

# CD4<sup>+</sup> T Cell–Mediated Antigen-Specific Immunotherapy in a Mouse Model of Cervical Cancer

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## Abstract

**A major agenda for tumor immunology is the generation of specific immune responses leading to the destruction of incipient and frank neoplasia. In this report, we show that a novel HPV16 E7 fusion protein can produce objective therapeutic responses against incipient cervical cancer in genetically engineered mice that express in the cervix the HPV16 early region genes implicated as causative agents in human cervical cancer. Although nonresponsive toward the HPV16 E7 oncoprotein in the CD8<sup>+</sup> T-cell compartment by virtue of MHC haplotype, the mice were capable of mounting an induced CD4<sup>+</sup> T-cell response against E7, and in addition developed spontaneous anti-E7 antibodies. HPV16/CD4<sup>-/-</sup> mice showed increased tumor burden indicative of CD4-mediated immune surveillance. Seeking to enhance the CD4 response, we immunized mice bearing incipient cervical cancer with a recombinant protein fusing E7 with a mycobacterial heat shock protein. The incidences of cervical carcinoma and of high-grade dysplasia (CIN 3) were consequently reduced by comparison to control mice. Thus, an HPV16 E7 immunogen holds promise for noninvasive treatment and prevention of human cervical cancer.** (Cancer Res 2005; 65(5): 2018-25)

## Introduction

A class of “high risk” human papillomaviruses (HPV), notably including HPV16 and HPV18, are associated with cancer of the anogenital tract, particularly of the uterine cervix (1, 2). Cervical cancer typically arises in the transformation zone between the squamous epithelium of the cervix and columnar epithelium of the uterus, an anatomic site subject to metaplasia (3). Given that the oncogenes implicated in induction of cervical carcinogenesis (4) are delivered by viral infection, there has been longstanding interest in the role of natural immunity and in the potential of induced immunity, in preventing or resolving cervical neoplasias. There is now experimental evidence in mice supporting the concept of natural antitumoral immunity, or

“immune surveillance” (5). These observations are consistent with epidemiologic evidence that the immune system can prevent anogenital and cervical cancers in humans (6–11). The pathogenesis of anogenital cancers relies critically upon HPV E6 and E7 oncoproteins which inactivate the function of the p53 and retinoblastoma tumor suppressor proteins (4). In HPV-associated cervical cancer, immune responses to viral oncogenes can be observed in both peripheral blood and tumors (12, 13), indicating that, in addition to innate responses, acquired immunity is also invoked and may play a role in tumor surveillance. Collectively, these other data have motivated consideration of E7-based immunotherapeutic strategies. Several are currently being developed for both prophylactic and therapeutic treatment of cervical neoplasia, including virus-like particles, viral vectors, DNA vaccines, and heat shock protein (hsp) fusion proteins (14).

In this report, we used a mouse model of cervical carcinogenesis to study the response of the immune system toward incipient cervical neoplasia and to investigate the potential for hyperactivating the immune response as a therapeutic strategy. K14-HPV16 transgenic mice express the human papillomavirus type 16 (HPV16) early region genes in basal keratinocytes under the control of the human keratin 14 promoter (K14; ref. 15). If the normally cyclic estrogen levels are sustained at modestly elevated levels in female transgenic mice using time release 17 $\beta$ -estradiol (estrogen), cervical neoplasias and then invasive squamous cell carcinomas develop in the cervix (16, 17). Cervical carcinogenesis in this model transpires in ~80% to 90% of mice over a 4-month period following initiation of estrogen control at 1 month of age; typically, cervical cancer is scored at 7 months of age. Concurrently, the mice develop skin dysplasias consequent to expression of the HPV16 oncogenes in the epidermis; the course of cervical carcinogenesis precedes the infrequent progression of the skin dysplasias into squamous cell carcinomas between 7 and 12 months of age, and the skin pathology is not dependent on or affected by estrogen (18).

Among the various HPV-targeted immunotherapeutic strategies in development, we chose to evaluate an immunogen composed of the E7 oncoprotein fused to a heat shock protein. Microbial heat shock proteins have been shown to be highly immunogenic, both alone, and when conjugated to relevant target antigens (19); as such, hsp fusion proteins hold promise as adjuvant-free vaccines for both infectious disease and cancer (20). In particular, a recombinant protein has been developed that fuses the *Mycobacterium bovis* Bacillus Calmette-Guerin hsp65 to HPV16 E7 [(h)hspE7], and this immunogen has been used to treat s.c. transplant tumors arising from inoculation of an E7-expressing murine tumor cell line TC-1 (21). In those studies, (h)hspE7 was shown to elicit both E7-specific IFN- $\gamma$

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release by CD4<sup>+</sup> T cells, and selective cytolytic killing of E7-expressing tumor cells by CD8<sup>+</sup> T cells. In the present study, we assessed the ability of (h)hspE7 to induce regression of primary cervical carcinomas arising *de novo* in the cervix of female HPV16 mice. Our studies indicate that (h)hspE7 can prime E7-specific IFN- $\gamma$  release by CD4<sup>+</sup> T cells and induce regression by a CD4-dependent mechanism in this mouse model, encouraging the potential of HPV antigen-based immunogens for prevention and treatment of cervical cancer.

## Materials and Methods

**Mouse Breeding and Hormone Treatment.** The generation of K14-HPV16 transgenic mice (15) and standardization of 17 $\beta$ -estradiol delivery for cervical carcinogenesis has been previously reported (16, 17). Briefly, s.c. continuous release pellets that deliver 17 $\beta$ -estradiol at a dose of 0.05 mg over 60 days (Innovative Research of America, Sarasota, FL) were implanted in the dorsal back skin of heterozygous 1-month-old K14-HPV16 female transgenic mice. Subsequent pellets were implanted at 3 and 5 months of age for a total of 6 months of hormone treatment. K14-HPV16 mice were maintained in the FVB/n background (FVB/n; The Jackson Laboratory, Bar Harbor, ME). The generation of *CD4* homozygous null animals has been reported (22). CD4<sup>-/-</sup> mice were bred 14 generations into FVB/n before intercrossing with HPV16 transgenic mice (N21 FVB/n) to produce HPV16/CD4<sup>-/-</sup> mice. To minimize potential effects of genetic drift, the crosses to maintain the HPV16 line and CD4<sup>-/-</sup> backcrosses to FVB/n were all carried out at the same time using a common FVB/n stock obtained from The Jackson Laboratory. Mice were housed in a pathogen-free barrier facility under the approval of the University of California at San Francisco Committee on Animal Research.

**Tissue Preparation and Histology.** For paraffin sections, reproductive tracts from transgenic animals were immersion-fixed in 3.75% paraformaldehyde and PBS or 10% zinc-buffered formalin followed by dehydration through graded alcohols and xylene, and embedded in paraffin. Five- and 10- $\mu$ m-thick paraffin sections were cut using a Leica 2135 microtome, deparaffinized and rehydrated through an alcohol series then subjected to H&E staining for histopathology. For frozen sections, the reproductive tract was embedded without fixation in OCT compound (Tissue-Tek, GMI, Ramsey, MN) and frozen on dry ice. Ten-micrometer-thick frozen sections were cut using a Leica CM1900 cryostat. Sections were air-dried, fixed in acetone, and subjected to H&E staining. The characterization of neoplastic stages based on H&E staining have been previously reported and was done in a blinded fashion on separate occasions by two investigators (16, 17). Tumor volume was determined using the following formula:  $V = 2/3 \times A \times Z$ , where *A* is the cross-sectional area of the tumor determined using a Zeiss Axioskop 2 plus equipped with a Hamamatsu-Orca digital camera and imaged using Improvion OpenLab software, and *Z* is the depth of tumor calculated through serial sections.

**Antigens and Immunization.** Generation and purification of (h)E7 (referred to as E7), hsp65, and (h)hspE7 [referred to as (h)hspE7] have been previously described (21). Endotoxin levels were measured by *Limulus* ameobocyte lysate assay (BioWhittaker, Walkersville, MD) and all preparations contained <4.3 endotoxin units/100  $\mu$ g protein [for (h)hspE7, 1.4 nmol = 100  $\mu$ g]. Mice were immunized s.c. with an equimolar amount (1.4 nmol) of (h)E7, hsp65 or (h)hspE7 in 0.2 mL PBS/5% glycerol as a vehicle. Control mice received vehicle alone. Sequences for peptides E7 p44-63, E7 p18-38 (23), and Tag p362-384 (ref. 24; Genemed Synthesis, Inc., South San Francisco, CA) have been previously reported. Ovalbumin (Sigma-Aldrich, St. Louis, MO) was used as a protein control for (h)E7. Concanavalin A (Con A; Sigma) was used as a positive control.

**Lymphokine Assays.** Spleens were disrupted using glass homogenizers, washed with PBS, and quantified by hemocytometer. Splenocytes were cultured in 96-well half-well flat-bottomed microculture plates (BD Falcon, San Jose, CA) with  $6.0 \times 10^5$  cells per well in 0.2 mL HL-1 serum-free media (BioWhittaker) and no antigen or 1.4  $\mu$ mol/L HPV16 E7. Splenocytes were stimulated with 0.25  $\mu$ g/mL Con A as a positive control. Cultures were set

up in triplicate and incubated at 37°C in 5% CO<sub>2</sub>/95% air (5% CO<sub>2</sub>) for 72 hours. The supernatants were analyzed for IFN- $\gamma$  and IL-5 by ELISA kits (R&D Systems, Minneapolis, MN). Reference curves were generated for each assay using recombinant IFN- $\gamma$  and IL-5 as standards. Absorbance of substrate was determined at 405nm on a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

**Lymphocyte Proliferation Assays.** FVB/n and HPV16 mice were immunized s.c. with 50  $\mu$ g of HPV16-E7 emulsified in a total volume of 50  $\mu$ L complete Freund's Adjuvant at the base of the tail (25). The mice were sacrificed 10 days after immunization and the draining lymph nodes (inguinal and peri-aortic) were removed. Lymph nodes were disrupted using glass homogenizers, washed with PBS, and quantified by hemocytometer. Lymph node cells were cultured in 96-well flat-bottomed microculture plates (Falcon) with  $1.0 \times 10^6$  cells per well in 0.2 mL HL-1 serum-free media (BioWhittaker) and titrated doses (0.7 and 0.35  $\mu$ mol/L) of HPV16-E7. Lymph node cells were stimulated with 0.25  $\mu$ g/mL Con A as a positive control. Cultures were set up with replicates in triplicate and incubated under standard conditions for 5 days with a pulse of 0.5  $\mu$ Ci of [<sup>3</sup>H]thymidine 6 hours before harvest onto glass fiber filters (Wallac, GMI). Incorporated [<sup>3</sup>H]thymidine was quantified by scintillation counting on an LKB Betaplate counter.

**Antibody Quantification.** HPV16-E7 was bound to Maxisorb microtiter plates (Nalge Nunc International, Rochester, NY) at 25  $\mu$ g/mL in PBS. Remaining binding sites were blocked with PBS/3% bovine serum albumin. Control plates were blocked with PBS/3% bovine serum albumin. ELISA assays were conducted on these plates. Serial dilutions (1:5) of serum from nontransgenic FVB/n mice and HPV16 mice of varying ages were prepared in PBS/3% bovine serum albumin and plated on HPV16-E7 plates in duplicate. Purified mouse anti-HPV16-E7 (8C9, Zymed, South San Francisco, CA) was used to generate a standard curve for antibody quantification. Biotinylated goat anti-mouse immunoglobulin G (IgG), human absorbed (Southern Biotechnology Associates, Inc., Birmingham, AL) was used at 0.25  $\mu$ g/mL to detect bound IgG. Streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA) was used at a 1:5,000 dilution. Absorbance of *o*-phenylenediamine (Zymed) substrate was determined at 450 nm on a Thermomax microplate reader (Molecular Devices) and data analyzed using Softmax v. 2.35 (Molecular Devices).

**Reverse Transcription-PCR.** Total RNA was made from nontransgenic thymus and HPV16 thymus and skin using a RNeasy kit (Qiagen, Valencia, CA). Total RNA was reverse transcribed by using random hexamers (Invitrogen Life Technologies, Carlsbad, CA) and Moloney murine leukemia virus reverse transcriptase (Life Technologies). Specific primers were used to amplify cDNAs: HPV16-E6/E7 (sense 5'-AGAAGTGAATGTTTCAGGACC-CACAG-3' and antisense 5'-TCTGCAACAAGACATACATCGACCGG-3') and  $\beta$ 2-microglobulin (sense 5'-CACGGAGAATGGGAAGCCGAA-3' and antisense 5'-TCCACACAGATGGAGCGTCCAG-3') as an internal control. Thirty-five cycles (HPV16-E6/E7) and 30 cycles ( $\beta$ 2-microglobulin) were done with a PTC-200 thermocycler (MJ Research, Waltham, MA) as follows: 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. PCR products were run on 1.5% agarose gels. Gels were photographed using an IS-1000 Digital Imaging System (Alpha Innotech Co., San Leandro, CA).

**Immunohistochemistry.** Frozen sections were used for matrix metalloproteinase-9 (MMP-9) and CD4 immunohistochemistry. Dilution used for rabbit anti-MMP-9 (a gift of Dr. Zena Werb, Department of Anatomy, University of California, San Francisco, CA) was 1:1,000 and rat anti-mouse CD4 (H129.19; BD Biosciences, San Jose, CA) was 1:200 in a blocking solution containing PBS pH 7.4 and 0.25% blocking reagent (Perkin Elmer, Boston, MA). Incubation with primary antibody was overnight at 4°C. After incubation with a biotinylated secondary antibody (goat anti-rabbit IgG, goat anti-rat IgG, or goat anti-mouse IgM, 1:200; Pierce Biotechnology, Rockford, IL) for 30 minutes at ambient temperature, antigens were revealed with 3,3'-diaminobenzidine (Sigma-Aldrich) according to the manufacturer's instructions. Sections were counterstained in 1% methyl green, dehydrated in *iso*-butanol and xylene, mounted in Cytoseal 60 (Stephens Scientific, Riverdale, NJ), and visualized with Nomarski optics. All images were digitally captured on a Nikon Microphot-FX microscope

equipped with a DC-330 CCD color camera (DAGE-MTI, Michigan City, IN) and imaged using Improvion OpenLab software.

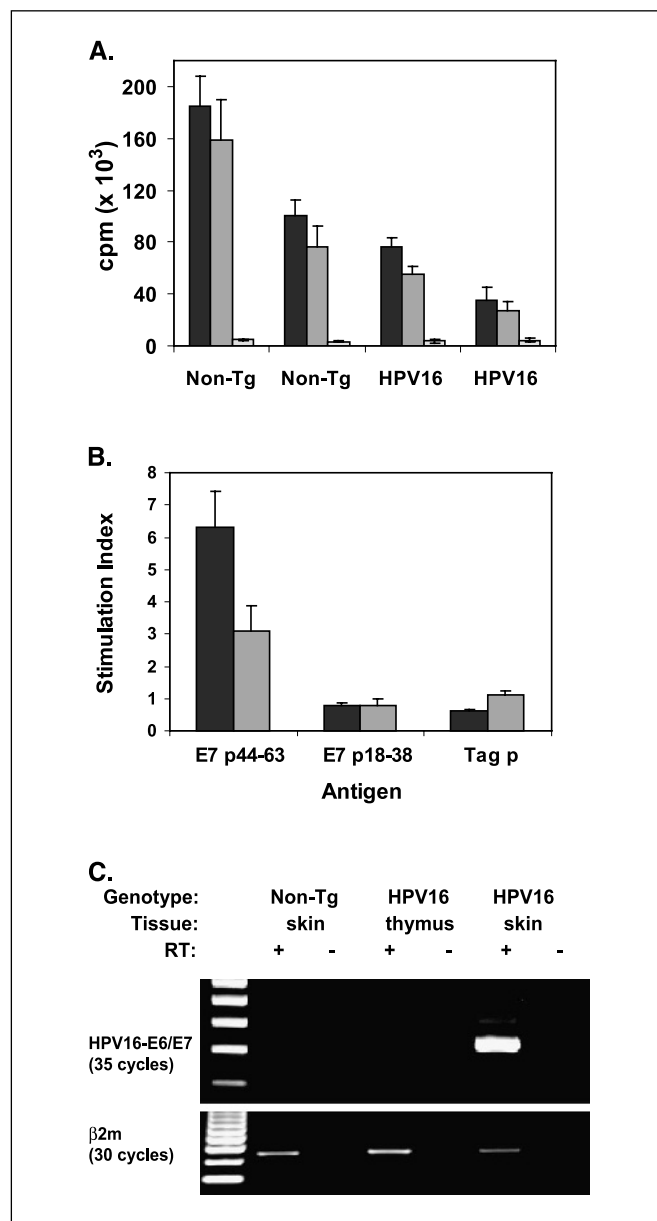
**Statistical Analysis.** Tumor burden was analyzed using a Mann-Whitney test (InStat v1.12, GraphPad Software). Tumor-free survival was analyzed using a  $\chi^2$  test (Prism v3.0a, GraphPad Software). Infiltrating MMP9<sup>+</sup> and CD4<sup>+</sup> cells was analyzed using two-tailed paired and unpaired Student's *t* tests, respectively (Prism v3.0a, GraphPad Software, San Diego, CA).

## Results

**Partial Self-Tolerance to HPV16 E7 in K14-HPV16 Mice.** As a precedent to testing antigen-specific immunotherapies directed against cervical cancer, we sought to assess the capability of K14-HPV16 transgenic mice to mount an induced immune response to the E7 oncoprotein. In contrast to the case in humans, the HPV16 oncoproteins are transmitted through the germ line in these transgenic mice, and thus it was conceivable that self-tolerance to the E7 oncoprotein was induced, as has been reported for other HPV transgenic mice (26). Non-transgenic and HPV16 mice were immunized with recombinant HPV16 E7 protein in complete Freund's adjuvant. Given that the HPV16 oncogenes are expressed both in skin and cervix, we chose to perform immune function assays using the lymph nodes draining the site of immunization. Lymph node cells showed demonstrable T-cell proliferation *ex vivo* (Fig. 1A), although the proliferation response was consistently diminished in HPV16 mice compared with nontransgenic littermates. There is an immuno-dominant class II (I-A<sup>q</sup>) restricted epitope of HPV16 E7, which includes amino acids p44-63 (23). Therefore, we immunized nontransgenic and HPV16 mice with the E7p44-63 peptide in complete Freund's Adjuvant. As was observed with whole E7 protein, both transgenic and nontransgenic mice responded to p44-63, but HPV16 mice had a diminished response compared with nontransgenic mice (Fig. 1B). These results indicate a state of partial self-tolerance toward HPV16 E7 in the form of reduced peripheral CD4<sup>+</sup> T-cell responses. This difference in responsiveness could be due to a lower precursor frequency of E7-specific T cells, to reduced responsiveness within the responding clones (anergy), or to the activity of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. (We did not in this study assess the potential involvement of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in the immunologic phenotype, a possibility that deserves future investigation.)

In regard to CD8<sup>+</sup> T cells, it has been documented that FVB/n (H-2<sup>q</sup>) mice, the background strain for K14-HPV16 model, cannot mount a class I restricted CD8<sup>+</sup> T-cell response to HPV16 E7 (27). As such, the HPV mice can be considered ignorant (or passively self-tolerant) of E7 in the cytotoxic T-cell compartment.

A major mechanism of self-tolerance involves the deletion of developing T cells responsive toward ubiquitous as well as many tissue-selective antigens as a result of their expression in the thymus (28, 29). Indeed, the endogenous *Keratin 14* gene and several K14 promoter-driven transgene constructs have been shown to be expressed in the cortical epithelium of the thymus (30–32). Motivated by these considerations, thymi from 1-day-old neonatal HPV16 mice were tested for expression of E6/E7 mRNA. No E6/E7 mRNA was detected by reverse transcription-PCR analysis (Fig. 1C), suggesting that the observed state of reduced responsiveness toward E7 in the CD4<sup>+</sup> T-cell compartment was unlikely to involve thymic deletion of E7-specific T cells. In contrast, several HPV16 E6/E7 transgenic mouse lines generated by Lambert et al. demonstrably express E7 in the thymus (26). This difference between the two transgenic lines may involve integration dependent effects on thymic gene expression, as has been observed



**Figure 1.** HPV16 mice can mount an immune response to the HPV16-E7 oncoprotein upon immunization. **A**, proliferative response of pooled inguinal and peri-aortic lymph node cells from normal nontransgenic (*Non-Tg*) and HPV16 mice immunized 10 days previously with HPV16-E7 emulsified in complete Freund's adjuvant. Cells were cultured in 0.70  $\mu$ mol/L HPV16-E7 (*black columns*), 0.35  $\mu$ mol/L HPV16-E7 (*grey columns*), or no antigen (*white columns*), in HL-1 medium. **B**, proliferative responses of lymph node cells from nontransgenic mice (*black columns*) and HPV16 mice (*grey columns*). The mice were immunized with HPV16 E7 p43-62 peptide in CFA, boosted with p43-62 in IFA, and restimulated *in vitro* with the immunizing peptide, E7 p43-62, an irrelevant E7 peptide, E7 p18-38, or an irrelevant Tag peptide. Lymph node cells were stimulated with Con A as a positive control. All mice displayed similar Con A responses (data not shown). Lymph node cells were incubated for 5 days before a 6-hour pulse with [<sup>3</sup>H] thymidine. Columns, mean of triplicate wells; bars,  $\pm$ SD. HPV16 mice show a significant decrease in E7 p43-62-specific proliferation compared with nontransgenic controls ( $P = 0.0159$ ; unpaired *t* test). Both experiments are representative of three assays with equivalent results. **C**, HPV16 E6/E7 mRNA is not expressed in the thymus of HPV16 neonatal mice. Total RNA from 1-day-old HPV16 thymus, HPV16 skin, and nontransgenic skin was prepared and reverse transcribed into cDNA. PCR for E6/E7 and  $\beta$ 2-microglobulin done on the cDNA from the three tissues. The PCR product was visualized following electrophoresis through a 1.5% agarose gel; +RT and -RT displayed for each tissue.

with other transgenes (e.g., the alternative tolerance or autoimmunity in independent lines of RIP1-Tag transgenic mice; refs. 33, 34). We infer, given the lack of detectable thymic expression of E7 in the K14-HPV16 line studied herein, that a peripheral mechanism is responsible for the partial tolerance observed, although we formally cannot exclude possible contributions from rare (and undetectable) E7-expressing cells in the thymus. In any event, the skin in K14-HPV16 mice (Fig. 1C), and the uterine cervix in females (16), both express E6/E7 mRNA, which we infer elicits the modestly attenuation in responsiveness to E7 protein.

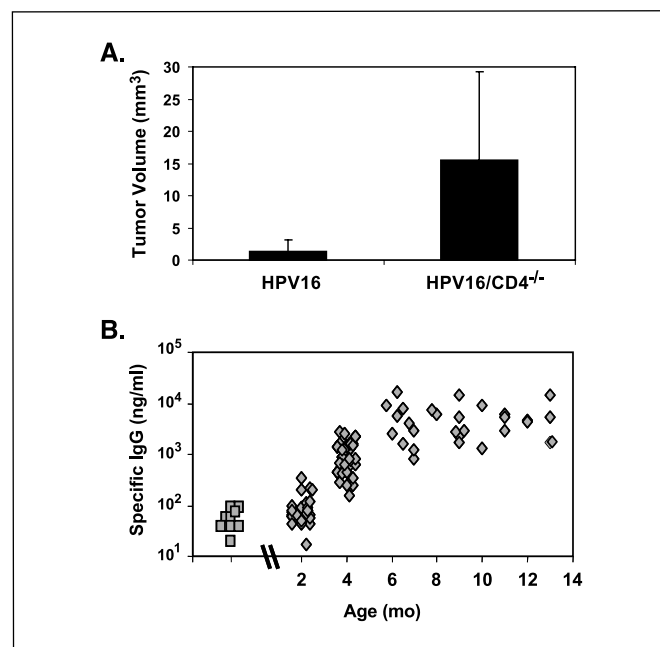
#### Immune Surveillance of the Cervix in HPV16 Mice.

Epidemiologic studies in humans have shown that the incidence and severity of HPV-associated cervical or anogenital neoplasias are higher in HIV-positive immunocompromised humans (6, 7), suggesting that immune surveillance normally serves to restrict development of HPV-induced neoplasias in immunocompetent individuals. Therefore, bolstered by the results of the lymph node proliferation assay (a measure of CD4<sup>+</sup> T-cell reactivity) indicating that HPV16 mice were capable of mounting an immune response against exogenous E7 antigen, we took a genetic approach to assess immune surveillance, by generating HPV16 mice carrying a homozygous disruption of the *CD4* gene (22). Female HPV16 ( $n = 10$ ) and HPV16/CD4<sup>-/-</sup> ( $n = 9$ ) mice were implanted with estrogen, allowed to age, and their reproductive tracts analyzed for pathology at 7 months of age, using well-established criteria (17). Remarkably, HPV16/CD4<sup>-/-</sup> mice showed a ~10-fold increase in tumor burden as compared with HPV16 controls ( $P = 0.0017$ , Wilcoxon test; Fig. 2A). The incidence of tumors in HPV16/CD4<sup>-/-</sup> mice was 100% as compared with an 80% incidence in control HPV16 mice. Because the penetrance of invasive cancer was already quite high in the untreated mice, the 20% increase is not statistically significant. In addition, HPV16/CD4<sup>-/-</sup> mice averaged 1.7 physically separate cervical tumors per mouse, whereas immune-competent HPV16 mice had on average 1.0 tumors per mouse ( $P = 0.0896$ , Wilcoxon test). The increases in tumor incidence, in tumor number, and in tumor burden in mice lacking CD4<sup>+</sup> T cells are collectively persuasive that a functionally antagonistic immune response normally arises in response to E7 expression in the context of incipient cervical neoplasia in this mouse model, much as is inferred to be the case in human cervical cancer (7).

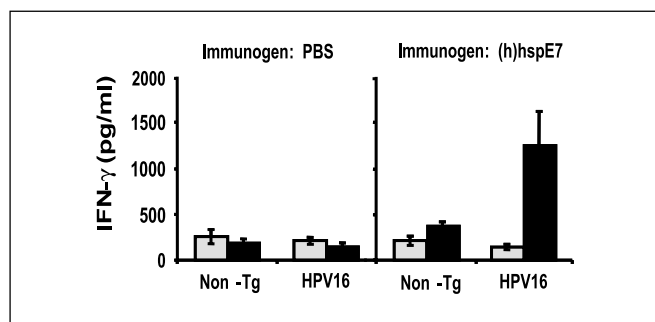
Based on the evidence of immune surveillance in the cervix, we evaluated K14-HPV16 mice for evidence of spontaneous immunity towards endogenously expressed E7 by assaying for autoantibodies. Serum from nonestrogen implanted HPV16 mice of varying ages was analyzed for E7-specific IgG. Spontaneous antibodies to HPV16-E7 were detected in all animals  $\geq 4$  months of age (Fig. 2B), indicating the mice were mounting a spontaneous HPV-specific immune response. While this E7-specific IgG response implied the presence of E7-specific T-cell help for class switching, we were unable, in the context of a concurrent study of the immunologic variables of skin carcinogenesis, to detect E7-specific T-cell proliferative responses in the draining lymph nodes to the skin. Thus, when lymph node cells from the nodes draining dysplastic skin lesions or tumors were stimulated with recombinant HPV16 E7, and supernatants analyzed for IFN- $\gamma$  production, no measurable response toward the HPV16 E7 protein was observed (35). These results suggest that E7-reactive T cells represent a small fraction of the T cells populating the draining

lymph nodes of the skin. We have not directly analyzed the lymph nodes draining the uterine cervix in these mice but anticipate a similar situation therein.

**(h)hspE7 Primes an IFN- $\gamma$  Response in HPV16 Mice.** The spontaneous immunity towards E7 and the evidence for cervical immune surveillance supported the proposition that this mouse model might be amenable to evaluation of therapeutic strategies based on hyperactivating the immune response against HPV16 E7. Among various antigen-specific immunotherapeutic strategies in preclinical development, we chose to examine this postulate with one in particular. (h)hspE7 is a recombinant protein fusing the *M. bovis* Bacillus Calmette-Guerin hsp65 with HPV16 E7; inoculation of this protein into mice induced regression of palpable s.c. tumors expressing the HPV16 E7 tumor antigen, without an exogenous adjuvant (21). (h)hspE7 has been shown to prime both T<sub>H</sub>1 and cytotoxic T-cell responses in mouse strains capable of mounting an E7-specific CD8<sup>+</sup> T-cell response, as well as to elicit humoral immunity (21). We first sought to assess the antigenicity of this protein in the FVB genetic background of the HPV16 model, recognizing the lack of immunologic recognition of E7 by the H-2<sup>d</sup> class I molecules. Nontransgenic FVB mice and HPV16 mice were immunized with PBS or (h)hspE7 and boosted 14 days later. Fourteen days after the boost, splenocyte cultures were prepared and analyzed for IFN- $\gamma$  production following *in vitro* stimulation with native E7, to reveal the E7-specific component of the induced response (Fig. 3). Splenocytes from nontransgenic mice inoculated with (h)hspE7 produced minimal levels of IFN- $\gamma$  upon *ex vivo* E7 stimulation. In contrast, splenocytes from HPV16 mice immunized with (h)hspE7 released markedly higher levels of



**Figure 2.** Spontaneous tumor immunity and immune surveillance in HPV16 mice. *A*, HPV16/CD4<sup>-/-</sup> mice ( $n = 9$ ) develop a larger cervical tumor burden than HPV16/CD4<sup>+/+</sup> mice ( $n = 10$ ;  $P = 0.0017$ ; Wilcoxon test). Tumor burden was determined as described in the methods. *B*, sera from normal nontransgenic mice (squares,  $n = 10$ ) and HPV16 mice of varying ages (diamonds,  $n = 103$ ) were assayed for antibody reactivity to HPV16-E7 by ELISA. Point, IgG titer of a mouse of the indicated age. E7-specific IgG titer was quantified by comparison to a positive control antibody, purified mouse anti-HPV16-E7 (8C9).



**Figure 3.** (h)hspE7 primes IFN- $\gamma$  responses. Splenocytes from nontransgenic and HPV16 mice immunized with either PBS or (h)hspE7 were cultured with no antigen (white columns) or HPV16 E7 (black columns) in HL-1 media. Supernatants were assayed for IFN- $\gamma$  release by ELISA. Splenocytes were stimulated with Con A as a positive control. All mice displayed similar IFN- $\gamma$  production in response to Con A (data not shown). Columns, mean of triplicates; bars,  $\pm$ SE. Representative of three assays with equivalent results.

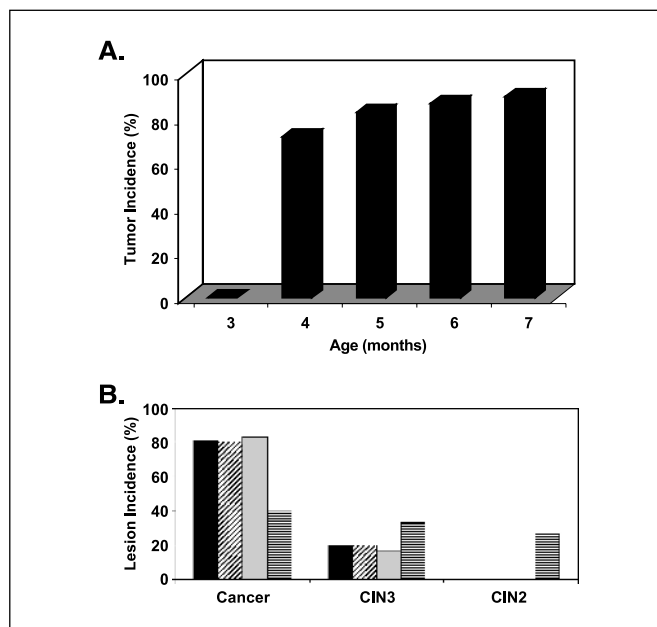
IFN- $\gamma$ . Thus, (h)hspE7 amplified a preexisting E7-specific IFN- $\gamma$  response in HPV16 transgenic mice.

### Treatment with (h)hspE7 Elicits CD4<sup>+</sup> T Cell-Dependent Regression of Cervical Neoplasia and Incipient Cancer.

Motivated by the genetic evidence that spontaneous CD4<sup>+</sup> T helper responses were limiting tumor growth in the HPV16 mice and by the data demonstrating that (h)hspE7 could hyperactivate the anti-E7 immune response, we investigated the impact of (h)hspE7 immunotherapy in animals with moderate to severe cervical intraepithelial neoplasia (i.e., having the histologic equivalent to CIN 2 and CIN 3 observed in HPV+ patients) and nascent cervical carcinomas. To help guide therapeutic trial designs, we conducted longitudinal analysis of cervical cancer incidence in the HPV16 model. HPV16 female mice were implanted with 60-day time release estrogen pellets at 1, 3, and 5 months of age and monitored for cancer incidence at monthly intervals from 3 to 7 months of age (Fig. 4A). We observed no instances of cancer in 3-month-old female mice, but by 4 months of age, 72% of the HPV16 mice had invasive cervical cancers, which increased to 90% over the next 3 months, concomitant with increasing tumor burden. For the current trial, we immunized HPV16 female mice at 5.5 months when the tumor incidence is 83% to 87%. Mice were immunized with 1.4 nmol of the (h)hspE7 fusion protein, or as a control native hsp65 protein lacking the E7 specificity. The dosage of (h)hspE7 had been optimized in the transplant model involving the TC-1 tumor cell line (21). Two weeks after the initial immunization, the mice were boosted with an equivalent dose of antigen. The trial was terminated 4 weeks subsequent to the 2nd inoculation (i.e., at 7 months of age) and outcome evaluated. In the first trial, histopathologic analysis of mice treated with (h)hspE7 revealed a significantly decreased incidence of tumors, with only 17% of mice showing evidence of cervical cancer ( $P = 0.0109$ , Fisher's exact test; Table 1). In contrast, HPV16 mice treated with PBS (vehicle) or hsp65 had incidences of 81% and 83%, respectively, within the range of untreated historical controls. In a second trial, we observed a less pronounced decrease, to a 56% incidence of cancer in (h)hspE7-treated mice. The second trial also included an additional group of animals that received 1.4 nmol of native E7 protein, which produced no decrease in tumor incidence (Table 1). Although (h)hspE7-treated mice in this second

experiment failed to show a statistically significant decrease in incidence relative to PBS-treated controls (in part due to the small cohort size), the lower incidence (56% versus  $\sim$ 80% in controls) is clearly suggestive. We assessed mean tumor volume in the second trial, and found no difference in treated versus control cohorts ( $1.6 \pm 0.9 \text{ mm}^3$  in the five (h)hspE7-treated mice that developed tumors versus of  $1.6 \pm 1.9 \text{ mm}^3$  among the eight PBS-treated controls). A third trial was conducted using a five-fold higher dose of (h)hspE7 (7 nmol). The (h)hspE7-treated mice showed a significant decrease in tumor incidence (20%;  $P = 0.0061$ , Fisher's exact test, Table 1). Collectively, these results indicate that the (h)hspE7 immunotherapy regresses HPV-induced cervical carcinogenesis in a transgenic mouse model, and suggest that dosing levels may be significant in reliably achieving an efficacious response.

Squamous carcinogenesis of the cervix in the HPV16 mouse model is a multistep process wherein distinctive lesional stages seem temporally similar to the case in humans (36). Thus, we asked whether mice lacking overt cervical carcinomas had premalignant lesions, and if so, of what histologic grade. Indeed, all treated mice lacking carcinomas had cervical dysplasia; thus, the vaccine was not eliminating all neoplastic tissue. There was, however, indication of antagonistic effects against dysplastic lesions. All mice in the various trial cohorts were scored based on the highest pathologic grade amongst the focal lesions detected in the cervix, recalling that all mice at the beginning of the trial probably had either CIN 3 lesions or cervical carcinomas. In the PBS-, hsp65-, and E7-treated cohorts, every mouse that had not progressed to invasive



**Figure 4.** (h)hspE7 regresses neoplastic lesions. A, incidence of cervical cancer in female HPV16 mice at 3 months ( $n = 15$ ), 4 months ( $n = 18$ ), 5 months ( $n = 35$ ), 6 months ( $n = 15$ ) and 7 months ( $n = 40$ ) of age is shown. B, (h)hspE7-treated mice (horizontal-striped columns,  $n = 26$ ) presented with fewer tumors and advanced dysplasias (CIN 3), concomitant with an increased frequency of less aggressive CIN 2 lesions, when compared with control PBS-treated (black columns,  $n = 15$ ), hsp65-treated (cross-hatch columns,  $n = 15$ ), or E7-treated (gray columns,  $n = 6$ ) mice ( $P = 0.0294$ ,  $\chi^2$  Contingency test). The mice were scored according to the most advanced lesion detected upon serial sectioning of the entire cervix; notably, all mice at the start of the trial had cancer ( $\sim$ 80%) or CIN 3 lesions.

**Table 1.** (h)hspE7 immunotherapy in the hPV16 transgenic mouse model of cervical cancer

Genotype	Treatment	Dose (nmol)	Trial	Tumor incidence (%)	Statistics <i>P</i>
HPV16	PBS	—	1	81 (13/16)	—
		—	2	80 (8/10)	—
HPV16	hsp65	1.4	1	83 (5/6)	1.0000
		1.4	2	78 (7/9)	1.0000
HPV16	E7	1.4	2	83 (5/6)	1.0000
HPV16	(h)hspE7	1.4	1	17 (1/6)	0.0109
		1.4	2	56 (5/9)	0.2524
		7.0	3	20 (1/5)	0.0061
HPV16/CD4 <sup>-/-</sup>	PBS	1.4	1	100 (9/9)	0.1553
HPV16/CD4 <sup>-/-</sup>	(h)hspE7	1.4	1	86 (6/7)	0.7633

NOTE: HPV16 mice were implanted with 17 $\beta$ -estradiol pellets as described. At 5.5 and 6 months of age, the mice were treated with hsp65, (h)hspE7, E7, or PBS. At 7 months of age, or 6 weeks after starting the immunotherapy, the mice were sacrificed and analyzed histopathologically. All mice treated with hsp65 and (h)hspE7 were screened for IgG responses to hsp65 after immunization. Only mice with evidence of humoral immunity to hsp65 were included in the data analysis (data not shown). Statistical analysis is by  $\chi^2$  test, and each group is compared to PBS controls for that trial. In trial 3, statistical analysis is compared to the historical PBS control data.

cervical cancer had severe dysplasia (CIN 3) at the end of the trial (Fig. 4B). In contrast, ~27% of mice treated with (h)hspE7 had only moderate dysplasia (CIN 2) at the end of trial ( $P = 0.0294$ ,  $\chi^2$  contingency test), whereas the remainder still had CIN 3 lesions (33%) or cervical cancer (40%). It is unclear whether these CIN 2 lesions are newly developing lesions that arose following the elimination of the CIN 3 and overt cancers present at the start of the trial, or represent regression of the latter lesions to a more benign state. To gain an insight into the mechanism of action of (h)hspE7, we assessed treated and control cervixes immunohistochemically for several diagnostic cell markers. We have previously shown in the epidermis of HPV16 mice that stromal infiltration of premalignant lesions by MMP-9-expressing innate immune cells is associated with more rapid malignant progression (35, 37). Therefore, we analyzed the cervix from 6.5-month-old mice immunized at 5.5 and 6 months of age with hsp65 control (Fig. 5A;  $n = 4$ ) or (h)hspE7-treated mice (Fig. 5B;  $n = 4$ ) for MMP-9 expression. A statistically significant decrease in the number of MMP-9<sup>+</sup> cells was observed in the mice treated with (h)hspE7 (Fig. 5C;  $P = 0.044$ , paired Student's  $t$  test).

To understand the potential role of CD4<sup>+</sup> T cells in the mechanism of (h)hspE7 action, we conducted immunohistologic analysis for CD4-expressing cells in the cervix of 6.5-month-old hsp65 control (Fig. 6A;  $n = 9$ ) and (h)hspE7-treated mice (Fig. 6B;  $n = 8$ ). The CD4<sup>+</sup> T-cell infiltrate was found primarily in the cervical stroma, where we observed a statistically significant increase in infiltrating T cells in those mice receiving the (h)hspE7 immunotherapy (Fig. 6C;  $P = 0.021$ , unpaired Student's  $t$  test). To clarify the role of CD4<sup>+</sup> T cells in the observed efficacy against cervical cancer and CIN 3 lesions in mice treated by (h)hspE7 immunization, we similarly treated HPV16/CD4<sup>-/-</sup> mice with (h)hspE7. HPV16/CD4<sup>-/-</sup> female mice on sustained estrogen were immunized with (h)hspE7 at the same 5.5-month time point as their immune-competent HPV16 controls. The CD4-deficient HPV16 mice treated with (h)hspE7 showed no evidence of tumor regression, presenting with an 86% incidence of cervical cancer at the 7-month experimental end point (Table 1). It should be noted that the CD4-deficient HPV16 mice would

likely begin the trial with a larger tumor burden based on the data in Fig. 2. Nonetheless, these data are consistent with a CD4-dependent mechanism for regression of cervical tumors in the HPV16 transgenic mouse.

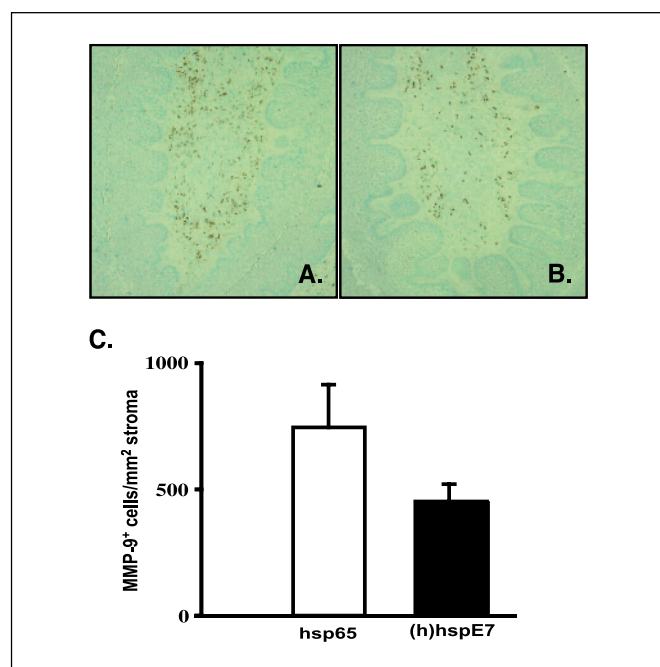
In addition, we sought to analyze natural killer cell infiltration. Unfortunately, the available antibody reagents to detect mouse natural killer cells proved ineffectual in immunohistochemical analysis<sup>3</sup>; thus, we were unable to assess the infiltration of, or changes in abundance or character of, natural killer cells. It will be of interest to analyze their possible contribution to the therapeutic efficacy of this vaccine in future work, using flow cytometry and/or improved reagents.

## Discussion

This study has shown that an antigen-specific immune activating protein has efficacy when used to treat mice with incipient cervical carcinomas. Fusion of the HPV16 E7 oncoprotein to a highly immunogenic mycobacterial heat shock protein, hsp65, produced an immunogen with intrinsic adjuvant activity that we have shown to be capable of changing the course of cervical carcinogenesis in a mouse model mimicking key features of the human disease. The responses seen are particularly encouraging given the strong similarities of the mouse model to the human condition. The strengths of the model are the relative synchronicity and high penetrance of cervical carcinogenesis (which allows assembly of cohorts for therapeutic trials), the appearance of carcinomas in the transformation zone of the cervix much as in humans, and the *de novo* development of those cancers via progenitor stages that are histologically analogous to the CIN lesions used to grade neoplastic development in humans. In regard to immunologic phenotype, there is no evidence for thymic expression of E7 mRNA, and the mice are nontolerant in the humoral compartment, as evidenced by the appearance of spontaneous anti-E7

<sup>3</sup> Our unpublished observations and L. Lanier, personal communication.



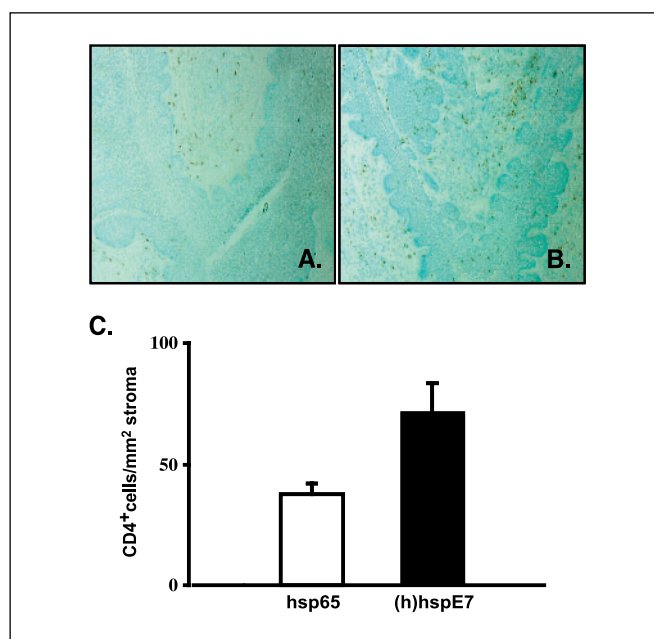


**Figure 5.** Reduced numbers of infiltrating MMP-9<sup>+</sup> cells in neoplastic cervix of (h)hspE7-treated mice. Immunolocalization of MMP-9<sup>+</sup> cells in cervical biopsies from (A) control hsp65-treated ( $n = 4$ ) and (B) (h)hspE7-treated ( $n = 4$ ) animals. Quantitation of MMP-9<sup>+</sup> cells/mm<sup>2</sup> (C) was obtained by counting the total number of brown-stained cells (MMP-9<sup>+</sup>) and dividing that by the total stromal area calculated using Improvision OpenLab software. Columns, mean; bars,  $\pm$ SE. A 40% reduction in the number of MMP-9<sup>+</sup> cells/mm<sup>2</sup> was observed in (h)hspE7-treated cervix compared with the hsp65 controls ( $P = 0.044$ , paired Student's  $t$  test).

antibodies concomitant with neoplasia in the skin and cervix, as well as by the enhanced cervical tumor phenotype seen when CD4<sup>+</sup> T cells are genetically ablated. In contrast, the mice are constitutionally tolerant of the E7 oncoprotein in the CD8 compartment by virtue of MHC haplotype. To assess the role of CD8<sup>+</sup> T-cell responses to E7 in the model, we are engaged in the lengthy process of producing HPV16 mice congenic for the H-2b MHC, whose class I molecules will bind E7 epitopes and mediate CD8<sup>+</sup> T-cell responses to E7, but are otherwise inbred into FVB, to maintain the permissive modifier loci that engender squamous carcinogenesis by the HPV oncogenes with the qualifier that endogenous immune responses to E7 in the model could alter carcinogenesis.

One benefit of our investigation of an HPV16 E7 protein-based immunogen using a mouse model of cervical cancer lacking the capability to mount an E7-specific CD8<sup>+</sup> T-cell response is that we have been able to unambiguously document the functional importance of antigen-specific CD4<sup>+</sup> T cells. The data show that CD4<sup>+</sup> T cells spontaneously antagonize cervical carcinogenesis, as revealed by the *CD4<sup>+</sup>* gene knockout, and show that CD4<sup>+</sup> T cells can be mobilized by (h)hspE7 to reverse the course of cervical carcinogenesis in a significant fraction of treated mice. Although we do not provide any conclusive evidence of the ultimate effector mechanism, we did observe an increase in the CD4<sup>+</sup> T cells infiltrating the stroma adjacent to the premalignant lesions in the (h)hspE7-treated mice. Given the recent reports demonstrating the importance of IFN- $\gamma$  in tumor immune surveillance, it is reasonable to hypothesize that IFN- $\gamma$  producing E7-specific helper T cells are the effector cell (38). The importance of CD4<sup>+</sup>

T cells is provocative for two reasons. First, most tumor immunologists consider CD8<sup>+</sup> T cells to be the primary effectors of immune surveillance and prime targets for achieving effective tumor immunity with therapeutic and preventative immunostimulatory treatments. Second, we have documented in these same K14-HPV16 transgenic mice a much different situation for the skin cancers that subsequently develop in males and females irrespective of the sustained estrogen that selectively enhances cervical carcinogenesis. CD4<sup>+</sup> T cells, largely activated against bacteria infecting neoplastic skin, have been found to serve as functional enhancers, not antagonists, of epidermal progression (35). A hallmark of the immune enhancement observed in the epidermis is the infiltration of MMP-9-expressing granulocytes (35, 37). However, the hsp-E7 immunotherapy actually results in fewer MMP-9-expressing cells in the cervical microenvironment, again suggesting we have achieved a tumor-antagonistic, not a tumor-promoting, immune response. Notably, the importance of MMP-9 for cervical carcinogenesis has recently been evaluated using gene knockout mice and pharmacologic inhibitors, revealing MMP-9 to be a functionally important enhancer of angiogenesis, tumor formation, and tumor growth (39). As such, one could envision that the activated antigen-specific CD4<sup>+</sup> T cells in the cervix are suppressing infiltration of MMP-9 expressing macrophages as part of their mechanism of tumor immunity. Thus, two squamous epithelial microenvironments undergoing carcinogenesis induced by expression of the same oncogenes in the same mouse line are differentially affected by CD4<sup>+</sup> T cells: predominantly tumor antigen-nonspecific CD4<sup>+</sup> T cells enhance progression in the skin, whereas tumor antigen-specific CD4<sup>+</sup> T cells limit progression in the cervix. We do not consider this nonspecific immune enhancement in the skin to be incongruous with cervical immunotherapies based on specific immune



**Figure 6.** Increased numbers of infiltrating CD4<sup>+</sup> cells in (h)hspE7-treated mice. Immunolocalization of CD4<sup>+</sup> cells in the neoplastic cervix of (A) control hsp65-treated ( $n = 9$ ) and (B) (h)hspE7-treated ( $n = 8$ ) animals. Quantitation of CD4<sup>+</sup> cells/mm<sup>2</sup> (C) was obtained as in Fig. 5. Columns, mean; bars,  $\pm$ SE. A 46% increase in CD4<sup>+</sup> cells/mm<sup>2</sup> was observed in (h)hspE7-treated mice compared with the hsp65 controls ( $P = 0.021$ , unpaired Student's  $t$  test).

stimulation, in light of the results presented here, but the potential for counterbalancing effects of "tumor enhancement" by CD4<sup>+</sup> T cells should nevertheless be kept in mind. In conclusion, we have shown for the first time in a mouse model of *de novo* cervical carcinogenesis that an antigen-specific immunogen can produce objective responses, encouraging the potential of such strategies for treating premalignant and malignant lesions in the human cervix.

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