Induction of MAGE-A3 and HPV-16 immunity by Trojan vaccines in patients with head and neck carcinoma

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ABSTRACT: *Background.* We performed a pilot study using Trojan vaccines in patients with advanced squamous cell carcinoma of the head and neck (SCCHN). These vaccines are composed of HLA-I and HLA-II restricted melanoma antigen E (MAGE)-A3 or human papillomavirus (HPV)-16 derived peptides, joined by furin-cleavable linkers, and linked to a ''penetrin'' peptide sequence derived from HIV-TAT. Thirty-one patients with SCCHN were screened for the trial and 5 were enrolled.

Methods. Enrolled patients were treated with 300 μg of Trojan peptide supplemented with Montanide and granulocyte-macrophage colony-stimulating factor (GM-CSF) at 4-week intervals for up to 4 injections.

Results. Following vaccination, peripheral blood mononuclear cells (PBMCs) from 4 of 5 patients recognized both the full Trojan constructs and constituent HLA-II peptides, whereas responses to HLA-I restricted peptides were less pronounced.

Conclusion. This treatment regimen seems to have acceptable toxicity and elicits measurable systemic immune responses against HLA-II restricted epitopes in a subset of patients with advanced SCCHN. © 2012 Wiley Periodicals, Inc. *Head Neck* **34:** 1734–1746, 2012

KEY WORDS: SCCHN, vaccine, HLA-I, HLA-II, immune response

The therapeutic options for patients with advanced squamous cell carcinoma of the head and neck (SCCHN) whose surgical extirpation and/or treatment with chemoradiotherapy fail are generally limited to observation or palliative chemotherapy.[1] In order to improve the outcomes for this patient population, investigators have explored the use of immunotherapy in the form of peptide vaccines in an attempt to stimulate therapeutic antitumor immune effector responses. Traditional peptide cancer vaccines have focused on priming HLA class I (HLA-I) restricted responses, adhering to the premise that efficient stimulation of antigen specific CD8 T cells, is both necessary and sufficient for effective antitumor immunity.[2] However, the limited clinical efficacy of HLA-I restricted vaccines in isolation[3] has increased attention on the potential utility of simultaneously stimulating HLA class II (HLA-II) mediated CD4 responses, which may be necessary for the development and/or maintenance of antitumor immunity.[4,5] A first step to capitalize on this strategy is to develop vaccines against well-characterized antigens which use both HLA-I and HLA-II peptide-specific sequences.

In order to both generate HLA-I and HLA-II restricted T-cell responses in humans and overcome historic problems associated with extracellular proteolysis of short HLA-I peptides, we used a "Trojan'' peptide approach. Trojan peptide-based vaccines contain a "penetrin'' peptide sequence derived from HIV-TAT (RKKRRQRRR) which allows the entire peptide to translocate through the cell membrane and penetrate directly into the endoplasmic reticulum and Golgi apparatus (Golgi) where they can form peptide-HLA complexes.[6] In addition, this strategy uses furin-cleavable linkers to join multiple HLA-I and HLA-II peptide-epitopes, enabling release of the individual peptide-epitopes in the Golgi, where furin endopeptidase resides.[7] These Trojan peptide-

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Additional Supporting Information may be found in the online version of this article.

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Conflict of interest: Dr. Strome is a major stockholder in Gliknik, a biotechnology company. Gliknik has licensed the rights to this vaccine technology from the University of Maryland. Dr. Strome receives royalties from the licensure of this technology. Dr. Celis receives royalties from the Mayo Clinic College of Medicine as an inventor of the Trojan peptide vaccines.

based vaccines seem to be relatively resistant to proteolysis and do not require proteosomal processing and transport by Transporter associated with antigen processing (TAP), because they penetrate directly to the endoplasmic reticulum and Golgi.[6] Perhaps more importantly, these functional attributes are associated with improved outcomes in both prophylactic and therapeutic mouse tumor models.[7]

For purposes of this study, we designed 2 separate Trojan constructs using previously described HLA-I and HLA-II T cell epitopes of the human papillomavirus type 16 (HPV-16) and melanoma antigen E (MAGE)-A3 tumor associated antigens. MAGE is a cancer testis antigen originally found in melanomas but later identified in other epithelial cell malignancies,[8] while HPV-16 is a papillomavirus which is associated with SCCHN of the oropharynx and hypopharynx.[9,10] The choice of HPV-16 was premised on several considerations including its high prevalence in SCCHN, tumor specificity, and the presence of well-defined peptide epitopes.[9–13] Similarly, MAGE-A3 was selected based on its high levels of expression in SCCHN and the availability of well-characterized HLA-I and HLA-II immunostimulatory epitopes.[14–16] Before injection, the Trojan peptides were solubilized in Montanide ISA 51 and granulocyte macrophage colony stimulating factor (GM-CSF; Sargramostim), as a means to promote dendritic cell migration to the site of vaccination and to enhance antigen presentation.[17] In the first group of patients, both an HLA-A*02 positive genotype and expression of HLA-A*02 on the tumor cell surface were mandated for study entry. This original study design was premised on the fact that the HLA-I peptides in the vaccine are HLA-A*02-specific and could only target tumors expressing this HLA-I allele.[18] However, based on our interval findings that the peptides predominantly stimulated responses against the HLA-II epitopes, whose allele restrictions are recognized to be promiscuous, the inclusion criteria were modified to include all patients with either MAGE-A3 or HPV-16-positive tumors.

Thirty-one patients with recurrent or metastatic SCCHN were screened for this pilot study, and 5 were enrolled. Three patients received the MAGE-A3 and 2 patients received the HPV-16 vaccine. Although 1 patient with a known stable brain metastases experienced a serious adverse event (SAE), the Trojan vaccines were well tolerated and toxicities in general did not require or prolong hospitalization. In 4 of 5 immunized patients, durable interferon-γ (IFN-γ) responses were observed in response to both the intact Trojan peptide and the HLA-II-specific peptide. In addition, Trojan peptide-specific immunoglobulin G (IgG) was detected in plasma from 2 patients. By Elispot, Trojan peptide-specific HLA-I IFN-γ-producing cells were identified in the peripheral blood of 1 of the MAGE-A3 immunized patients (patient 3). To the best of our knowledge, this study is the first to report the use of Trojan peptide vaccines in humans and suggests that this strategy might provide a useful platform for priming antigen-specific T cell responses in patients with head and neck carcinoma.

MATERIALS AND METHODS

Patient population, study protocol, and treatment schedule

Between December 2005 and March 2007, 31 patients were screened for their eligibility to enroll in a phase I clinical study designed to evaluate toxicity and immunogenicity of 2 Trojan (HPV-16 and *MAGE-A3*) peptide-based vaccines. All experimental work related to human subjects in this study was approved by the University of Maryland's Institutional Review Board. Written informed consent was provided according to the Declaration of Helsinki. Enrollment criteria included (1) patients with recurrent or metastatic SCCHN not amenable to treatment with standard therapy or patients who refused standard treatment, (2) the presence of the HLA-A*02 genotype, and (3) either *MAGE-A3* or HPV-16 expression in the tumor. Importantly, based on interim data analysis demonstrating that the immune responses were predominantly HLA-II restricted, we eliminated the HLA-A*02 restriction in the latter part of the study.

The vaccine dose used in the trial was 300 μg of Trojan peptide, with 1.2 mL of Montanide ISA 51 (Seppic, France) and 100 μg/m^2 of GM-CSF) (Berlex, Seattle, WA). This vaccine dose was chosen because it is well within the range of previous peptide vaccine trials for cancer and it was well tolerated by the majority of patients while providing evidence of immunological efficacy.[2] Peptides were reconstituted with 0.5 mL of sterile water for injection and each vial was mixed with Montanide ISA 51 and GM-CSF in an empty vial. The total contents of the mixture were drawn up in a syringe and pushed back into the vial. This mixing step was repeated 25 times to make unified emulsions, and final emulsions were drawn up in multiple syringes for injection. All vaccinated patients were injected subcutaneously in the inguinal region with the appropriate Trojan peptide vaccine at 4-week intervals for up to 4 injections as indicated in Figure 1. The primary indications for giving fewer than 4 injections were drug-related toxicity (patient 1) or disease progression (patients 2, 3, 4, and 5). After the final injection, patients returned for follow-up visits every 3 months.

Trojan peptide-based vaccines and individual peptides

The *MAGE-A3* Trojan peptide and the HPV-16 Trojan peptide were manufactured by Multiple Peptide Systems (San Diego, CA) and processed and vialed by Merck Biosciences AG (Laufelfingen, Switzerland). Patients were immunized with either the *MAGE-A3* or the HPV-16-specific Trojan peptide vaccine. Specific sequences of the Trojan vaccines and individual antigens to determine vaccine-induced immune responses are listed in Table 1. The peptides were studied under IND #10927 (NCI Clinicaltrials.gov #NCT00257738).

Isolation of peripheral blood mononuclear cells and tissue/tumor infiltrating lymphocytes

Whole blood samples were collected before the initiation of treatment, within 24 hours before each vaccination, and then at defined follow-up intervals. PBMCs were isolated by Ficoll gradient centrifugation. Tissue/tumor infiltrating lymphocytes (TILs) were isolated as described by Whiteside et al,[19] cryopreserved in RPMI-1640 supplemented with 10% normal human AB serum (NHS) and 10% dimethyl sulfoxide using an automated cell freezer (Gordinier Electronics, Roseville, MI) and stored in the vapor phase of liquid nitrogen for later use. If possible, PBMC/TIL isolated from separate procurement dates were

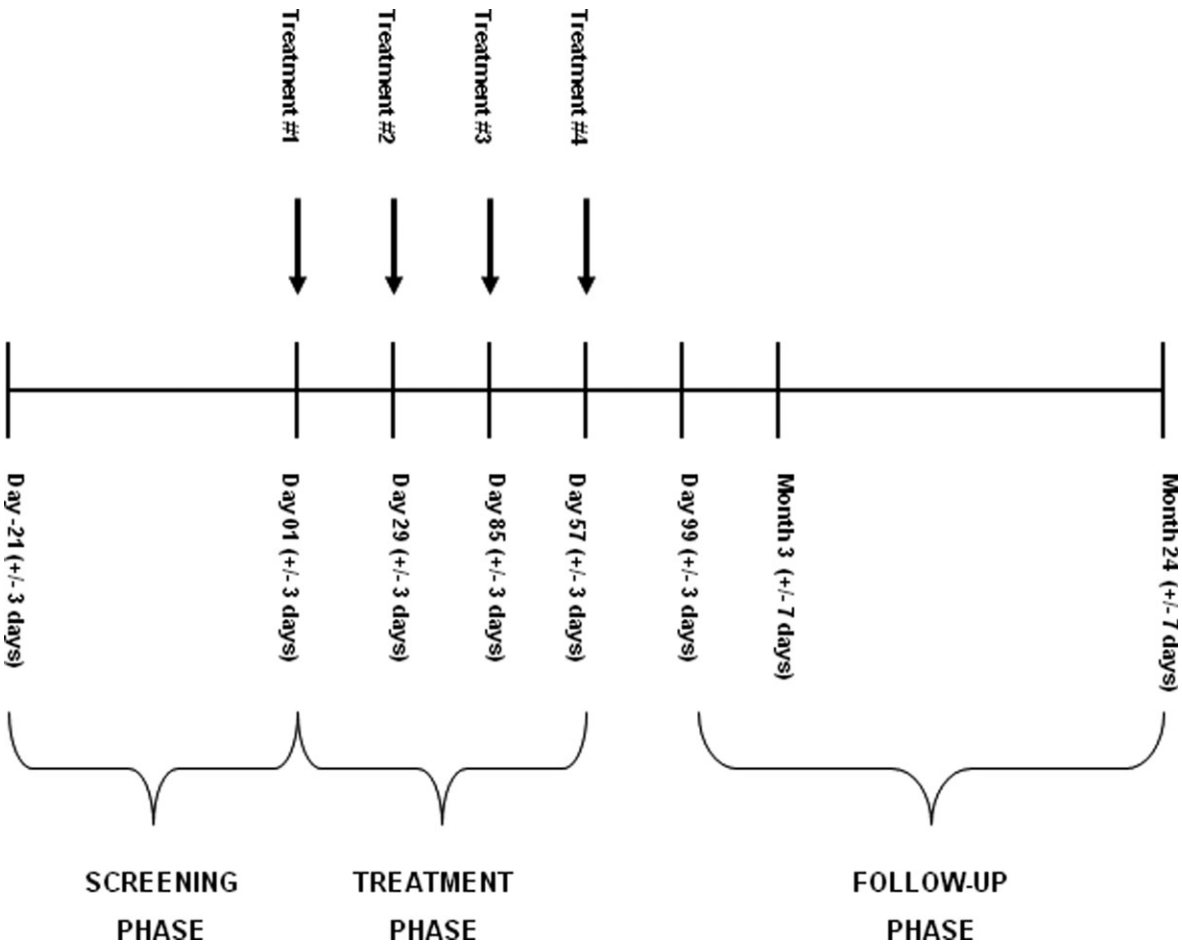

FIGURE 1. Immunization schedule. All enrolled patients were immunized subcutaneously in the inguinal region at 4-week intervals for up to 4 injections. After the final immunization, patients returned for follow-up visits every 3 months.

simultaneously thawed and assayed to assess in vitro function. All experiments were performed in RPMI-1640 supplemented with 10% NHS, 2% HEPES, 1% P/S, and 1% L-Glutamate (hereafter indicated as cRPMI).

TABLE 1. Trojan vaccines and individual peptides.

Antigen	Amino acid sequence[*,†,‡]	HLA-restriction
MAGE-Trojan	KVAELVHFL/**RVKR**/*FLWGPRALV*/***RVKR***/*VIFSKASSSLQL*/*RKKRRQRRR*	A2, DR4/DR7
MAGE-CTL$_1$	KVAELVHFL	A2
MAGE-CTL$_2$	FLWGPRALV	A2
MAGE-HTL	VIFSKASSSLQL	DR4/DR7
HPV-Trojan	TLGIVZPI/**RVKR**/*PAGQAEPDRAHYNIVTFZZKZD*/*RKKRRQRRR*	A2, DR1/DR4/DR13/DR15
HPV-CTL	TLGIVZPI	A2
HPV-HTL	PAGQAEPDRAHYNIVTFZZKZD	DR1/DR4/DR13/DR15
Control	YIGEVLVSV	HA-2[§]

Abbreviations: MAGE, melanoma antigen E; HPV, human papillomavirus.
[*] RVKR; furin-cleavable linkers are indicated in boldface.
[†] RKKRRQRRR; HIV-TAT translocating domain is indicated in italics.
[‡] Z = cyclophenyl alanine.
[§] Human minor histocompatability antigen HA-2.

HLA typing

DNA was extracted from a portion of the PBMC using a QIAamp DNA mini kit (Qiagen) and the alleles of genes encoding HLA-ABDR were determined by the Micro SSP HLA-ABDR DNA typing kit (One Lambda, Canoga Park, CA) per the manufacturers' instructions. All HLA-A*02-positive patients were further analyzed for the genes encoding HLA-A*02 by the Micro SSP HLA-A*02 DNA typing kit (One Lambda) per the manufacturers' instructions.

Melanoma antigen e-a3 and human papillomavirus-16 polymerase chain reaction

Total RNA was extracted from squamous cell tumor biopsies using a Qiagen RNeasy Mini kit according to the manufacturers' recommendations followed by cDNA synthesis using an iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). The melanoma antigen gene family (MAGE) consists of 12 highly homologous genes encoded on human chromosome Xq28.[20] *MAGE-A3* (NM_005362) is highly homologous to *MAGE-A1*, *2*, *4*, *5*, *6*, and *12* in the region of the Trojan peptide. We observed that amplification and sequencing of *MAGE-A3* from tumor samples using previously published primers

for *MAGE-3*, was not specific for *MAGE-A3*.[15] Therefore, new polymerase chain reaction (PCR) primers were designed. For specific amplification of *MAGE-A3* from cDNA primers, MA3_F4 AAC GAG CGA CGG CCT GAC G and MA3_R4 GTG GAA ACT AAG GGA TGC were designed, which yield a PCR product of 1221 bp. For specific amplification of *MAGE-A3* from genomic DNA primers, MA3_F1 GTG GAA ACT AAG GGA TGC and MA3_R4 were designed, which yield a PCR product of 736 bp. Primer MA3_R4 is specific for *MAGE-A3* and forms a 3′ C:C mismatch with the sequences of *MAGE-A2*, *4*, *5*, *6*, and *12*, which is predicted to decrease PCR efficiency by greater than 99%.[21] To confirm the specificity of the amplified products, total RNA and genomic DNA were extracted from 3 different SCCHN cell lines, TU159, 012SCC, and HW12, which express multiple MAGE proteins. An *Eco*RI restriction enzyme recognition site is present in nucleic acid 1017 of *MAGE-A3* (NM_005362) but not in the potentially amplified sequence of any of the other MAGE genes. The specificity of the amplification was tested by restriction digestion of the PCR products with an *Eco*RI restriction enzyme followed by direct sequencing of PCR products (Supplemental Figure 1A, online only). Complete digestion of the PCR product indicated specific amplification in all samples tested, compared with nonspecific amplification with previously published *MAGE-3* primers (Supplemental Figure 1B, online only). Amplification of HPV-16 cDNA was performed using primers E7_F GCT CAG AGG AGG AGG ATG AA and E7_R GCC CAT TAA CAG GTC TTC CA to amplify HPV16 E7 (255 bp product).[10] PCR products were then verified using direct sequencing (Supplemental Figure 1C, online only). All PCRs were performed using ABGENE 2X ThermoPrime ReddyMix (Thermo Fischer Scientific, Rockford, IL) with 1.5 m*M* MgCl2 and a PCR protocol that consisted of 10 cycles that began with an annealing temperature of 63°C and decreased by 0.5°C per cycle followed by 32 cycles with an annealing temperature of 58°C and a 1-minute elongation time.

Immunohistochemistry

For HLA-A*02 immunohistochemical detection, frozen sections were cut at 5-micron thickness from the patient and control tissues and mounted on charged slides. The slides were incubated in cold acetone for 10 minutes before processing in a DAKO Autostainer with an anti-HLA-A*02 antibody (SB03-111, kindly provided by Dr. S. Ferrone, University of Pittsburgh) and AEC substrate as per the manufacturer's instructions. Staining with lymphocyte markers CD3, CD8, and CD68 (all from Ventana, Tucson, AZ) and CD4 (Biocare, Concord, CA) was performed on formalin-fixed paraffin-embedded tissue. Deparaffinization, rehydration, and epitope retrieval were performed following standard procedures. Staining was performed on a Ventana Benchmark-XT stainer using an AP detection system.

Trojan-specific t-cell response monitoring

Interferon-γ recall elispot assay. A 96-well nitrocellulose-bottom (Elispot) plates (Millipore, Molsheim, France) were precoated with IFN-γ at a final concentration of 15 μg/mL (Mabtech, Nacka, Sweden). Plates were then washed 6 times with sterile phosphate-buffered saline (PBS) and blocked with 100 μL/well of RPMI 1640 supplemented with 10% NHS. After 60 minutes of incubation, the blocking media was discarded and 2×10^5 PBMC were plated in triplicate wells. Next, Trojan and individual peptides were added to appropriate wells at a final concentration of 10 μg/mL and plates were incubated at 37°C, 5% CO_2. After 48 hours of incubation, cell supernatants were discarded and Elispot plates developed using a Mabtech development kit. In brief, plates were washed 6 times with 1X PBS and subsequently incubated for 2 hours at room temperature with 100 μL/well of biotinylated anti-human IFN-γ (7-B6-1; Mabtech) monoclonal antibody at a final concentration of 1 μg/mL. After 2 hours, plates were washed 6 times with 1X PBS and incubated for 1 hour with 100 μL/well of Streptavidin-HRP (Mabtech) at room temperature. Finally, Elispot plates were washed 6 times with 1X PBS and developed for 30 minutes with 100 μL/well of peroxidase substrate AEC kit (Vector Laboratories, Burlingame, CA), followed by rinsing under running tap water. Plates were stored overnight in the dark at room temperature, and spots were counted using a VersaScan microplate reader (Velocity 11, Palo Alto, CA). To assess precoating efficiency, cells were stimulated with Concanavalin A (CON-A; 1 μg/mL) and wells with cells alone and control peptide served as background control and negative controls, respectively. The mean total of IFN-γ spot-forming cells (SFC) in triplicate wells were determined based on the difference between the frequency of spots obtained with the vaccine-specific peptides and the control peptide and is expressed as number of SFC as indicated in the Figure legends. Specific sequences of the Trojan vaccines, individual vaccine-specific antigens and control antigen to determine vaccine-induced immune responses are listed in Table 1.

Interferon-γ restimulation elispot assay

To amplify T cell responses, PBMC were resuspended at 2×10^6 cells/mL in cRPMI supplemented with GM-CSF (50 ng/mL) and interleukin (IL)-4 (25 ng/mL). Next, PBMC were plated at 2×10^6 per well in 24-well plates and half of the wells were restimulated with the appropriate Trojan vaccine (10 μl/mL) and incubated at 37°C, 5% CO_2. After 48 hours of incubation, cells were harvested, resuspended at 1×10^6 cells/mL in cRPMI supplemented with IL-7 and IL-15 (both at 5 ng/mL) and plated at a final concentration of 10^6 cells/well in a 24-well plate and incubated at 37°C, 5% CO_2. After 7 days, cells were harvested, washed, and plated at 1×10^5 cells/well in triplicate wells in 96-well IFN-γ precoated Elispot plates. Trojan vaccine and control peptides were added to the appropriate wells (10 μg/mL) and the plates cultured for 24 hours at 37°C, 5% CO_2. After 24 hours of incubation, Elispot plates were developed as described above.

Melanoma antigen e-trojan and human papillomavirus-trojan immunoglobulin g enzyme-linked immunosorbent assay

Plasma samples from the vaccinated patients were assayed for antibodies against either MAGE or HPV-

Trojan peptides by enzyme-linked immunosorbent assay (ELISA). In brief, ELISA plates (NUNC 445101, Raskilde, Denmark) were coated overnight at 4°C with 1 μg of MAGE or HPV-Trojan vaccine per well. Negative control wells included no vaccine, vaccine alone, secondary antibody alone, and vaccine plus secondary antibody. After overnight incubation and subsequent washing, plasma samples were diluted in blocking buffer (as indicated in the Figure legend) and 100 μL of diluted plasma was added to triplicate wells. After 1 hour of incubation at room temperature, plates were washed 5 times with PBS supplemented with 0.05% Tween 20 (1X PBST) and incubated for another hour with 100 μL of peroxidase-conjugated goat anti-human IgG (dilution 1:1000, Kirkegaard & Perry, Gaithersburg, MD). Finally, 100 μL of 3,3′, 5,5′-Tetramethylbenzidine (Kirkegaard & Perry) substrate was added and plates were incubated for up to 30 minutes at room temperature. Optical density (OD) values were monitored at 650 nm to not exceed 1.3. Reactions were stopped with 100 μL 1N HCl and OD values determined at 450 nm. Baseline levels of samples tested with no vaccine were subtracted from those with vaccine. OD values at 450 nm were graphed and reported.

Tetramer analysis

Phycoerythrin-labeled tetramers were assembled by Beckman Coulter (Fullerton, CA). Tetramers specific for *MAGE-A3* CTL_1 (KVAELVHFL), *MAGE-A3* CTL_2 (FLWGPRALV), HPV-16 CTL (TLGIVZPI), and Control HLA-A*02 restricted tetramer (YIGEVLVSV) were used. For tetramer staining, 20 μL aliquots of tetramer were directly added to 10^6 PBMC. Cells were incubated for 30 minutes at room temperature in the dark. Next, 5 μL aliquots of CD3 and CD8 monoclonal antibody were added for surface staining, and the cells were incubated for an additional 30 minutes at 4°C in the dark. After incubation, cells were washed twice with fluorescence activated cell sorting (FACS) buffer, resuspended in 500 μL of FACS buffer, and analyzed within 2 hours of staining. A minimum of 100,000 events were acquired using a BD LSR II flow cytometer and analyzed with BD FACS DIVA Software (BD Biosciences).

Statistics

The number of IFN-γ producing PBMC observed in separate Elispot wells were treated as events that occurred in a pseudo time-period. Poisson regression was, therefore, used to test if there were any significant differences in the mean events between prevaccine and postvaccine time-points for various vaccine and epitope treatments in each patient. The analysis was implemented with the SAS 9.2 (SAS Institute, Cary, NC) GENMOD procedure with Poisson distribution. Over-dispersion (variance > mean) of the model was adjusted by using the "dscale'' option in the model statement. The significance level was set at 5%. Any $p < .05$ was considered to be significant.

RESULTS

Patient characteristics, hla results, and melanoma antigen e-a3/human papillomavirus-16 expression

A total of 31 patients were screened for enrollment in this pilot study, and the demographic data on all screened study patients is provided in Table 2. All screened patients had histologically proven SCCHN and either had treatment failure or had refused standard therapy. HLA typing was performed on all potential study patients, followed by a tumor biopsy to determine *MAGE-A3*, HPV-16, and HLA-A*02 expression within the tumor. Among the 31 patients who were HLA typed, 11 patients were determined to be HLA-A*02 positive. Nine of the HLA-A*02 positive patients and 2 patients who were HLA-A*02 negative were subjected to a tumor biopsy. Two patients who were HLA-A*02 positive were found to be negative for HLA-A*02 at the tumor level (Supplemental Figure 2, online only). These findings are consistent with previous studies from us and other authors showing that loss of HLA-I expression is a relevant mechanism of immune escape in SCCHN.[22]

Of the 11 patients who underwent biopsy, 4 were shown to express *MAGE-A3* and 2 were proven to express HPV-16. One of these patients expressed both antigens. In contrast, 4 patients expressed neither of the required antigens. In summary, 5 patients with SCCHN were enrolled (including 1 "single use exemption'' secondary to a diagnosis of myelodysplastic syndrome) in this pilot study and treated with up to 4 immunizations with 1 of the Trojan vaccines (Table 3).

Clinical outcomes and toxicities

None of the 5 patients immunized demonstrated a complete response or partial response by Response Evaluation

TABLE 2. Demographics of screened study patients.

Characteristic	Value
Total number of patients	31
Male	25
Female	6
Median age, y; (range, y)	56 (39–79)
Stage of disease	IV
Primary site:	6
Oral	
Oropharynx	11
Hypopharynx	4
Larynx	5
Nasal/paranasal cavities	2
Nasopharynx	1
Unknown	1
HLA phenotype: HLA-A*02$^+$ PBMC	11/31 (35%)
African American	2/10 (20%)
White	9/21 (38%)
HLA-A2$^+$ tumor	7/9 (78%)
Tumor antigen expression*: MAGE-A3$^+$	4/11 (36%)
HPV-16$^+$	2/11 (18%)
MAGE-A3$^+$/HPV-16$^+$	1/11 (9%)

Abbreviations: PBMC, peripheral blood mononuclear cells; MAGE, melanoma antigen E; HPV, human papillomavirus.
* Including 2 tumor biopsies of a patient with HLA-A*02$^-$.

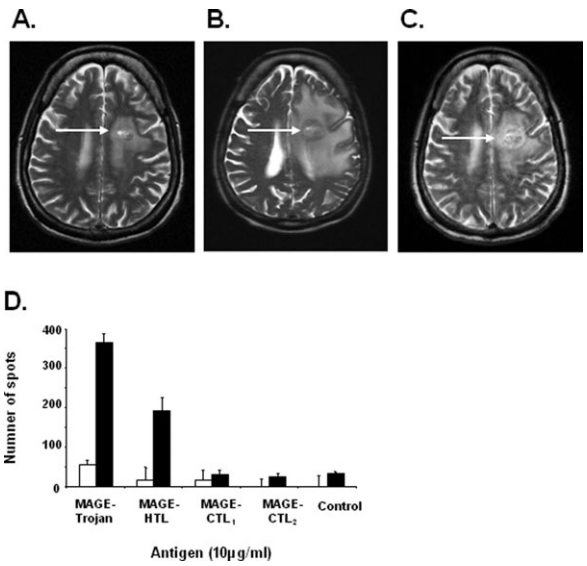

FIGURE 2. The T2-weighted brain MR images of patient 1 and Trojan vaccine-specific immunity. Left image (A) obtained 8 weeks before vaccination (January 30, 2006), middle image (B) obtained during neurological event (April 21, 2006), and right image (C) obtained after resolution of neurological symptoms (July 14, 2006). Arrows indicate the site of the cerebral metastasis. Peripheral blood mononuclear cells (PBMCs) were restimulated once with melanoma antigen E (MAGE)-Trojan vaccine and cultured in the presence of interleukin (IL)-7 (5 ng/mL) and IL-15 (5 ng/mL). After 7 days, cells were evaluated for MAGE Trojan-specific HLA-I and HLA-II peptide (D) specificity by interferon-γ (IFN-γ) Elispot assay. Number of spots per 100,000 PBMCs is shown.

Criteria in Solid Tumors (RECIST) criteria. Patient 5 is alive at 31 months after trial entry. Patient 1 lived for 27 months, whereas patients 3, 4, and 2 died at 24, 8, and 4 months, respectively. Patient 1 received only 1 vaccine and was removed from study due to an SAE. A follow-up CT scan 5 months after vaccine administration showed stable disease. Patient 2 was removed from the study after 2 vaccinations due to disease progression. Patients 3 and 4 showed disease progression by CT scan 2 months after trial entry. Patient 5 showed stable disease after 2 vaccinations and underwent tumor resection 3 months after the final vaccination. Importantly, our survival data must be taken in context with the fact that these patients received other surgical and/or chemotherapeutic regimens after vaccination.

National Institutes of Health Common Toxicity Criteria were used to determine the clinical toxicities associated with the Trojan vaccines. Toxicities observed after the first immunization and worst toxicity observed after any immunization are summarized in Table 4 and Table 5, respectively. One patient experienced an SAE which is particularly noteworthy. Specifically, this patient with a known stable brain metastases (Figure 2A) developed right-sided hemiplegia, right-sided facial weakness, and speech impairment 24 days after her first vaccination. A T2-weighted MRI revealed marked progression of increased signal surrounding the cerebral metastasis consistent with increased cerebral edema (Figure 2B). In addition, there was a rim of decreased signal that developed along the periphery of the metastasis, which, although nonspecific, is an imaging feature that could be consistent with the development of surrounding cellular/lymphocytic infiltration. With steroid treatment, these findings subsided and her neurological symptoms resolved (Figure 2C). In vitro Elispot interrogation of PBMC, demonstrated strong recognition of both the Trojan and HLA-II restricted peptides postimmunization (Figure 2D). We postulate that this cerebral edema is the result of a vaccine-induced inflammatory reaction, although direct biopsy evidence is lacking.

Trojan peptide-based vaccines induce robust and long lasting systemic vaccine-specific hla-ii t cell responses

To evaluate the vaccine-specific T cells responses, PBMC obtained from patients at baseline, within 24 hours before each vaccination, and at completion or discontinuation of study were evaluated both directly ex vivo and after in vitro sensitization using IFN-γ Elispot. In the absence of prior in vitro exposure (hereafter called "recall Elispot''), Trojan vaccine-specific postimmunization T cell responses were observed in PBMC from patient 3 ($p = .0045$, $p = .0100$, and $p < .0001$ after vaccination number 2, 3, and 4, respectively), patient 4 ($p = .0016$ after vaccination number 3) and patient 5 ($p < .0001$, $p = .0270$, $p = .0001$, $p = .0004$, and $p = .0007$ after vaccination number 1, 2, 3, 4 and after 3 months of follow-up, respectively). Furthermore, transient Trojan-specific HLA class II responses were observed in PBMC from patient 3 after vaccination number 2 ($p = .0021$) and after vaccination number 3 in PBMC from patient 4 ($p = .0041$). Examples of *MAGE-A3* (patient 3) and HPV-16 (patient 5) recall assays are shown in Figure 3A and 3B, respectively.

TABLE 3. Demographics and HLA status of immunized patients.

Patient no.	Age, y	Sex	AJCC stage	HLA ABDR*	HLA A2 (tumor)	Trojan HTL IFN-γ	Trojan IgG response
1	50	F	IV	A2, A24, B77, B44, DR4, DR12	60% to 80%	+	-
2	65	M	IV	A2, A66, B7, B71, DR15, DR9	60% to 80%	-	-
3	70	M	IV	A2, A-, B7, B13, DR15, DR-	40% to 60%	+	+
4†	47	M	IV	A3, A33, B-, B35, DR1, DR17	N/A	+	-
5	55	M	IV	A2, A3, B7, B35, DR12, DR15	80% to 100%	+	+

Abbreviations: AJCC, American Joint Committee on Cancer; IFN- γ, interferon-γ; IgG, immunoglobulin G; F, female; M, male; N/A, not applicable.
* Trojan-vaccine specific HLA-I and II restrictions are underlined.
† Single use exemption, due to a secondary diagnosis of myelodysplastic syndrome.

TABLE 4. Toxicity after the first immunization.

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Diarrhea	1			
Nausea		1		
Vomiting	1	1		
Edema-head and neck		1		
Chills		1		
Dehydration		1		
Infection		1		
BUN		1		
Pain-musculoskeletal		1		
Pain-injection site		1		
Soft tissue-joint function-gout		1		
Hemorrhage/bleeding	1			
Cardiac-ischemia infarction		1		
Hemoglobin-low		1		
Total	3	12	0	0

Abbreviation: BUN, blood urea nitrogen.

Trojan-specific immunity was significantly enhanced when PBMC were subjected to a single in vitro exposure with the respective Trojan vaccine. Specifically, MAGE or HPV Trojan-specific immunity was detected in 4 of 5 immunized patients ($p < .0001$ in all 4 patients at any given time-point) after 1 single in vitro restimulation. Examples of *MAGE-A3* (patient 3) and HPV-16 (patient 5) in vitro sensitization assays are shown in Figures 3C and 3D, respectively. Importantly, in vivo priming and additional ex vivo amplification also resulted in robust Trojan vaccine-induced responses to the HLA-II peptide in these 4 patients (Figure 3C and 3D). These responses seemed to peak after the second or third vaccination with $p < .0001$ at any given time-point. Quantitative levels of immune responses observed at baseline, after the second immunization, and after the fourth immunization in both patients who completed the full vaccination schedule are summarized in Supplemental Figure 3, online only.

TABLE 5. Worst toxicity observed during any immunization.

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Nausea			1	1
Vomiting		2		
Dyspnea		1	1	
Fatigue		1		
Edema-head and neck				1
Chills		1		
Infection			1	
Neuropathy-motor hemifacial				1
Neuropathy-cranial nerve VI			1	
Pain-musculoskeletal		1	2	
Pain-injection site		1		
Soft tissue-joint function-gout		1		
Hemorrhage/bleeding		1		
Cardiac-ischemia infarction		1		
Hemoglobin				1
Diplopia			1	
Upper respiratory-fistula	1			
Total	1	10	7	4

To exclude nonspecific immune responses, in 2 *MAGE-A3* vaccinated patients (patients 1 and 2) the HPV-Trojan vaccines were added to the recall Elispot assay as an additional internal control. In these 2 assays, nonspecific responses were absent (data not shown). In addition, the HIV-TAT sequence was tested in a restimulation HPV-16 Elispot assay (patient 4), and no significant differences in SFC were observed between wells containing the HIV-TAT or negative control peptides (data not shown).

In contrast, by recall Elispot, no systemic responses could be detected to the HLA-I epitopes present in the Trojan vaccines. One transient Trojan vaccine-specific immune response was detected after in vitro restimulation in PBMC from patient 3 with $p = .0210$ (MAGE-CTL_1) and $p = .0430$ (MAGE-CTL_2) after receiving 3 immunizations. In general, the lack of response was not secondary to antigen-specific T-cell anergy, as tetramer analysis revealed no robust and long-lasting increase in the number of antigen-specific CD8 T cells over time (Supplemental Figure 4, online only). These data demonstrate that the Trojan peptide vaccines, when administered in combination with Montanide ISA 51 adjuvant and GM-CSF, induce systemic immunity to both the entire Trojan construct and the HLA-II restricted epitopes. Furthermore, the fact that the responses to the intact Trojan constructs were higher than the responses to their constituent epitopes suggests that natural cleavage of the Trojan peptides creates novel HLA-II restricted epitopes. Alternatively, it is possible that the observed responses were biased by our methodology, where we initially stimulated with the Trojan construct and used the HLA-I or HLA-II restricted peptides for restimulation. Further studies will be required to clarify this issue.

Trojan peptide-based vaccines induce antigen-specific immunoglobulin g responses

It is well established that under most conditions antibody class switch recombination (CSR) requires T cell help. Furthermore, regulatory T cells are recognized to inhibit antigen-specific CSR by both direct suppression of B-cell function and indirect inhibition of the T helper response.[23] Therefore, in order to understand the functional activity of the CD4 T-cell responses, we interrogated the IgG titers to the Trojan peptide vaccines. Vaccine-induced immune responses were demonstrated in patient 3 (*MAGE-A3* Trojan-specific IgG) and patient 5 (HPV-16 Trojan-specific IgG; Figures 4A and 4C, respectively). These responses were durable up to follow-up month 3 and, in 1 case, were observed even at a 1/5000 dilution. Furthermore, IgG subtyping of the patient with the highest titers (patient 5) revealed that these responses were IgG1 (data not shown). Interestingly, despite the presence of vaccine-induced T-cell responses to the HLA-II epitope in patients 1 and 4, no Trojan-specific IgG was detected (Figures 4B and 4D). These data demonstrate that the Trojan peptide vaccines are capable of eliciting high titer IgG responses in select patients, suggesting that the peptide-induced CD4 cell responses in these patients are capable of supporting antigen-specific CSR.

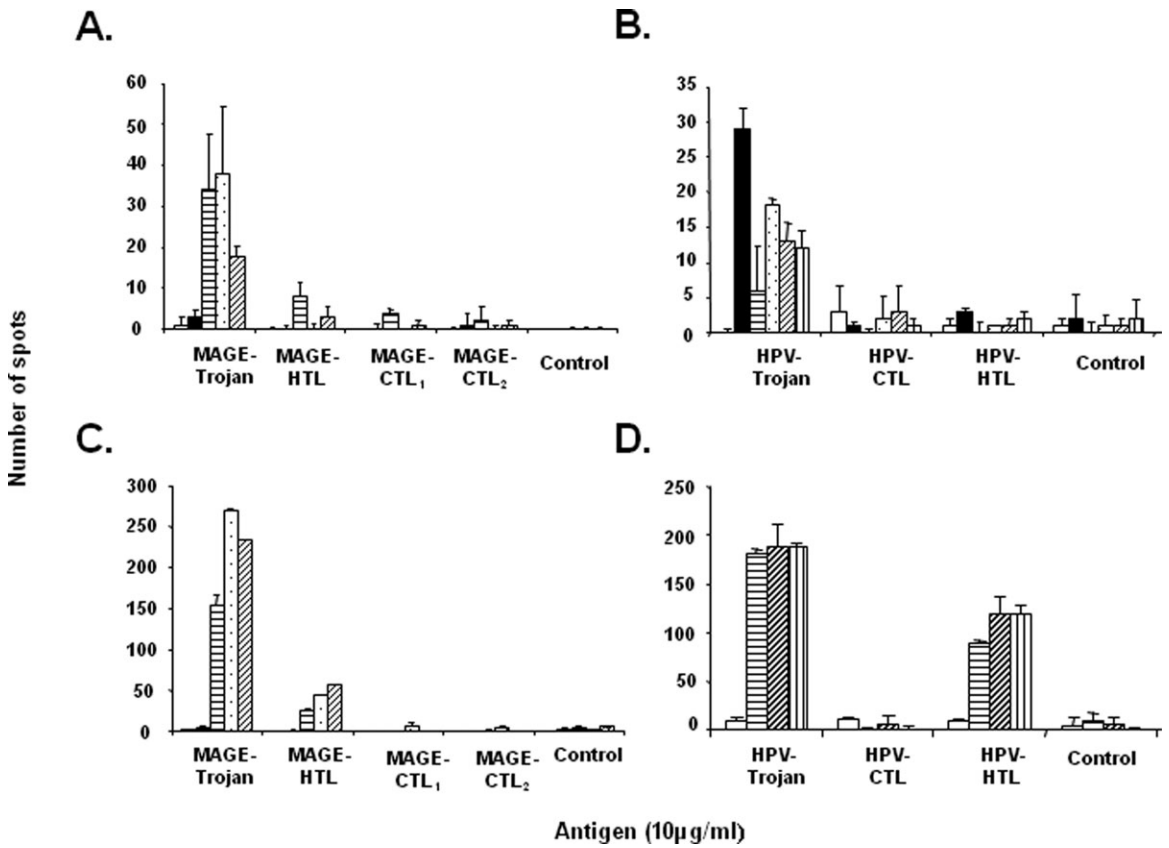

FIGURE 3. Trojan peptide-based vaccines induce Trojan-specific precursors in vivo. Peripheral blood mononuclear cells (PBMCs) collected before immunization and at indicated time points after immunization were evaluated for Trojan specificity by interferon-γ (IFN-γ) Elispot assay. Direct ex vivo measurement of T-cell reactivity against melanoma antigen E (MAGE)-Trojan (A) human papillomavirus (HPV)-Trojan (B) and individual Trojan-specific HLA-I and HLA-II peptides (A and B). Number of spots per 200,000 PBMCs is shown. Error bars represent SD from triplicate wells. Control values are shown without subtraction from experimental values. PBMCs were restimulated once with MAGE-Trojan or HPV-Trojan vaccine and cultured in the presence of interleukin (IL)-7 (5 ng/mL) and IL-15 (5 ng/mL). After 7 days, cells were evaluated for MAGE-Trojan (C), HPV-Trojan (D), and individual Trojan-specific HLA-I and HLA-II peptide (C and D) specificity by IFN-γ Elispot assay. Number of spots per 100,000 PBMC is shown. Error bars represent SD from triplicate wells. * $p < .05$.

Antigen-specific t cells accumulate at the site of vaccination

In order to further define the nature of the immune response elicited by this vaccine, we next characterized the phenotype and function of cells located at the site of vaccination. Two patients developed palpable inflammatory lesions at the site of immunization (Figure 5A). One patient consented to a biopsy of this region (performed 19 days after vaccine #2), and the inflammatory nature of the response was characterized phenotypically by both immunohistochemistry and flow cytometry and functionally by IFN-γ Elispot. Analysis of hematoxylin-eosin–stained sections revealed granuloma formation, consistent with the well characterized association between Montanide ISA 51 adjuvant and granulomas.[24] As demonstrated in Figure 5B, the majority of cells recruited to the site of injection were $CD68^+$ monocytes/macrophages, with a smaller population of $CD3^+$ T cells. Subsequent flow cytometric analysis of these cells revealed that the majority of this $CD3^+$ cell population was $CD4^+$, with a limited number of $CD8^+$ T cells (Figure 5C). Importantly, from a functional perspective, immune cells obtained from the vaccination site, specifically recognized the HPV-Trojan vaccine ($p < .0001$) and HPV-HLA-II epitope ($p < .0001$), as determined by an IFN-γ restimulation Elispot assay (Figure 5D). These data demonstrate that vaccination induces ongoing antigen-specific immune responses at the site of vaccination.

Antigen-specific t cells accumulate within the tumor

In order to understand the nature of the immune response within the tumor microenvironment, we analyzed the cells from an involved lymph node of 1 patient who underwent a neck dissection posttreatment. Importantly, this patient had metastatic neck disease and refused standard of care treatment options before vaccination. In order to validate this decision, the patient met with physicians from medical oncology, radiation oncology, and otorhinolaryngology–head and neck surgery, each of whom carefully explained the ramifications of this decision to the patient. Furthermore, the patient underwent a psychiatric evaluation and was judged competent to decline treatment and sign the informed consent. Two months after completing the full vaccine protocol,

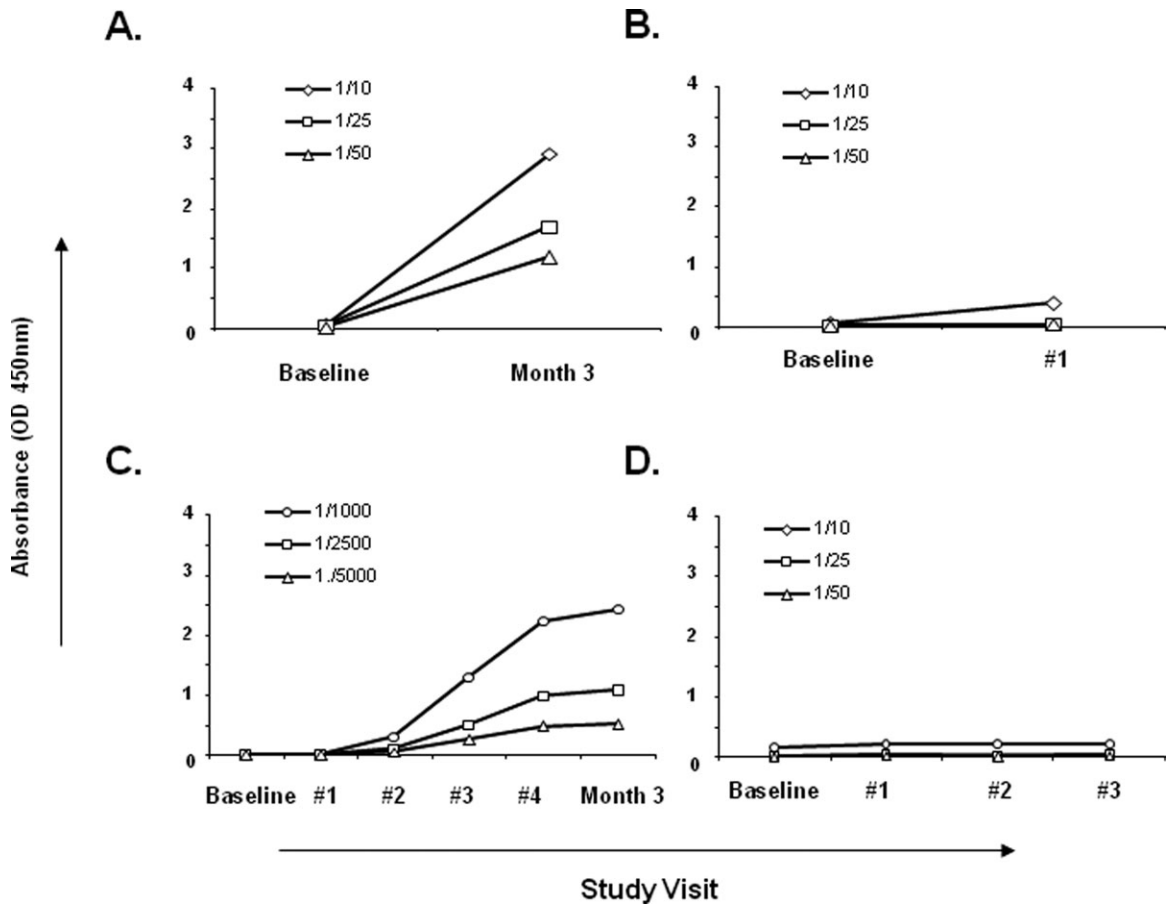

FIGURE 4. Vaccination with melanoma antigen E (MAGE)-A3 and human papillomavirus (HPV)-16 specific Trojan vaccines elicits immunoglobulin G (IgG) antibody responses in patients with squamous cell carcinoma of the head and neck (SCCHN). Trojan-specific IgG was quantified in plasma obtained before immunization and at various time-points after immunization as indicated. MAGE-A3 Trojan (A and B) and HPV Trojan (C and D) specific IgG was quantified by enzyme-linked immunosorbent assay (ELISA). Data represent mean optical density (OD) readings from triplicate wells derived from 3 different plasma dilutions.

without an objective response as determined by RECIST criteria, the patient agreed to undergo a therapeutic neck dissection.

Interestingly, in the pretreatment biopsy, there was a notable absence of $CD4^+$ T-cell infiltration and a limited number of $CD8^+$ cells. In sharp contrast, in the posttreatment biopsy, there were abundant $CD4^+$ and $CD8^+$ cells throughout the specimen (Figure 6A). Furthermore, there were a qualitatively greater number of apoptotic cells in the posttreatment specimen as demonstrated by Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining. Importantly, these apoptotic cells were judged to be tumor, rather than infiltrating lymphocytes, by our head and neck pathologist (J. Papadimitriou) who reviewed the slides.

Because it could be argued that these observed phenotypic differences were simply a result of sample error secondary to tumor heterogeneity, we sought to determine the antigen specificity of the TIL. A 48-hour recall experiment demonstrated no significant IFN-γ release in response to the peptide antigens and the more sensitive restimulation studies could not be accurately completed secondary to poor cell viability. Therefore, we used tetramer staining as a surrogate to determine the presence of $CD8^+$ cells specific for the HPV-CTL epitope (Figure 6B). While the percentage of tetramer positive CD3 T cells in the peripheral blood was 0.4%, the percentage in the lymph node was 8.1%. Interestingly, preliminary studies from this sample revealed that the majority of T cells had a senescent ($CD27^-CD28^-$) phenotype (data not shown).[25] Taken in concert, these data indicate that vaccine-specific immune cells accumulate at the site of immunization and in the tumor microenvironment. Furthermore, the lack of a recall response to the peptides in combination with the presence of $CD27^-CD28^-$ senescent cells within the tumor microenvironment, suggests that the effector function of these antigen-specific T cells might be impaired by tumor host interactions.

DISCUSSION

This pilot study tested Trojan peptide-based vaccines in 5 patients with recurrent or metastatic SCCHN. This novel vaccine contains HLA-I and HLA-II restricted peptide epitopes from *MAGE-A3* or HPV-16. This study is, to the best of our knowledge, the first to explore therapeutic vaccination with *MAGE-A3* and HPV-16-derived

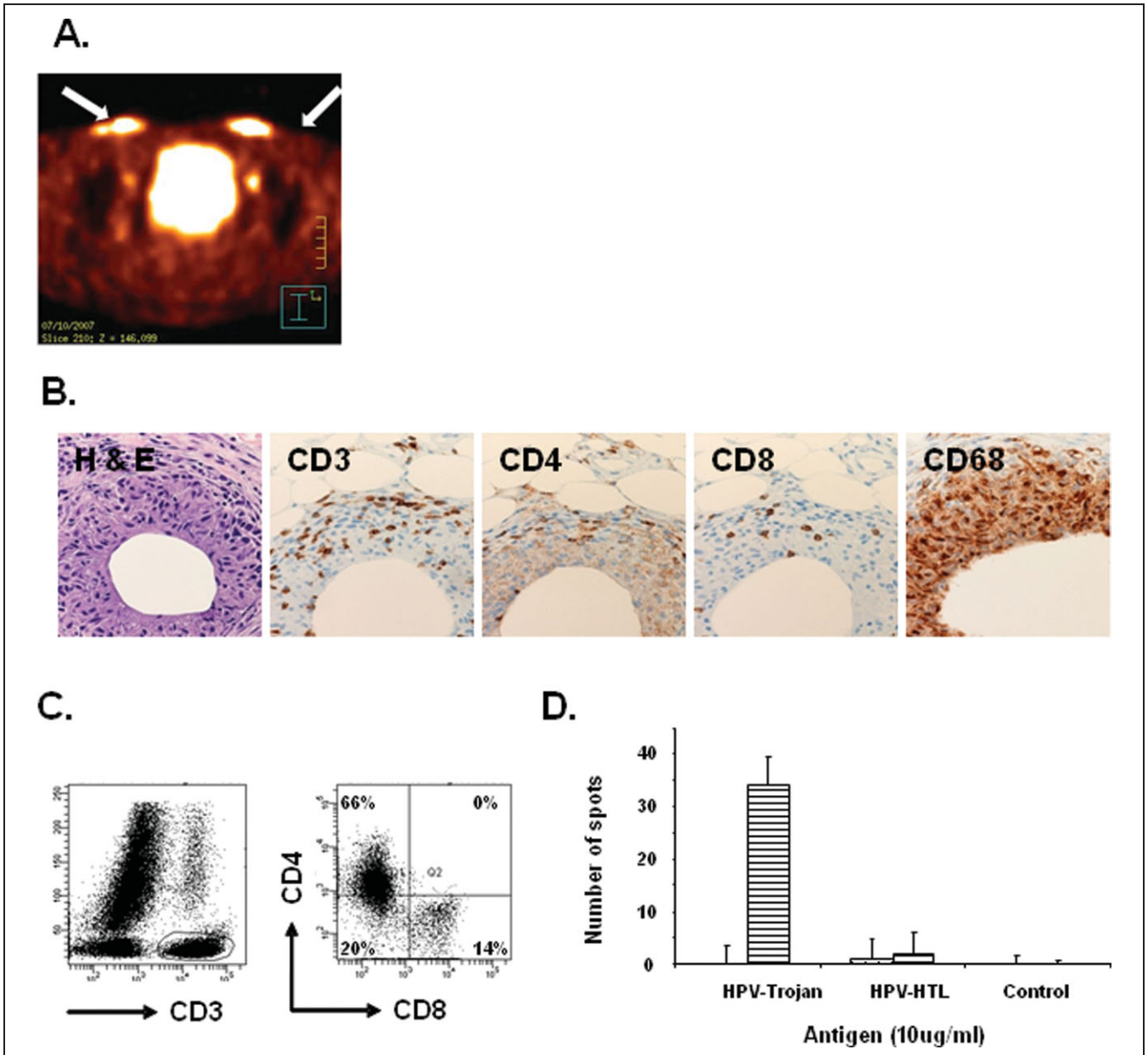

FIGURE 5. Trojan-specific precursor frequency at the injection site exceeds precursor frequency in the peripheral circulation. Frozen section slides were generated from a granuloma derived from the site of immunization and stained by immunohistochemistry with a variety of leukocyte-specific markers. The granuloma was biopsied 19 days after the second vaccination. Positron emission tomography (PET) scan revealing high levels of inflammation at the injection sites as indicated by arrows (A); high numbers of antigen-presenting cells ($CD68^+$) and $CD3^+$ T cells infiltrated the site of immunization (B); part of the granuloma was enzymatically digested into a single cell suspension and evaluated for Trojan vaccine-specificity (C). As measured by flow cytometry, the majority of $CD3^+$ T cells in the granuloma were found to be $CD4^+$ cells. Next, cells were directly ex vivo evaluated for Trojan-specificity by interferon-γ (IFN-γ) Elispot. High numbers of Trojan-specific cells were found at the site of injection (D). In contrast, at the same time interval, no Trojan-specific responses were detected in cells peripheral blood mononuclear cells (PBMCs) derived from the peripheral circulation. Number of spots per 50,000 cells (granuloma) and 100,000 PBMCs is shown. Error bars represent SD from triplicate wells. * $p < .05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

HLA-I and HLA-II-restricted T-cell epitopes in recurrent or metastatic SCCHN, and the first to use Trojan constructs for the treatment of human malignancy.

Several features of our study are important to consider, both as they relate to the interpretation of our results and to the design of subsequent clinical studies using Trojan peptide vaccines for the treatment of SCCHN. First, from a clinical perspective, none of the patients developed objective clinical responses by modified RECIST criteria. From a toxicity perspective, the Trojan vaccines were well tolerated and toxicities in general did not require hospitalization. Furthermore, 1 patient developed significant cerebral edema 24 days after the first vaccination which was associated with hemiplegia and an immune response to both the Trojan and HLA-II restricted peptides. These findings, while clinically intriguing, mandate

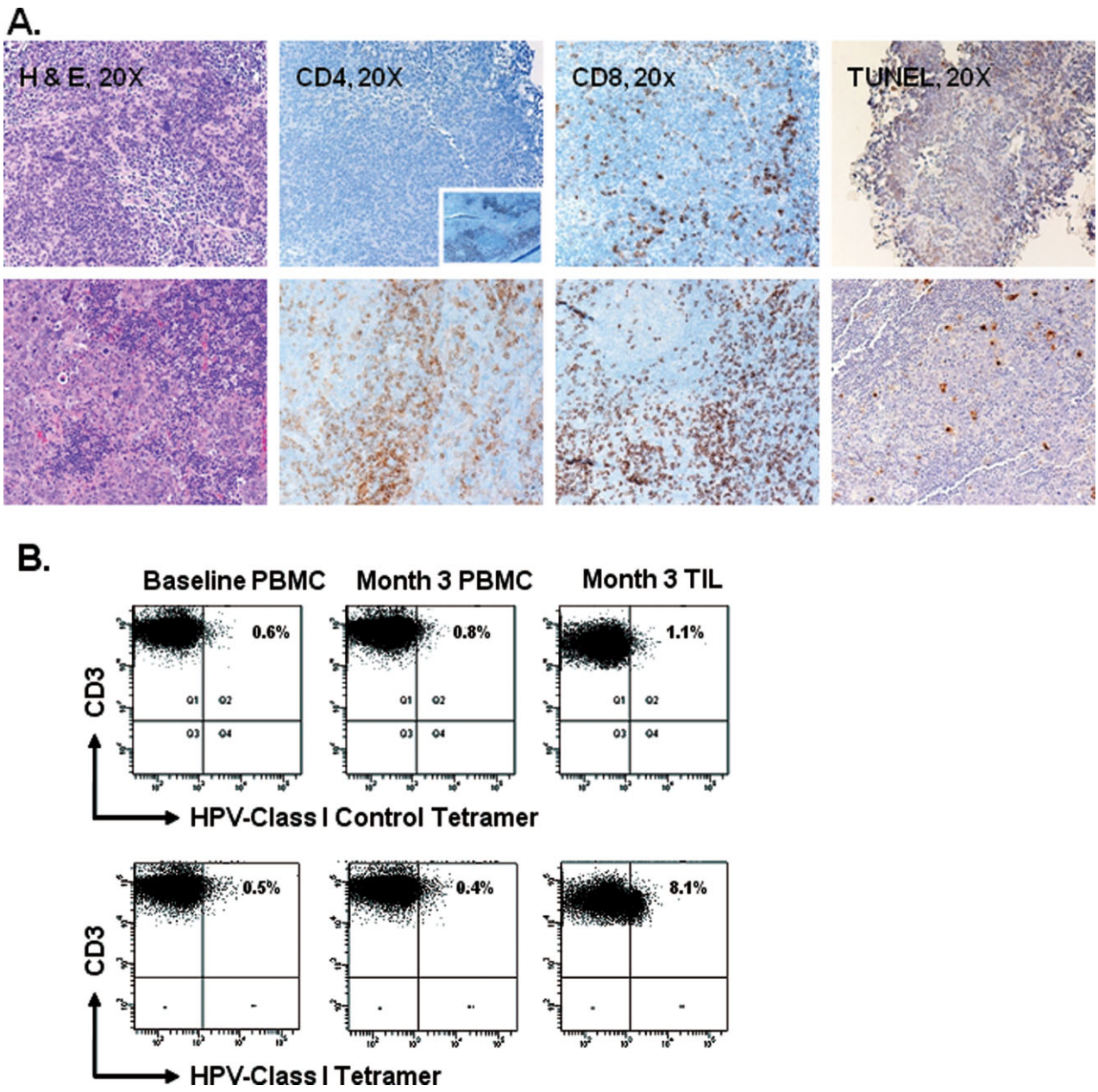

FIGURE 6. The $CD8^+$ and $CD4^+$ T cells infiltrate the tumor microenvironment during immunization and a subset appears to be human papillomavirus (HPV)-specific. Photomicrographs of immunohistochemical staining of tumor tissue harvested before and after immunization with HPV-Trojan vaccine. High numbers of $CD8^+$ and $CD4^+$ cells were found in the tumor microenvironment after immunization (A). Representative sections from CD4 and CD8 staining of the neck metastasis slides before and 3 months after the last vaccination are shown. Positive controls were positive for all experiments (inset CD4). Part of the neck metastasis, collected at 3 months after immunization, was enzymatically digested into a single cell suspension tumor infiltrating lymphocytes (TILs). Peripheral blood mononuclear cells (PBMCs) collected before and PBMCs and TILs collected after 3 months of immunization were directly ex vivo stained with HPV-16 specific HLA-A*0201 tetramer and analyzed by flow cytometry for Trojan HLA-I specificity (B). In comparison to PBMCs collected at the same time-interval, TILs derived from the tumor microenvironment after 3 months of immunization were found to be HPV-16 specific, 0.4% and 8.1%, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

caution in using Trojan vaccines in patients with known brain metastases.

Second, in 4 of 5 immunized patients, the Trojan vaccines stimulated systemic T-cell responses to both the whole constructs and the constituent HLA-II epitopes. Furthermore, Trojan vaccine-specific IgG antibodies were detected in plasma from 2 of these patients, suggesting that these CD4 T cells were able to support a humoral immune response. In contrast, the 1 HLA-I-specific IFN-γ-producing response was transient. Furthermore, staining of TIL from a tumor resected posttreatment from 1 patient, demonstrated a significant increase in the number of tetramer positive cells when compared with correlate PBMC. Taken in concert, our data suggest that Trojan

peptide vaccines, when administered in combination with Montanide ISA 51 adjuvant and GM-CSF, induce both antigen-specific cellular and humoral immunity, with a biased systemic response to the HLA-II epitopes. Furthermore, although there were no objective responses to the vaccine, it is notable that 3 patients survived for 24, 27, and 31 (still alive) months posttherapy, in a setting where the average survival is approximately 6 months. Enthusiasm for these clinical observations must be tempered by small numbers, a potential selection bias, and the fact that these patients received additional therapy after vaccination.

The induction of functional systemic responses to the HLA-II peptides in 4 of 5 patients without durable HLA-I responses in PBMC was surprising. These findings are particularly striking because in 3 patients, responses to the Trojan constructs were observed in primary recall assays without restimulation. Although patients with end-stage disease are traditionally considered to be immunosuppressed by virtue of their large tumor burdens, this was not reflected by total white blood cell counts, levels of $CD3^+$ cells, $CD3^+CD8^+$ cells (Supplemental Table 1, online only) nor the ability to develop Trojan-specific immune responses (Table 3).

Although it is possible that these responses were predominated by the presence of regulatory T cells (Tregs), we do not think this is likely. Specifically, the Elispot assays used IFN-γ as a readout. Whereas Tregs are recognized to be induced by this cytokine, they do not generally produce IFN-γ.[26] Furthermore, while Tregs suppress CSR,[24] 2 of the treated patients developed IgG antibody responses to the Trojan peptides. Thus, our findings demonstrate that this vaccine strategy may elicit functional cellular and humoral systemic immune responses to both the entire Trojan constructs and their constituent CD4 restricted epitopes. These observations are particularly relevant in light of a recent report demonstrating that adoptive transfer of antigen-specific CD4 cells can mediate regression of metastatic melanoma[27] and animal studies showing that antigen-specific CD4 cells can effectively mediate direct regression of HLA-II positive melanomas.[28,29]

Several possible explanations may account for the lack of durable HLA-I responses in our study. First, recent data suggest that patients with SCCHN have a high frequency of HLA-II precursors to *MAGE-A3* in the absence of prior vaccination, suggesting that this cell population may be readily responsive to peptide stimulation.[30] In addition, because SCCHN are recognized to escape the immune response through deletion/anergy of HLA-I type effectors through Fas-FasLigand (Fas-FasL) interactions, it is likely that a limited number of antigen-specific precursors are available for subsequent expansion.[31] Second, 2 of the 4 patients who were HLA*A02 positive were determined to be HLA*A0205 and HLA*A0206, respectively. Although data are limited, it is thought that HLA-I epitope recognition is reduced in these patients, potentially influencing the development of HLA-1 restricted T-cell immunity.[32] Finally, it is now well established that HLA-I immune responses are more prevalent in lymph nodes, suggesting that our decision to study PBMC may have biased our outcome.[33] Further bias may have also been introduced in our experimental design, where we restimulated in vitro with the Trojan vaccine, rather than the individual epitopes.

Interestingly, in the 1 patient from whom we were able to compare pretreatment and posttreatment biopsies, there was a profound increase in both $CD4^+$ and $CD8^+$ T cell responses at 3 months after vaccination. Furthermore, TIL from this patient demonstrated a high percentage of antigen-specific T cells in the postvaccination biopsy. Importantly, the fact that these TILs did not secrete IFN-γ in a recall assay and demonstrated a largely $CD27^-CD28^-$ phenotype (data not shown), suggests that they were biologically exhausted or senescent.[25] This idea is consistent with previous observations that $CD27^+/CD28^+$ T cells are responsible for the therapeutic effects in patients with melanoma responding to adoptive T cell transfer.[34] However, data from patients with colon cancer, demonstrate that the presence of both CD27/CD28 double negative and double positive CD8 cells in the tumor microenvironment are independently associated with reduced metastases.[35] Further studies will be required to definitively characterize the function of these antigen-specific T cells.

Two of the major difficulties experienced within patient accrual in this pilot study included the unexpected low frequency of HLA-A*02 expression among this patient population and relatively quick deterioration of most patients with recurrent or metastatic SCCHN. In fact, HLA-A*02 is frequent in all ethnic groups and is found in approximately 35% and 50% of African Americans and whites, respectively.[36] Surprisingly, only 20% of African Americans and 38% of whites were found to be HLA-A*02-positive in our total study population (Table 2).

Interestingly, a clear increase in *MAGE-A3* HLA-II-specific T cells was observed in 1 study patient who was determined to express HLA-DR*15. Although HLA-DR*04 and HLA-DR*07 specificity for the MAGE-HTL epitope, incorporated in our MAGE-Trojan vaccine, has previously been reported,[16] it suggests that this MAGE HLA-II epitope is promiscuous and immunogenic in patients who are not DR*04 and/or DR*07. Based on the low percentage of HLA-A*02 expression in this population, the limited tolerance of these patients to nearly 4 months of vaccine delivery and the induction of functional responses to both the Trojan vaccine and the HLA-II restricted epitopes, we are currently conducting a phase I clinical trial in which patients are immunized every 2 weeks and in which HLA-A*02 is not required for study entry. We anticipate that these changes will increase the feasibility of our approach without inhibiting efficacy.

In summary, this pilot study demonstrates the feasibility of using Trojan peptide vaccines in combination with Montanide ISA 51 adjuvant and GM-CSF for the treatment of patients with advanced SCCHN. These data add to a growing body of literature demonstrating the efficiency of using long peptides to prime antitumor immune responses.[11] The fact that these Trojan constructs were effective in stimulating responses against both the parental vaccine as well as its HLA-II restricted epitopes, limits traditional feasibility concerns associated with the use of HLA-I restricted peptides. Furthermore, while our conclusions must be viewed in the context of limited sample size, the presence of antigen-specific cells both at the vaccine site and within the tumor microenvironment, suggests that these Trojan peptide-primed cells may home to the tumor site. From a clinical perspective, the ability of

this vaccine strategy to induce humoral and cellular responses with limited toxicity, may allow it to serve as a solid foundation on which to add additional immunomodulators. Alternatively, these Trojan peptides might be effective as "monotherapy'' to limit the risk of disease recurrence after standard therapy for patients with high-risk SCCHN. Our ongoing studies are exploring both of these options as a means to both limit the treatment-related morbidity and prolong the survival of our patients afflicted with SCCHN.

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