The Journal of Immunology

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J Immunol 2002; 169:350-358; ;
doi: 10.4049/jimmunol.169.1.350
http://www.jimmunol.org/content/169/1/350

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Established Human Papillomavirus Type 16-Expressing Tumors Are Effectively Eradicated Following Vaccination with Long Peptides[1]

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Peptide-based vaccines aimed at the induction of effective T cell responses against established cancers have so far only met with limited clinical success and clearly need to be improved. In a preclinical model of human papillomavirus (HPV)16-induced cervical cancer we show that prime-boost vaccinations with the HPV16-derived 35 amino-acid long peptide $E7^{43-77}$, containing both a CTL epitope and a Th epitope, resulted in the induction of far more robust E7-specific $CD8^+$ T cell responses than vaccinations with the minimal CTL epitope only. We demonstrate that two distinct mechanisms are responsible for this effect. First, vaccinations with the long peptide lead to the generation of E7-specific $CD4^+$ Th cells. The level of the induced E7-specific $CD8^+$ T cell response proved to be dependent on the interactions of these Th cells with professional APC. Second, we demonstrate that vaccination with the long peptide and dendritic cell-activating agents resulted in a superior induction of E7-specific $CD8^+$ T cells, even when T cell help was excluded. This suggests that, due to its size, the long peptide was preferably endocytosed, processed, and presented by professional APCs. Moreover, the efficacy of this superior HPV-specific T cell induction was demonstrated in therapeutic prime-boost vaccinations in which the long peptide admixed with the dendritic cell-activating adjuvant oligodeoxynucleotide-CpG resulted in the eradication of large, established HPV16-expressing tumors. Because the vaccine types used in this study are easy to prepare under good manufacturing practice conditions and are safe to administer to humans, these data provide important information for future clinical trials. ***The Journal of Immunology*****, 2002, 169: 350–358.**

Oncogenic, so-called high-risk types of human papillomavirus (HPV),[4] such as HPV16, are the main causative factor in the pathogenesis of cervical carcinomas (1). The majority of these cancers expresses the HPV16-derived E6 and E7 oncoproteins (2), which are therefore attractive targets for T cell-mediated immunotherapy (3). Several observations, such as the spontaneous regression of most premalignant cervical lesions (4–6) and the infiltration of $CD4^+$ T cells and macrophages in regressing genital warts (7, 8), suggest that therapeutic vaccination may prevent progression from oncogenic HPV infection through cervical intraepithelial neoplasia to cervical cancer. This notion is supported by the fact that an increase in HPV infection was found in immunosuppressed (9) and immunodeficient (10) patients.

Over the years, various vaccines containing exactly fitting MHC class I-binding peptides have been tested for their therapeutic efficacy against both virally and nonvirally induced cancers (11, 12). However, the observed immunological and clinical responses were rather meager. Therefore, ways to develop more powerful cancer vaccines should be thoroughly explored. In a preclinical model we previously achieved considerable success when mice were vaccinated with a H-$2D^b$-restricted HPV16 E7 CTL epitope ($E7^{49-57}$, RAHYNIVTF) emulsified in IFA (13). Immunized mice developed CTL-mediated protection against a subsequent challenge with an otherwise lethal dose of HPV16 E7-expressing tumor cells. Based on these encouraging findings several HPV peptide-based vaccines comprising minimal CTL epitopes were tested in patients with progressive disease (12, 14, 15). Although moderate T cell responses were observed, the clinical benefit was only modest. This may be due to an impaired immune system, which is commonly found in patients diagnosed with cancer, but failure of the vaccines to induce strong sustained immunity is more likely to be the cause. Recent preclinical data demonstrated that tumor-specific $CD4^+$ Th cells critically contribute to the development and efficacy of antitumor responses (16–20). The effectiveness of these Th cells probably lies in their capacity to deliver essential activation signals to professional APCs, such as dendritic cells (DC), needed for an optimal priming of tumor-specific CTL (21–24). In addition, Ag-specific $CD4^+$ T cells may provide CTL with essential growth stimuli during the effector phase (25). In line with this notion, several studies show that effective CTL priming can be induced by the inclusion of Th epitopes in peptide vaccines (26–29). Another strategy to power up vaccines is the use of molecularly defined strong DC-activating adjuvants such as oligodeoxynucleotide (ODN)-CpG (30–32), monophosphoryl lipid A (MPL) (33, 34), anti-CD40 Ab (35), and GM-CSF (36).

We have recently developed a clinical grade vaccine against HPV16 that consists of 32- to 35-aa-long overlapping peptides, together covering the HPV16 E6 and E7 protein sequences. The

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Received for publication February 27, 2002. Accepted for publication April 29, 2002.

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[1] This work was supported by grants from the Dutch Organization for Scientific Research (Grant 920-03-094 to S.Z.), the Cancer Research Institute (to S.H.v.d.B.), the Dutch Cancer Society (RUL 99-2024 and 2000-2200 to R.O. and C.J.M.M.), and the European Community (Grant QLK2-CT-2000-00470).

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[4] Abbreviations used in this paper: HPV, human papillomavirus; DC, dendritic cell; MPL, monophosphoryl lipid A; ODN, oligodeoxynucleotide; CD40L, CD40 ligand.

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design of these long peptides was based on the presence of Th and CTL epitopes (3, 37–39), but we also speculated that, due to the length of the peptides, direct binding to MHC class I molecules would not take place. As a consequence, endocytosis, processing, and presentation by professional APC would be required to induce HPV16-specific CTL, and this would circumvent the potential hazard of peptide-induced tolerance (40).

In a preclinical mouse tumor model we analyzed the T cell response to one of these 35-aa-long peptides, containing both a HPV16 E7-derived Th epitope and the HPV16 $E7^{49–57}$ CTL epitope. As demonstrated earlier (26), the physical linkage between these two epitope types may potentiate specific CTL responses. Vaccination with the long peptide resulted in markedly improved CTL responses in comparison to the minimal essential CTL epitope vaccine. In-depth analysis revealed that two distinct mechanisms were responsible. Moreover, vaccination with the long peptide and the DC-activating agent ODN-CpG induced strong antitumor immunity that eradicated established tumors with sizes that otherwise resulted in the death of mice within ~5 days.

Materials and Methods

Mice and cell lines

C57BL/6 (B6, H-2^b) mice were obtained from IFFA Credo (Paris, France). MHC class $II^{-/-}$ B6 mice were purchased from Taconic Farms (Germantown, NY) and $CD40^{-/-}$ B6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Tumor cell line TC-1 was generated by retroviral transduction of lung fibroblasts of C57BL/6 origin by HPV16 E6/E7 and c-H-*ras* oncogenes (41). Tumor cell line 13.2 was derived from MEC (B6) transformed with adenovirus type 5-derived E1 protein in which the H-$2D^b$ E1A epitope was replaced with the HPV16 $E7^{49–57}$ CTL epitope. Isolation and culture of the HPV16 E7-specific CTL clone 9.5, the p53-specific CTL clone 1H11, and the nonrelevant 9.42 CTL clone have been described elsewhere (42–44). D1 cells are long-term growth factor-dependent immature splenic DC derived from C57BL/6 mice and were cultured as described (45, 46).

Peptides

Peptides were generated as described before (13). The purity of the peptides was determined by reverse-phase HPLC and was found to be routinely >90% pure. Peptides were dissolved in 0.5% DMSO in PBS and, if not used immediately, stored at −20°C. The nomenclature refers to the numbers of the first and last amino acid. The HPV16-derived peptide $E7^{49–57}$ (RAHYNIVTF) was selected because it was identified as an H-$2D^b$ CTL epitope (13). The $E7^{43–77}$ 35-residue long peptide (GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR), which represents one of the peptides of a good manufacturing practice-grade vaccine against HPV16-induced tumors currently tested in a clinical trial, contains the $E7^{49–57}$ CTL epitope with its natural flanking sequences. (CTL epitope underlined). HPLC and mass spectrometry demonstrated that no small fragments representing the minimal CTL epitope were present in this peptide lot. The DRAHYNIVTF sequence, which is encompassed in this peptide, represents a Th epitope (47).

Tetramers and Abs

PE-labeled H-$2D^b$ epitope $E7^{49–57}$ (RAHYNIVTF)-containing tetramers were constructed and used for the analysis of peptide-specific CTL immunity as described earlier (48). FITC-labeled anti-CD8b.2 Ab (Ly-3.2; clone 53-5.8), allophycocyanin-labeled anti-CD4 Ab (L3T4; clone RM4-5), and PE-labeled anti-IFN-γ Ab (clone XMG1.2) (BD PharMingen, San Diego, CA) were used in the various FACS procedures.

Adjuvants

IFA was obtained from Difco (Detroit, MI). Montanide ISA 51 was purchased from SEPPIC (Paris, France). CpG-ODN 1826, sequence TTCAT GACGTTCCTGACGTT, was provided by Coley Pharmaceutical (Langenfeld, Germany) and used at a their suggested optimal working concentration of 50 μg/mouse. GM-CSF was obtained from PeproTech (Rocky Hill, NJ) and used at a concentration of 4 μg/mouse based on the work of Disis et al. (36). The FGK-45 hybridoma cells producing stimulatory anti-CD40 Ab were provided by Dr. A. Rolink (University of Basel, Basel, Switzerland). MPL was provided by Corixa (Seattle, WA) and used at the suggested optimal concentration of 10 μg/mouse.

Immunization strategies

C57BL/6 mice were injected s.c. with either 50 μg $E7^{49–57}$ short peptide or 150 μg $E7^{43–77}$ 35-residue long peptide dissolved in PBS to achieve similar molar levels of the $E7^{49–57}$ CTL epitope in both cases. Combinations with various adjuvants were tested. In the case of IFA and Montanide, the dissolved peptides were emulsified in 50% of these respective substances. ODN-CpG, MPL, and GM-CSF were all dissolved in PBS and mixed with the peptides before s.c. vaccination. The total injected volume was 200 μl/mouse. Anti-CD40 Ab was dissolved in PBS and injected separately from the peptides i.v. on days 0, 1, and 2 at an amount of 100 μg per injection (a total volume of 200 μl/mouse) as described before (35). Spleens were harvested after 10 days. In the prime-boost experiments with IFA, mice were boosted with identical vaccines 50 days after priming to allow the formation of memory CTL and Th cells (49). In these experiments spleens were harvested 10 days after the booster immunization. In the therapeutic antitumor experiments, tumor-bearing mice were vaccinated at the time tumors were palpable in all mice and 14 days later, considering the aggressive outgrowth of TC-1 tumors in untreated mice.

T cell cultures

T cells were obtained from immunized mice by culturing spleen cells (5×10^6 cells/well of a 24-well plate) in complete medium in the presence of 0.5×10^6 $E7^{49–57}$-expressing cells (tumor cell line 13.2) or, when indicated, in the presence of D1 cells. Before use, the D1 cells were incubated for 16 h with the $E7^{43–77}$ long peptide and subsequently activated by adding LPS (10 μg/ml) for 6 h and then thoroughly washed. Complete medium consists of IMDM (BioWhittaker, Walkersville, MD) supplemented with 8% FCS, 100 IU/ml penicillin, 2 mM glutamine (ICN, Aurora, OH), and 30 μM 2-ME (Merck, Darmstadt, Germany). Cultures were maintained at 37°C in humidified air containing 5% CO_2. No exogenous IL-2 was added. On day 6, dead cells were removed from the culture by centrifugation over a Ficoll density gradient and remaining cells were seeded in 24-well plates at 1×10^6 cells/well. On day 7 tetramer staining or intracellular cytokine staining was performed.

Tetramer staining

Spleen cultures, stimulated with tumor cell line 13.2 for 7 days, were transferred at 40×10^4 cells per well to 96-well V-bottom microtiter plates and washed twice with PBS/BSA 0.5%. Subsequently, PE-labeled $E7^{49–57}$-containing tetramer was added. After 30 min of incubation at room temperature, cells were washed twice with PBS/BSA 0.5% and incubated with FITC-labeled anti-CD8b Ab for 30 min. Subsequently cells were washed twice in PBS/BSA 0.5%, suspended in PBS/BSA 0.5% containing propidium iodide (0.5 μg/ml) and transferred to tubes. Cell samples were analyzed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). A total of 20×10^4 events were analyzed for fluorescence intensity. Debris was gated out using the propidium iodide staining and subsequently the $CD8^+$ fraction was gated. Mean background tetramer staining of similarly cultured and stained cells from nonimmunized control mice was found to be <1% of $CD8^+$ cells (0.94%; SD, 0.36%). As a positive control the HPV16 E7-specific CTL clone 9.5 was used.

Intracellular cytokine staining

Spleen cultures were stimulated with D1 cells pulsed with 35-mer $E7^{43–77}$ for 7 days. Subsequently, the percentage of $CD8^+$ and $CD4^+$ IFN-γ-producing T cells was measured as described before (48). LPS-activated nonpulsed D1 cells or D1 cells pulsed with short peptide or the long peptide (5 μg/ml) were used as stimulator cells.

Cytokine ELISA

The production of IFN-γ by the CTL clones 9.5, 1H11, and 9.42 was measured as described elsewhere (50).

Statistics

The survival of mice receiving various treatments was statistically analyzed using the Kaplan-Meier test. Where indicated the survival was significantly prolonged ($p < 0.05$).

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Results

Prime-boost vaccinations with the HPV16 E7$^{43–77}$ 35-aa-long Th and CTL epitope-containing peptide result in a robust CD8$^+$ T cell response

Mice (B6) were vaccinated once with either the minimal CTL epitope HPV16 E7$^{49–57}$ (short peptide) or the HPV16 E7$^{43–77}$ 35-residue long peptide, admixed with IFA. Ten days after vaccination, the spleens were harvested and subjected to an extra round of in vitro stimulation that magnifies but does not alter the hierarchy of in vivo induced CD8$^+$ T cell responses before the percentage of E7$^{49–57}$ peptide-specific CD8$^+$ T cells was determined by H2-D^b E7$^{49–57}$ (RAHYNIVTF) tetramer staining (48). Because immunization with short peptide in IFA has been shown to induce E7$^{49–57}$-specific CD8$^+$ T cells that are able to protect mice from a subsequent tumor challenge (13), but which were not demonstrable by tetramer analysis directly ex vivo, we decided to use one round of in vitro stimulation to discriminate nonresponding mice from those with low/high T cell frequencies.

In both groups, three of nine mice responded to the vaccine while generally 5% of the CD8$^+$ T cells stained with tetramers (Fig. 1, *A* and *B*), suggesting that the vaccines perform equally well when injected once.

To induce a more vigorous E7$^{49–57}$-specific CD8$^+$ T cell response, both vaccines were used in a prime-boost regimen, which allowed primed T cells to form memory T cells before the response was boosted by a second vaccination 50 days later (49). Mice vaccinated with the minimal CTL epitope showed CD8$^+$ T cell responses of similar strength as those seen after one vaccination (Fig. 1*C*). In contrast, mice vaccinated with the long peptide displayed a more robust E7$^{49–57}$-specific CD8$^+$ T cell response in 9 of 11 mice, with high numbers of E7$^{49–57}$-specific CD8$^+$ T cells (mean, 19%; range, 5–40%) (Fig. 1*D*). These experiments demonstrate that vaccination with the long peptide in a homologous prime-boost regimen is superior to vaccination with the minimal CTL epitope and suggest that the formation of Th cells, by the intrinsic Th epitope present in the long peptide (47), contributes considerably to the level of the CD8$^+$ T cell responses.

The high E7$^{49–57}$-specific CD8$^+$ T cell response is dependent on MHC class II-restricted Th type 1 cells and CD40-CD40L interactions

To directly assess whether CD4$^+$ Th cells were induced after priming, allowing the formation of memory Th cells before the second vaccination, wild-type B6 mice were vaccinated once with the long peptide in IFA. Subsequently the percentage of E7$^{43–77}$-specific IFN-γ-producing CD4$^+$ T cells was measured by intracellular cytokine staining. Whereas no responses over background were observed in naive mice (data not shown), 5% or more of the CD4$^+$ T cells from vaccinated mice specifically responded upon stimulation with the long E7$^{43–77}$ peptide (Fig. 2*A*). To further demonstrate that the impressive CD8$^+$ T cell responses, detected following prime-boost vaccinations with the long E7$^{43–77}$ peptide, were in fact enhanced by MHC class II-restricted E7-specific CD4$^+$ Th cells, MHC class II$^{-/-}$ mice were prime-boosted with the long peptide vaccine. The number of E7$^{49–57}$-specific CD8$^+$ T cells detected in the MHC class II$^{-/-}$ mice (Fig. 1*E*) was far lower than found in B6 mice after two vaccinations and comparable to that found after one vaccination with the long peptide (Fig. 1*B*). These data not only indicate that MHC class II-restricted CD4$^+$ Th type 1 responses are induced after one vaccination but also suggest that these E7$^{43–77}$ peptide-specific Th cells are required for inducing vigorous CD8$^+$ T cell responses. Most likely, professional APC that process and present the long peptide upon the booster immunization are activated through CD40-CD40 ligand (CD40L) interactions by E7$^{43–77}$ peptide-specific Th cells (47), and this will lead to enhanced CD8$^+$ T cell activation.

To study the role of CD40-mediated activation of APC by Th cells in the outcome of the CD8$^+$ T cell response, CD40$^{-/-}$ mice

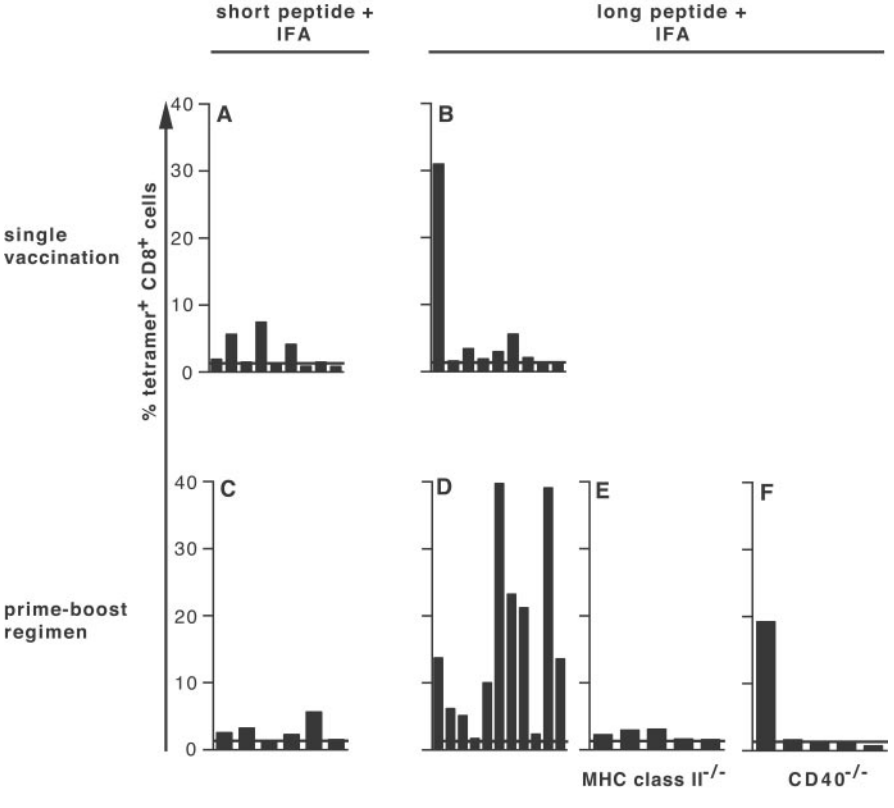

FIGURE 1. High E7$^{49–57}$-specific CD8$^+$ T cell responses are detected following prime-boost vaccinations with the HPV16 long peptide. Mice (B6) were either vaccinated once with the E7$^{49–57}$ peptide (*A*) or the E7$^{43–77}$ long peptide (*B*), or primed and boosted 50 days later with the E7$^{49–57}$ peptide (*C*) or the E7$^{43–77}$ long peptide (*D*). MHC class II$^{-/-}$ mice (*E*) and CD40$^{-/-}$ mice (*F*) were primed and boosted with the E7$^{43–77}$ long peptide. Peptides were emulsified in IFA. Ten days following the final vaccination spleens were harvested and T cell cultures were stimulated with the E7$^{49–57}$-expressing tumor cell line 13.2. Subsequently, the percentage of E7$^{49–57}$ tetramer-positive CD8$^+$ cells was determined by FACS analysis. Each bar represents an individual mouse. Horizontal lines indicate background responses plus SD of naive mice.

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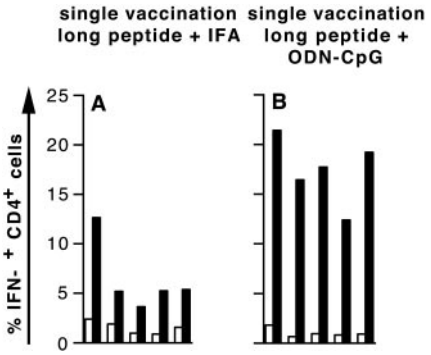

FIGURE 2. E7-specific $CD4^+$ Th type 1 cells are induced following priming with the $E7^{43–77}$ long peptide. Mice (B6) were vaccinated s.c. with the long peptide admixed with IFA (*A*) or ODN-CpG (*B*). Spleens were harvested and cultures were stimulated with D1 cells pulsed with the $E7^{43–77}$ long peptide. Subsequently, the percentage of $CD4^+$ IFN-γ-producing cells was analyzed by FACS. Open bars, The percentage of IFN-γ-producing $CD4^+$ T cells stimulated with nonpulsed D1 cells; filled bars, The percentage of IFN-γ-producing $CD4^+$ T cells stimulated with the long peptide-pulsed D1 cells. Each set of two bars represents an individual mouse. Spleens of naive mice were taken along as controls and stimulated in the same way, but the percentage of IFN-γ-producing cells did not exceed the background on nonpulsed D1 cells (data not shown).

were vaccinated twice with the long peptide (Fig. 1*F*). As expected, the number of $E7^{49–57}$-specific $CD8^+$ T cells induced did not exceed that seen after one vaccination with the long peptide in wild-type B6 mice (Fig. 1*B*). In both groups of mice, one of the tested mice displayed a high percentage of tetramer-positive $CD8^+$ T cells, but this has to be attributed to individual variation.

Single vaccinations with the $E7^{43–77}$ peptide and various DC-activating agents result in vigorous $CD4^+$ and $CD8^+$ T cell responses toward HPV16 E7

If the Th-mediated activation of professional APC is important for the observed robust $CD8^+$ T cell response, direct activation of DC by itself should be sufficient to raise adequate $CD8^+$ T cell responses. To address this point, B6 mice were vaccinated once with the long peptide or the minimal $E7^{49–57}$ peptide in combination with various DC-activating agents. IFA and Montanide (a human grade IFA) have been used extensively by us and others in previous studies. Mice vaccinated with either the short peptide or the long peptide combined with anti-CD40 Ab or GM-CSF in PBS displayed no or minimal $CD8^+$ T cell responses (Fig. 3, *A* and *B*). However, the combination of GM-CSF and IFA resulted in moderate $E7^{49–57}$-specific $CD8^+$ T cell responses when the long peptide was administered (Fig. 3*C*). Mice receiving the minimal peptide mixed with MPL or ODN-CpG showed clear-cut $CD8^+$ T cell responses in several mice (Fig. 3, *D* and *E*), although the number of responders (4 of 9 and 4 of 12 mice, respectively) was not increased compared with mice vaccinated with IFA (Fig. 1*A*). In contrast, vaccination with the long peptide admixed with MPL or ODN-CpG resulted in $E7^{49–57}$-specific $CD8^+$ T cell responses in all mice. Moreover, the level of the detected responses was high (up to 40% of stimulated $CD8^+$ T cells) in the majority of animals.

To confirm that both functional $CD8^+$ and $CD4^+$ T cell responses were triggered following a single vaccination with the long peptide and DC-activating agents, the numbers of $CD8^+$ and $CD4^+$ IFN-γ-producing cells were measured upon stimulation with the short and long peptides, respectively. High numbers of IFNγ-producing $CD8^+$ T cells were detected, confirming that the $CD8^+$ T cells detected by the H2-D^b $E7^{49–57}$ tetramers were functionally active (data not shown). Compared with the use of IFA as adjuvant (Fig. 2*A*), the use of the DC-activating agent (ODN-CpG) raised even higher numbers of $E7^{43–77}$-specific IFN-γ-producing $CD4^+$ T cells (15–20% of stimulated $CD4^+$ T cells; Fig. 2*B*).

Our prime-boost experiments with the long peptide (Fig. 1, *D–F*) suggested that the induction of high numbers of HPV-specific $CD8^+$ T cells was the result of the activation of APC by HPV-specific $CD4^+$ T cells. Therefore, direct activation of DC should be able to bypass a lack of T cell help and as such no difference in the numbers of HPV-specific $CD8^+$ T cells among MHC class $II^{-/-}$ mice, $CD40^{-/-}$ mice, or wild-type mice after vaccination with the long peptide and a DC-activating agent is expected. To address this question, mice were vaccinated with the long peptide and ODN-CpG. As shown in Fig. 3, *F* and *G*, robust $CD8^+$ T cell responses were detected in all types of mice after one vaccination. Together with the experiments shown in Fig. 1, these experiments show that proper precursor T cells are present in all types of mice. Furthermore, it demonstrates that activation of professional APC is an important aspect in the induction of high numbers of HPV-specific $CD8^+$ T cells. Furthermore, the observation that in this Th-independent setting vaccination with the long peptide is superior to vaccination with the short peptide suggests that, compared with the minimal CTL epitope that may bind to MHC class I molecules present on all nucleated cells, the long peptide is preferentially processed and presented by professional APCs.

Vaccinations with the long $E7^{43–77}$ peptide and the DC-activating adjuvant ODN-CpG can effectively eradicate HPV16-expressing tumors

Numerous studies show that protection of C57BL/6 mice against HPV16 E7-expressing tumors is entirely dependent on $E7^{49–57}$-specific $CD8^+$ T cells (13, 41, 42, 51). These $CD8^+$ CTL are able to kill the HPV16 E7-expressing epithelial tumor cell line TC-1 (42, 51). Because it was shown that the percentage of $E7^{49–57}$ tetramer-positive $CD8^+$ T cells correlated with cytotoxicity (48), the antitumor efficacy of the short peptide or the long peptide vaccine admixed with ODN-CpG, which consistently resulted in high numbers of $CD8^+$ T cells, was tested by therapeutically vaccinating tumor-bearing mice in the contralateral flank (Fig. 4). Vaccinations with the short or the long peptide combined with IFA were taken along for comparison (Fig. 4). Notably, the vaccinations were given at the time that tumors were palpable in all mice (day 10). Based on a pilot experiment in which tumors treated with one single vaccination occasionally progressed after initial regression (data not shown), a booster vaccination was administered 14 days later to fully sustain E7-specific Th and CTL immunity. Compared with nonvaccinated mice (Fig. 4*A*), administration of the peptides in IFA showed some delay in tumor growth but all mice had died by day 50 (Fig. 4, *D* and *E*). In both groups treated with either the short or the long peptides combined with ODN-CpG, inhibition of tumor growth was seen 8–12 days after treatment (Fig. 4, *B* and *C*). Complete eradication of the TC-1 tumor was observed in three of nine mice treated with the short peptide and ODN-CpG (Fig. 4*B*) and in 2 of 10 control mice who, as a control, received two injections of ODN-CpG peritumoral (Fig. 5). In all of the control mice injected with ODN-CpG in the contralateral flank, tumor growth was comparable to nontreated animals (Fig. 5). In contrast, 8 of 10 mice treated with the long peptide and ODN-CpG completely eradicated their tumors, some of them sizing up to 250 mm^3 (Fig. 4*C*). The mice with the two largest tumors (up to 500 mm^3) initially succeeded in reducing the tumors to a size that was not palpable anymore, but eventually the tumors became detectable again and progressive tumor growth developed. One of these mice died spontaneously, whereas the other was killed to investigate the

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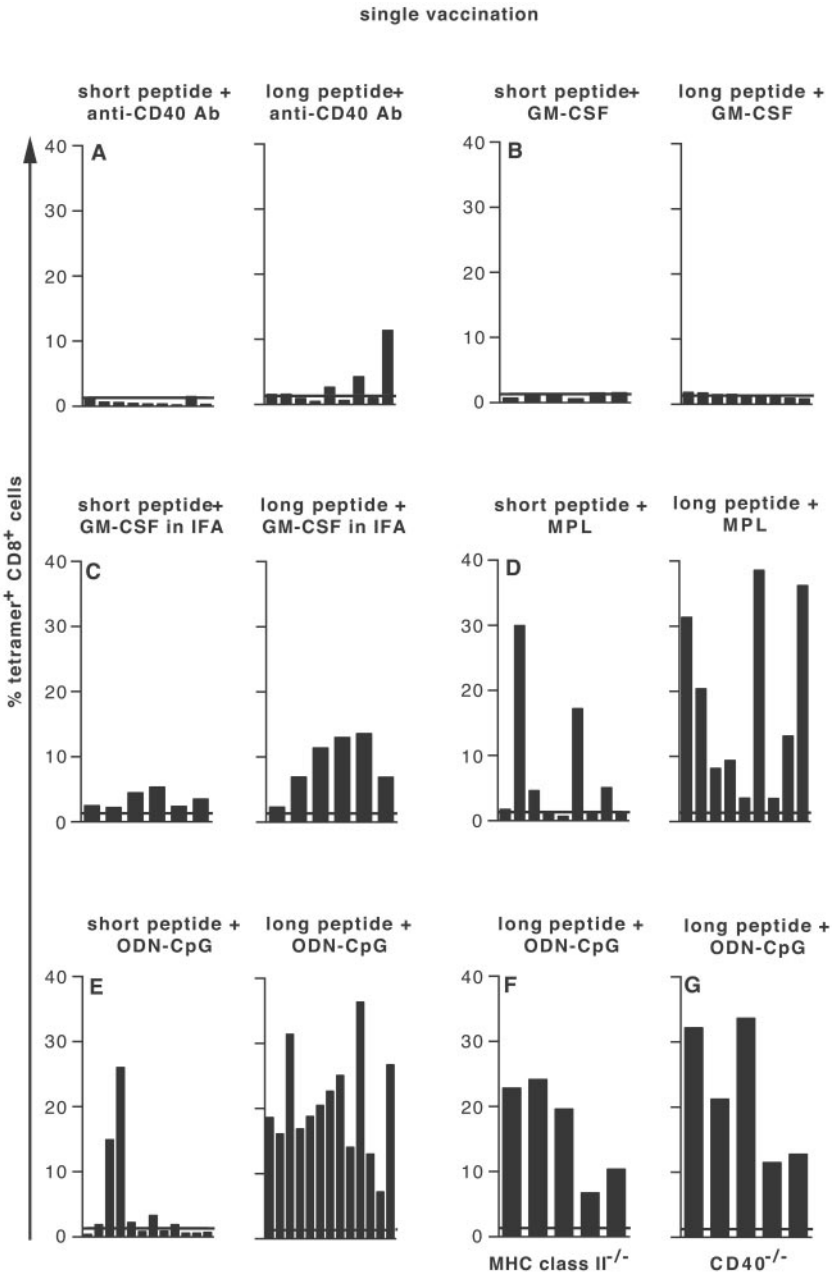

FIGURE 3. One vaccination with the E7$^{43–77}$ long peptide combined with various DC-activating adjuvants results in high CD8$^+$ T cell responses. Mice (B6) were vaccinated once with the short E7$^{49–57}$ peptide or the long peptide. Anti-CD40 Ab (*A*), GM-CSF (*B*), GM-CSF plus IFA (*C*), MPL (*D*), and ODN-CpG (*E*) were used as adjuvants. Similarly, MHC class II$^{-/-}$ mice (*F*) and CD40$^{-/-}$ mice (*G*) were vaccinated once with the long peptide and ODN-CpG. Spleens were harvested 10 days after priming and cultures were stimulated with the E7$^{49–57}$-expressing tumor cell line 13.2 for 1 wk. Subsequently, the percentage of E7$^{49–57}$ tetramer-positive CD8$^+$ cells was determined by FACS analysis. Each bar represents an individual mouse. Horizontal lines indicate background responses plus SD of naive mice.

tumor, bearing in mind the possibility that recurrence may have resulted from escape mechanisms. The recognition of this excised tumor by two tumor-specific CTL clones was compared with that of the original in vitro growing tumor cell line (Fig. 6). Clearly, the recognition of the original tumor cells was far higher in comparison to the excised tumor, indicating that escape may have occurred through reduced CTL killing. Additional FACS analysis showed a 2-fold decreased expression of K^b and D^b MHC class I molecules on the cell surface of the excised tumor (data not shown). Because TC-1 already expresses low levels of K^b and D^b on its surface, further down-regulation of the MHC class I molecules may be one of the multiple mechanisms through which this tumor could have escaped from the immune system.

Comparison of the survival time of TC-1-bearing mice showed that there was no significant difference between mice treated with the minimal CTL epitope mixed with IFA or ODN-CpG, mice treated with the long peptide in IFA, or untreated animals (Fig. 7). Mice treated with the long peptide admixed with ODN-CpG survived significantly longer (Kaplan-Meier analysis, $p < 0.05$). These in vivo data are consistent with the in vitro analysis of the CD4$^+$ and CD8$^+$ T cell responses after vaccination with the peptides in IFA or mixed with ODN-CpG.

Together these data demonstrate that it is essential to activate DC and induce high numbers of tumor-specific T cells to obtain effective antitumor responses.

Discussion

In this study we show that the HPV16 E7-specific CD8$^+$ T cell response is far more vigorous after vaccination with a HPV16 E7-derived 35-residue long peptide than following vaccination with the minimal CTL epitope. Our data suggest that two distinct DC-related mechanisms lie at the basis of this result. First, the long peptide contains both a CTL epitope and a Th epitope. Prime-boost vaccinations of wild-type, MHC class II$^{-/-}$, and CD40$^{-/-}$ mice show that the CD40-CD40L interactions between APC and E7-specific Th cells contribute considerably to the level of the CD8$^+$

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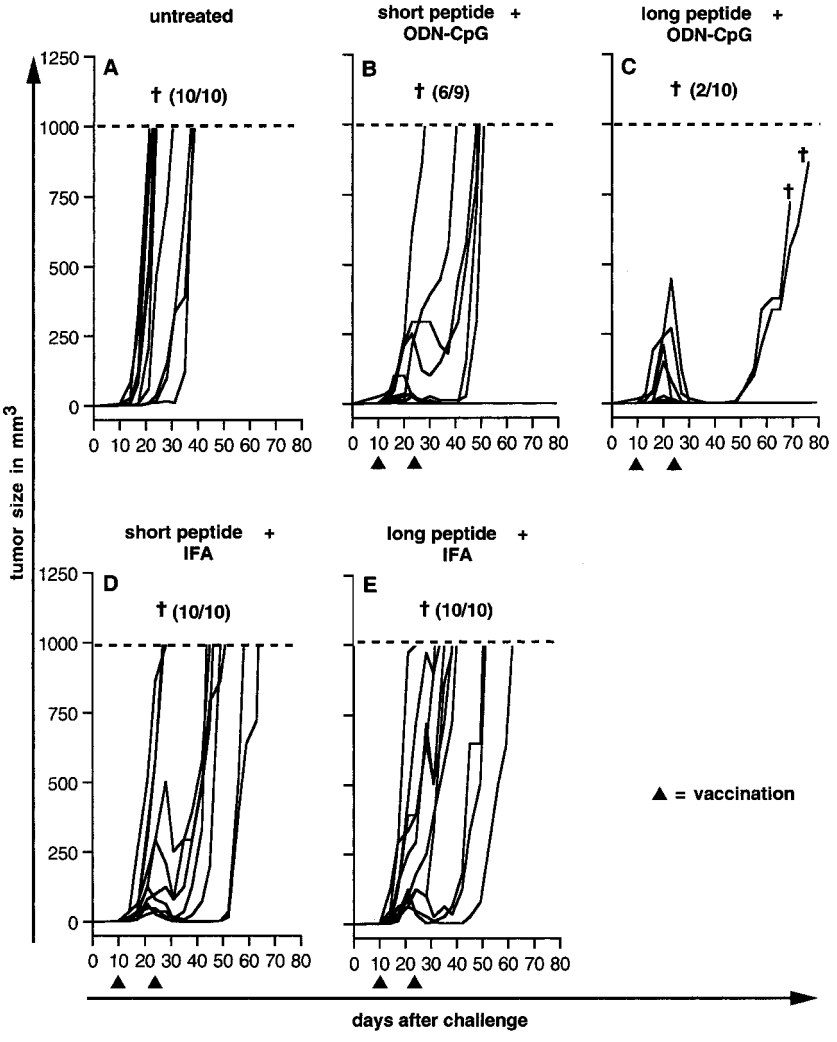

FIGURE 4. Prime-boost vaccinations with the $E7^{43–77}$ long peptide combined with the DC-activating agent ODN-CpG can eradicate large established tumors. B6 mice ($n = 10$) were challenged on day 0 with 5×10^4 HPV16-expressing tumor cells (TC-1) and primed, when all tumors were palpable, on day 10 and boosted 14 days later with either ODN-CpG plus the short peptide (*B*), with ODN-CpG plus the long peptide (*C*), or with IFA plus the short peptide (*D*) and IFA plus the long peptide (*E*). Untreated mice were taken along as a control (*A*). Mice were killed when the tumor size reached 1000 mm^3. One of the mice in *B* developed an i.p. tumor that could not be measured over time. This animal was killed and excluded from the experiment. One of the two mice that developed progressive tumors in *C* died spontaneously, while the other was killed before the tumor reached 1000 mm^3 to investigate the tumor.

T cell response. Second, under circumstances excluding T cell help, a comparison of the $CD8^+$ T cell responses induced by either the minimal CTL epitope (nine residues) or the long peptide combined with DC-activating agents demonstrates that vaccination with the long peptide results in far better responses. This suggests that compared with the short minimal CTL epitope the long peptide is much better presented by professional APC. Additionally, we show that prime-boost vaccinations with the long peptide and the strong DC-activating adjuvant ODN-CpG lead to the complete eradication of established tumors. These data provide a scientific basis for the use of long overlapping peptides, alone or in combination with strong DC-activating agents, in future clinical trials.

The high efficacy of our long peptide vaccine may partly result from the fact that the Th and CTL epitope are physically linked to each other, although we have not tested this in the current study. The potential advantage of epitope linkage lies in the increased chance for simultaneous presentation of both the MHC class I- and class II-restricted epitopes on the surface of a single APC, thereby facilitating the delivery of cognate T cell help to CTL priming. A direct comparison of vaccines that used a mixture of nonlinked CTL and Th epitopes with vaccines that used identical, but physically linked, CTL and Th epitopes demonstrated that the latter resulted in more vigorous CTL responses (26–29). Naturally occurring epitope linkage can be found in most protein Ags, including the HIV-1 envelope protein (52), the IDDM-associated GAD65 protein (53), and the Her-2/neu protein (54). Interestingly, recent vaccinations with Her-2 peptides, comprising both CTL and Th epitopes, resulted in enhanced Her-2-specific Th and CTL activity in cancer patients (54). Our experiments in wild-type B6 and MHC class $II^{-/-}$ mice show that simultaneous vaccination with both CTL and Th epitopes is advantageous for the development of a strong E7-specific $CD8^+$ T cell response. In addition, the experiments in $CD40^{-/-}$ mice demonstrate that particularly the interaction of CD40-CD40L between E7-specific Th cells and APC is responsible for this boost of the $CD8^+$ T cell response.

Comparison of the $CD8^+$ T cell responses induced after a single vaccination with the short peptide or the 35-residue long peptide in combination with DC-activating adjuvants revealed another interesting property of the long peptide vaccine. Under these circumstances the contribution of E7-specific Th cells is negligible because at priming their number is too low to exert this function. Consequently, only differences in physical properties or kinetics of the peptides are likely to play a role. Because of its size, which excludes direct binding to MHC class I molecules, the long peptide has to be taken up by professional APC that are able to process exogenously derived Ags for presentation in MHC class I molecules (55). We demonstrate that direct activation of APC with DC-activating agents, bypassing the need for T cell help, only marginally affects the outcome of the E7-specific $CD8^+$ T cell response when the minimal peptide is used, whereas DC activation induces a superior E7-specific $CD8^+$ T cell response when the long peptide is administered. To confirm that T cell help was not

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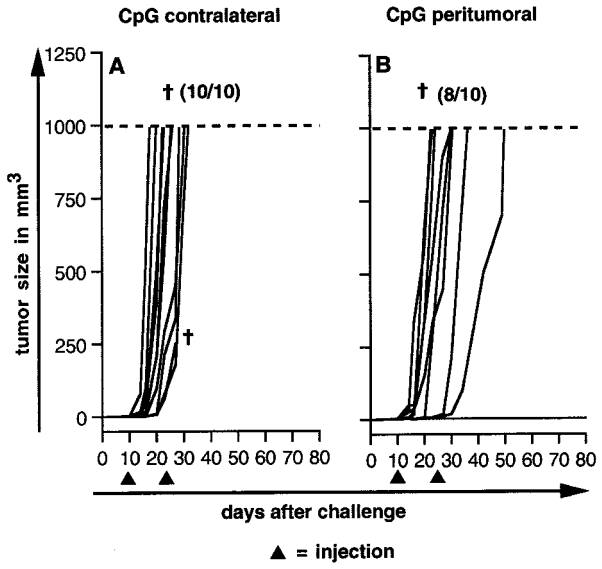

FIGURE 5. Peritumoral injection of ODN-CpG has only marginal effects on tumor growth. B6 mice were challenged on day 0 with 5×10^4 HPV16-expressing tumor cells (TC-1) and injected, when all tumors were palpable, on day 10 and 14 days later with ODN-CpG, either in the contralateral flank (*A*) or peritumoral (*B*). The data of untreated mice are not shown but were similar to those depicted in Fig. 4*A*. One of the mice in *A* died spontaneously. Two mice in *B* were tumor free when the experiment was terminated at day 80. Compared with untreated mice there was no statistically significant prolongation of survival.

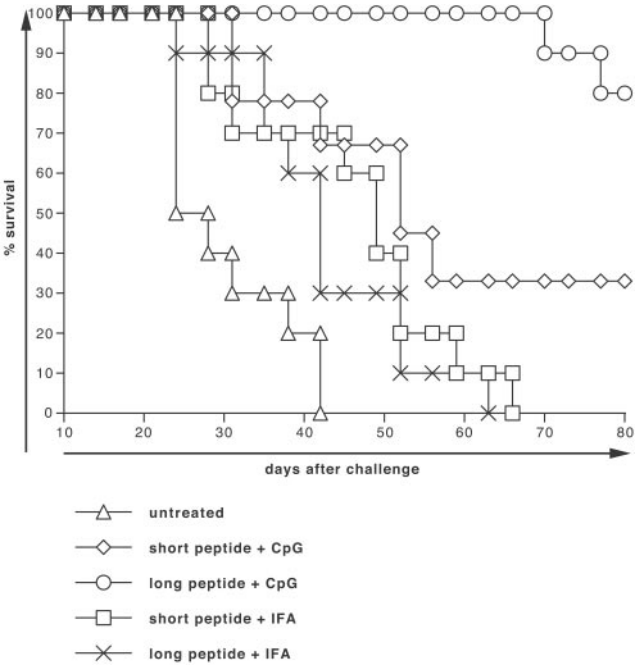

FIGURE 7. Vaccination with the E7$^{43-77}$ long peptide and ODN-CpG results in prolonged survival. The mice described in Fig. 4 were killed when the tumor size reached 1000 mm^3 to avoid unnecessary suffering. Notably, one of the mice treated with the long peptide plus ODN-CpG (see Fig. 4*C*) was killed to investigate the tumor before it had reached 1000 mm^3. The survival of mice treated with ODN-CpG and the long peptide was significantly increased compared with untreated mice ($p < 0.05$). Only two mice developed tumors while eight were totally tumor free at the time the experiment was terminated (at day 80).

responsible for this effect, the long peptide was coinjected with the DC-activating adjuvant ODN-CpG in MHC class II$^{-/-}$ and CD40$^{-/-}$ mice and, as expected, the observed CD8$^+$ T cell responses were comparable to those seen in wild-type mice. The superior CD8$^+$ T cell-inducing capacity of long peptides plus DC-activating agents over short peptides can be explained by the fact

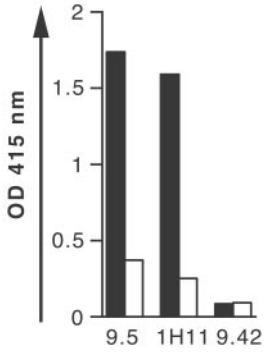

FIGURE 6. A progressively growing HPV16-expressing tumor displays tumor escape mechanisms. The tumor of one of the two mice that developed progressive tumors despite vaccination with the long peptide plus ODN-CpG (see Fig. 4*C*) was excised and tested after two in vitro passages for recognition by two tumor-specific CTL clones by measuring IFN-γ production. The original in vitro growing HPV16-expressing tumor cell line TC-1 was used for comparison. The CTL clone 9.5 recognizes the HPV16 E7$^{49-57}$ peptide in a D^b context, while the CTL clone 1H11 recognizes a p53-derived K^b-restricted epitope that is highly expressed by TC-1 as we have previously shown (33). The CTL clone 9.42 recognizes a nonrelevant epitope and was used as a control. IFN-γ was added to both in vitro tumor cell cultures 48 h before testing to achieve maximum expression of MHC class I. Indicated is IFN-γ production after stimulation with the original TC-1 tumor cell line (filled bars) and with the excised tumor (open bars).

that minimal CTL epitopes show no distinction between MHC class I molecules present on professional APC or the MHC class I molecules on other nucleated cells. In the absence of expression of proper costimulatory molecules, such nonprofessional APC have been shown to exert tolerizing influences on CTL precursors (40). Additionally, long peptides need to be taken up and processed by professional APC. The use of specific DC-activating agents affected especially CD8$^+$ T cell induction by the long peptide vaccine, suggesting that the long peptides are indeed taken up and processed by professional APC. This may create an intracellular reservoir for continuous supply of MHC class II- and MHC class I-bound peptides to the cell surface and as such lead to sustained CD8$^+$ T cell activation. Exact MHC class I-binding short peptides have a short half life at the cell surface and DC loaded in vivo with such peptides will not benefit from resupply to the cell surface from internally processed stores of Ag (56). Finally, recent experiments performed in our department indicate that ODN-CpG not only activates DC but can also improve the MHC class I peptide processing pathway, leading to an increased number of available CTL epitopes on the cell surface of the DC (F. Ossendorp M. Camps, A. Sijts, P. Kloetzel, and C. J. M. Melief, unpublished observations).

We and others have completed phase I/II vaccination studies in which HPV16$^+$ patients were vaccinated with CTL epitopes admixed with Montanide (12, 15), a human grade of IFA with similar properties (57), or admixed with Montanide plus a nonlinked universal Th epitope (14). All together, no or marginal HPV16 CTL responses were detected in these patients. Our data show that minimal peptide epitopes mixed with mineral oil based adjuvants, such as Montanide or IFA, trigger only marginal CD8$^+$ T cell responses in prime-boost regimens. As shown here, the inclusion of a Th

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epitope markedly improves the vaccine. Increasing the size of the peptide, forcing it to be presented by professional APC, is another way to enhance the vaccination efficacy. In addition, the strength of the vaccine can be further improved by the use of DC-activating adjuvants. We demonstrate that the DC-activating agents MPL (a detoxified form of LPS that can be used in humans) (33), ODN-CpG, and the DC-activating chemokine GM-CSF in IFA help increasing the levels of $CD8^+$ T cell responses induced by the long HPV16 E7-derived peptide. Interestingly, this DC activation also results in increased levels of E7-specific $CD4^+$ Th cells, which may additionally enhance and sustain HPV-specific CTL responses. Indeed, the combination of the DC-activating agent ODN-CpG and the long peptide proved to result in complete eradication of established tumors following prime-boost vaccinations. These data are in line with the results of a recent study, which demonstrated that ODN-CpG markedly enhance the number of tumor-specific CTL (58).

Based on our data, we conclude that vaccination with long peptides containing both CTL and Th cell epitopes and DC-activating agents can contribute to the design of stronger HPV vaccines. We have now initiated a phase I/II study in which a clinically grade long (32–35 residues) overlapping peptide vaccine, covering the entire HPV16 E6 and E7 protein, is used to vaccinate patients with HPV16-induced cervical or vulvar lesions.

Acknowledgments

We thank W. Benckhuisen for peptide synthesis and Dr. F. Ossendorp and Dr. T. van Hall for critical reading of the manuscript.

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