Immune Responses to a Class II Helper Peptide Epitope in Patients with Stage III/IV Resected Melanoma

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ABSTRACT

The importance of CD8+ cytolytic T cells for protection from viral infection and in the generation of immune responses against tumors has been well established. In contrast, the role of CD4+ T-helper cells in human infection and in cancer immunity has yet to be clearly defined. In this pilot study, we show that immunization of three resected, high-risk metastatic melanoma patients with a T-helper epitope derived from the melanoma differentiation antigen, melanoma antigen recognized by T cells-1, results in CD4+ T-cell immune responses. Immune reactivity to that epitope was detected by DR4-peptide tetramer staining, and enzyme-linked immunospot assay of fresh and restimulated CD4+ T cells from patients over the course of the 12-month vaccine regimen. The postvaccine CD4+ T cells exhibited a mixed T-helper 1/T-helper 2 phenotype, proliferated in response to the antigen and promiscuously recognized the peptide epitope bound to different human leukocyte antigen-DRB alleles. For HLA*0401+ patient, antigen-specific CD4+ T cells recognized human leukocyte antigen-matched antigen-expressing tumor cells, secreted granzyme B, and also exhibited cytolytic activity that was MHC class II-restricted. These data establish the immunogenicity of a class II epitope derived from a melanoma-associated antigen and support the inclusion of class II peptides in future melanoma vaccine therapies.

INTRODUCTION

Human melanoma cells express antigens recognized by CD8+ cytotoxic T lymphocytes (CTL) and CD4+ helper T lymphocytes (1). These include antigens generated by unique, random mutations that are usually patient specific as well as differentiation-related or cancer-testis antigens that are commonly expressed on melanomas in many patients (2–6). The most widely shared antigens recognized by CTLs are derived from melanocyte differentiation proteins including tyrosinase, gp100, tyrosinase-related protein-1, and Melan-A/melanoma antigen recognized by T cells-1 (MART-1). In vivo reactivity by CD8+ CTLs against MHC class I-restricted epitopes derived from these antigens have been demonstrated in a number of clinical trials in which patients were vaccinated with peptides in aqueous solution or with various adjuvants (7–11). In contrast, induction of cognate CD4+ helper T lymphocytes by vaccination has not been pursued as extensively, and in the few clinical trials in which CD4+ T cells were induced (12–14) there was limited functional characterization of resulting effector cells.

There is a strong rationale for the induction of CD4+ helper T lymphocytes in the generation of antitumor immunity. There is accumulating evidence that T-helper (Th) cells, in particular the Th1-type, modulate the effectiveness and long-term survival of memory CD8+ CTLs. This may occur by (1) helping to initiate antigen-specific CD8+ T cells by activating dendritic cells (DC) via CD40–CD40L interaction and by (2) secreting cytokines such as interleukin (IL) 2 (15–19). Furthermore, CD4+ Th1- and Th2-type cells have also been shown to possess direct lytic activity against antigen-expressing tumor cells (20, 21). Herein we describe a small, 12-patient Phase II pilot study of a melanoma peptide vaccine that included MHC class I- and class II-restricted epitopes derived from the differentiation antigens tyrosinase, gp100, Melan-A/MART-1, and the cancer-testis antigen melanoma antigen-3. No clear evidence of CD8+ T cell immune responses to the class I-restricted peptides was found. Only the class II-restricted MART-11,73 peptide was found to be immunogenic, as our study indicates that this helper epitope induced both Th1- and Th2-type CD4+ T cells in the 3 patients vaccinated with this peptide. Furthermore, it displayed the potential to bind and be recognized by patients with different human leukocyte antigen (HLA)-DR subtypes. The immunogenicity and MHC-binding promiscuity of this peptide described herein make it a potentially useful antigen for additional evaluation and eventual inclusion in future melanoma peptide vaccines.

In this trial we used adjuvant AS02B, which consists of a combination of monophosphoryl A and QS-21 in an oil/water emulsion. This adjuvant has demonstrated low toxicity in a previous cancer vaccine trial (22) and has been described as a T-helper cell adjuvant with the potential to induce mixed Th1/Th2 immune responses (23). Our data presented herein confirm that AS02B can induce Th1 and Th2 responses to a melanoma-associated peptide.

MATERIALS AND METHODS

Trial Eligibility. All of the patients had resected stages III and IV melanoma by the 2001 modified American Joint...
Commission on Cancer staging system and were rendered free of disease surgically. They were required to have a magnetic resonance imaging or computed tomographic scan of the head, as well as computed tomographic scan of the chest, abdomen, and pelvis showing no indication of disease within 4 weeks of therapy to verify that they were clinically free of melanoma. Eligibility criteria included age ≥18, creatinine <2.0 mg/dl, bilirubin <2.0 mg/dl, platelets ≥100,000 per cu mm or more, hemoglobin ≥9 g/dl, and total white blood cell ≥3,000 per cu mm. HIV, hepatitis C antibody, and hepatitis B surface antigen were all required to be negative. The 3 patients for which in vitro data are presented were HLA-DR4 positive by microtoxicity assay (Specialty Labs, Santa Monica, CA); additional subtyping was performed by PCR sequence-specific oligonucleotide probe (University of California, Los Angeles Immunogenetics Center). All of the patients were required to comprehend and sign an informed consent form approved by the Los Angeles County/University of Southern California Institutional Review Board. The clinical trial was conducted under an investigator new drug application IND BB 7038 obtained from the Food and Drug Administration.

**Schedule of Vaccinations.** Patients were immunized once monthly for the first 6 months, then every 3 months for the subsequent 6 months, for a total of eight vaccinations over 1 year. All of the injections were in a volume of 0.5 ml each and given deeply s.c.

**Screening for Vitiligo and Eye Changes.** All of the patients had a complete skin exam before therapy and at each visit for vaccination to screen for vitiligo. Slit lamp exams and iris photos were performed in all of the patients by an ophthalmologist before starting therapy, and hand-held ophthalmoscope retinal and iris exams were performed at each vaccination visit to assess ocular toxicity. No patient had evidence of ocular toxicity, and no patient developed vitiligo.

**Adjuvant.** AS02B was supplied by GlaxoSmithKline Biologicals (Rixensart, Belgium) in glass ampoules fitted with a 22-gauge needle for injection containing 0.5 ml of sterile adjuvant solution without preservative.

**Peptides.** Clinical grade peptides were synthesized by Clinalfa Pharmaceuticals (Läufelfingen, Switzerland). The peptide vaccine was administered as outpatient therapy. Peptide MART-151-73 was produced by solid phase synthesis using 9-fluorenylmethoxy carbonyl chemistry and reverse-phase high-performance liquid chromatography purification. Chemical identity was verified by mass spectrometry, and the finished, high-performance liquid chromatography purification. Chemically synthesized and pure, the synthetic peptides were reconstituted in 100% DMSO (10 mg/ml) and diluted in aqueous solution.

**Preparation of Peripheral Blood Mononuclear Cell Specimens.** Apheresis samples were taken within 48 h of the first vaccination (“prevaccine”) and within 2 weeks of the sixth vaccination (“postvaccine”). A final blood sample by venipuncture was taken on the day of the eighth vaccination if possible, or within 3–6 months thereafter (“postvaccine 12+ months”). Pre- and postvaccine pheresis samples were processed to purify peripheral blood mononuclear cells (PBMC) by sedimentation on Lymphoprep (Greiner Bio-One, Longwood, FL) and extensive washing in Hank’s Balanced Salt Solution. Cells were frozen in 40% human antibody serum (HS; Omega Scientific, Tarzana, CA), 50% RPMI 1640 (Invitrogen, Carlsbad, CA), and 10% DMSO (Sigma, St. Louis, MO). All of the cells were stored in a liquid nitrogen freezer at −168°C until use.

**Cell Lines.** The T2.DR4 cell line (a kind gift from Dr. Janice Blum, University of Indiana, Indianapolis, IN) was generated by transfection of HLA-DRβ1*0401 cDNA into T2 cells (TAP-deficient lymphoma). It is HLA-DM deficient, making its cell surface DRβ1*0401 molecules receptive to loading by exogenous peptide (24). HLA-DRβ1*0401/+MART-1+ (25) melanoma cell lines 624mel, 697mel, 1102mel, and 1359mel were generously provided by Dr. John Wunderlich (National Cancer Institute, NIH, Bethesda, MD). K562 cells (derived from a patient with chronic myelogenous leukemia) were used to inhibit natural killer cell-mediated background lysis during chromium-release assays. All of the cell lines were maintained in culture with RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Omega Scientific), 1-glutamine, nonessential amino acids, sodium pyruvate, and antibiotics (denoted CM for “complete media”; all from Invitrogen).

**Flow Cytometry Immunofluorescence Analysis of Tumor Cell Lines.** The following monoclonal antibodies were used to measure HLA-DR4 surface expression on cell lines 624mel, 697mel, 1102mel, and 1359mel: biotinylated anti-HLA-DR4 (One Lambda, Canoga Park, CA) and phycoerythrin-conjugated streptavidin (BD Biosciences, San Diego, CA). One-million cells from each cell line were washed twice with fluorescence-activated cell sorter buffer (PBS +1% bovine serum albumen) and labeled with 20 μg of biotinylated anti-HLA-DR4 antibody. Cells were incubated in the dark on ice for 30 min, washed twice with fluorescence-activated cell sorter buffer, and then labeled in the same manner with 20 μg of phycoerythrin-conjugated streptavidin. All of the samples were then washed twice and resuspended in fluorescence-activated cell sorter buffer for immediate analysis.

**HLA-DRβ1*0401 Tetramer Staining.** PBMCs (pre- and postvaccine) from patient 1 (DRβ1*0401+) were thawed and washed twice with Hank’s Balanced Salt Solution. For direct ex vivo staining, an aliquot of cells were resuspended in RPMI 10% HS-CM with IL-2 (2 units/ml). The cells were then plated (1 × 10^6/well) in 48 well flat-bottomed plates (Corning Inc., Corning, NY) and rested overnight at 37°C/5% CO2 to induce T-cell receptor up-regulation. For staining after an in vitro restimulation, another aliquot of cells were resuspended in RPMI 10% HS-CM and plated (1 × 10^6/well) in 48 well flat-bottomed plates (Corning) with the MART-151-73 peptide or an irrelevant DR4-binding peptide, HIV RT1*0401 (Ref. 26; 10 μg/ml each). The plates were then incubated at 37°C/5% CO2, and cells were fed with RPMI 10% HS-CM containing IL-2 (20 units/ml) as needed. On the ninth day of culture (1 day before staining), medium was replaced with RPMI 10% HS-CM containing 2 units/ml of IL-2 to induce T-cell receptor up-regulation.
Tetramer staining was performed by incubating $5 \times 10^5$ cells with 5 $\mu$L of DR61*0401-MART-151–73 iTag Tetramer (Beckman Coulter, San Diego, CA) in 50 $\mu$L of RPMI 10% HS-CM. For direct ex vivo staining, DR61*0401-Flu MP72–72 iTag (Beckman Coulter) was used as a negative control. After which, fluorescein isothiocyanate-labeled anti-CD4 and PC5-labeled anti-CD3 antibodies (5 $\mu$L each; Beckman Coulter, Miami, FL) were then added for an additional 20 min at room temperature. Cells were then washed with wash buffer (PBS containing 1% bovine serum albumen and 0.1% Na azide) before fixing in PBS containing 0.5% formaldehyde. Samples were run on an EPICS XL Flow Cytometer to collect $\sim 1.5 \times 10^5$ CD4$^+$ events for each sample. Data analysis was performed through sequential gating on CD3$^+$ and CD4$^+$ populations to obtain the percentage of tetramer$^+$ in the total CD4$^+$ T-cell population.

**Generation of Dendritic Cells.** Pre vaccine PBMCs (1–1.3 $\times 10^9$) were thawed, washed twice with Hank’s Balanced Salt Solution, and adhered in T-150 flasks (Greiner Bio-One) for 2 h at 37°C/5% CO$_2$ in X-VIVO 15 medium (Cambrex, East Rutherford, NJ). Nonadherent cells were gently washed off with warm X-VIVO 15. The plastic adherent cells were cultured in X-VIVO 15 supplemented with 1000 units/ml each of recombinant human granulocyte macrophage colony-stimulating factor (Berlex, Richmond, CA) and recombinant human IL-4 (R&D, Minneapolis, MN). Six days later, recombinant human granulocyte macrophage colony-stimulating factor and recombinant human IL-4 were replenished (1000 units/ml each). On the seventh day of culture, “immature” DCs were harvested and placed (1 $\times 10^7$/ml) in 12-well plates (Corning) with X-VIVO 15. The DCs were pulsed with the MART-151–73 peptide (20 $\mu$g/ml) and activated (24 h) with a maturation mixture consisting of cytokines tumor necrosis factor-$\alpha$ (10 ng/ml), IL-1$\beta$ (10 ng/ml), IL-6 (100 units/ml), all from R&D, and prostaglandin E$_2$ (Sigma; 1 $\mu$g/ml). The next day, “mature” DCs were harvested with warm PBS (Mediatech, Herndon, VA), then washed with fresh X-VIVO 15 to remove residual cytokines and unbound peptide. The DCs were then irradiated (6000 Rads) using a $^{137}$Cs source (Gammeck 40; Atomic Energy of Canada, Ontario, CA), washed with X-VIVO 15 and used to stimulate autologous PBMCs.

**Lymphocyte Expansion with Synthetic Peptides.** Pre- and postvaccine PBMCs were thawed and plated (2 $\times 10^5$/well) in X-VIVO 15 with autologous peptide-pulsed mature DCs (1 $\times 10^5$/well) in 24-well plates (Greiner Bio-One). The cultures were supplemented with 10 ng/ml of IL-7 (R&D) on the initial day of plating and incubated at 37°C/5% CO$_2$. Three days later, nonadherent responder cells were transferred to new 24-well plates and supplemented with 20 units/ml of IL-2 (Chiron, Emeryville, CA). Nonadherent responder cells were collected on the tenth day for functional analysis by rinsing the wells with warm culture media.

**Th1/Th2 ELISPOT Assay.** ELISPOT IP plates (Millipore, Bedford, MA) were prepared by first prewetting the membranes with a 70% ethanol (Sigma) solution and rinsing three times with PBS. Antihuman-interferon (IFN)-$\gamma$ or antihuman-IL-5 (Mab 1-D1K and Mab TRFK5, respectively; Mabtech, Malmo, Sweden) capture antibody (10 $\mu$g/ml in PBS) were then added to each well, and the plates were stored overnight at 4°C. The next day, the ELISPOT plates were inverted to discard the capture antibody solutions and blocked (2 h) with RPMI 10% HS-CM at 37°C/5% CO$_2$. Uncultured, cryopreserved pre- and postvaccine PBMCs were thawed, washed twice with Hank’s Balanced Salt Solution, and treated with DNase I (4,000 IU; Roche, Basel, Switzerland) for 20 min in a 37°C waterbath. CD4$^+$ cells were affinity-purified using the magnetic activated cell sorting (Miltenyi Biotech, Bergish Gladbach, Germany) technique for positive selection ($\approx$95% purity as determined by flow cytometric analysis; data not shown) and added to the ELISPOT plates (10$^7$/50 $\mu$L/well in triplicates) after discarding the blocking solutions. The plates were then incubated for 2 h at 37°C/5% CO$_2$ to allow the CD4$^+$ cells to settle onto the membrane. The CD4$^+$ flow-through population from the respective prevaccine samples were collected and added to the plates as antigen-presenting cells (2 $\times 10^5$/50 $\mu$L/well). Relevant or irrelevant peptides were added at a final concentration of 10 $\mu$g/ml, and the plates were incubated (48 h) at 37°C/5% CO$_2$.

CD4$^+$ cells from DC-stimulated PBMCs (pre- and post-vaccine) were affinity-purified in the same manner as above and plated in dilutions of 10$^5$, 3.3 $\times 10^4$, and 1.1 $\times 10^4$/well. Autologous prevaccine PBMCs were thawed and used as antigen-presenting cells, and relevant or irrelevant peptides were added at a final concentration of 10 $\mu$g/ml. In addition to peptide-pulsed autologous PBMCs, antigen-presenting cells for in vitro restimulated CD4$^+$ cells from patient 1 (DRB1*0401$^+$) included the melanoma lines 624mel, 697mel, 1102mel, and 3A10, respectively (Mabtech) in PBS with 0.5% bovine serum albumen (Sigma). Plates were then washed four times with PBS/0.05% Tween 20, and an avidin-peroxidase complex (diluted 1:100; Vectastatin Elite Kit; Vector, Burlingame, CA) in PBS/0.05% Tween 20 was added for 1 h at room temperature. Unbound complex was removed by two successive washings in PBS/0.05% Tween 20 and three with PBS. Color development was performed with 3-amino-9-ethyl-carbazole (Sigma) for 4 min and stopped by rinsing the plates under running tap water. Spot numbers were determined with the aided of computer-assisted video imaging analysis (ELISPOT Reader System; Carl Zeiss, Oberkochen, Germany) as described previously (27). The highest readable dilutions gave $>600$ spots per well and were used in determining frequency of antigen-specific cells (normalized to 10$^5$ input cells for Th1/Th2 ELISPOT assays).

**Proliferation Assay.** PBMCs (pre- and postvaccine) were thawed, washed twice with Hank’s Balanced Salt Solution, and treated with DNase I (4,000 IU; Roche) for 20 min in a 37°C waterbath. CD8$^+$ cells were depleted using the magnetic activated cell sorting technique, and the CD8$^+$ fractions were resuspended in RPMI 10% HS-CM and plated (2 $\times 10^5$/well, replicates of 5) in 96-well round-bottomed cluster plates (Corning Inc.). Relevant or irrelevant peptides (10 $\mu$g/ml final conc-
centration) were added to each well, and the plates were incubated at 37°C/5% CO₂. Four days later, 1 μCi of [³H] thymidine (Perkin-Elmer, Boston, MA) was added to each well for the final 16 h of incubation. Cells were harvested onto fiberglass filters (Molecular Devices, Sunnyvale, CA), and radioactivity was assessed at 0.15% after subtraction of background (0.08%).

RESULTS

Demographics. Twelve patients (Table 1) with stages III/IV resected melanoma who were HLA-A1*, A3*, or B44* were treated with peptides tailored to their HLA class I haplotype. Within this cohort, 2 patients were HLA-DR4+, and, therefore, also received two DR4-restricted peptides, MART-1 51-73 and gp100 44-59. One patient was DR7* by genotypic analysis (DR4* by serological typing) and, therefore, was also eligible for vaccination with MART-1 51-73 and gp100 44-59. Only MART-1 51-73 was found to be immunogenic, because no immune responses were detected to gp100 44-59 or any MHC class I-restricted peptides. The in vitro data for the 3 patients vaccinated with MART-1 51-73 are described herein.

Toxicities and Clinical Outcomes. Vaccinations were given monthly for 6 months, then twice at 3-month intervals thereafter (eight vaccinations total). Toxicities of the overall trial for 12 patients were modest, with only 1 case of grade III/IV toxicity noted. The most common side effects were injection site pain and discomfort, as well as erythema, flu-like symptoms, and fatigue for several days after each injection (Table 2). All of the patients on the trial finished the yearlong vaccine regimen. Of the 3 vaccinated with the MART-1 51-73 peptide, patients 1 and 2 are alive and free of disease at 25 and 34 months after beginning the trial, respectively. Patient 3 relapsed 17 months after beginning the trial and is currently undergoing additional therapy.

Detection of MART-1 51-73-Specific T Cells Using HLA-DRβ1*0401 Tetramers. Antigen-specific CD3+CD4+ cells were detected at 0.15% after subtraction of background (0.08%).

Table 1 Patient demographics

<table>
<thead>
<tr>
<th>Total number of patients</th>
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<tbody>
<tr>
<td>Male</td>
<td>8</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
</tr>
<tr>
<td>Median age (years)</td>
<td>40</td>
</tr>
<tr>
<td>Mean time since diagnosis (mos.)</td>
<td>35.5</td>
</tr>
<tr>
<td>Stage of disease</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>9</td>
</tr>
<tr>
<td>Stage IV</td>
<td>3</td>
</tr>
<tr>
<td>Prior therapy</td>
<td></td>
</tr>
<tr>
<td>Surgery (sentinel node biopsy)</td>
<td>12</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>3</td>
</tr>
<tr>
<td>Hormonal</td>
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</tr>
<tr>
<td>Immunologic</td>
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<tr>
<td>Radiation</td>
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Table 2 Toxicities

<table>
<thead>
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<th>Toxicity/grade (N = 12)</th>
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<th>3/4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allergic reaction/hypersensitivity</td>
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<td></td>
</tr>
<tr>
<td>(including drug fever)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthralgia</td>
<td>4/1</td>
<td></td>
</tr>
<tr>
<td>Diarrhea (without colostomy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever (in the absence of neutropenia, where neutropenia is defined as AGC &lt; 1.0 × 10⁹/L)</td>
<td>2/0</td>
<td></td>
</tr>
<tr>
<td>Injection site pain</td>
<td>9/0</td>
<td></td>
</tr>
<tr>
<td>Dermatitis, focal (associated with high dose chemotherapy and bone marrow transplant)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>1/0</td>
<td></td>
</tr>
<tr>
<td>Fatigue (lethargy, malaise, asthenia)</td>
<td>7/0</td>
<td></td>
</tr>
<tr>
<td>Pigmentation changes (e.g., vitiligo)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>1/0</td>
<td></td>
</tr>
<tr>
<td>Rash/deśquamation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urticaria (hives, welts, wheals)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rigors, chills</td>
<td>1/0</td>
<td></td>
</tr>
<tr>
<td>Erythema</td>
<td>4/0</td>
<td></td>
</tr>
<tr>
<td>Ulceration</td>
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</table>

Granzyme B ELISPOT Assay. ELISPOT plates (BD Biosciences) were prepared by adding antihuman-granzyme B capture antibody (5 μg/ml; BD Biosciences) in PBS and stored overnight at 4°C. The next day, the ELISPOT plates were inverted to discard the capture antibody solutions and blocked for 2 h with RPMI 10% HS-CM at 37°C/5% CO₂. DC-stimulated, affinity-purified CD4+ cells were added to the ELISPOT plates (3.3 × 10⁵/50 μl/well in triplicate) after discarding the blocking solutions. T2-DR4 cells (10⁵/well) and autologous PBMCs (2 × 10⁵/well) were pulsed with peptides (16 h) and used as antigen-presenting cells. The plates were incubated (5 h) at 37°C/5% CO₂. A biotinylated antihuman-granzyme B secondary antibody (BD Biosciences) was used in the assay (2 μg/ml in PBS 10% fetal bovine serum). Washings, color development, and spot analysis were performed as described above for the Th1/Th2 ELISPOT assays.

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by direct *ex vivo* staining of the postvaccine sample from patient 1 (*DRB1*<sup>0401</sup>*), as shown in Fig. 1A. A single *in vitro* re-stimulation of PBMCs from this patient with the MART-1<sub>51–73</sub> peptide demonstrated elevated levels of MART-1<sub>51–73</sub>-specific CD3<sup>+</sup>/CD4<sup>+</sup> cells in the postvaccine and long-term (at 12+ months) samples. MART-1<sub>51–73</sub> tetramer staining in Fig. 1B indicates that specificity rose to >10% of CD3<sup>+</sup>/CD4<sup>+</sup> cells in postvaccine sample (after the sixth vaccination at month 6), with
prevacine samples at background levels compared with an irrelevant peptide-stimulated control culture. Specificity remained high after the eighth and final vaccination at month 12, with positive staining at >9%.

**Vaccination with MART-1<sub>51-73</sub> Peptide in Adjuvant AS02B Induces a Mixed Th1/Th2 CD4<sup>+</sup> Response.** In vitro ELISPOT analysis of fresh CD4<sup>+</sup> T cells from 2 of the 3 patients vaccinated with the MART-1<sub>51-73</sub> peptide epitope revealed that IFN-γ- and IL-5-secreting cells were induced in approximately a 1:1 ratio (Fig. 2, A and B) with very low backgrounds prevaccine. All of the negative control values are shown, without subtraction from experimental values. Restimulation of the PBMCs in all 3 of the patients using autologous peptide-pulsed mature DCs demonstrated both Th1 and Th2 recall responses postvaccine with prevaccine responses near background levels (Fig. 3, A and B). Subsequent booster immunizations were given at months 9 and 12 of the vaccine regimen and resulted in increased recall responses observed at or after the twelfth month of the trial for each patient, seen as the third bar for each patient in Fig. 3, A and B. Significant levels of cytokine-secreting cells were detected, rising to 2300+ IFN-γ spots and 2200+ IL-5 spots per 10<sup>5</sup> CD4<sup>+</sup> cells in patient 1.

Assays of proliferation to MART-1<sub>51-73</sub> by fresh pre- and postvaccine CD8<sup>+</sup>-depleted PBMCs were performed with the addition of [3H]thymidine at day 4 of incubation. Postvaccine samples for all 3 of the patients demonstrated higher proliferation to MART-1<sub>51-73</sub> than respective prevaccine samples as shown in Fig. 4, with values rising to ~40,000 cpm of thymidine for patient 1, 10,000 cpm for patient 2, and 4000 cpm for patient 3. Prevacine samples ranged between ~400 (patient 3) to 1200 (patient 1) cpm. Positive controls with phytohemagglutinin-treated cells were >50,000 counts/2<sup>10</sup> cells for all of the pre- and postvaccine samples. Of interest is that the highest proliferation response was in the patient who was DR<sup>β1</sup>*0401<sup>+</sup> (patient 1), which is consistent with the previously published binding data for MART-1<sub>51-73</sub> (23).

**Vaccination with MART-1<sub>51-73</sub> Induces CD4<sup>+</sup> T Cells That Recognize DR<sup>β1</sup>*0401<sup>+</sup>/MART-1<sup>+</sup> Tumor Cells.** To determine whether postvaccine CD4<sup>+</sup> T cells recognize the MART-1<sub>51-73</sub> epitope naturally presented by HLA-DR<sup>β1</sup>*0401<sup>+</sup>/MART-1<sup>+</sup> melanoma lines, PBMCs from patient 1 (DR<sup>β1</sup>*0401<sup>+</sup>) were restimulated once in vitro with autologous peptide-pulsed mature DCs. CD4<sup>+</sup> effectors were magnetic activated cell sorting-purified and tested for their ability to recognize cell lines 624mel, 697mel, 1102mel, and 1359mel. ELISPOT analysis revealed activation of IFN-γ-secreting CD4<sup>+</sup> T cells in response to these cell lines (Fig. 5), but formation of IL-5 spots were not detected (data not shown). These CD4<sup>+</sup> cells did not respond to the HLA-DR<sup>4</sup><sup>+</sup>, MART-1<sup>+</sup> cell line 888mel, suggesting that immunological reactivity against MART-1 was restricted by HLA-DR<sup>4</sup>.

The magnitude of the responses correlated with the relative concen-

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**Fig. 2** Measurement of CD4<sup>+</sup> T-cell reactivity against MART-1<sub>51-73</sub> in uncultured peripheral blood mononuclear cells. A, interferon (IFN) γ enzyme-linked immunospot (ELISPOT). B, interleukin (IL) 5 ELISPOT. Spots per 100,000 CD4<sup>+</sup> effectors is shown on the ordinate; bars, ±SD. A, IFN-γ ELISPOT; B, IL-5 ELISPOT.
Trations of surface HLA-DR4 expression in each of the respective cell lines (shown as mean channel fluorescence above each data set), which indicates that 1359mel and 1102mel generated the highest number of IFN-γ spots and also had the highest levels of HLA-DR4 expression by flow cytometry. These data suggest that MART-151–73 is naturally processed on DRβ1*0401* /MART-1* melanoma cell lines and that it can be recognized by IFN-γ-secreting, Th1-type CD4+ cells generated after a peptide vaccination.

Lytic CD4+ T Cells Specific for MART-151–73 Use the Perforin-Granzyme-Mediated Pathway. Recent published evidence has suggested that both Th1- and Th2-type T-cell clones established from melanoma patients were able to lyse autologous tumor cells (17, 18). To test this hypothesis, cytotoxicity assays using affinity-purified CD4+ cells from patient 1 against HLA-matched, peptide-pulsed and chromated T2.DR4 cells (DRβ1*0401*) were performed to assess whether the ELISPOT-positive CD4+ cells were also capable of lysing peptide-expressing targets. PBMCs were restimulated once in vitro with autologous mature DCs pulsed with MART-151–73. CD4+ cells were affinity-purified and tested for their ability to lyse T2.DR4 cells that were exogenously loaded with MART-151–73 in a 5-h chromium-release assay. Antigen-specific lysis was detected with maximal specific lysis at ~30% at effector:target ratios of 60:1 (Fig. 6A), which was completely abrogated by the addition of an anti-MHC class II antibody (TU39; Fig. 6B). These data suggest that lysis was mediated in a MHC class II-restricted manner. Interestingly, the addition of an anti-Fas antibody to the assay did not inhibit cell-mediated lysis (Fig. 6B).

To determine the possible mechanism(s) involved in CD4+ cell-mediated lysis of peptide-pulsed targets, a granzyme B ELISpot assay was performed on CD4+ cells specific for MART-151–73. Granzymes are a family of serine proteases (the most common being granzymes A and B) secreted by cytotoxic lymphocytes that activate caspase-dependent apoptotic pathways in target cells. CD4+ cells from patient 1 and T2.DR4 cells (Fig. 7, right) used in the chromium-release assays were plated together on ELISPOT plates for 5 h (as described in “Materials and Methods”). Autologous peptide-pulsed PBMCs were also included as targets (Fig. 7, left). The data in Fig. 7 indicate that MART-151–73-specific CD4+ T cells likely use the perforin-granzyme-mediated pathway to lyse their specific targets, because both postvaccine and long-term cells (at 12+ months) generated >300 Granzyme B-positive spots/3.3 × 10^4 input effectors using peptide-pulsed PBMCs as targets, and ≥150 spots/3.3 × 10^4 input effectors using peptide-pulsed T2.DR4 target cells. Prevaccine values were <10 spots, and the negative controls with irrelevant HIV peptide-pulsed target cells and MART-151–73-pulsed targets with no effectors were >10-fold less.

DISCUSSION

In this pilot melanoma vaccine trial, we evaluated the toxicity of and the cellular immune responses to a vaccine regimen of MHC class I- and class II-restricted melanoma-associated peptides. Patients with resected high-risk stages III and IV melanoma received class I peptides restricted to HLA-A1, A3, or B44 with class II-restricted peptides if their DR serological typing matched DR4 or DR11. The MHC class I peptide +/- MHC class II peptide regimen with adjuvant AS02B was not toxic, with primarily vaccine-related grade I and II toxicity seen. Injection site pain, flu-like symptoms, and fevers were common, but the regimen was otherwise felt to be well tolerated.

![Fig. 4](image_url) Incorporation of [3H]thymidine by previously uncultured, CD8−-depleted peripheral blood mononuclear cells stimulated with peptide for 5 days. Counts per minute (CPM) are shown on the ordinate; 200,000 cells/well were assayed in sextuplicate; bars, ±SD.

![Fig. 5](image_url) Measurement of reactivity against DRβ1*0401*/MART-1+ melanoma cell lines by IFN-γ ELISPOT. Reaction to DRβ1*0401*/MART-1+ melanoma cell lines by IFN-γ ELISPOT. Spots per 100,000 CD4+ effectors are shown on the ordinate. Relative concentration of surface human leukocyte antigen-DR4 (as determined by flow cytometric analysis) in each cell line are expressed numerically above each data set as mean channel fluorescence; bars, ±SD.
No clear evidence of immune responses to any class I-restricted peptides (tyrosinase\textsubscript{146-156}, tyrosinase\textsubscript{192-200}, melanoma antigen-3\textsubscript{167-176}, melanoma antigen-3\textsubscript{168-176}, gp100\textsubscript{17-25}, and gp100\textsubscript{57-65}) used in the trial were seen in fresh or in vitro restimulated PBMCs, despite previous reports of in vitro immunogenicity of these peptides (28–31). Adjuvant AS02B was found to not be strongly Th1-polarizing, possibly explaining the inability to generate CD8\textsuperscript{+} T-cell responses in the 3 patients receiving the MART-1\textsubscript{51-73} peptide. To remedy this issue, future class I+II peptide vaccine trials may use incomplete Freund’s adjuvant as an alternative adjuvant and possibly IL-12 to induce highly polarized Th1 responses.

The important finding of this trial was that the MART-1\textsubscript{51-73} epitope was immunogenic in a few patients who were serologically DR4\textsuperscript{+}. In multiple different immunological assays, postvaccine CD4\textsuperscript{+} T cells specific for MART-1\textsubscript{51-73} were detected in the 3 patients vaccinated with this peptide. Of note, another DR4-binding melanoma-associated epitope included in the vaccine, gp100\textsubscript{44-59}, was not immunogenic, because 0 of the 3 DR4\textsuperscript{+} patients who received it exhibited any detectable immune reactivity to that peptide in any assay (data not shown).

Soluble DR\textsubscript{B1}*0401-MART-1\textsubscript{51-73} tetramers bound specifically to the postvaccine CD3\textsuperscript{+}/CD4\textsuperscript{+} cells (patient 1) by direct \textit{ex vivo} staining. Significant levels of staining in the postvaccine and long-term samples were achieved after a single in vitro restimulation with the MART-1\textsubscript{51-73} peptide. MHC class I tetramers have become an invaluable immune monitoring tool for quantifying CD8\textsuperscript{+} T-cell responses in melanoma vaccine trials using class I-restricted peptides. Here we demonstrate the potential utility and practicality of using a tumor antigen-specific MHC class II tetramer for immune monitoring in a cancer vaccine trial. In contrast to class I tetramers, which allow for highly sensitive direct \textit{ex vivo} enumeration of antigen-specific CD8\textsuperscript{+} T cells in PBMCs with very low background staining, detection of antigen-specific CD4\textsuperscript{+} T cells by class II tetramers has been shown to require \textit{in vitro} expansion (32, 33). Recently, however, direct \textit{ex vivo} enumeration of antigen-specific CD4\textsuperscript{+} T cells in PBMCs by class II tetramers loaded with peptides derived from influenza (34) and hepatitis C virus (35) have been demonstrated. We show here that the tumor antigen-specific class II tetramer used in this study can also be used for direct \textit{ex vivo} staining. The generally low frequency of antigen-specific CD4\textsuperscript{+} T cells in peripheral blood combined with the low avidity between MHC class II-peptide complexes and T-cell receptors make their direct \textit{ex vivo} enumeration a challenge. CD4\textsuperscript{+} cell enrichment techniques and the enhanced cell acquisition and analysis rates of new flow cytometers will contribute to the resolution of this issue.

We detected an approximately equal ratio of Th1- and Th2-type CD4\textsuperscript{+} T-cell responses in the postvaccine samples of 3 of 3 serologically DR4\textsuperscript{+} patients who were vaccinated with the MART-1\textsubscript{51-73} peptide. These immune responses were identified directly in the fresh postvaccine PBMCs of 2 of the patients (1 and 2), whereas the third required \textit{in vitro} restimulation with the peptide before responses were evident. The observed gradient of responses in the 3 patients with all of the immunological assays correlates strongly with the expression of different HLA-DR alleles, with the highest response occurring in patient 1, who is DR\textsubscript{B1}*0401\textsuperscript{+}. These data are consistent with a previous report that the MART-1\textsubscript{51-73} peptide binds at least two DR4 alleles, DR\textsubscript{B1}*0401 and *0404, with stronger relative binding occurring on DR\textsubscript{B1}*0401 (24). Here we present evidence that the promiscuity of the peptide may extend beyond these two alleles, as patient 3 (DR7/DR13\textsuperscript{+}) by genotypic

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\textbf{Fig. 6 A}, cytotoxicity against peptide-pulsed T2.DR4 cells (DR\textsubscript{B1}*0401\textsuperscript{+}). Pre- and postvaccination peripheral blood mononuclear cells from patient 1 were restimulated once with autologous mature dendritic cells pulsed with MART-1\textsubscript{51-73}. CD4\textsuperscript{+} T cells were affinity-purified and assayed. \textit{B}, inhibition of lysis by anti-MHC-II antibody TU39 in postvaccination CD4\textsuperscript{+} cells. Effector:target (E:T) ratios are shown on the abscissa and percentage of lysis on the ordinate; bars, ±SD. Ab, antibody; E:T, effector:target.

\textbf{Fig. 7}, MART-1\textsubscript{51-73}-specific granzyme B secretion by \textit{in vitro} restimulated CD4\textsuperscript{+} cells from patient 1. Spots per 3.3 × 10\textsuperscript{4} CD4\textsuperscript{+} effectors are shown on the ordinate; bars, ±SD.
The observation of both IFN-γ and IL-5-secreting CD4+ cells in the uncultured and in vitro restimulated postvaccine PBMCs suggests induction of a bipolar T-helper cell response against MART-151-73. Recognition of HLA-matched, MART-1+ melanoma lines by the affinity-purified CD4+ lymphocyte population from patient 1 (DRB1*0401) indicates that MART-151-73 is a naturally processed epitope in HLA-DRB1*0401+ tumor cells. However, the CD4+ cells of this patient responded to the melanoma cell lines by secreting only IFN-γ but not IL-5. Furthermore, these CD4+ cells did not lyse the cell lines nor did they secrete granzyme B in response to them (data not shown). These data infer that possibly only Th1-type cells may have effector functions at the tumor site in vivo and only by secretion of cytokines and not direct lysis of tumor cell targets, unless tumor cells express higher densities of the appropriate MHC:peptide complex in situ. Elucidation of the effector functions and clinical significance of tumor antigen-specific, IL-5-secreting Th2-type CD4+ T cells is currently being pursued in our laboratory.

It has been reported previously that immune responses in cancer vaccine patients skewed toward Th2-type responses are correlated with disease progression in melanoma patients, whereas normal donors and patients who remained disease-free exhibited highly-polarized Th1 or mixed Th1/Th2 responses (36). Th1-type CD4+ T cells secreting IFN-γ appear important to the optimal generation and durability of tumor antigen-specific CD8+ CTLs in vivo and may also recruit these effector cells into the tumor microenvironment (20). We have demonstrated in our study that MART-151-73-specific, IFN-γ-secreting (Th1-type) CD4+ cells in 1 patient can recognize HLA-matched, MART-1+ melanoma cell lines in vitro, whereas their Th2-type counterparts failed to do so. This finding suggests that highly polarized Th2-type immune responses against tumors might be ineffective and that Th1-type cells may be important mediators of tumor regression in vivo. The evidence presented here, which corroborates previous clinical observations (36, 37), suggests that future peptide-based melanoma vaccines should be tailored toward inducing Th1-type responses.

T-cell clones of both the Th1-and Th2-type have been shown to have lytic function (20, 21). In our study, we found evidence that MART-151-73-specific CD4+ cells had lytic capability when the relevant peptide was exogenously pulsed onto HLA-DR-matched target cells. Moreover, these CD4+ cells secreted granzyme B in response to the relevant target, whereas the addition of an anti-Fas antibody was not able to inhibit lysis in chromium release assays. These data infer that cytotoxicity was likely mediated via the granzyme-perforin pathway. However, no detectable lysis or granzyme B secretion above background controls could be detected against HLA-matched, MART-1+ melanoma cell lines (data not sown). This is likely due to the relatively low concentration of the appropriate MHC:peptide complex on the surface of most melanoma cells, which may be insufficient to activate the lytic pathways in responding CD4+ effector cells. This level of relevant MHC:peptide complex on melanoma cells was sufficient for recognition by IFN-γ-secreting CD4+ cells, suggesting that the signaling threshold for the secretion of cytokines in Th1-type cells may be lower than that necessary to initiate lytic responses.

In summary, the MART-151-73 peptide is an immunogenic epitope for the induction of CD4+ helper T cells in vivo in vaccinated melanoma patients. Vaccination with this epitope in adjuvant AS02B resulted in mixed Th1/Th2 responses, and the peptide appears to bind promiscuously to multiple HLA-DR4 subtypes. These data suggest that this epitope may have therapeutic potential in melanoma patients who express MHC class II alleles other than HLA-DR4.

It is likely that this peptide is a naturally processed epitope in DRB1*0401+/MART-1+ melanoma tumors, as demonstrated by the ability of MART-151-73-specific, Th1-type CD4+ T cells to recognize DRB1*0401+/MART-1+ melanoma lines and not a HLA-DR4+/MART-1- cell line. Our findings provide a description of the generation of CD4+ helper T-cell effector functions in vivo after administration of a cancer vaccine and may be useful in the design of future candidate melanoma vaccines.
Immune Responses to a Class II Helper Peptide Epitope in Patients with Stage III/IV Resected Melanoma

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