Determinant Spreading Associated with Clinical Response in
Dendritic Cell-based Immunotherapy for
Malignant Melanoma¹

Lisa H. Butterfield, Antoni Ribas,
Vivian B. Dissette, Saral N. Amarnani,
Huong T. Vu, Denise Oseguera, He-Jing Wang,
Robert M. Elashoff, William H. McBride,
Bijay Mukherji, Alistair J. Cochran,
John A. Glaspy, and James S. Economou²

Department of Surgery, Division of Surgical Oncology [L. H. B.,
A. R., V. B. D., S. N. A., J. S. E.], Department of Medicine, Division
of Hematology/Oncology [A. R., H. T. V., D. O., J. A. G.],
Department of Biomathematics [H.-J. W., R. M. E.], Department of
Experimental Radiation Oncology [W. H. M.], Department of
Pathology and Laboratory Medicine [A. J. C.], and Department of
Microbiology, Immunology and Molecular Genetics [J. S. E.],
University of California Los Angeles Jonsson Comprehensive Cancer
Center, University of California at Los Angeles, Los Angeles,
California 90095-1782, and Department of Medicine, University of
Connecticut Health Center, Farmington, Connecticut [B. M.]

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 mononu-
clear cells for immunological analysis using IFN-γ ELIS.

Results: The frequency of MART-1/Melan-A (MART-1)
epitope-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 en-
cephalomyelitis, priming with a single encephalitogenic peptide
of the proteolipid protein generates T cells reactive with addi-
tional peptide epitopes from the same proteolipid protein (in-
tramolecular) 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 anti-
gens 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 mul-
tiple 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 APCs³
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

¹ The abbreviations used are: APC, antigen-presenting cell; DC, den-
dritic cell; PBMC, peripheral blood mononuclear cell; GM-CSF, gran-
ulocyte/macrophage colony-stimulating factor; IL, interleukin; NED, no
evidence of disease; AFP, α-fetoprotein; i.d., intradermal; UCLA, Uni-
versity of California Los Angeles; NIAID, National Institute of Allergy
and Infectious Disease; PE, phycoerythrin.

² The costs of publication of this article were defrayed in part by the
payment of page charges. This article must therefore be hereby marked
in accordance with 18 U.S.C. Section 1734 solely to
indicate this fact.

³ Supported by NIH/National Cancer Institute Grants ROI CA 77623,
ROI CA 79976, T32 CA75956, and K12 CA 79605 and the Monkarsh
Fund, Naify Fund, and Evelyn Kesslerman Research Fund (all
to J. S. E.). In addition, the support of UCLA General Clinical Research
Center (USPHS Grant M01-RR-0865) and the UCLA Jonsson Compre-
hensive Cancer Center Flow Cytometry Core Facility (NIH Grant CA
16042) is gratefully acknowledged. A. R. is a recipient of an American
Society of Clinical Oncology Career Development Award and NIH
Grant K23 CA93376.

Received 8/16/02; revised 10/28/02; accepted 10/29/02.
Received on April 13, 2017. © 2003 American Association for Cancer Research.
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 a complete response was observed in a patient in whom both intramolecular (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%; demonstrated intermolecular (MHC class I) and intramolecular (different antigens) determinant spreading in a patient in whom both intramolecular (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.

**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 the UCLA Peptide Synthesis Facility and gp100280–288 (YLEPGPVTA; Refs. 23 and 24), tyrosinase-1 (MLLAVLYCL; Ref. 25), and MAGE3271–279 (FL-WGPRALV; 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-Pharmacia, 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 × 10^9 cells/ml in RPMI 1640/5% autologous serum/lentamicin. 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.

**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 infectious 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 (KKQFLINLV; Ref. 21) and the MHC class II peptide MART-151–63 (RNGYRALMDKSLHVGQCALTRR; Ref. 22) from the UCLA Peptide Synthesis Facility and gp100280–288 (YLEPGPVTA; Refs. 23 and 24), tyrosinase-1 (MLLAVLYCL; Ref. 25), and MAGE3271–279 (FL-WGPRALV; Ref. 26) synthesized at the University of Connecticut Health Center (Farmington, CT).
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 x 10⁶ PBMCs incubated with 1 x 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-151, 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 x 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 MART-127,35 peptide, washed, and cultured on day 0 with IL-2 (10–25 ng/ml) and keyhole limpet hemocyanin (5 μg/ml) in RPMI 1640/10% AB serum at 3 x 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 x 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. Any background lysis of AFP549–557–pulsed targets was subtracted to determine MART-127,35–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 System. 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% 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% PBS, slides were placed on a DAKO Autostainer (DAKO Corp., Carpinteria, 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 (MBM-45; DAKO Corp.); (d) tyrosinase, 1:100 (T311; 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 antinmouse 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-1 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.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>18</td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
</tr>
<tr>
<td>Median age (range) (yrs)</td>
<td>65 (39–79)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>7</td>
</tr>
<tr>
<td>IV NED</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
</tr>
<tr>
<td>Sites of disease</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>6</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>7</td>
</tr>
<tr>
<td>Lung</td>
<td>2</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
</tr>
<tr>
<td>Other visceral</td>
<td>4</td>
</tr>
<tr>
<td>Prior therapy</td>
<td></td>
</tr>
<tr>
<td>Adjuvant IFN-α2b</td>
<td>5</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>5</td>
</tr>
<tr>
<td>Other immunotherapy</td>
<td>8</td>
</tr>
<tr>
<td>Hormonotherapy</td>
<td>2</td>
</tr>
<tr>
<td>Irradiation</td>
<td>3</td>
</tr>
</tbody>
</table>

Immunological Analysis of MART-1 peptide-specific Responses. PBMCs were obtained before, during, and after three biweekly vaccinations and analyzed for MART-1 recognition by species-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 postimmunization time points showed an increase in frequency of MART-127-35-reactive cells for the tetramer (Figs. 1 and 2) and IFN-γ ELISPOT assays (Fig. 3; P = 0.0001 and 0.0012, respectively) but no overall difference between mean pre- and postimmunization values for IFN-γ or IL-4 intracellular cytokine staining (Fig. 4) and microcytotoxicity assays (Fig. 5). Peak values did not have a clear relation with the number of immunizations, although there was a general trend of an increase early after the immunizations and a subsequent decrease in MART-127-35 reactivity over time.

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 tyrosinase 1–9. 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 EI 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_{127-135}/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.
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 points 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, and F3), increased vitiligo (B2 nd D2), and disease stabilization (C1) or in those receiving 107 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 immunopathogenic 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 and gp100280–288 and tyrosinase368–376D. Using the ELISPOT assay, Ranieri et al. (46) detected reactivity to two other HLA-A*0201-binding epitopes (gp100290–297 and tyrosinase218) and four HLA-DR4 class II epitopes (MART151–73 and gp100244–259 and gp100261–263 and tyrosinase26–70) in the one patient tested. Also, intramolecular 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 gp100260–284 epitope 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 after cross-priming by exogenous antigen presented by host APCs. 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).
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-antitumor effects. In conclusion, we attempted 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.


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


Updated version  Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/9/3/998

Cited articles  This article cites 72 articles, 34 of which you can access for free at: http://clincancerres.aacrjournals.org/content/9/3/998.full.html#ref-list-1

Citing articles  This article has been cited by 43 HighWire-hosted articles. Access the articles at: /content/9/3/998.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.