Determinant Spreading Associated with Clinical Response in Dendritic Cell-based Immunotherapy for Malignant Melanoma


ABSTRACT

Purpose: The purpose of this study was to determine the toxicity and immunological effects of three different doses and two routes of administration of autologous dendritic cells (DCs) pulsed with the MART-127–35 immunodominant epitope.

Experimental Design: Eighteen HLA-A*0201-positive subjects with stage III-IV melanoma received three biweekly i.v. or intradermal injections of ex vivo generated myeloid DCs pulsed with MART-127–35 epitope. Repeated blood samples were processed to obtain peripheral blood mononuclear cells for immunological analysis using IFN-γ ELISPOT, MHC class I tetramer, intracellular cytokine staining, and microcytotoxicity assays.

Results: The frequency of MART-1/Melan-A (MART-1) antigen-specific T cells in peripheral blood increased in all dose levels as assessed by ELISPOT and MHC class I tetramer assays, but without a clear dose-response effect. The intradermal route generated stronger MART-1 immunity compared with the i.v. route. MART-1-specific immunity did not correlate with clinical outcome in any of the four immunological assays used. However, analysis of determinant spreading to other melanoma antigens was noted in the only subject with complete response to this single-epitope immunization.

Conclusions: Intradermal immunization with MART-1 peptide-pulsed DCs results in an increase in circulating IFN-γ-producing, antigen-specific T cells. The frequency of these cells did not correlate with response. In contrast, spreading of immune reactivity to other melanoma antigens was only evident in a subject with a complete response, suggesting that determinant spreading may be an important factor of clinical response to this form of immunotherapy.

INTRODUCTION

Determinant spreading is a phenomenon best described in models of autoimmunity in which autoreactive T-cell responses, initiated by a single antigenic epitope, evolve into multiepitopic responses. In some models, such as experimental allergic encephalomyelitis, priming with a single encephalitogenic peptide of the proteolipid protein generates T cells reactive with additional peptide epitopes from the same proteolipid protein (intramolecular) and to other myelin antigens [intermolecular (1–3)]. In such models, the progression of autoimmune disease appears to depend on the acquisition of these new determinant specificities because induction of tolerance to these latter antigens abrogates relapses (4–7). Based on these models, it is thought that determinant spreading plays an important role in the pathogenesis of human autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, and insulin-dependent diabetes (8–12).

It is thought that the initiating self-antigen is presented by MHC class II molecules at the surface of professional APCs within the host tissue. This MHC class II epitope activates CD4+ T cells that cause chronic inflammation and target cell death, the debris from which contains secondary epitopes that are taken up by APCs and cross-presented. This phenomenon has traditionally been viewed as largely CD4+ T-cell-restricted.

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The abbreviations used are: APC, antigen-presenting cell; DC, dendritic cell; PBMC, peripheral blood mononuclear cell; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; NED, no evidence of disease; AFP, α-fetoprotein; i.d., intradermal; UCLA, University of California Los Angeles; NIAID, National Institute of Allergy and Infectious Disease; PE, phycoerythrin.
by virtue of the exogenous acquisition of antigen (13, 14). It is becoming clearer, however, that exogenous antigen can be taken up by DCs and be loaded onto MHC class I (15–17) and that determinant spreading can explain the acquisition of new CD8-specific CTL responses (18–20).

We report herein a clinical trial in which melanoma patients were immunized with immature myeloid DCs pulsed with a single HLA-A*0201-restricted epitope (MART-127–35) with the following observations: (a) pathological evidence of intratumoral (MHC class I and class II) and intermolecular (different antigens) determinant spreading were generated; (b) low DC doses (10^5 DCs/vaccination) could generate detectable antigen-specific T-cell responses; (c) immature DCs stimulated IFNγ-producing T cells; and (d) i.d. delivery was superior to i.v. delivery by immunological analysis using ELISPOT and MHC class I tetramers.

**MATERIALS AND METHODS**

**Study Design and Eligibility Criteria**

Eighteen patients with histologically proven malignant melanoma were enrolled in a Phase I, dose-escalating study to evaluate the toxicity and possible immunological effects of MART-127–35 peptide-pulsed autologous DCs. Increasing numbers of MART-1 peptide-pulsed DCs (10^5, 10^6, and 10^7) were given to groups of three patients either i.d or i.v. Patients received three biweekly vaccinations. Eligibility included patients with stage II–IV melanoma with or without measurable disease; MART-1 expression in the tumor by immunohistochemistry or reverse transcription-PCR; expression of the HLA-A*0201 allele; a Karnofsky performance status of 70% or greater; hematopoietic, hepatic, and renal function; and immune competence demonstrated by a positive skin delayed hypersensitivity test to at least one 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, and this trial was approved by the Institutional Review Board and the Internal Scientific Peer Review Committee at UCLA (IRB #95-08-375), and the Food and Drug Administration (BB IND #7122). Additionally, this trial underwent ad hoc reviews by the Jonsson Comprehensive Cancer Center Quality Assurance Committee.

**Peptide Synthesis and Characterization**

Clinical grade MART-127–35 (AAGIGILTV) peptide was synthesized at the UCLA Peptide Synthesis Facility. Clinical grade peptides were subjected to identity, purity, and potency testing (mass spectral analysis, microsequence analysis, high performance capillary electrophoresis, quantification of residual solvents, and freedom from adventitious infective agents). Potency was tested by the ability to generate HLA-A*0201-restricted MART-1-specific T-cell responses.

Peptides used in vitro for immunological monitoring included control non-melanoma epitope AFP249–255 (KQEFLINLV; Ref. 21) and the MHC class II peptide MART-151–73 (RNGYRALMDKSLHVGTQCALTR; Ref. 22) from the UCLA Peptide Synthesis Facility and gp100280–286 (YLEPGPVTA; Refs. 23 and 24), tyrosinase1-10 (MILLAVLYCL; Ref. 25), and MAGE271–279 (FLWGRALV; Ref. 26) synthesized at the University of Connecticut Health Center (Farmington, CT).

**Vaccine Preparation and Administration**

Patients underwent a single unmobilized leukapheresis processing 1 plasma volume. PBMCs were isolated by Ficoll-Hypaque (Amersham-Pharmaica, Piscataway, NJ) gradient centrifugation and cryopreserved in RPMI 1640 (Life Technologies, Inc., Rockville, MD) 20% heat-inactivated autologous serum/10% DMSO (Sigma, St. Louis, MO). Median PBMC yield was 8 × 10^9 (range, 5 × 10^9 to 1.4 × 10^10).

DCs were generated from adherent monocytes using GM-CSF and IL-4 following the procedure of Romani et al. (27). Briefly, PBMCs were thawed for each immunization, washed, and plated at 2.5–5 × 10^6 cells/ml in RPMI 1640/5% autologous serum/lentamycin. After adherence for 2 h at 37°C, nonadherent cells were gently removed, and adherent cells were cultured for 7 days with recombinant human GM-CSF (800 units/ml; Immunex, Seattle, WA) and recombinant human IL-4 (500 units/ml; Schering-Plough, Kenilworth, NJ). On the day of immunization, DCs were harvested, washed, and pulsed with 10–50 μg/ml MART-127–35 peptide in serum-free RPMI 1640 for 1–2 h. The MART-127–35 peptide, the leukapheresis product, and all reagents used for vaccine preparation were tested for Mycoplasma, endotoxin, and sterility. Samples of each DC vaccine were stained for cell surface markers CD86 (B7-2), HLA-DR, and CD14. Large, granular lymphocytes were gated on by forward and side scatter, and mean fluorescence intensity was determined for the entire DC population. The mean fluorescence and range were as follows: (a) CD86, 1129 (range, 199–2283; 11–32-fold over background); (b) HLA-DR, 3226 (range, 816–8508; 7–50-fold over background); and (c) CD14, 1158 (43–6186; 38-fold over background). 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. Based on these criteria, mean DC content of the vaccines was 32% (range, 8–70%). After confirming sterility results, DCs were prepared for immunization by washing and resuspending the appropriate numbers (10^5, 10^6, and 10^7) in 0.2 ml of saline for i.d. injection in the lower abdominal area or in 5.0 ml of saline for i.v. injection. Patients received pretreatment with 50 mg of diphenhydramine and 650 mg of acetaminophen (both p.o.) and were monitored for 2 h after immunization in the UCLA General Clinical Research Center.

**Immunological Monitoring**

**Tetramer Analysis.** MART-127–35/HLA-A*0201 tetramers were used for the detection of CD8+ T cell with the ability to recognize the MHC class I-restricted MART-127–35 peptide epitope (28). Initial preparations of MART-127–35 tetramers were provided by Drs. Mark Davis and Peter P. Lee at Stanford University (Stanford, CA), and early patient PBMC samples were independently tested in both laboratories. Subsequent tetramer preparations were obtained from the Tetramer Facility sponsored by the NIAID (NIAID, Emory University at...
Yerkes, Atlanta, GA). Each new MART-127–35/tetramer preparation was titrated using MART-1-restricted T-cell populations to determine the optimal staining concentration. PBMCs from each time point were thawed simultaneously, and 106 cells were stained with the tetramers and CD8-FITC (Caltag, Burlingame, CA) and antibodies used to gate out non-CD8+ T lymphocytes (tricolor-conjugated CD4, CD13, and CD19; Caltag). Staining was performed at room temperature for 30 min in the dark. The lymphocytes were gated on by forward and side scatter, and cells positive for CD4/CD13/CD19 were gated out. The positive cells are a distinct population of CD8-FITC+/tetramer-PE+ cells.

**IFN-γ ELISPOT.** To determine the frequency of peptide-specific cytokine-producing cells, the ELISPOT technique was used as described previously (29–31). PBMCs were thawed as described above, and T-cell restimulation was performed overnight with 1–2 × 106 PBMCs incubated with 1 × 105 JY cells pulsed with specific (MART-127–35) or nonspecific (AFP549–557) peptides. Unpulsed JY cells also served as a negative control. For determinant spreading studies, PBMCs were pulsed with MART-1-51–73, gp100280–288, or tyrosinase1–9 epitopes overnight and then added to precoated ELISPOT plates. Primary antibody (BD Pharmingen, San Diego, CA)-coated plates (Millipore, Bedford, MA) were incubated with restimulated cells (in duplicate at three dilutions) at 37°C for 24 h. After washes and staining with matched secondary antibody, the plate was developed, and colored spots, representing cytokine-producing cells, were counted once or twice manually and unblinded under a dissecting microscope. There were no spots detected for JY cells plated alone. In several studies, PBMCs were plated with JY cells without peptides, and “allo-specific” spots were routinely detected. Background spots to negative control AFP549–557 epitope were factored out. This control was necessary to account for variation between PBMC samples obtained at different times during the treatment of the patients. Measurement of MART-127–35-specific responses over time was generally performed once, due to limited PBMC supply. In 16 samples, the results were repeated with similar results. Determinant spreading in patient E1 was confirmed in six separate experiments with similar results.

**Intracellular Cytokine Staining.** To detect both cell surface phenotype and cytokine synthesis in a single cell, FastImmune analysis was performed (32). Two million thawed PBMCs were rested overnight and then restimulated for 6 h with specific (MART-127–35) or nonspecific (AFP549–557) peptides on 1 × 105 JY cells. The cells were treated with intracellular transport inhibitor brefeldin A (Sigma) for 4 h to increase levels of cytokines in the cytoplasm. Cells were stained for CD3, PerCP for 15 min at room temperature, followed by permeabilization and intracellular staining with IL-4-PE and IFN-γ-FITC according to the manufacturer’s instructions. Controls included nonrestimulated lymphocytes, cells maximally stimulated with phorbol 12-myristate 13-acetate (Sigma) and calcium ionophore (Sigma), and samples stained with isotype control antibodies (PharMingen). Background staining to AFP peptide restimulation and isotype antibodies was subtracted to determine MART127–35–specific IFN-γ and IL-4 staining.

**Cytotoxicity Assay.** The presence of MART-127–35 peptide-specific cytotoxicity was assessed on PBMCs after two rounds of ex vivo restimulation as described previously (31, 33, 34). Briefly, thawed patient PBMCs were pulsed with MART127–35 peptide, washed, and cultured on day 0 with IL-7 (10–25 ng/ml) and keyhole limpet hemocyanin (5 µg/ml) in RPMI 1640/10% AB serum at 3 × 106 cells/1.5 ml/well. Cells were restimulated weekly for 2 weeks. JY target cells were chromated and peptide-pulsed at 10 µg/ml for 2 h, and then target cells were washed, diluted to 5 × 104 cells/ml, and plated with various ratios of CTL. To control for nonspecific lysis, a 10–50-fold excess of unchromated K562 was added to target populations before adding to CTL. After 4–6 h at 37°C, supernatants were harvested and counted in a gamma counter. Triplicate wells were averaged, and the percentage of specific lysis was calculated as follows: [sample − spontaneous release]/[maximum release − spontaneous release]. Any background lysis of AFP549–557 pulsed targets was subtracted to determine MART127–35–specific lysis.

**Histology**

Specimens were fixed in 10% neutral buffered formalin and embedded in paraffin following standard procedures. Three-µm-thick sections were cut, and slides were deparaffinized in xylene and graded ethanol and brought to water and then stained with H&E (Fisher Scientific, Pittsburgh, PA). Immunohistochemical staining was performed following the DAKO EnVision Systems. Endogenous peroxidase activity was quenched by treating slides with 3% hydrogen peroxide in methanol for 10 min. Heat-induced epitope retrieval was performed on the slides using 1 mM EDTA (pH 8; for MART-1) or using 0.01 M citrate buffer (pH 6; for the rest of antibodies) and a vegetable steamer (Black & Decker). After heating for 25 min, cooling, and washing in 0.01 M PBS, slides were placed on a DAKO Autostainer (DAKO Corp., Carpenteria, CA) and then sequentially incubated in primary antibodies for 30 min. The following antibodies were used: (a) MART-1, 1:50 (Biocare Medical, Walnut Creek, CA); (b) S-100, 1:100 (DAKO Corp.); (c) gp100, 1:50 (HMβ-45; DAKO Corp.); (d) tyrosinase, 1:100 (T111; Novacastra Laboratories); (e) CD8, 1:50 (DAKO Corp.); (f) CD4, 1:100 (DAKO Corp.); (g) CD20, 1:1000 (DAKO Corp.); and (h) CD57, 1:30 (Becton Dickinson, San Jose, CA). Rabbit antimouse immunoglobulins (DAKO Corp.) were applied for 15 min, followed by EnVision + rabbit peroxidase (DAKO Corp.) for 30 min. Diaminobenzidine and hydrogen peroxide were used as the substrates for 10 min at room temperature. After washing in tap water, the slides were counterstained with hematoxylin, and coverslips were mounted. As a negative control, normal mouse serum (DAKO Corp.) was used in place of primary antibodies.

**Statistical Analysis**

Analysis of results of immunological assays was carried out using pretreatment samples and samples from trial day +14, +28, +35, +56, and +112, with day 0 as the date of the first immunization. The maximal value for each subject and each assay during days 14–56 was used as the measure for treatment response because only 50% of the subjects had samples from day +112. Analysis of mean pre- and posttreatment values for each assay was analyzed by the Wilcoxon signed-rank test.
Spearman correlation coefficient was used to evaluate the correlation between the maximum responses by any two assays. Univariate analysis was performed using the Wilcoxon rank-sum or the Kruskal-Wallis test to examine the association of the treatment response with each of the following factors: (a) gender (male versus female); (b) age (dichotomized as <60 years or ≥60 years); (c) dose (10^5, 10^6, and 10^7); (d) route (i.d. versus i.v.); (e) stage (III versus IV); (f) disease status (NED versus alive with disease); and (g) clinical response only for subjects with measurable disease (response versus stabilization plus progressive disease, or response plus stabilization versus progressive disease). A linear model was also developed to simultaneously correlate the above factors with treatment response. Stepwise procedure was used for factor selection. All tests are two-sided. The Ps reported here were not adjusted for multiple testing. Due to the small sample size, the Ps are approximate.

**RESULTS**

**Patient Characteristics.** Eighteen HLA-A*0201-positive patients with stage III or IV MART-1-positive melanoma were enrolled in this trial in which MART-127-35 peptide-pulsed DCs (10^5, 10^6, and 10^7) were administered i.d. or i.v. in cohorts of three patients in a dose-escalation fashion. DCs were generated from GM-CSF/IL-4 differentiated monocyte precursors that were deemed immature by the following phenotypic criterion: HLA-DR high; CD86 (B7-2) high; and CD14-positive large granular lymphocytes. Previously, we have shown that the DCs made by this methodology are immature but have high levels of MHC class I and II antigens, CD80 and CD86, CD54, and CD40 and are more stimulatory in allogeneic and autologous mixed lymphocyte reaction.

Patient characteristics are shown in Table 1. Mean age was 60 years (range, 39–79 years). Twelve patients were male, and six were female. Eight subjects had no measurable disease (seven subjects had stage III disease, and one subject had stage IV with NED after surgical excision). Ten subjects had stage IV disease with measurable metastatic lesions at the following sites: nodes (seven subjects); skin (six subjects); lung (three subjects); liver (two subjects); intestine (one subject); breast (one subject); and adrenal glands (one subject). Pretreatment in the seven subjects with stage III disease included surgery (seven subjects), IFN-α2b (three subjects), biochemotherapy [cisplatin, 1,3-bis(2-chloroethyl)-1-nitrosourea, dacarbazine, IFN-α2b, and IL-2; two subjects], irradiation (one subject), and other experimental vaccines (one subject). Pretreatment in 11 subjects with stage IV disease included surgery (11 subjects), recombinant cytokines (IL-2, IL-2 + histamine hydrochloride, or IFN-α2b; 7 subjects), external beam irradiation (2 subjects), biochemotherapy (2 subjects), chemotherapy (2 subjects), tamoxifen (2 subjects), and other experimental vaccines (2 subjects).

**Safety and Toxicity.** Forty-nine doses of vaccine were administered. Two subjects did not complete the vaccination schedule, one due to the development of clinically evident brain metastasis 1 week after the initial vaccination (C3), and one due to rapid disease progression after leukapheresis (F1). One vaccine administered i.v. caused hypertension requiring hospital admission; this preparation grew a *Pseudomonas* species bacteria. DC immunizations resulted in no local reaction at the injection site and no constitutional symptoms. Two subjects had worsening of preexisting vitiligo (B2 and D2), but none of the subjects developed vitiligo de novo. One subject (D1) developed alopecia 1 month after the final immunization but completely recovered within 2 months. This subject had completed adjuvant IFN-α2b therapy 3 months before enrollment and had normal levels of thyroid hormones.

**Clinical Outcome.** Median follow-up for the whole group is 18 months (range, 1–28 months). Of 10 subjects with measurable disease, 1 patient (E1) with >80 s.c. metastases has a durable complete response (24+ months), first noted 1 month after the third vaccination. Two subjects had disease stabilization of prior progressive metastases in lymph nodes (subject A1, 12 months, died at 28 months) and lung (subject C1, 4 months, died at 23 months). One subject (F1) had rapid disease progression after leukapheresis and before receiving any vaccination. The remaining six subjects (A2, A3, B1, B2, B3, and F3) had disease progression after completing the full vaccine schedule, and all have died. Of eight subjects with NED at trial entry, one (C3) developed clinically significant central nervous system metastasis after the first immunization. Two subjects progressed: patient D1 with stage III melanoma developed lung metastasis at 8 months and is alive with disease at 27+ months; and patient D2 with resected stage IV melanoma developed nodal metastasis at 7 months and is alive at 25+ months. Five subjects with stage III disease (C2, D3, E2, E3, and F2) have had no evidence of relapse at a median of 24+ months (range, 18–27+ months) of follow-up.

**Immunological Analysis of MART-127-35 Specific Responses.** PBMCs were obtained before, during, and after three biweekly vaccinations and analyzed for MART-1 recognition by HLA class I tetramer, IFN-γ ELISPOT, IFN-γ and IL-4 intracellular cytokine staining, and short-term cytotoxicity. These assays were chosen to cover a wide range of functional assays for cytotoxicity and cytokine production (ELISPOT and intracellular staining) as well as simple enumeration of peptide-specific cells in peripheral blood. Intracellular staining was chosen as a secondary cytokine assay due to its ability to detect
cell surface (CD3) as well as type 1 and type 2 cytokine (IFN-γ and IL-4), despite having lower repeated sensitivity compared with the ELISPOT.

Baseline MART-127–35 reactivity in peripheral blood was low to undetectable in the majority of subjects, as determined by the four immunological assays. Samples from available time points were thawed and immediately analyzed for the presence of CD8+ T cells with affinity for MART-127–35 tetramers. X axis, patient identifiers. Y axis, CD8 and MART-127–35 double positive events over the total CD8+ T-cell population. Z axis, time points of PBMCs.

In selected subjects with accessible metastatic lesions, pre- and postvaccination samples of melanoma tissue and tumor-infiltrating lymphocytes were analyzed. In a subject with a complete response (E1), postimmunization biopsies of s.c. metastasis revealed a dense lymphocytic infiltrate within the melanoma lesions (Fig. 6, A–F), consisting largely of CD8+ T cells with some CD4+ cells (Fig. 6, G–J). In this subject, biopsy of a residual lesion 2 months after the final immunization revealed a complete disappearance of melanoma cells and a local decrease in epidermal melanocytes (without clinically evident vitiligo).

Univariate and multivariate analysis was conducted to determine the relation between the results of the immunological assays and several parameters that may be predictive for immunological responses. Univariate analysis of postvaccination MART-127–35 reactivity was not correlated with gender, age, dose, initial stage, and disease status for either tetramer or...
ELISPOT assays. The i.d. immunizations resulted in higher levels of MART-127–35-reactive cells by the functional IFN-γ ELISPOT (P = 0.032), which was not confirmed by univariate analysis of MHC class I tetramer data. However, multivariate analysis using dose, route, and disease status as possible predictive factors confirmed that i.d. administration of DC vaccines resulted in an enhancement of MART-127–35 reactivity compared with i.v. delivery by both ELISPOT and tetramer (P = 0.03 and P = 0.07, respectively). The effect of the dose of MART-127–35 peptide-pulsed DCs on immunological assays revealed discordant results, with the tetramer assay suggesting an increase in MART-127–35-specific effectors with increasing vaccine doses (Fig. 2, P = 0.01), with a trend toward an opposite effect of vaccine dose when analyzed by IFN-γ ELISPOT (Fig. 3, P = 0.07). In subjects with measurable melanoma lesions at trial entry, the correlation between results of immunological assays and clinical outcome was studied. In this small group of subjects receiving different doses of vaccines by two routes, no correlation could be drawn between these two variables.

Overall conclusions from this extensive in vitro analysis of MART-127–35 reactivity after MART-127–35/DC immunizations suggest that the tetramer and ELISPOT assays were the most instructive in following the immune activation in peripheral blood after immunizations, with the i.d. route resulting in higher levels of circulating MART-127–35-specific cells. However, neither of these assays served as a good surrogate for clinical activity in the current Phase I clinical trial.

**Melanoma-specific Determinant Spreading.** Determinant spreading is believed to be the main immunopathogenic event in several systemic and organ-specific autoimmune diseases. Because detailed analysis of MART-127–35 reactivity could not explain the differences between a subject with a complete response in this trial and the other nine subjects without clinical benefit, we tested whether MART-127–35-specific lymphocytes served as a driver clone for a multideterminant response. Cryopreserved PBMCs from day +75 post-vaccination were analyzed for reactivity to the HLA-DR4-restricted MART-151–73 epitope as evidence for intramolecular determinant spreading within the MART-1 tumor antigen protein sequence. This is a MHC class II-binding epitope that is unlikely to be processed and presented by melanoma cells but may have been cross-presented by host APCs in this DR4+ patient. To study the possibility of intermolecular determinant spreading, PBMCs were also restimulated with the HLA-A*0201-binding melanoma epitopes gp100280–288 and tyrosinase1–9, Reactivity to these epitopes, which were not present in the DC vaccines but were expressed by the patients’ melanoma cells, could also be cross-presented by APCs from debris released by killed melanoma cells. As a negative control, cells were also restimulated with the HLA-A*0201-binding non-melanoma epitope AFP549–557. A series of seven ELISPOT studies were performed analyzing patient E1 reactivity to these additional melanoma epitopes. The results are summarized in Fig. 7. A consistent pattern was noted, with posttreatment peripheral leukocytes from this subject producing IFN-γ when restimulated with HLA-matched melanoma-derived epitopes for gp100, tyrosinase, and the MHC class II MART-1 epitope. No
such reactivity was noted in pretreatment samples. PBMC samples were also tested from eight other subjects who did not have a clinical response, but who had metastatic disease (six of eight subjects) or increased vitiligo (two of eight subjects) or received the same DC dose and route (one of eight subjects). No other patient tested had a detectable response to the other epitopes before or after vaccination. Therefore, determinant spreading can occur in human subjects after DC-based single-epitope immunizations, which correlates with clinical response in this small series of subjects.

DISCUSSION

The results of this trial show that immunization with immature DCs pulsed with a single MHC class I peptide can generate robust peptide-specific T-cell responses in most patients, even with small numbers of DCs ($10^5$) and without side effects. The i.d. delivery route was superior to the i.v. delivery route, and IFN-γ ELISPOT and MART-1$_{27-35}$/MHC class I tetramer assays were instructive as an immunological end point of successful vaccination. However, T-cell responses to the immunizing peptide epitope were not predictive of clinical response, despite the four assays used and the numerous time points assessed during the treatment. Our results should be interpreted with caution because the performance specifications

Fig. 6 Tumor regression and lymphocytic infiltrate in patient E1. Photographs were taken at the times shown to monitor the appearance of the s.c. tumor (A–C). Biopsies obtained were stained with H&E to identify tumor deposits and lymphocyte infiltration (D–F). To characterize the nature of the lymphocytic infiltrate, the biopsy at day +130 was stained for the cell surface markers shown (G–J).

Fig. 7 Determinant spreading. PBMCs were assayed for the presence of IFN-γ-producing cells when restimulated overnight with the HLA-DR4 MART-1$_{111-123}$ epitope and the HLA-A*0201-binding melanoma epitopes gp100$_{260-288}$ and tyrosinase$_{1-9}$. As a negative control, cells were also restimulated with the HLA-A*0201-binding non-melanoma epitope AFP$_{549-557}$. Only subject E1 had detectable reactivity to MART-1$_{111-123}$, gp100$_{260-288}$, and tyrosinase$_{1-9}$ ($P = 0.11$, not significant due to the small number of events).
for the immunological assays used in the current trial monitoring have not been established. Additionally, the limited number of subjects, although adequate for determining safety and toxicity in a Phase I trial, does not allow us to make inferences with adequate confidence about the treatment effects on immune responses, the correlation between vaccine schedule or dose and immune responses, and the relationship between immune and clinical responses. The real value of our attempts to detect large differences in immunological outcomes is the exploration of strong trends that may lead to further investigation.

Whereas some recent immunotherapy trials have found concordance between immunological monitoring assays and clinical response (35, 36), most have not (37–41). Many clinical trial immunological monitoring studies have also been limited by few PBMC samples and one or two assays performed, many only testing pretreatment and posttreatment time points. Among studies in which several assays were performed on multiple time point PBMC samples, one found a good correlation between tetramer enumeration of peptide-specific T cells and their function (42), one found no correlation between assays (43), another found correlation between cytokine assays but not with delayed-type hypersensitivity (44). In this trial, we found limited concordance between the levels of MART-1 peptide-specific T cells measured by the most sensitive assays, MHC class I tetramer and IFN-γ ELISPOT. This is not unexpected because the tetramer enumerates circulating cells regardless of function, and the ELISPOT detects peptide-specific cells that secrete IFN-γ. Recently, a thorough analysis of three MART-1 peptide-immunized patients showed T-cell phenotype and activity fluctuations over time, with some peptide-specific cells secreting IFN-γ and GM-CSF, whereas others secreted only IL-2 (45); hence, as expected, all active peptide-specific cells may not secrete a single cytokine.

The complete responder (E1) in this series developed HLA-A*0201-restricted responses to two additional melanoma antigens (gp100 and tyrosinase) expressed by her tumor as well as a HLA-DR4-restricted MART-1 epitope. Determinant spreading was not found in other patients with measurable disease (B1, B2, B3, C1, C3, and F3), increased vitiligo (B2 nd D2), and disease stabilization (C1) or in those receiving 10^7 DCs i.d. (E2). We hypothesize that the MART-127–35-specific driver clone in E1 generated melanoma cell lysis, resulting in endogenous priming with new melanoma-derived epitopes (MART-1 MHC class II epitope, gp100, and tyrosinase) by endogenous APCs. Therefore, the driver clone activated a cascade of tumor-specific autoreactive T-cell clones. This study provides support that cancer vaccines may take advantage of the immunopathogenetic mechanisms that lead to serious autoimmune diseases to generate clinical responses.

An example of intramolecular determinant spreading has been described previously in a complete responder in another DC-based clinical trial (46). In this trial, subjects received s.c. and i.v. injections of DCs pulsed with the HLA-A*0201 melanoma-derived epitopes MART127–35, gp100280–288, and tyrosinase368–376. Using the ELISPOT assay, Ranieri et al. (46) detected reactivity to two other HLA-A*0201-binding epitopes (gp100290–297 and tyrosinase349–355) and four HLA-DR4 class II epitopes (MART131–37, gp10044–59, gp100615–633, and tyrosinase56–70) in the one patient tested. Also, intermolecular determinant spreading has been reported in one subject immunized with MART127–35 and tyrosinase368–376 peptide-pulsed DCs and subsequent spreading to the gp100290–297 epitope (47), whereas another subject immunized with gp100290–297 emulsified in incomplete Freund’s adjuvant showed subsequent spreading to MAGE-12170–178 (48). Taken together with the results of our trial, determinant spreading may be an important phenomenon in responders to DC-based immunotherapy. We did not test all known HLA-A*0201 epitopes, hence spreading to other antigens or epitopes might have occurred in E1 or other patients.

Determinant spreading is a normal feature of protective immune responses to infectious agents, allowing recognition of multiple antigenic targets (3). The immune system depends on diversification to adequately protect against dangerous non-self. It is also possible that it may also play a role in protection against dangerous self processes such as cancer. Therefore, immune responses leading to tumor regression may use the same mechanisms involved in the immunopathogenesis of aggressive autoimmune diseases (1, 2). Most melanoma-derived antigens are lineage-specific proteins, which are differentially recognized by T cells when expressed by malignant as opposed to benign pigmented cell (49–52). A continuum exists between antitumor and anti-self responses because the development of clinical vitiligo has been associated with clinical response to IL-2-based melanoma therapy (49).

In experimental models of autoimmune diseases, a spreading hierarchy has been defined, starting with a determinant that has the largest pool of high-avidity T cells that serve as a driver clone for spreading to determinants with progressively smaller and low-avidity T-cell precursor clones (1, 53, 54). This phenomenon had been generally believed to be unique to CD4+ T-cell responses after cross-priming by exogenous antigen presented by host APCs. It is now clear that cross-priming can induce CD8+ T-cell responses (15), and the possibility of MHC class I determinant spreading has been studied. In a model of tumor immunity, a single MHC class I tumor-derived epitope generated protective immunity that was clearly linked to intramolecular spreading to other determinants derived from the same tumor (13). In HLA-A*0201 positive subjects with melanoma (as well as healthy donors), anti-MART127–35 reactive T cells can be detected at higher frequencies compared with other melanoma-derived epitopes (55), and the analysis of HLA-A*0201 restricted tumor-infiltrating lymphocytes from subjects with spontaneous or IL-2-induced responses (56) suggests that the MART127–35 epitope is immunodominant within this prevalent HLA subtype. Therefore, the observations in the responsive subject described in this report may reflect a similar spreading hierarchy from the high-avidity large T-cell pool of MART127–35-reactive T cells to other lower avidity and less frequent T cells.

There was no attempt to mature DCs in this trial, which had a phenotype consistent with immature DCs. Other clinical trials have clearly documented immunological and clinical responses with antigen-loaded immature DCs in human subjects (57–68). Similarly, in vivo immunization with immature DCs generates antitumor responses in murine models (69, 70). However, a trial attempting to immunize against influenza with peptide-pulsed immature DCs raised the concern that immune tolerance might...
Determinant Spreading in MART-1 Peptide/DC Trial

be induced (71). Furthermore, another trial directly comparing coinjection of immature and mature DCs loaded with different antigens reported a consistent pattern of superior immune activation with mature DCs (47). The discussion between the relative value of mature and immature DCs is hampered by the lack of uniform criteria to define these populations, which develop in a continuum, and the multiple factors that may lead to functionally different DCs. These include the type of tissue culture media; the presence or absence of serum; the use of autologous, allogeneic, or xenogeneic serum; the tissue culture flasks; and the number of DC manipulations (data not shown; Ref. 72). It is widely believed that matured DCs are superior to immature DCs when attempting to generate human CTLs in vitro, given the clear superior antigen-presenting phenotype of mature DCs (73). The same rationale may not hold true when using DCs in vivo. The beneficial effects of ex vivo maturation may be counterbalanced by a decreased ability to traffic to lymph nodes (74) or may be superfluous after maturation signals from the trauma of injection, local inflammatory cells, or early encounter with CD4 T cells in vivo. Hence, when used in vivo, there are too little comparative data and too many variables in DC preparation to conclude that a particular degree of maturation is required for antitumor effects.

In conclusion, we attempted to generate anti-melanoma responses using autologous DCs pulsed with an epitope present in the patient’s melanoma lesions. A clear expansion of MART-1-specific T cells was evident in most patients by tetramer and ELISPOT analysis. Detailed immunological and statistical analysis of epitope-specific responses failed to correlate with clinical responses. This may be due in part to a low frequency of T cells and too many variables in DC preparation to conclude that a particular degree of maturation is required for antitumor effects.

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Determinant Spreading Associated with Clinical Response in Dendritic Cell-based Immunotherapy for Malignant Melanoma


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