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Adenovirus MART-1–engineered Autologous Dendritic Cell Vaccine for Metastatic Melanoma

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Summary

We performed a phase 1/2 trial testing the safety, toxicity, and immune response of a vaccine consisting of autologous dendritic cells (DCs) transduced with a replication-defective adenovirus (AdV) encoding the full-length melanoma antigen MART-1/Melan-A (MART-1). This vaccine was designed to activate MART-1–specific CD8⁺ and CD4⁺ T cells. Metastatic melanoma patients received 3 injections of 10⁶ or 10⁷ DCs, delivered intradermally. Cell surface phenotype and cytokine production of the DCs used for the vaccines were tested, and indicated intermediate maturity. CD8⁺ T-cell responses to MART-1₂₇₋₃₅ were assessed by both major histocompatibility complex class I tetramer and interferon (IFN)- γ enzyme-linked immunosorbent spot (ELISPOT) before, during, and after each vaccine and CD4⁺ T-cell responses to MART-1₅₁₋₇₃ were followed by IFN- γ ELISPOT. We also measured antigen response breadth. Determinant spreading from the immunizing antigen MART-1 to other melanoma antigens [gp100, tyrosinase, human melanoma antigen-A3 (MAGE-A3)] was assessed by IFN- γ ELISPOT. Twenty-three patients were enrolled and 14 patients received all 3 scheduled DC vaccines. Significant CD8⁺ and/or CD4⁺ MART-1–specific T-cell responses were observed in 6/11 and 2/4 patients evaluated, respectively, indicating that the E1-deleted adeno-virus encoding the cDNA for MART-1/Melan-A (AdV-MART1)/DC vaccine activated both helper and killer T cells in vivo. Responses in CD8⁺ and CD4⁺ T cells to additional antigens were noted in 2 patients. The AdVMART1-transduced DC vaccine was safe and immunogenic in patients with metastatic melanoma.

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Dendritic cells (DCs) are the key physiologic stimulators of naive and primed cells to an antigen.^{1,2} Many types of myeloid DCs have been tested clinically with objective clinical responses, both partial and complete. Many current immunotherapy efforts use myeloid DCs pulsed with synthetic T-cell epitopes.^{3–6} Such strategies are limited by identification of specific human leukocyte antigen (HLA)-restricted epitopes, binding strength, and/or kinetics of peptide binding, as well as the paucity of well-characterized major histocompatibility complex (MHC) class II-restricted epitopes. DCs that have been genetically engineered to express a full-length tumor antigen can stimulate T cells to a variety of class I and class II epitopes.^{7–9} This provides for continuous synthesis of the transgene^{10,11} and a polyclonal CD8 and CD4 T-cell response, which may lead to stronger, broader T-cell activation.

MART-1/Melan-A (MART-1) is an immunogenic melanosomal-derived self antigen expressed in normal and malignant pigmented cells, including >90% of melanomas.^{12,13} The proximal MART-1 promoter was characterized by our group.¹⁴ For the most common MHC class I HLA type HLA-A*0201 (A2.1), the immunodominant peptide (MART-1₂₇₋₃₅) has been identified and extensively studied, although other peptides, including MART-1₅₃₋₆₂ may also be processed and presented.^{15–17} This immunodominant MART-1₂₇₋₃₅ peptide has been shown to bind other HLA-A subtypes of the A2 superfamily (A2.2, 2.4, 2.5, 2.6, 2.9, and A69.1, and also HLA-B35 and –B45), hence patients who are not HLA-A2.1+ may be able to mount CD8⁺ T-cell responses to this antigen.^{17–20} MHC class II-restricted DR4²¹ and DQ5²² DR11 and DR52 epitopes have also been described.²³

We reported the results of a phase 1 dose-escalation clinical trial testing vaccination with autologous MART-1₂₇₋₃₅ peptide-pulsed DC.⁴ The goal of this study was to determine the most favorable dose and route of peptide-loaded DC administration. Immunization with the single MART-1₂₇₋₃₅ peptide pulsed onto granulocyte macrophage-colony stimulating factor (GM-CSF)/interleukin (IL)-4-differentiated DCs resulted in peptide-specific immune activation at all the doses and both the routes were tested, although 10⁷ DCs administered intradermally (ID) seemed to be superior for both the immune and clinical activity. Of the 18 patients enrolled (10 with measurable disease), there was 1 complete response (CR) and 2 disease stabilizations (SD). We observed that the frequency of circulating MART-1₂₇₋₃₅-specific CD8⁺ T cells did not correlate with the clinical outcome. However, vaccine-induced acquisition of CD8⁺ and CD4⁺ T-cell responses to antigens not encoded by the vaccine but expressed by the tumor, an immunologic phenomenon termed determinant spreading, was observed exclusively in the patient with a CR.

A subsequent phase 2 study of MART-1₂₇₋₃₅ peptide-pulsed DC, in which 10 stage II to IV melanoma patients received the same MART-1₂₇₋₃₅ peptide-pulsed autologous DC vaccine (ID, 10⁷ DC/injection), produced a CR in just 1 patient with immunologic findings consistent with determinant spreading to tyrosinase.²⁴ As in the earlier phase 1 trial, most of the other 9 subjects were successfully immunized against MART-1, but did not have evidence of determinant spreading. Taken together, these phases 1 and 2 data provide supporting evidence that the immunologic phenomenon of determinant spreading may play an important role in clinical response to immunotherapy.

We have studied adenovirus (AdV)-mediated engineering of DC with full-length tumor antigens for CD8⁺ and CD4⁺ T-cell activation.^{7,10,25,26} The benefits of this strategy include

antigen-specific activation of both CD8⁺ cytotoxic and CD4⁺ helper T cells, AdV-induced intermediate level maturation of DCs²⁷ (Vujanovic, '08 *submitted*), long-term antigen expression by the DCs (days and weeks instead of hours for peptides²⁸), potent activation of CD8⁺ T cells specific for immunodominant and subdominant epitopes from a tumor antigen,⁹ and broad activation of tumor antigen-specific CD4⁺ T cells which produce the Th1 cytokines interferon (IFN)- γ and IL-2.²⁹ Together, these data indicate that adenovirally-transduced DC (AdV/DC) are a potent vehicle for T-cell activation. We have also conducted studies in murine melanoma models, testing this strategy and defining the mechanism.^{26,30–33}

Recent literature has identified crosstalk between DCs and natural killer (NK) cells as important for shaping the immune response. DCs, which take up pathogenic material and become activated, can activate NK cells. Conversely, NK cells can improve DC function through both cytokine secretion and cell-cell contact.^{34–37} In murine models testing E1-deleted adenovirus encoding the cDNA for MART-1/Melan-A (AdVMART1)-transduced DC vaccines, we have identified a critical role for NK cells.³⁸ NK function has not yet been studied in human antigen-engineered DC clinical trials.

Herein we report the first clinical trial with an antigen engineered DC-based vaccine. This antigen-engineered DC vaccine was able to activate both CD8⁺ and CD4⁺ T cells specific for the MART-1 melanoma antigen in a subset of patients.

MATERIALS AND METHODS

Study Design and Eligibility Criteria

This was a phase 1/2, single site, dose-escalating study to evaluate the toxicity and immunologic effects of autologous DCs transduced with the MART-1 gene in subjects with melanoma. Two doses of AdVMART1/DC (10⁶, 10⁷ DCs) were given to groups of 3 patients ID. Patients over the age of 18 received 3 biweekly vaccinations. Study groups were stratified according to the HLA subtype. Eligible patients were HLA-A2.1, 2.2, 2.4, 2.5, 2.9, or 69.1 positive by DNA subtyping for HLA-restricted trial groups A to C, and other HLA subtypes for groups D to F, had biopsy-proven malignant melanoma, which expressed MART-1 mRNA as determined by RNA PCR or immunostaining.

Eligibility included patients with metastatic melanoma, a Karnofsky performance status of >70%, adequate hematopoietic, hepatic and renal function, and immune competence demonstrated by a positive skin delayed hypersensitivity test to at least 1 recall antigen (candida, tetanus toxoid, or mumps). Exclusion criteria included prior therapy < 4 weeks before trial entry, untreated central nervous system lesions, pregnancy, or concurrent immunosuppressive conditions or therapy. All subjects provided signed informed consent. The trial protocol and consent, and all modifications introduced during its conduct, were approved by the Institutional Review Board and the Internal Scientific Peer Review Committee at the University of California Los Angeles (UCLA, IRB no. 97-07-074), the NIH Recombinant DNA Advisory Committee (RAC; Human Gene Transfer Protocol no. 9811-269) and the Food and Drug Administration (BB IND no. 7122). Additionally, this trial underwent *ad hoc* reviews by the Jonsson Comprehensive Cancer Center Quality Assurance Committee (JCCC QA).

Clinical Response

Patients with measurable disease had serial measurement of indicator lesions, which were considered for response according to RECIST criteria, at 6 weeks, and thereafter. At least 2 unidimensional target lesions were identified for response evaluation.

AdVMART1 Virus

AdVMART1 contains the 400-bp MART-1 cDNA, originally cloned by RT-PCR, and driven by the CMV promoter/enhancer in a pAC-CMVpLpA AdV type 5 backbone.⁷ The virus was prepared by recombination with pJM17, which contains the 35-kb AdV genome, deleted in the E1 region, in 293 cells, which provide the E1 genes in *trans*. Clinical grade GMP (good manufacturing practice) vector was prepared at Molecular Medicine, LLC (San Diego, CA). The criteria for lot release of GMP AdVMART1 vector was as shown (supplementary Table 1). The Food and Drug Administration requested full-length virus sequencing, and the entire 36 kb recombinant adenoviral vector, including the transgene MART-1, was sequenced and 99.90% of the genome was found to match the reference sequences in the NCBI nucleotide database.

AdVMART1-transduced DCs

A single unmobilized leukapheresis processing 1 plasma volume, was performed to obtain peripheral blood mononuclear cells (PBMCs), which were isolated by Ficoll-Hypaque (Amersham-Pharmacia, Piscataway, NJ) gradient centrifugation and cryopreserved in RPMI 1640 (Gibco BRL, Gaithersburg, MD) plus 20% heat-inactivated autologous serum and 10% DMSO (Sigma, St Louis, MO). DCs were generated from adherent PBMCs using GM-CSF (800 U/mL, Amgen/ImmuneX, Seattle, WA) and IL-4 (500 U/mL, Schering-Plough, Kenilworth, NJ) as previously described.⁴ On the day of immunization, DCs were harvested, washed, and transduced with AdVMART1 in 2% autologous serum RPMI 1640 for 2 hours. For patients A1 to B6, vaccines were prepared fresh, initiating a new culture for each vaccine. After confirming the sterility results and DC phenotypes according to IND 7122 lot release criteria, DCs were prepared for immunization by washing and resuspending 10^7 DCs in 0.2 mL of saline. The leukapheresis cell product and all the reagents used for vaccine preparation were tested for mycoplasma, endotoxin, bacterial and fungal sterility, and the final product was confirmed to contain cells with DC phenotype by flow cytometry. The final product was tested for endotoxin and mycoplasma contamination after all manipulations had been completed (the DC vaccine was loaded into a syringe and kept at 4°C for 2 hours to allow time for testing), and only when confirmed to be free of contaminants was administered to the research subjects. For patients B7 to B20, a single batch of DC was prepared and cryopreserved. For delivery, each vial was thawed, counted, and checked for viability (trypan blue), then injected (in the cryopreservation medium). Patients received pretreatment with 50 mg of diphenhydramine and 650 mg of acetaminophen, both orally, and an ID test dose consisting of a $1/10^4$ dilution of the final vaccine. After half an hour observation for possible allergic reactions, the full dose was injected ID in the lower abdominal region above the groin.

DC Phenotype Analysis

For release testing of each DC vaccine, fresh samples were stained immediately for cell surface markers CD86 (B7-2), HLA-DR, and CD14 (all from Caltag, Burlingame, CA). Large, granular lymphocytes were gated on by forward and side scatter and mean fluorescence intensity was determined for the entire DC population. The percentage of DCs in the total cell population was determined by the percentage of CD86⁺/HLA-DR⁺ large, granular lymphocytes of the total events (over the threshold size), and had to be >30%. Several patients did not receive all the 3 vaccines. Patients A2, B5, B9 were not fully vaccinated because of early progression, B8 had a spontaneous regression before immunization, the leukapheresis from A3 did not pass sterility tests and could not be used, and the vaccines from B19 and B20 did not pass 70% viability criteria. For additional phenotypic characterization of the DC vaccines, cryopreserved samples were thawed and stained with a panel of antibodies: CCR6, CCR7 (BD-Pharmingen, San Diego, CA), CD40, CD80 and CD83 (Caltag) as well as repeated staining with CD14, CD86, and HLA-DR.

DCs of healthy donors were stained in parallel, and 10,000 large granular lymphocyte events were collected. Phenotyping was not repeated after AdVMART1 transduction, nor after the thawing of cryopreserved vaccines.

DC Culture Cytokine Analysis

On day 7 of each DC vaccine, three 1 mL cell-free medium supernatant samples were removed after pelleting the DCs, and frozen at -80°C until use. To characterize the cytokines present in the DC vaccine cultures, for the first 4 patients (A1 to B1), ELISAs (all from BD Pharmingen) were performed for IL-1 β (3.9 pg/mL sensitivity), IL-6 (2.3 pg/mL), IL-10 (62.5 pg/mL), IL-12 p70 (7.8 pg/mL), IL-15 (1.9 pg/mL), and tumor necrosis factor (TNF)- α (3.9 pg/mL). For other tested patients (B5-B20), Luminex multiplex cytokine analysis was performed for GM-CSF (4 pg/mL), IL-4 (8 pg/mL), IL-6 (11 pg/mL), IL-8 (19 pg/mL), IL-10 (48 pg/mL), IL-12p70 (10 pg/mL), IFN- γ (4 pg/mL), IFN- α (15 pg/mL), TNF- α (4 pg/mL), human interferon-inducible protein (IP-10: 12 pg/mL), monokine induced by IFN- γ (MIG: 6 pg/mL), and monocyte chemotactic protein-1 (MCP-1: 5 pg/mL) (all from Biosource). The Luminex assay was performed in duplicate by the UPCI Immunological Monitoring Facility. Healthy donor DC culture supernatants were assayed in parallel. Two different aliquots of the culture supernatant from B6 were run together as an additional measure of reproducibility.

Immunologic Testing

Immune response testing was conducted on cells from pretreatment, day 14 (wk 2), day 28 (wk 4), day 35 (wk 5), day 56 (wk 8), and day 112 (wk 16). The following immunologic tests were conducted using these samples, which were batched, thawed, and tested at the same time to address concerns over assay variability.

Enzyme-linked Immunosorbent Spot

To determine the frequency of peptide-specific cytokine-producing cells, the enzyme-linked immunosorbent spot (ELISPOT) technique was used as previously described.^{4,39} PBMCs were thawed, treated with DNase I (Sigma), plated to remove adherent cells for 2 hours, then separated by first, positive selection for CD8⁺ cells (Miltenyi Biotec), and then by negative selection to isolate CD4⁺ cells (Miltenyi Biotec). The CD8⁻/CD4⁻ flow-through fraction was used for NK cell analysis by flow cytometry. For CD8⁺ responses, K562/A2.1 (provided by Wolfgang Herr, Mainz, Germany) were used. The K562/A2.1 were pulsed with specific peptides (10 $\mu\text{g/mL}$) for 1 to 2 hours (or no peptide), then washed and used as antigen presenting cells. K562/A2.1 cells plated alone also served as a negative control. For determinant spreading studies, cells were pulsed with class I human melanoma antigen (MAGE)-A3/6₂₇₁₋₂₇₉, gp100₂₈₀₋₂₈₈ or tyrosinase₁₋₉ epitopes. In some cases, responses to native MART-1₂₇₋₃₅ and to the 10-mer anchor-modified peptide ELAGI-GILTV were compared. For CD4⁺ T-cell responses, in HLA-DR4⁺ patients, T2/DR4 cells (provided by Janice Blum, University of Indiana, Indianapolis, IN) were used as K562/A2.1, above, pulsed with class II MART-1₅₁₋₇₃, or gp100₄₄₋₅₉ peptides, or controls [nonspecific (AFP₅₄₉₋₅₅₇) peptide or no peptide] and then were added to precoated ELISPOT plates. For several patients, responses to AdVMART1-transduced autologous DCs (from vaccines) were tested. AdV viral protein responses were tested as above, using an HLA-A2.1-restricted peptide (AdV hexon₇₁₁₋₇₂₁) or to HLA-DR AdV hexon₇₉₆₋₈₂₅, 856-885, 901-930 epitopes, pooled together.

Plates (Millipore, Bedford, MA) were incubated at 37°C for 24 hours and developed (AEC, Vector Labs) and spots were counted twice, manually under a dissecting microscope and using the computer-based Immunospot system (CTL Technology Ltd, version 3.0). The results were similar for manual and computer-based counting. There were no spots detected

for K562/A2.1 or T2/DR4 cells plated alone. Measurement of MART-1₂₇₋₃₅-specific responses over time was generally performed once, because of limited PBMC supply. Criterion for positive response was: an increase of at least 2-fold, for at least 2 consecutive time points tested, where wells with less than 10 total spots were eliminated as nonspecific noise. For determinant spreading assessment, pretreatment cells were compared with a single postvaccine time point (day 56 or day 112) to allow for responses to additional antigens to develop. The day +56 to 112 time point with the highest number of available PBMC was used.

Tetramer Analysis

MART-1₂₇₋₃₅/HLA-A*0201 tetramers (Coulter, Immunomics) were used for the detection of CD8⁺ T cell with the ability to recognize the MHC class I-restricted MART-1₂₇₋₃₅ peptide epitope. PBMCs or CD8⁺ selected cells (Miltenyi Biotec) from each time point were thawed simultaneously and 10⁶ cells were stained with the tetramers and a panel of other antibodies, which included CD8-FITC (Caltag) and antibodies used to gate out non CD8⁺ T lymphocytes [tricolor-conjugated CD4, CD13, and CD19 (Caltag) or iMASC (Coulter/Immunomics)]. Staining was performed at room temperature for 30 minutes in the dark. The lymphocytes were gated on by forward and side scatter, cells positive for CD4/13/19 were gated out, and CD8⁺ cells were gated on. The positive cells were a distinct population of CD8-FITC+/tetramer-PE+ cells. Analysis was performed on all samples in both CellQuest (Beckman Coulter) and FCS Express (version 2, DeNovo Software, www.denovosoftware.com) software. Criterion for positive response was an increase of at least 2-fold over baseline frequency, at 2 consecutive time points at least.

NK Phenotyping

Samples of CD8⁻/CD4⁻ lymphocytes were stained for NK phenotype and analyzed by flow cytometry. NK cells were identified as CD56^{hi}/CD16^{neg} or CD56^{dim}/CD16^{hi} lymphocyte subsets and analyzed separately. They were surface stained for activation markers CD25 and CD69, then fixed (0.25% paraformaldehyde), permeabilized (0.1% saponin), and stained intracellularly with granzyme B-FITC. Changes in patients' cells over time were followed. Healthy donors were tested in parallel.

Regulatory T Cells Phenotyping

Samples of CD4⁺ lymphocytes were stained for regulatory T cells (Treg) phenotype and analyzed by flow cytometry. Treg cells were identified as CD3⁺/CD4⁺/CD25^{hi} lymphocytes and analyzed separately. They were surface stained for markers CCR7 and CTLA-4, then fixed, and permeabilized and stained intracellularly with Fox-P3-FITC. Changes in patients' cells over time were followed. Healthy donors were also tested in parallel.

Biostatistical Analysis

The association between clinical response and the measures of immune response was assessed with a 2-sided Fisher exact test, and *P* values < 0.05 were considered significant.

RESULTS

Patient Characteristics

Twenty-three stage IV metastatic melanoma patients were accrued into this trial (Table 1). The primary trial end point was to evaluate the safety of administering AdVMART1-transduced DCs in patients with melanoma. The secondary end points were to evaluate the immunologic and clinical responses of patients. Twelve patients had received prior immunotherapy, including IFN- α 2b (6), IL-2 (2), biochemotherapy (4), and/or other

experimental vaccines (4). Only 5 patients had received prior chemotherapy. All patients had positive reaction to tetanus and/or candida in a baseline delayed type hypersensitivity skin test. Of the 23 patients enrolled, 5 did not receive any vaccines (A3, contaminated leukapheresis; B8, spontaneous response; B9, early central nervous system progression; B19 and B20, vaccines were below the 70% viability criterion). Of the 18 vaccinated patients, 1 (B15) received 1 vaccine, and 3 (A2, B5, and B12) received 2 vaccines, all because of early progression.

Toxicity

Directly before each immunization, patients received an ID injection of $>10^2$ and $<10^4$ AdV-MART1/DC to test for hypersensitivity. None of the patients exhibited a reaction to this test dose. Vaccines consisted of 0.5 mL of AdVMART1/DC suspension in either saline (fresh vaccines, A1 to B6) or cryopreservation media (B7 to B20), which was injected ID in the flank region below the axillae or above the groin. Vaccines were very well tolerated. There was no evidence of local skin reactions at the injection site and no systemic grade II-IV toxicities, other than worsening vitiligo in 1 subject with preexisting vitiligo (B4).

Clinical Outcome

One patient entered the clinical trial with an unconfirmed diagnosis of multiple mediastinal metastasis by computed tomographic pathologic criteria and marked fluorodeoxyglucose uptake by positron emission tomography scan while receiving adjuvant IFN- α 2b therapy. However, these lesions were not biopsied at baseline. The nodal lesions were completely resolved and this patient has not relapsed [56+ mo overall survival (OS)]. Given the lack of baseline pathologic confirmation of metastatic melanoma, this patient is labeled as not evaluable for response. Four patients had SD (B2, 36 mo OS; B10, 42 mo OS; B13, 33 mo OS; and B14, 27 mo OS). Another patient was rendered no evidence of disease (NED) after additional surgery postvaccination (B4, 58+ mo OS). Finally, 1 enrolled patient experienced a spontaneous response in scalp metastasis, which preceded vaccination, (B8), and was not vaccinated (Table 2).

DC Vaccine Phenotype

Release criteria for the vaccines included testing for cell surface levels of the MHC class II molecule HLA-DR, the monocyte marker CD14 and the costimulatory molecule B7.2 (CD86) (see Materials and Methods). These cells were additionally stained for the costimulatory marker CD80, the CD40 receptor (critically involved in the crosstalk between DCs and T cells), the chemokine receptors CCR6 and CCR7 (lymph node trafficking, also a marker of DC maturation), and the DC maturation marker CD83 (Fig. 1). Examples from 3 patients, with a range of DC phenotypes, are shown. Analysis of the surface phenotype of DC preparations indicated some inpatient and interpatient variability. DC preparations were initiated from PBMCs previously obtained and cryopreserved from a single unmobilized leukapheresis, and were supplemented with heat-inactivated autologous serum obtained at baseline. For patients A1 to B6, an independent DC culture was initiated for each vaccine. CD14 was universally expressed in the adherent monocyte precursors from which DCs were differentiated using our protocol,⁴⁰ and persisted at varying levels in the loosely adherent population of cells harvested as DCs (Fig. 1). The DCs generated using this protocol had low levels of CD83, suggesting an immature phenotype. They also had low levels of the CCR6 and CCR7 trafficking receptors, and variable levels of the CD40 receptor. Taken together, there was batch-to-batch and patient-to-patient variability in DC surface phenotype between different sequential DC preparations from the same subject. These data highlight the potentially variable phenotype of predominantly immature DC preparations, despite uniform reagents and procedures (also seen in our previous trials^{4,5,24}). To reduce these effects, and to minimize the use of clinical grade AdVMART1, we amended

the clinical protocol to allow for large, single batch productions of DCs, and delivery of cryopreserved and thawed vaccines. This batched protocol was followed for patients B7 to B20.

DC Vaccine Viability

The vaccines which were frozen as a single batch of transduced DC, then thawed, retested for viability, and delivered (in the cryopreservation medium) had a lower average viability than those which were prepared as single AdVMART1/DC vaccines from a cryopreserved leukapheresis. As mentioned, the last 2 vaccines prepared (patients B19 and B20) did not meet the 70% level and were not administered. To evaluate this issue, several experiments were performed comparing the 2 methods of vaccine preparation. We tested HLA-DR and CD86 surface expression and in addition, tested for early apoptotic cells with a combination of annexin-V and propidium iodide (PI) staining and flow cytometric analysis, in addition to the trypan blue exclusion assay.

DCs prepared from fresh PBMCs or cryopreserved PBMCs are similarly viable before transduction (>90%), and the HLA-DR and CD86 surface expressions are similar for DCs prepared by both procedures (not shown). When tested at the average time of delivery (for fresh AdVMART1/DC from frozen PBMCs, 3 hours after transduction; for thawed AdVMART1/DC, 20 minutes after thaw), the fresh AdVMART1/DC were 25% apoptotic (PI+/annexin-V+), whereas the thawed AdVMART1/DC were up to 50% apoptotic (not shown). Although most of the vaccines prepared for patients B7 to B20 passed the 70% viable by trypan blue exclusion criterion and were able to be delivered, the original procedure followed (A1 to B6) resulted in superior viability and was more reliable.

DC Cytokine Production

To determine the cytokine profile of the DC cultures, cell-free supernatants from the DC flasks were collected at the end of the 7-day culture period and quantitated for various cytokines by ELISA or Luminex assay. We initially performed ELISA assays on supernatants from individual vaccines. Figure 2A shows examples of cytokines detected in 3 different vaccines from A1, 2 from A2 and B1, and 1 from A3. The data was similar across individual vaccines made from a given patient, although not identical. Later, to improve the sensitivity and expand the number of cytokines from the limited samples, we used the multiplex Luminex assay. Data from this analysis is shown in Figure 2B. Our analysis revealed that culture supernatants from DC preparations generated in this protocol had low levels of IL-12p70 (indicating immature status), IL-1 β , IL-15, IFN- γ and IFN- α (0 to 46 pg/mL). Some DC cultures contained TNF- α (0 to 214 pg/mL, B6 had the highest level), most had variable levels of inflammatory cytokine IL-6 (36 to 573 pg/mL), and regulatory cytokine IL-10 (0 to 500 pg/mL), cytokines that have been previously correlated with an immature phenotype of DCs (IL-6 and IL-10⁴¹). Several chemokines were tested by Luminex, including IL-8, IP-10 (CXCL10, ligand for CXCR3), MCP-1 (CCL2, ligand for CCR2), and MIG (CXCL9, ligand for CXCR3) (Fig. 2B). Each of the 12 tested cultures was positive for most of the chemokines assessed. IL-8 (28 to 3629 pg/mL) and MCP-1 (235 to 25,333 pg/mL) were the highest and showed the most diverse levels. Again, B6 DC cultures had the highest levels for MCP-1 (>2.7 fold higher than the next highest culture), as well as IP-10. CXCR3 on CD8⁺ T cells was recently found to be associated with melanoma patient survival⁴² and MIG expression was highest in B5 (116 pg/mL) and B6 (191 pg/mL). It should be noted, however, that these cytokines/chemokines represent what was present in the DC cultures before AdVMART1 transduction and cryopreservation. We have previously found that AdV transduction reduces IL-10 production by DCs by 41% to 59% after 24 to 48 hours²⁷; Vujanovic, '08, (*submitted*).

Immunologic Responses

PBMCs obtained from baseline, and before the second (d+14), and third vaccine (d+28) and at 3 time points after the immunizations (d+35, d+56, d+112) were thawed in batches for each patient and analyzed for the presence of MART-1–reactive CD8⁺ and CD4⁺ T cells. Readouts included enumeration of CD8⁺ T cells with a T-cell receptor recognizing HLA-A*0201 tetramers folded with MART-1₂₇₋₃₅ peptide and the ability to produce the immune stimulatory cytokine IFN- γ upon overnight ex vivo stimulation with MART-1 peptides by ELISPOT, which was our primary assessment of immune response to the vaccine.

ELISPOT

The ELISPOT assay allows enumeration of antigen-specific cells by cytokine production on antigen reexposure. Therefore, it is considered a functional assay reflecting the frequency of CD8⁺ or CD4⁺ T cells activated by the vaccines. A positive response was defined as an increase of at least 2-fold over baseline at 2 or more consecutive time points, such that at least 10 spots average were counted in the wells. By these criteria, 6 of 13 evaluable patients had an increase in IFN- γ –producing CD8⁺ T cells after DC administration (A1, B1, B2, B14, B16, and B18) (Table 3, examples of different patterns observed in Fig. 3). These data indicate that the AdVMART1/DC vaccine successfully activated MART-1–specific CD8⁺ T-cell responses. These responses took several forms, examples of which are shown in Figure 3. They included undetectable MART-1 responses at baseline, becoming positive after 1 to 3 vaccines, preexisting MART-1 immunity boosted after vaccination, and minor, transient responses. Two of the 13 did not receive all the 3 vaccines, had only baseline and day+14 time points, and could not be positive by these criteria (A2 and B5). These are similar to the responses detected by others and ourselves in other published trials.

Our previous 2 DC-based trials in melanoma, testing MART-1₂₇₋₃₅ peptide-pulsed DC,^{4,24} used less stringent statistical criteria for positive ELISPOT immune response (an increase of at least 1.5-fold, at any single time point). Although these 2 separate clinical trials cannot be compared, by the previous criteria, 12 of the 13 evaluable patients would be positive for IFN- γ ELISPOT responses here.

An important goal of testing AdVMART1/DC was to determine if expansion of MART-1–specific CD4⁺ helper responses took place (to potentially improve the function of CD8⁺ effector T cells). Although many patients may have had such responses after vaccination, our analysis was based on peptide reactivity, hence we were limited to the published DR4-restricted peptide, MART-1₅₁₋₇₃. Four patients who were typed as HLADR4⁺ (by either antibody testing or DNA PCR subtyping) were tested by ELISPOT to detect changes in MART-1₅₁₋₇₃–specific CD4⁺ T cell IFN- γ secretion after vaccination. Two of the 4 evaluable patients had increased MART-1–specific CD4 T-cell responses after vaccination (A1 and B11). The other 2 DR4⁺ patients had either nonsequential increases (B1) or increases falling short of 2-fold (B6). Therefore, in at least a subset of evaluable patients, we were able to expand these MART-1–specific CD4⁺ T cells. There were no correlations between clinical response and the CD8⁺ or CD4⁺ T-cell ELISPOT response results.

MHC Tetramer

MART-1₂₇₋₃₅ MHC tetramer frequency analysis revealed that AdVMART1/DC administration led to a clear expansion of MART-1₂₇₋₃₅–specific T cells in peripheral blood in 4 of the 14 evaluable patients (A1, B4, B6, and B13) by the criteria of increase of at least 2-fold over baseline at 2 or more consecutive time points. Again, A2 and B5 did not receive all 3 vaccines, had only baseline and day+14 time points, and could not be positive by these criteria. Five patients had increased MHC tetramer responses, which were less than 2-fold in magnitude and/or did not occur at 2 consecutive time points at least (B1, B2, B4, B14, and

B18). Five other evaluable patients (A2, B5, B7, B10, and B13) had no increases. Examples of the range of response patterns observed (A1, B4, B6 and B13) is shown in Figure 4, and the data obtained from all patients are summarized in Table 4.

As above, for IFN- γ ELISPOT data, the MHC tetramer responses did not correlate with clinical response, however, B6 did have the greatest number of time points, which were statistically significantly positive; all 5 postvaccine time points.

AdV Hexon Responses

We also wished to determine whether CD8 and/or CD4 T-cell responses specific for adenoviral hexon peptides could be detected. We had detected virus-specific responses in early preclinical in vitro studies⁷ and have detected AdV hexon peptide-specific responses in healthy donors (to the A2-restricted peptide and the DR peptides pooled) after multiple rounds of in vitro stimulation with AdV-transduced DC (data not shown). In 5 patients (B10, B11, B13, B14, and B18), we tested for these responses by IFN- γ or IL-2 ELISPOT, and none of the patients had net >10 spots per 10⁵ cells plated (data not shown).

Determinant Spreading

We have previously reported 2 metastatic melanoma patients with CRs after MART-1₂₇₋₃₅/DC immunization in whom immunologic monitoring of MART-1₂₇₋₃₅-specific T cells by tetramers and ELISPOT revealed that the magnitude of the T-cell response did not correlate with clinical regression.^{4,24} However, analysis of acquired reactivity to MHC class I and II-restricted melanoma peptides other than MART-1₂₇₋₃₅ (including MART-1₅₁₋₇₃ Tyros₁₋₉) clearly differentiated these patients from other patients who were successfully vaccinated against MART-1 but did not have tumor regressions, suggesting that the acquisition of increased immune response to tumor antigens not delivered in the vaccine, may be linked to clinical response. Therefore, a similar analysis was performed in this trial, and CD8 and CD4 T-cell responses to peptides derived from gp100, tyrosinase, and MAGE-A3 were tested by IFN- γ ELISPOT in 9 patients with sufficient available lymphocytes (Tables 3, 4 and Fig. 5).

Patients B3, B5, B7, and B10 [progressive disease (PD), PD, PD, and SD (6 mo, OS = 42 mo)] had too few spots detected to other peptides for analysis. B13 and B14 (SD and SD) had insufficient lymphocytes available. This allowed us to test responses to other antigens in A1, B1, B2, B4, and B6. Patients A1 and B1 (PD and PD) had MART-1 reactivity, as well as detectable reactivity to other melanoma antigens, but the frequency of these cells declined during the enrollment period. Patient B4 (PD, currently NED at 58+ mo OS after additional surgery) developed a low but detectable frequency of CD8⁺ T cells specific for gp100 (an increase of 25/10⁶ CD8⁺ cells). Patient B2 (SD, 36 mo OS) developed a 9-fold higher frequency (225/10⁶ increase) of CD8⁺ cells specific for tyrosinase. B6 (unevaluable, OS = 56+ mo.) had increasing frequencies of cells specific to gp100 (CD8⁺ and CD4⁺) and also developed a response to MAGE-A3. Taken together with our prior experience, these data continue to support a link between an increase in the breadth of antigens in the immune response and outcome after MART-1/DC immunization.

NK Cell Analysis

Several publications in recent years have indicated that DC and NK cells can have direct, functional impact on each other.^{34–37,43–45} Our own results in a murine model of AdVMART1/DC-based immunization support a functional role for NK cells in antitumor responses to this vaccination strategy.³⁸ We therefore tested patient lymphocytes for NK phenotype to determine whether the AdVMART1/DC vaccines had an impact on NK cells in vivo. We followed both CD56^{hi}/CD16^{neg} (regulatory or cytokine-producing) and

CD56^{dim}/CD16^{hi} (cytotoxic) NK subsets for frequency changes, activation marker (CD25, CD69), and granzyme B content changes in 10 patients (B1, B2, B3, B6, B10, B12, B13, B14, B16, and B18). Two examples of this flow cytometric analysis are shown in Figure 6. In 7 of 10 evaluable patients (B1, B3, B6, B10, B13, B14, and B16), we found increased intracellular granzyme B content in one or both the NK cell subsets [at least 1.5-fold over background, in 5/10 the increase was in both subsets (B3, B6, B10, B14, and B16)]. We also tested for up-regulation of activation markers CD25 and CD69. Because the blood samples were drawn at 2 weeks postvaccination, there was a concern that these transient markers may not have maintained this increase over that time period. We observed 5/10 had at least 1.5-fold increase CD25 expression (B2, B3, B6, B16, and B18) and 2/10 for increase CD69 expression (B2, B3). These exploratory studies indicate that, in a subset of immunized patients, the AdVMART1/DC vaccines may have also led to activation of circulating NK cells.

Regulatory T Cells

Recent literature has identified Treg as potent negative regulators of antitumor immunity. There are several different subsets of these cells, but the “natural” Tregs (CD4⁺, CD25^{hi}, Fox-P3⁺) are the most studied to date and these cells have been found to be at increased frequencies in some cancer patients.^{46,47} Therefore, we studied 6 patients for the frequency of circulating CD3⁺/CD4⁺/CD25^{hi} lymphocytes expressing intracellular Fox-P3 (of the total CD3⁺/CD4⁺ T cells) and also analyzed surface markers CCR7 and CTLA-4. We wished to determine whether these patients had increased baseline frequencies of Treg relative to healthy donors (which might have explained reduced immune and clinical responses *in vivo*), and to detect any modulation of Treg, which might occur with vaccination. In the limited sample of patients (6 with baseline and 5 of those with between 1 and 4 postvaccine time points available for analysis) and lymphocytes, we found no evidence for increased Tregs at baseline (compared with healthy donors) and no consistent modulation of their numbers or surface markers with vaccination (data not shown).

DISCUSSION

DCs were originally described as difficult to transfect efficiently with plasmids by CaPO₄, electroporation, and lipofection. As we¹⁰ and others⁴⁸ demonstrated in human and murine DC,^{49–52} AdV transduction is an efficient method of gene transfer to DC. We have found that immature monocyte-derived DCs transduced with AdV become more mature by phenotypic criterion (decreased CD14, increased CD83, CD86, HLA-DR), decreased secretion of endogenous IL-10, and increased IL-12 p70, as well as by efficient stimulation of antigen-specific, IFN- γ -producing T cells.^{27,53–56}

Most AdV use the coxsackie-adenovirus receptor for binding, and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins for uptake. Despite low levels of coxsackie-adenovirus receptor, DCs do express sufficient amounts of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and high AdV concentrations yield >95% transduction efficiency without cytopathic effect. AdV-transduced DCs continue to process and present antigenic peptides for at least 10 days, whereas peptide-pulsed DCs present at 50% maximal level by day 2.¹¹ In our initial report,¹⁰ we found that the AdV-transduced DCs remained highly positive for many important cell surface molecules and continuously produced the transgene for at least 7 days *in vitro*. We subsequently showed that AdV-transduced DCs also stimulated T cells that recognized AdV epitopes (which are recall antigens for most subjects),^{7,29} perhaps because of the leakiness of E1-deleted AdV or the processing of viral proteins transferred during transduction.^{57,58}

Prior comparison studies in murine and human *in vitro* preclinical studies had suggested that DC gene modification had a superior ability to stimulate antigen-specific immune responses

compared with DC peptide pulsing.^{7,32} More recent studies have substantiated this.^{59–61} Plasmid DNA transfection can be efficient with newer methods⁶² but in a head-to-head comparison, AdV vector was found to be superior.⁶³ In continuing investigations, we have also recently found broad and potent activation of multiple CD8 T-cell peptide specificities by AdV-engineered DCs⁹ and broad T_H1 function of CD4 T cells activated by AdV/DCs compared with protein-loaded DCs.²⁹ In a previous trial in which GM-CSF/IL-4 DCs were transduced with both AdVMART1 and AdVgp100, 1 of 17 evaluable patients had a complete clinical response (ASH 2002, Haluska et al⁶⁴) and 3/12 patients with vitiligo were reported⁶⁵ More recently, a trial testing melanoma tumor RNA transfected, matured DC reported successful immunization of half of the subjects and superiority of ID immunization compared with intranodal delivery.⁶⁶

The AdV-transduced DC manufacturing process was critical. Although initial preclinical studies showed that the AdVMART1/DCs were able to be cryopreserved then thawed, and could retain DC surface phenotype and sufficient viability (at least in healthy donor cells), the 2 procedures tested here were not equivalent in the trial setting, with cells from advanced stage melanoma patients, delivered in DMSO-containing medium, and when tested by PI/annexin-V staining. In future trials, our data supports use of fresh transduced DCs, prepared from either fresh or cryopreserved PBMCs.

Here, we report that AdVMART1-transduced DCs were able to activate and expand CD8⁺ and CD4⁺ T cells specific to MART-1. Patient B6 had the following immunological response to vaccination: her circulating MART-1₂₇₋₃₅-specific CD8⁺ T cells, as measured by MHC tetramer, were positive for all postvaccine time points (most positive time points but not the highest frequency). Her functional CD8 response (IFN- γ ELI-SPOT) was not considered positive (not 2-fold over baseline at consecutive time points), nor was her CD4 response to MART-1 positive (not achieving 2-fold increase). However, the breadth of her functional melanoma antigen-specific immunity increased >2 fold to gp100 (CD8 and CD4 T cells), and she underwent determinant spreading to MAGE-A3 reactivity (as defined by no detectable immunity prevaccine, and becoming positive after vaccination). She was clinically unevaluable because the positron emission tomography/computed tomography scan-identified metastases were not biopsied before vaccination.

There were 4 patients with SD and OS from 27 to 42 months (B2, B10, B13, and B14), and a patient with a baseline frequency of MART-1₂₇₋₃₅ of 2.9% (B4) who also is of particular interest. B2 had a positive CD8 IFN- γ ELISPOT result and showed CD8 T cell determinant spreading to a peptide from tyrosinase. B10 and B13 did not show responses by tetramer or ELISPOT and did not have sufficient viable lymphocytes available for further testing. B14 had a positive response by CD8 IFN- γ ELISPOT, but we could not test other antigens with the available cells. B4 had received several previous treatments, and had developed a robust CD8 T-cell response to MART-1 before enrollment. These cells were also functional by ELISPOT. Although the vaccines were able to boost these responses, the increases were not statistically significant. During the enrollment period, B4 developed a detectable CD8 response to gp100. B4 did progress with lymph node metastases (which were removed surgically) and has remained NED since then.

Considerable controversy exists in the literature regarding the optimal protocol and cytokine combination for the generation of immune-stimulatory clinical-grade DC, and the best phenotype of these cells.^{67–70} It has been suggested that DC cultures that have not undergone a final dedicated maturation step^{67,68,70} may induce antigen-specific tolerance rather than activation. Defining optimal DC vaccines is complicated by the continued evolution of this field, differences between human and murine DCs and the effort required to

test these vaccines clinically. Similarly, the most meaningful immunologic response, which is indicative of clinical outcome is also controversial.

This is the third report from our group in which we have observed a link between clinical response and determinant spreading. In our previous reports,^{4,24} we observed little baseline reactivity to antigens other than MART-1, and only detected significant increases in responses to nonvaccine antigens in the patients with a CR. In this trial, we detected new postvaccine responses to nonvaccine antigens in patients with long OS. Since those reports, the sensitivity of the ELISPOT assay has been improved (lower background to cell lines presenting peptides, higher quality antibodies and plates, use of purified T-cell subsets). These technical modifications may be responsible for an improved ability to detect lower frequency antigen-specific cells. This may indicate that the breadth of antigens responded to by lymphocytes (in a functional assay) is critical, and that de novo acquisition of new reactivity to melanoma antigens expressed by the tumor is impossible to determine unequivocally.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

AdVMART1/DC vaccine phenotypes. After DC harvest, cells were stained to analyze cell surface phenotype. DCs were gated on (as large, granular lymphocytes) by forward and side scatter, and the MFI of each marker was determined. Examples of DC vaccines from three different patients, B2, B6 and B13, are shown. DC indicates dendritic cell; MFI, mean fluorescence intensity.

FIGURE 2.

Vaccine DC cytokine production. Cell-free culture supernatants were saved from each DC vaccine culture and tested for the presence of cytokines and chemokines. Results are shown as pg/mL of cytokine. A, Multiple vaccine preparations from several patients were tested by ELISA for IL-15, IL-1 β , IL-12p70, TNF- α , IL-10, and IL-6. B, Batched DC vaccine preparation supernatants were tested by Luminex multiplex analysis for IL-12p70, TNF- α , IL-10, IL-6, IFN- α , IFN- γ , and chemokines MIG, IP-10, IL-8, and MCP-1. DC indicates dendritic cell; IFN, interferon; IP, human interferon-inducible protein; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MIG, monokine induced by IFN- γ .

FIGURE 3.

Examples of IFN- γ ELISPOT responses from CD8 $^{+}$ and CD4 $^{+}$ T cells in 4 patients. Thawed PBMCs from each time point were purified for CD8 $^{+}$ and CD4 $^{+}$ T cells, and these cells were plated overnight with peptide-pulsed antigen presenting cells and controls overnight, in IFN- γ coated plates. Net MART-1₂₇₋₃₅ or MART-1₅₁₋₇₃-specific responses per million cells plated (after subtraction of “no peptide” controls) are shown. A, CD8 and CD4 T-cell responses from the first patient vaccinated, A1, are shown. These responses met the statistical criteria for positive responses. B, CD8 and CD4 T-cell responses from patient, B6, are shown. These responses did not meet the statistical criteria for positive responses. C, CD8 T-cell responses from the 2 HLA-DR4-negative patients vaccinated, B3 and B4, are shown. These responses did not meet the statistical criteria for positive responses. ELISPOT indicates enzyme-linked immunosorbent spot; HLA, human leukocyte antigen; IFN, interferon; MART-1, melanoma antigen MART-1/Melan-A; PBMC, peripheral blood mononuclear cells.

FIGURE 4.

MHC tetramer analysis of CD8⁺ T-cell frequencies. Examples of 4 different patient MHC tetramer responses are shown. Data are presented as the frequency of tetramer-positive cells among the CD8⁺ T cells (after gating on live lymphocytes). The frequencies are shown over each available time point tested, from baseline (day 0) to day +56 or day +112. The statistically significant responses shown are for A1 and B6. B4 had a high preexisting level of tetramer-positive cells. B13 had no increase. MHC indicates major histo-compatibility complex.

FIGURE 5.

ELISPOT analysis of determinant spreading. IFN- γ ELISPOT frequencies of CD8⁺ and CD4⁺ T cells specific for MART-1, gp100, tyrosinase, and MAGE-A3–derived peptides for 5 of the 9 tested patients are shown. Solid black and gray bars show the vaccine encoded MART-1 CD8⁺ and CD4⁺ responses, respectively. Striped bars show commonly expressed antigens not present in the vaccines. Arrows show any detected increases from baseline to postvaccine time points in immunity to nonvaccine encoded antigens. B2 increased the CD8⁺ T-cell response to tyrosinase, B4 had a slight increase in CD8⁺ immune response to gp100, and B6 increased CD8⁺ immunity to gp100, activated newly detectable immunity to MAGE-A3 as well as showed increased CD4⁺ T-cell response to gp100. A1 and B1 had detectable responses to MART-1 and other antigens, but these frequencies decreased during the enrollment period. Other tested patients not graphed are described in Tables 3 and 4. ELISPOT indicates enzyme-linked immunosorbent spot; IFN, interferon; MAGE, melanoma antigen; MART-1, MART-1/Melan-A.

FIGURE 6.

Phenotypic analysis of circulating NK cells. The CD8⁺/CD4⁺ lymphocytes thawed in batches from several patients were stained for the CD56^{hi}/CD16^{neg} (regulatory or cytokine-producing) and CD56^{dim}/CD16^{hi} (cytotoxic) NK subset markers, and also for frequency changes, activation marker (CD25, CD69), and intracellular granzyme B content changes. The MFI for each of the NK subsets is shown, over time, from 2 patients. A, NK phenotype results for patient B3. B, NK phenotype results for patient B6. MFI indicates mean fluorescence intensity; NK, natural killer.

TABLE 1

Patient Characteristics

Patient [*]	DC [†]	HLA-A2.1 [‡]	HLA-DR4 [§]	Age	Sex [¶]	Previous Treatments [#]	Metastasis ^{**}
A1	10 ⁶	+	0403	40	M	Surgery, IFN	Skin
A2	10 ⁶	+	—	81	M	Surgery, Dartmouth regimen	Lung
A3	10 ⁶	+	—	50	F	Surgery, IL-2-histamine, MART1/DC, aCTLA-4	Skin, mediastinum-lung
B1	10 ⁶	+	0401	85	M	Surgery	Nodes, lung, liver, spleen
B2	10 ⁶	+	—	51	M	Surgery, MART1/DC, aCTLA-4	Lung
B3	10 ⁷	+	—	68	M	Surgery, XRT, IFN	Nodes, lung
B4	10 ⁷	+	04011	50	M	Surgery, XRT, IFN, Biochemo, aCTLA4	Nodes, lung, brain
B5	10 ⁷	+	—	41	M	Surgery, XRT, Biochemo	Subcutaneous muscle
B6	10 ⁷	+	DR4+	43	F	Surgery, IFN	Nodes ^{‡‡}
B7	10 ⁷	+	DR4+	50	M	Surgery, XRT, IFN, CancerVax, Temoz	Subcutaneous, nodes, lung, bone
B8	10 ⁷	+	—	65	M	Surgery	Skin
B9	10 ⁷	+	—	55	M	Surgery, IFN	Lung, pleura
B10	10 ⁷	+	—	60	M	Surgery, Biochemo, Temoz	Nodes, lung
B11	10 ⁷	3	DR4+	60	M	Surgery, Biochemo	Lung
B12	10 ⁷	+	—	78	M	Surgery	Lung, liver
B13	10 ⁷	+	—	58	F	Surgery, isolated limb perfusion	Skin
B14	10 ⁷	+	?	55	F	Surgery, MART1 pep/DC, IL-2-histamine, aCTLA4	Lung
B15	10 ⁷	+	—	65	M	Surgery, IFN, DTIC	Lung
B16	10 ⁷	+	—	80	M	Surgery	Lung, nodes
B17	10 ⁷	+	—	65	M	Surgery	Lung, spleen
B18	10 ⁷	+	—	74	M	Surgery, IFN	Nodes
B19	10 ⁷	+	DR4+	82	M	Surgery	Nodes, bone, liver
B20	10 ⁷	+	—	40	F	Surgery, XRT, temozolomide	Brain, liver, spleen, gallbladder, nodes, heart, and small bowel

* Patient designation.

[†] Number of DC per injection.[‡] Test result for HLA-A2.1.

^{\$}Test result for HLA-DR4.

//Patient age.

¶Patient sex.

#Previous treatments received before enrollment.

**Sites of metastatic disease at enrollment.

††B6 nodes were not biopsied, see Results section.

aCTLA4 indicates anticytototoxic T-lymphocyte-associated antigen 4; DC, dendritic cell; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; MART1, melanoma antigen MART-1/Melan-A; XRT, x-ray therapy.

Patient Outcomes

TABLE 2

Patient [*]	Toxicity [†]	Response (+56–112) [‡]	Response (After Study) [§]	PFS (mo)	OS (mo) [¶]	Site of Relapse/Progress [#]	Postvaccine Treatment ^{***}	Current Status ^{††}	Last, Follow-up ^{‡‡}
A1	Grade 1 chills/fever to leukemia.	PD	PD	0	14	Skin	IL-2 immunocytokine, aCTLA-4, Gleevec, Temoz	D	5/03
A2	—	PD	—	0	2	Brain	XRT	D	06/02
B1	—	PD	—	0	3	Nodes	—	D	08/02
B2	—	Stable disease	PD	2	36	Lung	aCTLA-4	D	07/05
B3	—	PD	—	0	2	Skin, nodes, brain, lung, liver, omentum	XRT, Temozo	D	10/02
B4	Worsening vitiligo	PD	—	0	58+	Nodes	Surgery	NED	06/07
B5	—	PD	—	0	1	Brain	XRT	D	10/02
B6	—	Unevaluable	Unevaluable	56+	56+	—	—	Unevaluable	06/07
B7	—	PD	PD	4	36	Liver	Revimid, Temodar	D	11/05
B10	—	Stable disease	NA	6	42	Nodes	Revimid, CP675,206	AWD	03/03
B11	—	PD	PD	3	17	CNS	XRT	D	07/04
B12	—	PD	PD	0	1	CNS	XRT	D	05/03
B13	—	Stable disease	PD	12	33	Skin	CP-675,206	D	01/06
B14	—	Stable disease	PD	9	27	Brain	XRT, Temodar and Thal, Abraxane	D	08/05
B15	Grade 1 skin rash	PD	PD	0	1	Brain	XRT, Temodar Abraxane and Thal,	D	11/03
B16	—	PD	PD	0	10	Brain	XRT, Temodar	D	08/04
B17	—	PD	PD	0	7	Lung, spleen	—	D	05/04
B18	—	PD	PD	0	5	Adrenal	—	D	04/04

^{*} Patient designation.

[†] Toxicity observed during trial period.

[‡] Clinical response at end of trial enrollment (days 56–112), PD = progressive disease; NA = not applicable (no longer enrolled), unevaluable; see Results.

[§] Clinical response at time of last follow-up (June 2007).

^{||} Progression-free survival duration in months, at final follow-up (June 2007 or earlier).

[¶] Overall survival duration in months, at final follow-up (June 2007 or earlier).

[#] Sites of melanoma progression.

^{***} Additional treatments received after vaccine enrollment period.

^{††}Current clinical status at last follow-up.
^{##}Date of final follow-up.

aCTLA4 indicates anticytototoxic T-lymphocyte-associated antigen 4; AWD, alive with disease; CNS, central nervous system; D, deceased; IL, interleukin; NED, no evidence of disease; PD, progressive disease; PFS, progression-free survival; OS, overall survival.

TABLE 3
Summary of Frequencies of T-cell Responses Prevaccines and Postvaccines to MART-1 and Other Melanoma Antigens

Patient *	MART-1 ₂₇₋₃₅ [†]	gp100 ₂₈₀₋₂₈₈ [‡]	Tyrosinase ₁₋₉ [§]	MAGE-A3 ₂₇₁₋₂₇₉ [¶]	MART-1 ₅₁₋₇₃ [§]	gp100 ₄₄₋₆₀	Result ¶
A1	350 to 120 [#]	0 to 0	60 to 0	0 to 0	135 to 39	90 to 69	No spreading
B1	0 to 67	0 to 0	747 to 165	0 to 0	16 to 0	8 to 0	No spreading
B2	0 to 0	270 to 0	0 to 225	—	—	—	Spreading to tyrosine
B3	—	—	—	—	—	—	NE (under 10 spots)
B4	50 to 225	0 to 25	200 to 0	50 to 0	65 to 50	0 to 5	Weak spreading to gp100
B5	—	—	—	—	—	—	NE (under 10 spots)
B6	135 to 135	15 to 30	0 to 0	0 to 60	170 to 225	60 to 135	Spreading to MAGE3, increases to gp100, both CD8 and CD4.
B7	—	—	—	—	—	—	NE (under 10 spots)
B10	—	—	—	—	—	—	NE (under 10 spots)
B13	10 to 52 (d+14)	—	—	—	—	—	Insufficient cells
B14	5 to 145	—	—	—	—	—	Insufficient cells

* Patient identifier.

[†]CD8⁺ T-cell response to vaccine antigen peptide.

[‡]CD8⁺ T-cell response to commonly expressed melanoma antigen peptide.

[§]CD4⁺ T-cell response to vaccine antigen peptide.

^{||}CD4⁺ T-cell response to commonly expressed melanoma antigen peptide from gp100.

[¶]Result of the assay (NE, not evaluable).

[#]T-cell frequencies per million CD8⁺ or CD4⁺ T cells are shown. Numbers are net spots (peptide-specific-control spots) at baseline and postvaccine (either day +56 or day +112 postvaccines unless otherwise noted). Samples in which there was an increase from baseline to postvaccine are in bold.

MAGE indicates melanoma antigen; MART-1, MART-1/Melan-A; NE, not evaluable.

Summary of Immunological Responses

TABLE 4

Patient [*]	No. Vaccines [†]	CD8 MART-1 ELISPOT [‡]	CD8 MART-1 Tetramer [§]	CD4 MART-1 ELISPOT	Determinant Spreading	NK Cell Phenotype [#]	Clinical Response (+56–112) ^{**}	PFS (mo) ^{††}	OS (mo) ^{‡‡}	Comment
A1	3	+ at 4 time points	+ at 4 time points	+ at 4 time points	None	ND	PD	0	14	—
A2	2	—	—	DR4 negative	NE	ND	PD	0	2	Early progression, only 1 postvaccine time point
B1	3	+ at 3 time points	—	—	None	Increased granzyme B	PD	0	3	One time point tetramer increase, CD4 ELISPOT not sequential
B2	3	+ at 2 time points	—	DR4 negative	+ for CD8 to tyrosinase	CD56 ^{hi} increased CD25 and CD69	Stable disease	2	36	Only slight tetramer increase
B3	3	—	+ at 2 time points	DR4 negative	NE	Increased granzyme B, CD56 ^{hi} increased CD25 and CD69	PD	0	2	Less than 2x CD8 ELISPOT increase
B4	3	—	—	NE	Small increase for gp100	ND	PD	0	58+	High baseline MART tetramer, increases <2x; baseline CD8 ELISPOT +, increases under 2x NED postsurgery
B5	2	—	—	DR4 negative	NE	ND	PD	0	1	Early progression, only 1 postvaccine time point
B6	3	—	+ at 5 time points	—	+ for CD8 to gp100 and Mage-A3, + for CD4 to gp100	Increased granzyme B and CD25	Unevaluable	7+	56+	Nonsequential CD8 and CD4 ELISPOT increases
B7	3	ND	—	ND	ND	ND	PD	4	36	—
B10	3	—	—	DR4 negative	ND	Increased granzyme B	Stable disease	6	42	—
B11	3	A2 negative	A2 negative	+ at 4 time points	ND	ND	PD	3	17	—
B12	2	ND	ND	DR4 negative	ND	—	PD	0	1	Early progression
B13	3	—	—	DR4 negative	ND	Increased granzyme B	Stable disease	12	33	One time point CD8 ELISPOT >2x only
B14	3	+ at 4 time points	—	? , ND	ND	Increased granzyme B	Stable disease	9	27	Increases in tetramer insufficient for +
B15	1	ND	ND	DR4 negative	ND	ND	PD	0	1	Early progression
B16	3	+ at 3 time points	+ at 2 time points	DR4 negative	ND	Increased granzyme B and CD25	PD	0	10	—
B17	3	ND	ND	DR4 negative	ND	ND	PD	0	7	—

Patient [*]	No. Vaccines [†]	CD8 MART-1 ELISPOT [‡] + at 3 time points	CD8 MART-1 Tetramer [§]	CD4 MART-1 ELISPOT	Determinant Spreading [¶]	NK Cell Phenotype [#]	Clinical Response (+56–112) ^{**}	PFS (mo) ^{††}	OS (mo) ^{‡‡}	Comment
B18	3		—	DR4 negative	ND	Increased CD25	PD	0	5	Only 1 time point >2x tetramer increase

^{*} Patient identifier.

[†] No. vaccines received.

[‡] CD8⁺ T-cell response to MART-1 antigen peptide by IFN- γ ELISPOT. Statistically significant increase from baseline to postvaccine are in **bold**.

[§] CD8⁺ T-cell response to MART-1 antigen peptide by MHC tetramer. Statistically significant increase from baseline to postvaccine are in **bold**.

^{||} CD4⁺ T-cell response to vaccine antigen peptide. Statistically significant increase from baseline to postvaccine are in **bold**.

[¶] CD8⁺ and CD4⁺ T-cell response to commonly expressed melanoma antigen peptides (determinant spreading) by IFN- γ ELISPOT. Statistically significant increase from baseline to postvaccine are in **bold**.

[#] NK cell phenotype changes.

^{**} Clinical response at end of trial enrollment (days +56–112) (in **bold**). PD = progressive disease; unevaluable; see Results.

^{††} Progression-free survival duration in months, at final follow-up (June 2007). Times of at least 12 months are in **bold**.

^{‡‡} Overall survival duration in months, at final follow-up (June 2007). Times of at least 12 months are in **bold**.

ELISPOT indicates enzyme-linked immunosorbent spot; MART, MART-1/Melan-A; ND, not determined; NE indicates no evidence; NK, natural killer; OS, overall survival; PFS, progression-free survival; —, negative response.