needles. In some experiments, peptides were dissolved in water with 20% DMSO, followed by emulsification with an equal volume of complete Freund's adjuvant (CFA). In other experiments with hybrid and CTL epitope co-immunization, peptides with CpG were emulsified with an equal amount of Incomplete Freund's adjuvant (IFA). Three weeks following immunization, splenic lymphocytes were obtained and ELISPOT assays were performed for IFN-γ secretion following *in vitro* stimulation with HPV16 E7(8–22) epitope-only peptide or Ii-Key/HPV16 E7(8–22) hybrids. In some experiments, an *in vivo* CTL [31,32] assay was performed against an H-2D^b-restricted HPV6 E7 CTL epitope (E7 49–57), (RAHYNIVTF) [33].

2.6. ELISPOT assay

ELISPOT kits were purchased from BD Pharmingen and utilized according to manufacturer's instruction. Bulk splenocytes ($0.5–1.0 \times 10^6$ cells/well) were obtained from pooled spleens of immunized animals in each group, followed by stimulation with 2.7 nmol of HPV16 E7(8–22) epitope-only peptide, Ii-Key/HPV16 E7(8–22) hybrids or CTL epitope (HPV6) in 96-well immunospot 200 plates for 42 h. Briefly, plates were coated overnight at 4°C with the cytokine capture antibodies. The plates were blocked with 10% fetal bovine serum (FBS) in RPMI-1640 buffer for 2 h at RT and washed four times with PBS containing 0.05% Tween-20 (wash buffer). Suspensions of splenocytes were added to the cytokine antibody pre-coated plates. After 36 h of culture, the plates were washed five times with wash buffer. Biotinylated cytokine detection antibodies (2 μg/ml) were added for 2 h at RT. The plates were washed four times with wash buffer and avidin horseradish peroxidase-conjugated (avidin-HRP) was added for 1 h at 1:100 dilution. Avidin-HRP was removed by washing four times with wash buffer and two times with PBS. The spots were developed with HRP-3-amino-9-ethylcarbozole substrate (BD Pharmingen) for 30 min at RT. The plates were washed twice with sterile water and dried for 1–2 h at RT. Immunospots were counted using an AID ELISPOT reader (Autoimmune Diagnosticka, Strassberg, Germany).

2.7. In vivo CTL assay

The *in vivo* CTL assay was performed according to established methods [31,32]. Briefly, splenocytes from naïve HLA-DR4-tg mice were labeled with 0.3 μM CFSE (CFSE^{high}) and 0.0325 μM CFSE (CFSE^{low}) in PBS at 37 °C for 15 min. Labeling was stopped by washing cells in complete medium. Cells (CFSE^{high}) were then pulsed with 50 μM HPV16 E7(49–57) CTL epitope in complete medium at 37 °C for 45 min, followed by 4× wash with PBS/0.1%FBS. Pulsed CFSE^{high} cells were then mixed with an equal number of un-pulsed CFSE^{low} cells and re-suspended in PBS at 4×10^7/ml. Mice were i.v. injected with 10^7 mixed cells/mouse and sacrificed 18–20 h after injection. Splenic cells were analyzed by flowcytometry to establish the ratio of CFSE^{low}:CFSE^{high} cells. The CFSE^{low}:CFSE^{high} ratios of experimental groups were first normalized by the ratio of the control groups. The CTL epitope-specific killing was then calculated using the formula 1-CFSE^{low}/CFSE^{high}.

2.8. Peptide binding to HLA-DR4+ H9 lymphoma cells

H9 cell were cultured in RPMI 1640 with 10% FBS (Hyclone) for binding studies. Specifically, 1×10^6 H9 cells were washed and re-suspended in 300 μl of culture medium containing the indicated concentration of peptides HPV7 (LHEYMLDLQPETTDLggYPYD-VPDYA) and HPV17 (LRMK-*ava*-EYMLDLQPETTDLggYPYDVPDYA), respectively. HPV7 is HPV1 extended in the C-terminus with a two-glycine spacer (small letters) and an HA tag, (YPYDVPDYA), which is recognized by the monoclonal antibody 12CA5 [29]. HPV 17 is HPV4 extended at the C-terminus with two-glutamine spacers and an HA tag. The cell and peptide mixture was then incubated at 37 °C for 1 h, followed by 3X wash with PBS. Mouse anti-HA tag monoclonal antibody (12CA5) was then added for 45 min on ice, with a 2× wash thereafter. FITC-conjugated anti-mouse IgG monoclonal antibody was added and incubated for an additional 45 min on ice prior to analysis by flowcytometry.

Table 1
HPV16 E7 epitope peptides used for this study. The HPV sequence used for algorithm prediction of high-scoring MHC class II epitopes was AAD33253 (GenBank/PubMed). HPV1 is the parent epitope-only sequence HPV16 E7(8–22) in which Y at position 11 is in pocket 1 of HLA-DR4 molecules, according to algorithm prediction. The Ii-Key core motif sequence LRMK was linked to different amino acids neighboring the predicted HPV16 E7(8–22) epitope to test the influence of spacer length between LRMK and the epitope on activity. The Ii-Key/epitope hybrids are represented by peptides HPV2–5. HPV6 is an HLA-A2-restricted CTL epitope embedded within HPV16 E7(8–22) [14,15]. HPV7 is HPV1 extended at the C-terminus with two-glycines (small letters) as a spacer followed by an HA tag, (YPYDVPDYA), which is recognized by the monoclonal antibody 12CA5 [29]. Similarly, HPV17 is HPV4 extended at the C-terminus with a two-glycine spacer (small letters) and an HA tag. HPV11 is an H-2D^b-restricted CTL epitope from HPV16 E7 protein at position of 49–57 [33].

Peptide	Position in HPV16 E7HPV16 E7	Sequence
HPV1	8–22	Ac-LHEYMLDLQPETTDL-NH_2
HPV2	8–22	Ac-LRMK-ava-LHEYMLDLQPETTDL-NH_2
HPV3	9–22	Ac-LRMK-ava-HEYMLDLQPETTDL-NH_2
HPV4	10–22	Ac-LRMK-ava-EYMLDLQPETTDL-NH_2
HPV5	11–22	Ac-LRMK-ava-YMLDLQPETTDL-NH_2
HPV6	11–20	Ac-YMLDLQPETT-NH_2
HPV7	8–22	Ac-LHEYMLDLQPETTDL ggYPYDVPDYA-NH_2
HPV17	10–22	Ac-LRMK-ava-EYMLDLQPETTDL ggYPYDVPDYA-NH_2
HPV11	49–57	RAHYNIVTF

2.9. Statistic analysis

A one-way ANOVA of GraphPad PRISM 4 software was used to analyze our data (to compare three or more sets of data grouped by a single factor or category).

3. Results

3.1. Prediction of HLA-DR4-restricted epitopes and design of Ii-Key/HPV16 E7(8–22) hybrids

Computer algorithms (*e.g.*, www.syfpeithi.de/scripts/MHCServer.dll/home.htm) [34] were used to analyze possible HLA-DR-restricted epitopes in the HPV16 E7 sequence (AAD33253 from GenBank/PubMed). Using this algorithm, it was determined that the peptide represented by amino acid residues 8–22 of the HPV16 E7 protein contained a possible HLA-DR4-restricted epitope (HPV1, see Table 1). Using this algorithm-predicted sequence we designed a series of Ii-Key/HPV16 E7 hybrids in which the Ii-Key core motif, LRMK, was linked to different amino acid residues of the HPV16 E7(8–22) at the N-terminus via a flexible polymethylene spacer *ava* (5-aminovaleric acid = 5-aminopentanoic acid). In an earlier publication [23], we comprehensively studied the influence of spacer length between the Ii-Key moiety and epitope peptide on the immune response to the hybrid, showing that a single-ava-linker was optimal. In the current study, we addressed this issue in a more fine-tuned manner by modifying the spacer distance by single amino acid increments: LRMK-ava was linked to amino acid residues located at amino acids −3, −2, −1, and 1 relative to the p1 site (Table 1).

3.2. Identification of the most potent Ii-Key/HPV16 E7(8–22) hybrid in HLA-DR4-tg mice

Ii-Key/epitope hybrids were initially tested *in vivo* for their potency in activating CD4+ T helper cells following immunization with the peptides listed in Table 1. Three weeks after immunization, mice were sacrificed and splenocytes were tested for IFN-γ secretion after *in vitro* re-stimulation using the HPV16 E7(8–22) epitope-only peptide or Ii-Key/HPV16 E7(8–22) hybrids. From Fig. 1, one can observe that splenocytes from HPV4 (LRMK-*ava*-EYMLDLQPETTDL) immunized mice exhibited the highest frequency of IFN-γ secreting T cells following *in vitro* stimulation with HPV16 E7(8–22). Specifically, splenocytes from HPV4 immunized mice contained approximately 5-fold more IFN-γ secreting cells compared to HPV1 (epitope-only peptide) immunized mice. While more potent than the epitope-only peptide HPV1, hybrids HPV2 and HPV3 had less activity than HPV4, suggesting that the linker space between the Ii-Key moiety and epitope was suboptimal. Hybrid HPV5 hybrid gave less activity than HPV1, suggesting the amino acid residue glutamic acid (E) is either an important part of the epitope or contributes optimally to the spacer distance between LRMK and the epitope. The pattern of immunization potency of HPV peptides is similar, whether the epitope-only peptide HPV1 or Ii-Key/HPV hybrid HPV4 is used for *in vitro* restimulation (Fig. 1). Restimulation with hybrids HPV2, 3, and 5 resulted in a similar pattern and sensitivity of detection of IFN-γ secreting cells (data not shown) as did HPV4. HPV6 (HLA-A2-restricted CTL epitope and core sequence for HLA-DR4 as predicted by computer algorithms) had neither immunization nor restimulation activities, suggesting that the flanking sequences were required for activity of the HLA-DR4 epitope. In non-transgenic C57BL/6 mice, the background strain for HLA-DR4-IE-tg mice, HPV4 did not increase the frequency of IFN-γ secreting splenocytes (data not shown).

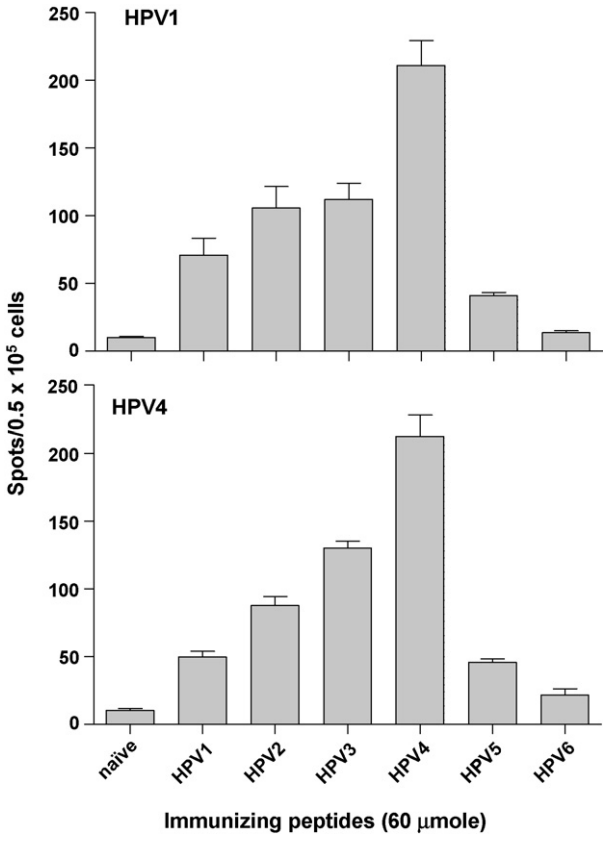


Fig. 1. Determine the most active HPV hybrid in HLA-DR4-tg mice. HLA-DR4-tg mice (3 mice/group) were immunized with 60 nmol of the indicated peptide in an equal volume of CFA (100 μl/mouse total volume). Three weeks after immunization, pooled splenocytes were obtained for IFN-γ ELISPOT following *in vitro* stimulation with the epitope-only peptide HPV1 or Ii-Key/epitope hybrids (HPV1–6, only the results of HPV1 (top) and HPV4 (bottom) are shown). The frequency of IFN-γ secreting cells in splenocytes from HPV4 immunized mice was approximately 5-fold greater than the frequency in splenocytes from HPV1 immunized mice. Each bar represents the mean ± SD from pooled splenocytes from three experimental wells. HPV4 vs HPV1: $p < 0.001$. HPV4 was also significantly more potent than HPV2 and HPV3 ($p < 0.001$), respectively. Comparable results were obtained from a repeat study.

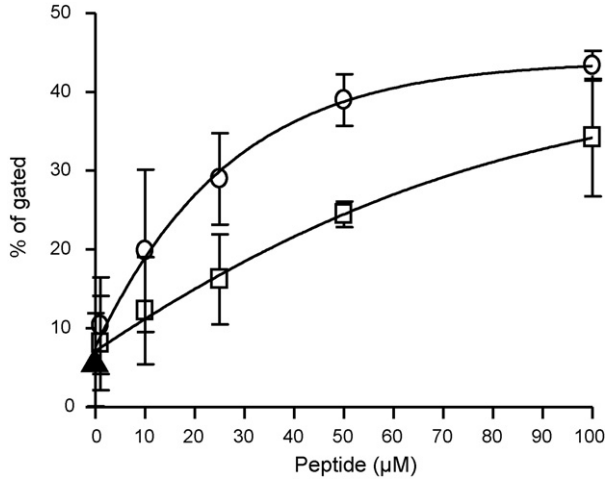


Fig. 2. Comparison of binding of Ii-Key/HPV16 E7 epitope hybrid vs HPV16 E7 epitope-only peptide. One million HLA-DR4+ H9 cells were incubated with increasing concentrations of the HPV1-HA tagged peptide HPV7 (□), or the HPV4-HA tagged peptide HPV17 (○), or no peptide (▲). After antibody staining and analysis by flow-cytometry, the percentage of positively gated cells was plotted against the peptide concentration. The gate was set such that ~95% of cells untreated with peptide fell outside of the gate. The data was from two independent experiments. The binding of HPV17 was significantly ($p < 0.0001$) higher than that of HPV7 over the range of concentrations tested.

3.3. Linkage of the Ii-Key motif to HLA-DR4 epitope increases the binding of that epitope to HLA-DR4 molecules

In order to reveal possible mechanisms for the increased potency of the HPV4 hybrid peptide, we determined the binding affinity of HPV1 compared to HPV4 in HLA-DR4+ H9 lymphoma cells. We synthesized two peptides (HPV7 and HPV17) linked to an HA tag, allowing us to monitor binding using an anti-HA monoclonal antibody. Specifically, HPV7 consisted of HPV1 extended at the C-terminus with a two-glycine residue spacer and an HA tag (YPYDVPDYA), while HPV 17 represented HPV4 similarly extended at the C-terminus with a two-glycine residue spacer and an HA tag. Different concentrations of the two peptides were first incubated with 1×10^6 HLA-DR4+ human lymphoma cells (H9 cells) for 1 h in culture medium. The binding efficiency was monitored by staining H9 cells with an anti-HA tag monoclonal antibody followed by a FITC-labeled secondary antibody. HPV17 was found to bind to HLA-DR4 molecules significantly better than HPV7 at the concentrations we tested (Fig. 2).

3.4. Ii-Key/HPV hybrid (HPV4) enhances the potency of a co-immunized HPV CTL epitope in a dose-dependent manner

Based on prior literature, we hypothesized that increased CD4+ T cell activity would induce a more robust CTL response following co-immunization of HPV4 and the H-2D^b-restricted CTL epitope HPV11 (HPV16 E7(49–57) (RAHYNIVTF) [33]. HLA-DR4-tg mice were immunized with HPV11 and varying concentrations of HPV4. The *in vivo* CTL assay demonstrated that the CTL epitope HPV16 E7(49–57) is immunogenic in HLA-DR4-tg mice to cause epitope-specific cell killing. When co-immunized with the HPV4 hybrid, the activity of HPV16 E7(49–57) was significantly enhanced in a dose-dependent manner. Maximum enhancement of HPV11-specific killing was observed in animals dosed with 30–60 nmol of HPV4 (Fig. 3). ELISPOT data suggests that the augmentation of CTL epitope activity is proportional to HPV4 activity (data not shown), indicating that MHC class II induced T helper activity is critical for the induction of CTL activity by peptide vaccine.

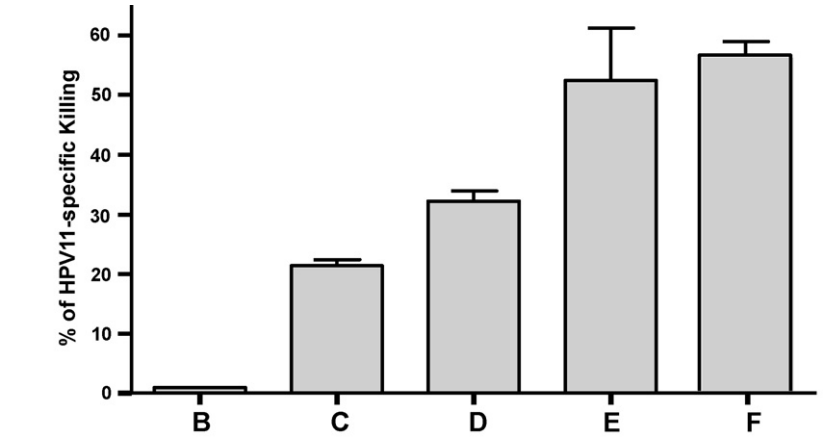
3.5. Ii-Key hybrid is more potent than the parent epitope-only peptide in augmenting the activity of a co-immunized HPV CTL epitope

Lastly, we wanted to test whether the Ii-Key hybrid HPV4 was more potent in enhancing the activity of the HPV CTL epitope than is the epitope-only HPV peptide (HPV1). HLA-DR4-tg mice were immunized with either the HPV11 (HPV16 E7(49–57)) CTL epitope peptide alone, the HPV11 CTL epitope with 30 nmol HPV1, or the HPV11 CTL epitope with 30 nmol HPV4. *In vivo* CTL analysis shows that HPV4 is more potent compared to HPV1 in enhancing the activity of the co-immunized CTL epitope. Specifically, the level of HPV11-specific cell killing is doubled in mice co-immunized with HPV4 and HPV11 compared to those co-immunized with HPV1 and HPV11 (Fig. 4).

4. Discussion

In this study we identified an active HLA-DR4-restricted epitope from the HPV16 E7 oncogene product, HPV16 E7(8–22) and showed that its vaccine potency could be significantly increased by linkage to the Ii-Key motif of the MHC class II invariant chain (Ii protein). In HLA-DR4-tg mice, one Ii-Key/HPV16 E7(8–22) hybrid (HPV4) is approximately 5-fold more potent than is the HPV16 E7(8–22) epitope-only peptide. Our results confirm previous studies showing that Ii-Key augments the activity of linked MHC class II epitopes [22–26]. We believe that IFN-γ was secreted by CD4+ Th lymphocytes because: (1) the embedded HPV6 (HLA-A2-restricted CTL epitope) stimulation gave no IFN-γ secretion in splenocytes from immunized mice (data not shown), suggesting that CD8+ T cells were not the source of IFN-γ; and, (2) HPV4 gave no enhanced activity in C57BL/6 mice, the background mice for HLA-DR4-tg mice (data not shown), indicating that the enhanced IFN-γ secretion is due to HLA-DR4-presentation of HPV4 to CD4+ T helper cells. These observations support prior studies demonstrating that isolated CD4+ T cells were indeed the major source of IFN-γ in cells from Ii-Key/epitope hybrid immunized mice [26]. HPV1, HPV4 and the other HPV hybrids gave similar results when used for *in vitro* stimulation (Fig. 1 and data not shown), indicating that the immune response was directed against the HPV epitope and not the LRMK moiety.

Co-immunization of the Ii-Key/HPV epitope hybrid together with a CTL epitope confirmed the concept that CD4+ T lymphocyte induction is important for the induction and maintenance of CTL activity in peptide vaccine design [19,20,35]. CD4+ cells play multiple roles in helping the activation of CD8+ CTLs, including the regulation of expression of crucial genes involved in CD8+ T cell memory and sensitivity to regulatory elements [36]. The importance of HPV-specific CD4+ T lymphocytes has been observed in a clinical study in which investigators found that a high percentage of HPV18 E6 epitope-specific CD4+ T lymphocytes correlated with favorable clinical outcome [37]. We have shown that both the intrinsic potency and dose of a MHC class II epitope correlates with specific *in vivo* cell killing elicited by a co-immunized CTL epitope (Figs. 3 and 4). In order to induce hybrid-specific Th1 activity which in turn would augment the CTL activity; we chose CpG as adjuvant for these experiments. Further, CpG is approved for human use (CFA is not). In addition, CFA contains bacterial proteins that may induce non-specific T helper cell activity which could obscure the antigen-specific help of CTL activity by the Ii-Key hybrid. The observation that linkage to Ii-Key greatly enhances the antigen-specific activity of HLA-DR-restricted epitopes [22–26] offers several advantages in peptide vaccine design: (1) the increased antigen-specific activity of Ii-Key/epitope hybrids may allow for less peptide and/or adjuvant to be used, reducing potential toxicity; (2) the generation of



	B	C	D	E	F
CpG	20 μg	20 μg	20 μg	20 μg	20 μg
HPV11	0	60 nmole	60 nmole	60 nmole	60 nmole
HPV4	0	0	15 nmole	30 nmole	60 nmole

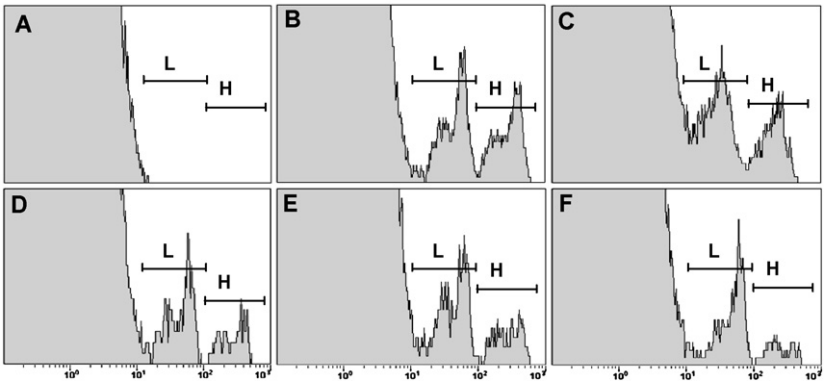
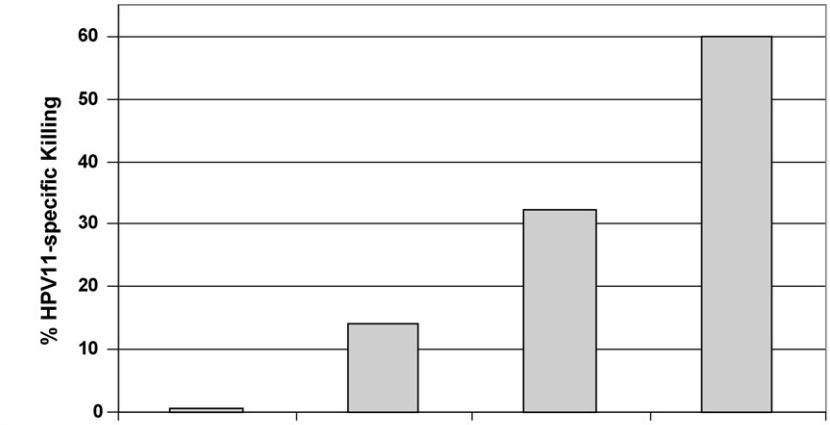


Fig. 3. Dose-dependent help by HPV4 in augmenting CTL activity towards cells presenting a MHC class I epitope. The mean ± SD of HPV 11-specific cell killing is shown in the top of the figure with data from two experiments (2 mice/group and 3 mice/group, respectively). HLA-DR4-tg mice were immunized with: no peptide (B); 60 nmol of HPV11 (HPV16 E7(49–57) an H-2D^b-restricted CTL epitope) (C); 60 nmol of HPV11 plus 15 nmol HPV4 hybrid (D); 60 nmol of HPV11 plus 30 nmol HPV4 hybrid (E); or 60 nmol of HPV11 plus 60 nmol HPV4 hybrid (F). Peptides were mixed with 20 μg of CpG and then emulsified in an equal volume of IFA in a total volume of 150 μl/mouse. Three weeks after immunization, HPV11-specific CTL activity was measured with an *in vivo* assay specified in Section 2. The top figure indicates the percentage of HPV11-specific CTL killing as determined by the ratio of CFSE^{low} to CFSE^{high} cells using the formula 1 − CFSE^{low}/CFSE^{high} following flowcytometry. The CFSE^{low}:CFSE^{high} ratios of experimental groups were first normalized by the ratio of the control groups. Groups 4 and 5 had similar activity to each other ($p > 0.05$) but were significantly more active than group 2 (both of $p < 0.001$ for groups 4 and 5 each) and group 3 ($p < 0.05$ and 0.01 for groups 4 and 5, respectively). The histograms for each group in one experiment (B–F) are shown below. The data shown are from pooled splenocytes (3 mice/group). The histogram of naïve splenocytes is shown in (A).

potent HLA-DR-restricted epitope hybrids from intermediate affinity, promiscuous HLA-DR-restricted epitopes may generate more potent yet still promiscuous HLA-DR hybrids; and, (3) increased peptide vaccine potency by optimal generation of an antigen-specific CD4+ and CD8+ immune response. Evidence for both the safety and potency of an Ii-Key hybrid in the clinic has been provided by a recent Phase I trial of an Ii-Key/HER2 hybrid in breast cancer patients [38]. In that study, the Ii-Key/epitope hybrid proved safe and well tolerated over a dose range from 100 μg to 1000 μg given at monthly intervals for 6 months. Of particular interest was the observation that the Ii-Key/epitope hybrid vaccine was able to generate a specific immune response in the absence of adjuvant when used at the highest dose [38]. Another important reason for the induction of strong antigen-specific CD4+ T helper lymphocytes in peptide vaccine design is that CD4+ T cells may have antigen-specific cytotoxic function in addition to their helper function [39]. The pan DR epitope PADRE has been used in both pre-clinical and clinical studies to augment the CTL activity in HPV peptide vaccine with some limited success [16,17]. In spite of generating active immune response to PADRE itself, increased CTL activity was not observed [16]. In another study by Kennedy and Celis [40] it was shown that the addition of an antigen-specific MHC class II epitope enhanced the survival rate in a murine tumor model, while the addition of an irrelevant MHC class II epitope (PADRE) did not.

Binding studies provide further evidence that Ii-Key/epitope hybrids interact more efficiently with HLA-DR4 molecules than do the epitope-only peptides. This is consistent with our prior hypothesis that the Ii-Key portion of an Ii-Key/epitope hybrid induces a conformational change of the HLA-DR molecule, allowing for increased MHC class II epitope accessibility. This potential mechanism of action was suggested by studies in which a series of related Ii-Key peptides were used to demonstrate that Ii-Key peptides bind to an allosteric site of HLA-DR1 molecules to enhance charging of an MHC class II epitope, hMBP(90–102), by the induction of a conformational change [22]. More recently, Chou et al. showed that Ii-Key functions similarly to HLA-DM in facilitating loading of an MHC class II epitope from hemagglutinin, HA(305–318), to HLA-DR1 molecules [41]. It is known that the interaction of DM with MHC class II molecules involves a conformational change [42,43]. Kinetic studies demonstrated that Ii-Key peptides act similar to DM in: (a) enhancing peptide binding to HLA-DR1, (b) dissociation of peptide-DR1 complexes, and (c) in maintaining a receptive



	A	B	C	D
CpG	20 μg	20 μg	20 μg	20 μg
HPV11	0	60 nmole	60 nmole	60 nmole
HPV1	0	0	30 nmole	0
HPV4	0	0	0	30 nmole

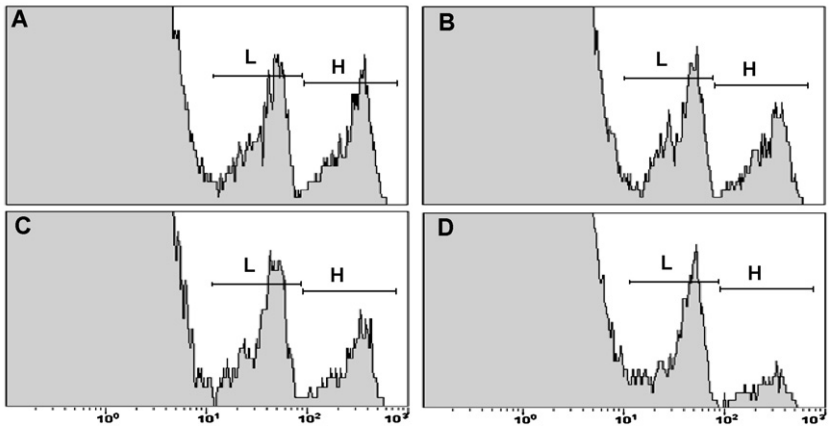


Fig. 4. Comparison of the activity of the Ii-Key/HPV epitope hybrid HPV4 and the epitope-only peptide HPV1 in helping specific CTL activity *in vivo*. HLA-DR4-tg mice were immunized with no peptide (A); 60 nmol of HPV11 (HPV16 E7(49–57), an H-2D^b-restricted CTL epitope (B); 30 nmol HPV1 plus 60 nmol HPV11 (C); or 30 nmol HPV4 plus 60 nmol HPV11. The peptides were administered as indicated for Fig. 3. Three weeks after immunization, HPV11-specific CTL activity was measured in an *in vivo* assay (see Fig. 3). The top figure indicates the percentage of HPV11-specific CTL killing. The histograms for each group are shown below. The data shown are from pooled splenocytes (3 mice for group (A) and 4 mice for groups (B–D), respectively).

conformation of empty DR1 [41]. Together, these studies strongly suggest that the Ii-Key motif in Ii-Key/MHC class II epitope hybrids may serve a similar function to DM in enhancing the potency of MHC class II epitopes. Further, this simple method for increasing the antigen-specific stimulation of T helper cells offers a practical method for generating safer yet more potent peptide vaccines for tumors and infectious diseases.

Ii-Key/HPV hybrid vaccine peptides offer the potential to serve as an effective immunotherapeutic treatment for HPV induced cancers. Such a vaccine would incorporate a potent Ii-Key hybrid from an HPV oncogene product as well as a CTL epitope(s) from the same virus. Several such HLA-A2-restricted CTL epitopes have been defined, some of which have been tested in the clinic with limited efficacy [14–17]. Our results clearly show that co-administration of an HLA-DR4-restricted Ii-Key/HPV hybrid with a CTL epitope significantly enhances specific cell killing. While roughly 20% of cervical cancer patients are HLA-DRB1*0401-positive [44], there is clearly the need to identify additional MHC class II epitopes with different HLA restrictions. In addition, screening PBMC from patients with HPV induced cancers may reveal allelic restrictions of HPV4 other than HLA-DRB1*0401. Prior studies using PBMC from patients with Her-2/neu positive cancers frequently have pre-existent CD4+ T helper cell responses to Ii-Key modified MHC class II epitopes [45]. Ideally, one would like a combination of Ii-Key/HPV hybrid peptides to cover 80–90% of cervical cancer patients. The higher antigen-specific potency of Ii-Key/epitope hybrids offers several advantages in designing a combined MHC class I/II epitope peptide vaccine as indicated above. The goal of all cancer therapy, be it immunotherapeutic or cytotoxic, has always been to maximize anti-cancer specificity; in this regard Ii-Key/epitope hybrids represent an important advance. The use of Ii-Key/HPV HLA-DR-restricted hybrid peptides plus HPV CTL epitope(s) could yield a safe and potent immunotherapeutic vaccine for HPV+ cancers, including cervical, skin and neck cancers [14–18,46]. Such a vaccine may be especially useful in the treatment of early stage patients [47].

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