

Prolonged Survival of Dendritic Cell–Vaccinated Melanoma Patients Correlates With Tumor-Specific Delayed Type IV Hypersensitivity Response and Reduction of Tumor Growth Factor β -Expressing T Cells

Mercedes N. López, Cristian Pereda, Gabriela Segal, Leonel Muñoz, Raquel Aguilera, Fermín E. González, Alejandro Escobar, Alexandra Ginesta, Diego Reyes, Rodrigo González, Ariadna Mendoza-Naranjo, Milton Larrondo, Alvaro Compán, Carlos Ferrada, and Flavio Salazar-Onfray

A B S T R A C T

Purpose

The aim of this work was to assess immunologic response, disease progression, and post-treatment survival of melanoma patients vaccinated with autologous dendritic cells (DCs) pulsed with a novel allogeneic cell lysate (TRIMEL) derived from three melanoma cell lines.

Patients and Methods

Forty-three stage IV and seven stage III patients were vaccinated four times with TRIMEL/DC vaccine. Specific delayed type IV hypersensitivity (DTH) reaction, ex vivo cytokine production, and regulatory T-cell populations were determined. Overall survival and disease progression rates were analyzed using Kaplan-Meier curves and compared with historical records.

Results

The overall survival for stage IV patients was 15 months. More than 60% of patients showed DTH-positive reaction against the TRIMEL. Stage IV/DTH-positive patients displayed a median survival of 33 months compared with 11 months observed for DTH-negative patients ($P = .0014$). All stage III treated patients were DTH positive and remained alive and tumor free for a median follow-up period of 48 months (range, 33 to 64 months). DTH-positive patients showed a marked reduction in the proportion of CD4+ transforming growth factor (TGF) β + regulatory T cells compared to DTH-negative patients (1.54% v 5.78%; $P < .0001$).

Conclusion

Our findings strongly suggest that TRIMEL-pulsed DCs provide a standardized and widely applicable source of melanoma antigens, very effective in evoking antimelanoma immune response. To our knowledge, this is the first report describing a correlation between vaccine-induced reduction of CD4+TGF β + regulatory T cells and in vivo antimelanoma immune response associated to improved patient survival and disease stability.

J Clin Oncol 27:945-952. © 2009 by American Society of Clinical Oncology

INTRODUCTION

Surgical treatment of malignant melanoma (MM) is successful in early stages, but if long distance metastases are detected, no therapeutic approach has proven to change the disease outcome.¹⁻³ MM is a relatively immunogenic cancer that expresses several identified melanoma-associated antigens (MAAs) recognized by the immune system.⁴ Despite its relatively low response rate (15.3%), with a median overall survival between 6 to 11 months, chemotherapy based on dacarbazine (DTIC) is commonly used to treat disseminated melanoma.^{2,3,5} Diverse phase III trials have explored the benefit of combine cytostatic drugs or recombinant

cytokines such as interferon alpha (IFN- α) and interleukin-2 (IL-2) with DTIC, observing increased toxicity rather than improved responses.^{2,3,5-7} Modern experimental immunotherapies against MM include the use of autologous dendritic cells (DCs) loaded with MAAs, as a natural adjuvant to activate an immune response against tumor cells.^{4,8-11}

DCs are professional antigen-presenting cells (APCs) that capture and present tumor-associated antigens to T cells generating strong and specific cellular immune responses in murine and human models.^{12,13} Several DCs-based immunotherapy protocols have been conducted for the treatment of MM, including one randomized phase III clinical trial.^{8-11,14} Discouragingly, in the last, no objective

From the Millennium Nucleus on Immunology and Immunotherapy, Disciplinary Program of Immunology, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile; Research Support Office, Clinical Hospital of the University of Chile, Santiago; and the Regional Hospital of Concepción, Concepción, Chile.

Submitted May 16, 2008; accepted October 2, 2008; published online ahead of print at www.jco.org on January 12, 2009.

Supported by grants from the Fund for the Promotion of Scientific and Technological Development (FONDEF DO211088), the National Fund for Scientific and Technological Development (FONDECYT 1060935 and 1070559), Millennium Nucleus on Immunology and Immunotherapy P04/030-F.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Flavio Salazar-Onfray, MD, Programa Disciplinario de Inmunología, Instituto de Ciencias Biomedicas, Facultad de Medicina, Universidad de Chile, Avenida Independencia 1027, Santiago, Chile; e-mail: fsalazar@immunotron.med.uchile.cl.

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

© 2009 by American Society of Clinical Oncology

0732-183X/09/2706-945/\$20.00

DOI: 10.1200/JCO.2008.18.0794

differences in tumor regression or survival were observed between patients treated with DCs or DTIC.¹⁴ The combined results of these studies strongly suggest that peptide-loaded DC immunization is insufficient to produce significant therapeutic effects.^{8-11,14}

Optimal tumor antigen delivery is one of the most important factors for DC-based immunotherapy success. For that reason, autologous tumor cell lysate, whole tumor cells, and mRNA have been tested as antigen providers for DCs.¹⁵⁻¹⁸ Furthermore, allogeneic melanoma cell lysates also constitute a valuable alternative.¹⁹⁻²¹ An advantage of this strategy is that it provides a standardized applicable source of melanoma-specific antigens, useful also in high-risk tumor-free patients.

Previously, we described an experimental approach based on the injection of autologous DCs pulsed with an allogeneic melanoma cell lysate.²² A significant increase in melanoma-specific IFN- γ production was observed *ex vivo* in half of tested patients. In addition, a significant correlation between positive tumor-specific delayed type IV hypersensitivity (DTH) reaction and both postvaccination short-term survival and progression-free survival (PFS) was observed.²² Several factors may explain this unequal treatment response among individuals. One of them may be the frequency of regulatory T lymphocytes (RTL) in patients. In fact, patients with diverse kind of cancer show correlations between increased number of CD4+CD25+ T cells and reduced survival.^{23,24} In addition, elimination of RTL by radiation or chemotherapy leads to improved tumor regressions after adoptive T cell transfer therapy in mice and humans.^{25,26}

In this follow-up phase II clinical report, the capability of autologous DCs pulsed with a novel allogeneic cell lysate TRIMEL to evoke effective antimelanoma immune responses was evaluated. Survival and PFS of immunized patients were also studied. DTH reaction induced by TRIMEL was tested as a way to distinguish responders from nonresponders, early after vaccination. Finally, we studied the correlation between melanoma-specific DTH response and patient survival and PFS, as well as the presence of regulatory T-cell frequencies in treated patients.

PATIENTS AND METHODS

Trial Eligibility and Patient Inclusion Criteria

This phase II, clinical trial performed from January, 2001, to December, 2007, was designed to address immunological efficacy, survival, and disease stability of patients vaccinated with tumor lysate-loaded DCs. Eligibility criteria for vaccination was previously described.²² The study was approved by the Bioethical Committee for Human Research of University of Chile, Faculty of Medicine. All patients were required to understand the study and sign an informed consent.

TRIMEL Cell Lysate and DCs Preparation and Characterization

The melanoma cell lysate (TRIMEL) used in the study was prepared as previously described (online only Appendix).²²

Peripheral blood mononuclear cells (PBMC), obtained at the Blood Bank Service of the University of Chile Clinical Hospital from Buffy coats (patients MT01 to MT06) or by leukapheresis (patients MT07 to MT50), were isolated as previously described.²² Monocytes were incubated for at least 2 days in serum-free AIM-V medium (therapeutic grade; Invitrogen Corporation, Grand Island, NY) in the presence of recombinant human IL-4 (rhIL-4; 500 U/mL; US Biologic, Swampscott, MA) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 800 U/mL; Shering Plough, Brinny Co, Ireland) and then loaded with 100 μ g/mL of TRIMEL in the presence of 20 U/mL of tumor necrosis factor- α (US Biologic, Swampscott,

MA, USA) to induce DCs maturation. After 24 hours additional incubation, DCs were recovered and cryopreserved using an automatic freezing system Cobe Spectra (Lakewood, CO).

TRIMEL/DC Administration to Patients

Patients were vaccinated intradermally (ID) with 1 mL of TRIMEL/DCs mixed with aluminum hydroxide (500 μ g; J.T. Baker, Phillipsburg, NJ) or Keyhole limpet hemocyanin (KLH; 100 μ g; Calbiochem, San Diego, CA) in the leg or arm closest to intact lymph nodes. The vaccination protocol consisted of four doses injected on days 0, 10, 30, and 50. A cohort of 12 patients were subcutaneously (SC) inoculated with 2.4×10^6 U/m² rhIL-2 (PROLEUKIN; Chiron Emeryville, CA) 2, 3, and 4 days after the second, third, and fourth vaccinations. Patients MT03, MT08, MT10, and MT34 were revaccinated with a second round of vaccines, 1 year after the first cycle.

Skin Test and IFN- γ Assays

All patients were assessed for *in vivo* DTH reactions to the TRIMEL cell lysate 1 month after the end of therapy. Skin tests were performed injecting SC 200 μ L of TRIMEL (2 mg/mL) and 100 μ L of control antigens KLH (1 mg/mL) or MULTITEST cell-mediated immunity (CMI; Pasteur-Mérieux, Lyon, France) in saline solutions at different site. Saline solution alone (100 μ L) was used as a negative control. A positive reaction was defined as skin erythema or induration \geq 5 mm at 48 hours after injection. Elispot for IFN- γ release was developed as previously described (Appendix).²²

Analysis of Regulatory T-Cell Populations

PBMCs from treated stage IV melanoma patients (14 DTH positive and 14 DTH negative) were collected before therapy, after second injection, and after completion of the vaccination protocol. Antibodies used in the analysis were anti-foxp3 (clone PCH101, 37.5 μ g/1 $\times 10^6$ cells; eBioscience, San Diego, CA); phycoerythrin (PE)-conjugated anti hIL-10 (clone JES3-9D7 37.5 μ g/1 $\times 10^6$ cells; eBioscience); anti-LAP (TGF- β 1; clone 27,232, 1.5 μ g/1 $\times 10^6$ cells; R&D Systems, Minneapolis, MN); PE Cy5.5-conjugated anti-CD4 (clone RPA-T4, 50 ng/1 $\times 10^6$ cells; eBioscience); PE-conjugated anti-immunoglobulin (IgG) 1 and fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG F(ab)2 (dimer of an antigen-binding fragment); (2 μ g/1 $\times 10^6$ cells; R&D Systems). For intracellular IL-10 and TGF- β measurement, 1 $\times 10^6$ cells/mL were incubated overnight with 100 ng/mL LPS in 1 mL GIBCO RPMI Medium 1640 (Sigma-Aldrich, St Louis, MO) 100% fetal bovine serum followed by 4 hours of incubation with 1 μ g/mL Brefeldin A (eBioscience). Cells were then fixed, permeabilized, and stained using the respective monoclonal antibodies (mAbs), according to manufacturer instructions. Cells were finally stained with anti-CD4 mAb or control mAbs and analyzed using FACscan cytometer (Becton Dickinson, Franklin Lakes, NJ). The data were evaluated using freeware WinMDI program (<http://facs.scripps.edu/software.html>).

Clinical Response Criteria

Patients were defined as immunologic responders if they displayed activity against TRIMEL in DTH assays. Stable disease (SD) was defined as less than 25% change in tumor size and absence of new lesions during a 6-week period. Inversely, progressive disease (PD) was defined as more than 25% increase in the perpendicular diameter of any measurable tumor, appearance of new lesions by imaging examination, and/or worsening of general condition. PFS was defined as the number of months that patient showed SD after first vaccine dose. Postvaccination survival is the number of months that patients survived after first vaccine dose.

Statistical Analysis

Survival curves estimations were calculated by Kaplan-Meier method and log-rank test. For subgroup analysis, the χ^2 test was used to compare proportions of categoric variables, unless the frequency of expected events was fewer than 5, in which case, Fisher's exact test was used. For continuous variables, the *t*-test was used after confirming normal distribution with the Shapiro-Wilk test. In the case of non-normal distributions the Wilcoxon rank sum test was applied. To evaluate the proportion of T-helper cell populations of responder and nonresponder subgroups, paired *t*-test or the sign test was used depending on normal or non-normal distribution, respectively. Statistical analyzes were performed using Stata 7.0 software (Stata Corp, College Station, TX). Statistical significance was considered at a *P* value less than .05.

Prolonged Survival of DC-Treated Melanoma Patients

Table 1. Characteristics of Patients and Immunologic Response to Therapy

Code	Sex	Stage	Additional Treatment	Adverse Effects	DTH CTRL (mm)	DTH Lysate (mm)	PFS (months)	Survival (months)
MT01	F	IV	Surgery of subcutaneous metastasis	No	NT	10 (+)	23	26
MT02	M	IV	None	Fever	NT	(-)	18	21
MT03	M	IV	None	No	7 (+)	7 (+)	20	26
MT04	M	IV	None	No	8 (+)	10 (+)	0	18
MT05	F	IIIC	None	No	7 (+)	10 (+)	64	64
MT06	F	IV	Local radiotherapy	No	7 (+)	(-)	1	5
MT07	F	IV	None	No	6 (+)	(-)	0	7
MT08	F	IV	Surgery of transient metastasis + IL-2	Low fever	6 (+)	(-)	0	11
MT09	M	IV	Surgery of lung metastasis + IL-2	No	5 (+)	12 (+)	58	58
MT10	F	IIIC	IL-2	No	10 (+)	18 (+)	57	57
MT11	M	IV	Radiotherapy	No	NT	NT	0	*
MT12	M	IV	IL-2	No	NT	(-)	24	36
MT13	F	IIIC	Local radiotherapy + IL-2	No	10 (+)	45 (+)	52	52
MT14	M	IIIC	IL-2	No	8 (+)	8 (+)	48	48
MT15	M	IV	Surgery of in transit metastasis + IL-2	Low flu like symptoms	6 (+)	9 (+)	22	36
MT16	F	IIIB	None	Headache, flu like symptoms	(-)	13 (+)	45	45
MT17	M	IV	IL-2	Slight nauseas, low appetite	NT	NT	Fast progression	*
MT18	F	IV	Surgery of lung metastasis and transient metastases	No	3 (-)	6 (+)	23	33
MT19	M	Uveal MM IV	Local radiotherapy	Slight migraine	(-)	(-)	0	5
MT20	F	Uveal MM IV	Local radiotherapy	No	2 (-)	7 (+)	6	10
MT21	F	IV	Surgery of transient metastasis and local radiotherapy	No	(-)	7 (+)	41	41
MT22	M	IV	IL-2	No	NT	NT	0	*
MT23	M	IV	Local radiotherapy + IL-2	No	6 (+)	2 (-)	7	11
MT24	M	IV	IL-2	No	8 (+)	(-)	7	12
MT25	M	IV	Local radiotherapy	No	NT	NT	0	*
MT26	M	IV	Surgery of compromised area + IL-2	No	7 (+)	14 (+)	39	39
MT27	M	IV	None	Slight migraine	3 (-)	5 (+)	39	39
MT28	F	IV	Local radiotherapy	No	6 (+)	10 (+)	0	5
MT29	M	IV	Surgery of lung and in transit metastasis	No	8 (+)	16 (+)	36	36
MT30	F	IV	Surgery of liver metastasis + local radiotherapy	Tiredness	NT	7 (+)	35	35
MT31	F	IV	Surgery of metastasis + local radiotherapy	Low flu-like symptoms	5 (+)	(-)	22	35
MT32	M	IIIC	Local radiotherapy	No	10 (+)	20 (+)	35	35
MT33	M	IIIC	None	No	6 (+)	40 (+)	33	33
MT34	F	IV	None	No	(-)	3 (-)	7	15
MT35	F	IV	IFN- α , surgery of transient metastasis	No	(-)	(-)	22	27
MT36	F	IV	Local radiotherapy	No	9 (+)	(-)	7	16
MT37	M	IV	None	No	(-)	(-)	0	3
MT38	F	IV	None	Local erythema	9 (+)	17 (+)	10	15
MT39	F	IV	None	Local erythema	(-)	(-)	7	15
MT40	M	IV	Local radiotherapy	No	3 (-)	(-)	0	3
MT41	M	IV	None	No	5 (+)	10 (+)	18	20
MT42	M	IV	Local radiotherapy	No	6 (+)	3 (-)	0	6
MT43	F	IV	Surgery of transient metastasis	No	5 (+)	07 (+)	12	16
MT44	F	IV	Surgery of local tumor mass + local radiotherapy	No	6 (+)	10 (+)	2	8
MT45	M	IV	Surgery of local tumor mass + local radiotherapy	No	5 (+)	7 (+)	11	11
MT46	M	IV	None	No	(-)	(-)	0	6
MT47	F	IV	None	No	NT	NT	0	6
MT48	F	IV	Surgery of in transit metastasis	No	5 (+)	6 (+)	0	7
MT49	M	IV	Surgery of tumor compromised tissues and local radiotherapy	No	5 (+)	6 (+)	5	5
MT50	F	IV	Surgery of tumor compromised tissues and local radiotherapy	No	6 (+)	7 (+)	3	3

Abbreviations: DTH, delayed type IV hypersensitivity; DTH CTRL, DTH against KLH or/and antigens of *Streptococcus*; PFS, progression-free survival; F, female; M, male; NA, not available; NT, not tested.

*Deceased before complete treatment.

RESULTS

Patient Characteristics and Postvaccination Survival

Fifty melanoma patients, 43 stage IV and seven stage III, high-risk patients, according to American Joint Committee on Cancer (AJCC) criteria, were included in the protocol (Table 1). The median follow-up period was 38 months. Only 11 patients (22%) showed mild secondary reactions, including local pain at the inoculation zone and/or mild fever; these reactions were more common in patients receiving IL-2. No adverse reaction was detected in the rest of patients (Table 1).

The overall median survival of the stage IV patients ($n = 43$) was 15 months (Fig 1A), and the 5-year estimated survival of 12% (Fig 1A). Furthermore, the PFS rate after the first immunization was 7 months (Fig 1B). All seven stage III patients remained free from tumor relapse during a median follow-up period of 48 months (range, 33 to 64 months; Table 1 and Fig 1A, dotted line). Median survival was directly related with disease severity according to AJCC classification: median survival of 7 months in patients with M1c disease ($n = 15$), 20.5 months in patients with M1b disease ($n = 13$), and 27 months in patients with M1a disease ($n = 15$; $P = .0246$; Fig 1C).

Melanoma-Specific Immune Responses

DTH reactions against TRIMEL ≥ 5 mm in diameter were detected in 62.2% of vaccinated patients (28 of 45). The remaining patients had no reaction ($n = 14$) or a reaction less than 5 mm ($n = 3$; Table 1). Positive DTH reactions against the TRIMEL and/or control antigens were observed in 80% of tested patients (Table 1). TRIMEL-specific DTH responder (DTH positive) and nonresponder (DTH negative) did not differ in age, sex, HLA haplotype, primary tumor or metastasis location, type of vaccine (long- or short-time produced DCs), hematologic condition, or DTH response against control antigens (Table 2). Since all stage III patients were DTH positive (Table 2), the entire group was excluded from the analysis of DTH response-associated survival. Interestingly, survival curves of stage IV/DTH-positive patients were three-fold more prolonged than IV/DTH-negative patients (Fig 2A). Stage IV/DTH-positive patients had a median survival of 33 months compared with 11 months for the stage IV/DTH-negative patients ($P = .0014$; Fig 2A). PFS rates for stage IV/DTH positive was 22 months after vaccination compared with 7 months for stage IV/DTH-negative patients ($P = .0017$; Fig 2B). These groups did not differ in clinical status or treatment strategy (Table 2).

We have previously observed increased IFN- γ secretion in PBMCs from postvaccinated patients.²² Here, patients whose PBMCs showed IFN- γ production against melanoma cell lines also recognized MAAs; and in at least two cases, the tested PBMCs also recognized autologous melanoma cells in vitro (online only Appendix Fig A1A). However, no correlation between survival and ex vivo IFN- γ production was noticed (Fig A1B).

Regulatory T-Cell Populations in Patients

The frequency of RTL populations; CD4+foxp3+ T cells (Treg), CD4+TGF β + T cells (TH3), and CD4+IL-10 producer T cells (TR1) was independently studied in DTH-positive and DTH-negative patients. Similar frequency of RTLs was detected in the PBMC of both groups before treatments (Figs 3 and 4). However, the frequency of Treg slightly but significantly decreased in DTH-positive patients

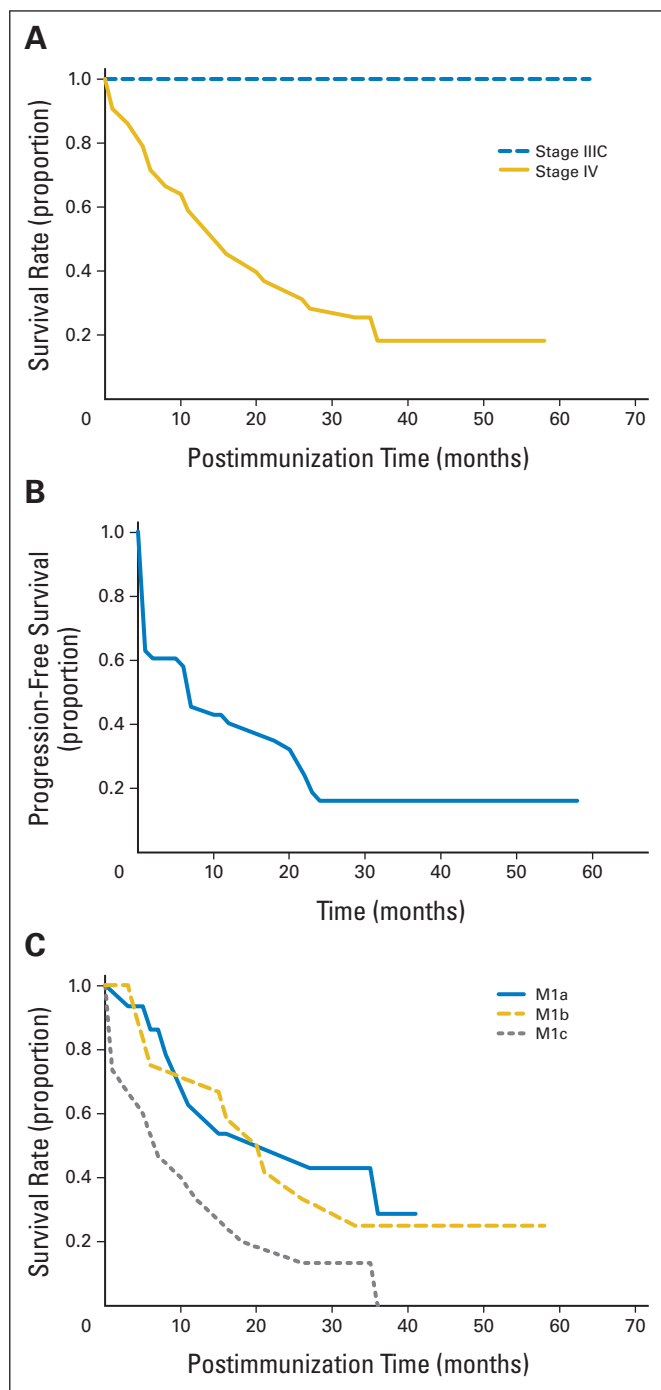


Fig 1. Postimmunotherapy survival and progression-free survival (PFS) according to the stage of disease and metastasis type of malignant melanoma (MM) patient. Stage III and IV MM patients (A) survival curves, and stage IV patients (B) PFS curve, after immunotherapy with autologous DC pulsed with TRIMEL ($n = 43$). (C) Stage IV MM patient survival curves comparing the different metastases categories. M1a, patients with in transit skin metastases ($n = 15$); M1b: patients with lung metastasis ($n = 13$); and M1c: patients with metastases in organs other than lungs ($n = 15$). The differences between M1c survival curves and both M1a and M1b curves are statistically significant ($P < .05$).

after vaccinations from 7.15% to 6.39% ($P = .0061$; Fig 3A, left). On the contrary, DTH-negative patients significantly increased the frequency of Treg lymphocytes after two and four immunizations (from 5.32%, to 5.98%, and 6.28%, respectively; $P < .0001$; Fig 3A, right). A differential evolution of TR1 frequency was also observed. While

Table 2. Demographic Characteristics of Patients Included in the Study

Variable	DTH Negative	DTH Positive	P
DTH to TRIMEL (n = 45)	17	28	NA
DTH to KLH or <i>Streptococcus</i> (n = 42)			.0581*
Positive	7	5	
Negative	8	22	
Sex (n = 45)			.0672‡
Male	8	15	
Female	9	13	
Mean of age, years	54.8	47.4	.0611†
SD	3.7	2.8	
AJCC criteria (n = 45)			
Stage III	0	7	.02609*
Stage IV	17	21	NA
Clark level (n = 29)			.0298‡
II	1	0	
III	4	3	
IV	7	14	
Breslow mean, mm (n = 26)	3.73	2.96	.1272†
SD	0.59	0.37	
Uveal melanoma (n = 45)	1	1	.61818*
HLA-A2+ (n = 25)	8	17	.0126‡
Primary tumor location (n = 43)			.0933‡
Extremities	8	11	
Head and neck	4	6	
Thorax	5	9	
Metastasis (n = 45)			.0354‡
M1a (transit skin)	6	15	
M1b (lung)	5	8	
M1c (other organs)	6	5	
Previous treatments (n = 4)			.4887*
IL-2	1	1	
IFN- α	1	1	
Treatment with IL-2 (n = 10)	4	7	.6024*
Treatment with short-time produced DCs (n = 10)	4	7	.5382*
Coadjuvant (n = 45)			.0330‡
AI (OH) ₃	4	4	
KLH	12	18	
Both	1	6	
Secondary effects	5	5	.2931*
ELISPOT to IFN- γ (n = 20)			.1749*
Positive	5	5	
Negative	2	8	
Retired from protocol	1	0	.3777*

Abbreviations: DTH, delayed type IV hypersensitivity; SD, standard deviation; AJCC, American Joint Committee on Cancer; IL, interleukin; IFN, interferon; DC, dendritic cells.
*Fisher's exact test.
†t test.
‡ χ^2 test.

DTH-positive patients showed a constant frequency of TR1 cells (2.61% before; 2.71% after two and 2.70% after four immunizations; Fig 3B, left), DTH-negative patients showed a slight, but significant increase of TR1 cells from 2.89% to 3.05% after two and 3.98% after last immunization ($P = .0017$; Fig 3B, right).

TH3 lymphocytes, which express membrane-bound TGF- β , strikingly differed between DTH-positive and DTH-negative patients (Fig 4). Although DTH-positive patients underwent transient increases in their TH3 frequency after the second immunization (from 2.41% to 4.25%), they finally showed a sharp fall to an average of 1.54% after the fourth vaccination, representing a total of 40% de-

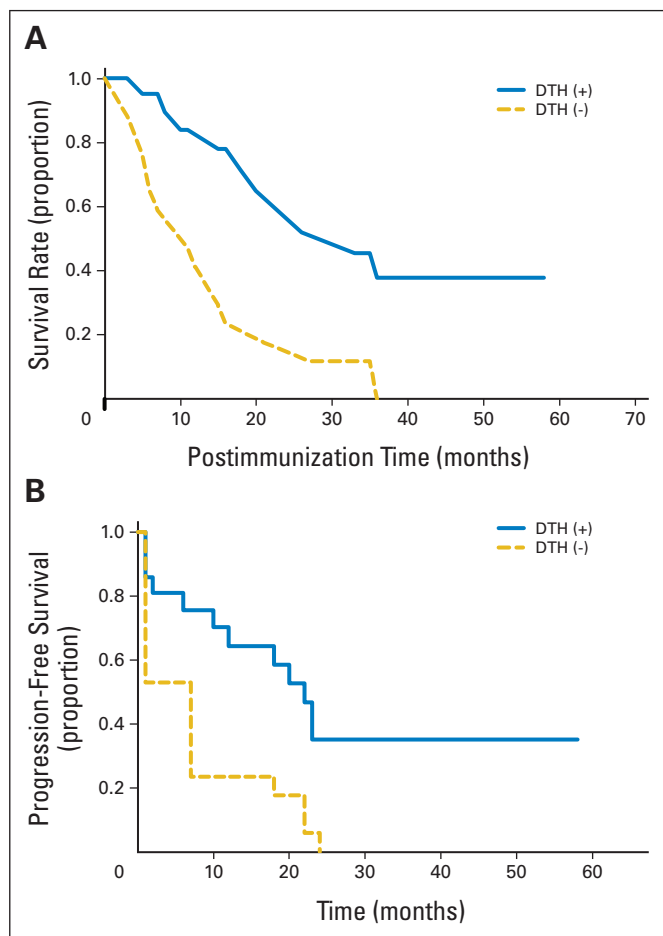


Fig 2. Postimmunotherapy survival, progression-free survival (PFS), and delayed type IV hypersensitivity (DTH) response of stage IV malignant melanoma (MM) patients. (A) Stage IV MM patients survival curves and (B) patients PFS curves after immunotherapy with autologous dendritic cells (DCs) pulsed with TRIMEL and grouped according to their DTH response; n = 21 DTH positive; n = 17 DTH negative. The differences between the survival curves in both graphs are statistically significant ($P < .01$).

crease respect to prevaccination status ($P = .0065$; Fig 4, left). In contrast, DTH-negative patients showed a constant rise in the frequency of TH3 cells from 2.79% before, to 4.23% after two and 5.78% after four immunizations, meaning a final two-fold increase in the TH3 population respect to prevaccine status ($P = .00001$; Fig 4, right). To conclude, we found that DTH-negative patients had four times higher frequency of TH3 cells (5.78%) compared with DTH-positive patients (1.54%) after treatment ($P < .00001$; Fig 4), while the proportion of total CD4+ T cells did not differ significantly between the DTH-positive and DTH-negative groups during the time points analyzed (data not shown).

DISCUSSION

The current work is a follow-up phase II study that confirms the effectiveness of TRIMEL-loaded DC-based immunotherapy to treat stage III and IV melanoma patients. To our knowledge, this is the first report demonstrating a correlation between positive immune response induced by DC vaccination and improved long-term patient survival in late-stage melanoma patients. Furthermore, our results suggest that DTH reaction against TRIMEL antigens constitute an

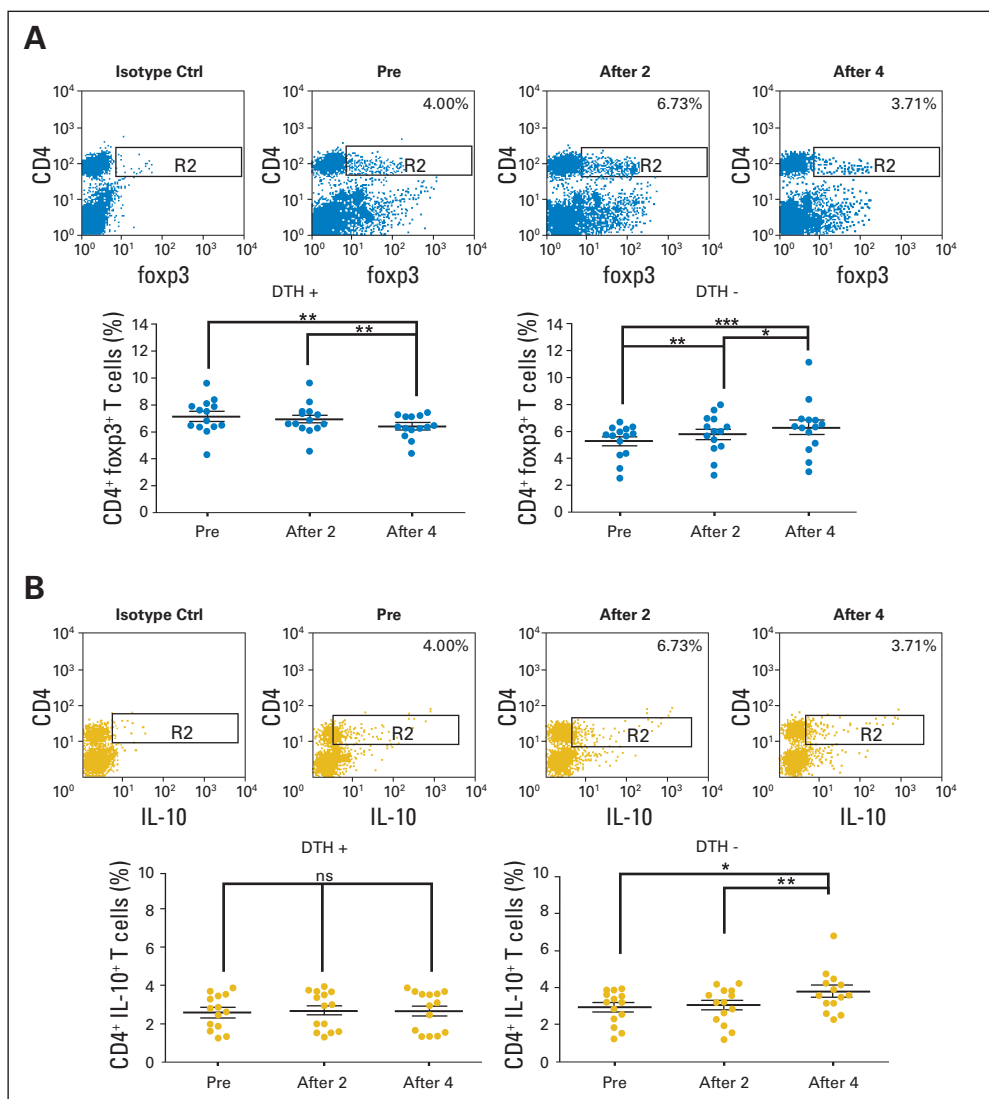


Fig 3. Variation of CD4⁺foxp3⁺ T cells (Treg) and CD4⁺ IL-10 producer T cells (TR1) proportions in delayed type IV hypersensitivity (DTH)-positive and DTH-negative patients during treatment. Peripheral blood mononuclear cells (PBMC) of DTH-positive and DTH-negative patients were collected before vaccination (pre), after the second immunization (after 2), and after a complete vaccination cycle (after 4), and were analyzed by flow cytometry. Upper dot-plots show the analysis on one patient, as an example, and the lower graphs show the analysis of 28 patients (14 DTH positive and 14 DTH negative). NS, not statistically significant. (*) $P < .05$; (**) $P < .01$; (***) $P < .001$.

excellent clinical response predictor, since this immune reaction directly correlates with patient survival and lack of disease progression. In addition, our results show for the first time, the impact of DCs immunization on RTL populations, particularly in the frequency of CD4⁺TGFβ⁺ T cells and its relationship to the treatment outcome.

Until now, several studies have shown encouraging data and conclusions based on increases in antivaccine immunity, but none have been accompanied by durable clinical improvements in metastatic disease.^{8-11,14} This lack of clinical efficacy observed in vaccine-based clinical trials has caused much disappointment in the medical and scientific community.^{27,28} Furthermore, the use of conventional oncologic definitions for clinical responses, although useful for the measurement of therapy outcome, results evidently insufficient as a unique parameter to evaluate the clinical benefits of tumor-disseminated cancer patients. In this study, although only sporadically objective clinical responses were observed (online-only Appendix Table A1), significant prolonged survivals were achieved and detected. Our findings are in line with other studies where increased patient survival does not correlate to potent objective tumor regressions.^{29,30}

Clinical trials using synthetic peptides as antigens have failed to produce objective clinical responses or improvements in patient survival,^{8,11,14} probably due to tolerance induction by high affinity peptides, tumor escape by clones lacking antigen expression, or absence of immunologic danger signals associated to the antigens. In contrast, DCs fused or loaded with autologous tumor or tumor lysate have been used to induce stronger and more extensive immunologic responses against tumors.^{15-18,29-31} However, those approaches are limited to a reduced proportion of patients having tumors at accessible sites. For that reason, we designed a protocol that included TRIMEL, which not only provides a standardized and widely applicable source of specific MAAs, but also induces an enhanced in vitro maturation of DCs, as previously described.^{22,32}

Recently, numerous evidences indicate that danger signals, mediated by damage-associated molecular patterns (DAMPs), are delivered by stressed or dying tumor or stroma cells, thereby activating DCs.³³⁻³⁶ The presence of DAMPs in TRIMEL may explain the improved in vivo immunogenicity induced by our DCs in several treated patients. In fact, 62% of patients showed DTH reactivity

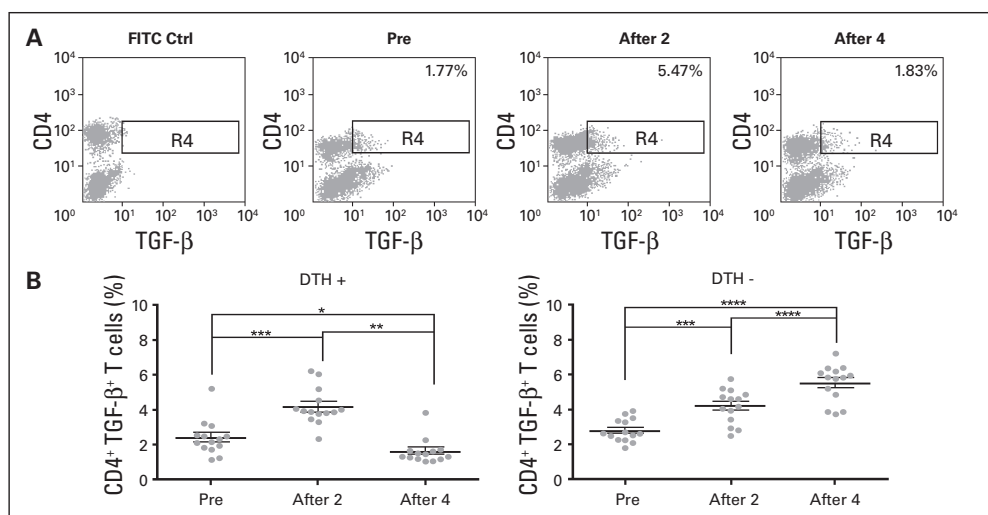


Fig 4. Transforming growth factor (TGF)- β producing T-cell proportions in delayed type IV hypersensitivity (DTH)-positive and DTH-negative patients after TRIMEL/DC treatment. Peripheral blood mononuclear cells (PBMC) of DTH-positive and DTH-negative patients were collected before vaccination (pre), after the second immunization (after 2) and after a complete vaccination cycle (after 4) were intracellularly stained and analyzed by flow cytometry. Upper dot-plots show the analysis on one patient, as an example, and the lower graphs show the analysis of 28 patients (14 DTH positive and 14 DTH negative). NS, not statistically significant. (*) $P < .05$; (**) $P < .01$; (***) $P < .001$.

against TRIMEL after one complete cycle of vaccination. DTH reactivity against control antigens in most of patients discard that the absence of TRIMEL-specific DTH response was related to a general immunosuppressive status (Table 1). The specificity of the immune response induced by our therapy can also be indirectly deduced by analysis of patient survival. Indeed, the postvaccination median survival of stage IV patients in our study was 4 to 9 months longer than the overall median survival observed in published analyses of stage IV patients.^{1-3,14,37} Although increased survival appears to be independent of vaccine-induced tumor regressions, DTH-positive patients with minimal tumor mass, such as stage III patients or surgically pretreated stage IV patients showed the best clinical performance, supporting other findings that combined tumor resection and adjuvant immunotherapy.³⁸ Remarkably, seven stage III patients remained tumor free for a follow-up median of 48 months after immunizations, when only 30% to 40% patients in that stage was expected to survive according to AJCC (online-only Appendix Fig A2). All these evidences suggest that the specific immune response induced by TRIMEL/DC vaccine impact patients' general condition and quality of life by controlling tumor metastatic dissemination rather than by destruction of established tumor.

Demographic comparison between DTH-positive and DTH-negative groups indicates that prolonged survival of DTH-positive patients is due to treatment effects rather than patient selection (Table 2). Additional evidence, concerning vaccine-induced immune response and survival, was obtained studying the differential frequency of RTL in patients. In fact, the marked reduction in the frequency of TH3 cells, and the slight decrease in Treg cells in DTH-positive patients compared with increased RTL in DTH-negative patients imply that the basal immunological status of patient, or maybe variations in genes associated to the immune response pathway play an important role in the development of a more immunogenic versus a more tolerogenic response. Beside the increased Treg frequency observed in ovarian cancer patients,^{23,24} it has been recently demonstrated that TGF β is overexpressed in melanoma patients compared with healthy donors and that increased levels can be induced by immunization with MAA-associated gp96.³⁹ Elevated concentration of TGF β has been correlated with the specific inhibition of cytotoxic T lymphocytes and natural killer cell-mediated cytotoxicity.^{40,41} Tumor cells, suppressor

T lymphocytes, and a new subset of myeloid CD14⁺ cells may be responsible for this cytokine production.⁴² The correlation between melanoma-specific DTH-positive reaction and the reduction of TGF β -expressing T cells indicates that these cells are able to limit the immunologic antimelanoma response. Moreover, the level of soluble TGF β and/or TH3 cells in melanoma patients potentially constitutes an easy and relevant prediction factor for the outcome of DC-based therapy.

This study raises important questions about future trials. It may be reasonable to prospectively evaluate the real potential of TRIMEL/DC-based therapy in a randomized and controlled phase III protocol, in terms of survival and adverse effects, in order to unambiguously establish the clinical benefit of cellular immunotherapy in melanoma.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Mercedes N. López, Carlos Ferrada, Flavio Salazar-Onfray
Financial support: Flavio Salazar-Onfray
Administrative support: Flavio Salazar-Onfray
Provision of study materials or patients: Mercedes N. López, Rodrigo González, Milton Larrondo, Alvaro Compán, Carlos Ferrada
Collection and assembly of data: Mercedes N. López, Cristian Pereda, Gabriela Segal, Raquel Aguilera, Fermín E. González, Alejandro Escobar, Rodrigo González
Data analysis and interpretation: Mercedes N. López, Cristian Pereda, Gabriela Segal, Leonel Muñoz, Raquel Aguilera, Fermín E. González, Alejandro Escobar, Diego Reyes, Ariadna Mendoza-Naranjo, Carlos Ferrada, Flavio Salazar-Onfray
Manuscript writing: Leonel Muñoz, Alexandra Ginesta, Ariadna Mendoza-Naranjo, Flavio Salazar-Onfray
Final approval of manuscript: Mercedes N. López, Cristian Pereda, Gabriela Segal, Leonel Muñoz, Raquel Aguilera, Fermín E. González, Alejandro Escobar, Alexandra Ginesta, Diego Reyes, Rodrigo González, Ariadna Mendoza-Naranjo, Milton Larrondo, Alvaro Compán, Carlos Ferrada, Flavio Salazar-Onfray

REFERENCES

1. Balch CM, Soong SJ, Gershenwald JE, et al: Prognostic factors analysis of 17,600 melanoma patients: Validation of the American Joint Committee on Cancer melanoma staging system. *J Clin Oncol* 19:3622-3634, 2001
2. Eigentler TK, Caroli UM, Radny P, et al: Palliative treatment of disseminated malignant melanoma: A systematic review of 41 randomised clinical trials. *Lancet Oncol* 4:748-759, 2003
3. Eggermont MM: Randomized trials in melanoma: An update. *Surg Oncol Clin N Am* 15:439-451, 2006
4. Boon T, Coulie PG, Van den Eynde BJ, et al: Human T cell responses against melanoma. *Annu Rev Immunol* 24:175-208, 2006
5. Quirt I, Verma S, Petrella T, et al: Temozolomide for the treatment of metastatic melanoma: A systematic review. *Oncologist* 12:1114-1123, 2007
6. Falkson CI, Ibrahim J, Kirkwood JM, et al: Phase III trial of dacarbazine versus dacarbazine with interferon alpha-2b versus dacarbazine with tamoxifen versus dacarbazine with interferon alpha-2b and tamoxifen in patients with metastatic malignant melanoma: An Eastern Cooperative Oncology Group study. *J Clin Oncol* 16:1743-1751, 1998
7. Keilholz U, Punt CJ, Gore M, et al: Dacarbazine, cisplatin, and interferon-alfa-2b with or without interleukin-2 in metastatic melanoma: A randomized phase III trial (18951) of the European Organisation for Research and Treatment of Cancer Melanoma Group. *J Clin Oncol* 23:6747-6755, 2005
8. Nestle FO, Aljaghi S, Gilliet M, et al: Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 4:328-332, 1998
9. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al: Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* 4:321-327, 1998
10. O'Rourke MG, Johnson MK, Lanagan CM, et al: Dendritic cell immunotherapy for stage IV melanoma. *Melanoma Res* 17:316-322, 2007
11. Ridgway D: The first 1000 dendritic cell vaccines. *Cancer Invest* 21:873-886, 2003
12. Porgador A, Gilboa E: Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. *J Exp Med* 182:255-260, 1995
13. Paglia P, Chiodoni C, Rodolfo M, et al: Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. *J Exp Med* 183:317-322, 1996
14. Schadendorf D, Ugurel S, Schuler-Thurner B, et al: Dacarbazine versus vaccination with autologous peptide-pulsed dendritic cells in first-line treatment of patients with metastatic melanoma: A randomized phase III trial of the DC study group of the DeCOG. *Ann Oncol* 17:563-570, 2006
15. Kyte JA, Kvalheim G, Lislerud K, et al: T cell responses in melanoma patients after vaccination with tumor-mRNA transfected dendritic cells. *Cancer Immunol Immunother* 56:659-675, 2007
16. Trefzer U, Herberth G, Wohlan K, et al: Tumour-dendritic hybrid cell vaccination for the treatment of patients with malignant melanoma: Immunological effects and clinical results. *Vaccine* 23:2367-2373, 2005
17. O'Rourke MG, Johnson M, Lanagan C, et al: Durable complete clinical responses in a phase I/II trial using an autologous melanoma cell/dendritic cell vaccine. *Cancer Immunol Immunother* 52:387-395, 2003
18. Nagayama H, Sato K, Morishita M, et al: Results of a phase I clinical study using autologous tumor lysate-pulsed monocyte-derived mature dendritic cell vaccinations for stage IV malignant melanoma patients combined with low dose interleukin-2. *Melanoma Res* 13:521-530, 2003
19. Palucka AK, Ueno H, Connolly J, et al: Dendritic cells loaded with killed allogeneic melanoma cells can induce objective clinical responses and MART-1 specific CD8+ T-cell immunity. *J Immunother* 29:545-557, 2006
20. Vilella R, Benítez D, Milà J, et al: Pilot study of treatment of biochemotherapy-refractory stage IV melanoma patients with autologous dendritic cells pulsed with a heterologous melanoma cell line lysate. *Cancer Immunol Immunother* 53:651-658, 2004
21. Salcedo M, Bercovici N, Taylor R, et al: Vaccination of melanoma patients using dendritic cells loaded with an allogeneic tumor cell lysate. *Cancer Immunol Immunother* 55:819-829, 2006
22. Escobar A, López M, Serrano A, et al: Dendritic cell immunizations alone or combined with low doses of interleukin-2 induce specific immune responses in melanoma patients. *Clin Exp Immunol* 142:555-568, 2005
23. Curiel TJ, Coukos G, Zou L, et al: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10:942-949, 2004
24. López M, Aguilera R, Pérez C, et al: The role of regulatory T lymphocytes in the induced immune response mediated by biological vaccines. *Immunobiology* 211:127-136, 2006
25. Dudley ME, Wunderlich JR, Yang JC, et al: Adoptive cell transfer therapy following non-myceloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 23:2346-2357, 2005
26. Antony PA, Piccirillo CA, Akpınarli A, et al: CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol* 174:2591-2601, 2005
27. Rosenberg SA, Yang JC, Restifo NP: Cancer immunotherapy: Moving beyond current vaccines. *Nat Med* 10:909-915, 2004
28. Andrews DM, Maraskovsky E, Smyth MJ: Cancer vaccines for established cancer: How to make them better? *Immunol Rev* 222:242-255, 2008
29. Hersey P, Menzies SW, Halliday GM, et al: Phase I/II study of treatment with dendritic cell vaccines in patients with disseminated melanoma. *Cancer Immunol Immunother* 53:125-134, 2004
30. Morton DL, Foshag LJ, Hoon DS, et al: Prolongation of survival in metastatic melanoma after active specific immunotherapy with a new polyvalent melanoma vaccine. *Ann Surg* 216:463-482, 1992
31. Ridolfi R, Petrini M, Fiammenghi L, et al: Improved overall survival in dendritic cell vaccination-induced immunoreactive subgroup of advanced melanoma patients. *J Transl Med* 4:36, 2006
32. Mendoza-Naranjo A, Saéz PJ, Johansson CC, et al: Functional gap junctions facilitate melanoma antigen transfer and cross-presentation between human dendritic cells. *J Immunol* 178:6949-6957, 2007
33. Matzinger P: Friendly and dangerous signals: Is the tissue in control? *Nat Immunol* 8:11-13, 2007
34. Bianchi ME: PAMPs and alarmins: All we need to know about danger. *J Leukoc Biol* 81:1-5, 2007
35. Sauter B, Albert ML, Francisco L, et al: Consequences of cell death: Exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 191:423-434, 2000
36. Chen Z, Moyana T, Saxena A, et al: Efficient antitumor immunity derived from maturation of dendritic cells that had phagocytosed apoptotic/necrotic tumor cells. *Int J Cancer* 94:539-548, 2001
37. Khan MA, Andrews S, Ismail-Khan R, et al: Overall and progression-free survival in metastatic melanoma: Analysis of a single-institution database. *Cancer Control* 13:211-217, 2006
38. Young SE, Martinez SR, Essner R: The role of surgery in treatment of stage IV melanoma. *J Surg Oncol* 94:344-351, 2006
39. Filipazzi P, Valenti R, Huber V, et al: Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol* 25:2546-2553, 2007
40. Lee JC, Lee KM, Kim DW, et al: Elevated TGF-beta1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. *J Immunol* 172:7335-7340, 2004
41. Thomas DA, Massagué J: TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 8:369-380, 2005
42. Salazar-Onfray F, López MN, Mendoza-Naranjo A: Paradoxical effects of cytokines in tumor immune surveillance and tumor immune escape. *Cytokine Growth Factor Rev* 18:171-182, 2007

Acknowledgment

We thank Miguel O'Ryan, MD, and Andrea Villablanca, PhD, for critical review of the manuscript, and Manuel Salazar and Marisol Briones for technical help.

Table A1. Characteristics of Patients and Objective Clinical Response to Therapy

Code	Clark/Breslow (mm)	Primary Tumor Location	Metastasis	Vaccine Type	Objective Clinical Response	HLA-A2
MT01	NA	Right upper thorax	Transient in mammary gland and pulmonary nodules	First cycle DC + ALOH/ second DC + KLH	Regression of a single SC metastasis in mammary gland (PR)	(+)
MT02	Clark III, Breslow 4.8	Left thigh	Left inguinal lymph nodes, lung and skin	DC + ALOH	Regression of one skin metastasis after vaccination (PR)	(-)
MT03	Clark IV, Breslow 2.3	Left shoulder	Lung and liver	First cycle DC + ALOH; second DC + KLH + IL-2	No	(+)
MT04	Clark IV, Breslow NA	Right cheekbone	Cervical lymph nodes and right malar bone	DC + KLH	No	(+)
MT05	Breslow 0.98 mm, Clark NA	Left shoulder	Negative cervical nodes and positive armpit (4) lymph nodes	DC + KLH	No	(-)
MT06	Clark IV, Breslow NA	Left knee	Lung with multiple pleural metastases	DC + KLH	No	(+)
MT07	Breslow 4.3, Clark NA	Back	Armpit lymph nodes, liver	DC + KLH	No	(+)
MT08	Clark III, Breslow 2	Left foot sole	Left inguinal lymph nodes and multiple in transit metastasis	DC + KLH + IL2 two complete cycles	No	(+)
MT09	Clark IV, Breslow 2.9	Scalp	Lung metastasis	DC + KLH + IL-2	No	(+)
MT10	Primary not found	Primary not located	Positive inguinal sentinel node, positive popliteal lymph nodes (4) and soft tissues affected	First cycle DC + ALOH, second cycle DC + KLH + IL2	No	(+)
MT11	Not apply	Retro-orbital	Liver, multiple bone metastasis	DC + KLH	No	ND
MT12	Clark IV, Breslow NA	Right foot sole	Liver	DC + ALOH + IL-2	No	(-)
MT13	Clark III, Breslow NA	Left cheekbone	Infra parotid lymph node and left lower jugular group	DC + KLH + IL-2	No	(-)
MT14	Clark NA, Breslow 3.6	Left shoulder	Left cervical lymph nodes 4/9	DC + KLH + IL-2	No	(-)
MT15	Clark IV, Breslow NA	Abdomen	Left inguinal affected lymph nodes, in transit metastasis	DC + KLH + IL-2	No	(+)
MT16	Clark III, Breslow NA	Left thigh	Left inguinal lymph nodes 2/12	DC + KLH	No	(+)
MT17	Clark IV, Breslow NA	Left cheek	Multiple brain metastases, regional sub scapular	DC + KLH + IL-2	No	(-)
MT18	Clark IV, Breslow 2.11	Left ankle	Lung and transient metastasis	DC + KLH + ALOH	No	(+)
MT19	Not apply	Right eye	Lung, liver, scalp, and retroperitoneal	DC + KLH	No	(+)
MT20	Not apply	Right eye	Local orbital; liver micro metastasis	DC + KLH	No	(+)
MT21	Clark IV, Breslow NA	Scalp	Left cervical Lymph nodes and scalp transient metastasis	DC + KLH + ALOH	No	(+)
MT22	Clark IV Breslow NA	Right big toe	Lung and liver	DC + ALOH + IL-2	No	(+)
MT23	Clark IV, Breslow NA	Right cheek	Regional lymph nodes, right orbital	DC + ALOH + IL-2	No	(-)
MT24	Clark III, Breslow 3.5	Left dorsal shoulder	Left armpit lymph nodes 4 of 10 with surrounded compromised tissues and local infiltration; develops brain metastasis	DC + ALOH + IL-2	No	(-)
MT25	Clark NA, Breslow 5.5	Right shoulder	Right cervical lymph node; brain metastasis	DC + KLH	No	(+)
MT26	Breslow 5.6, Clark NA	Left thigh	Left inguinal area; lymph nodes, Compromise of local soft tissues; muscle compromise and transient metastasis	DC + ALOH + IL-2	No	(+)
MT27	Clark NA, Breslow 1.3	Supra umbilical abdomen	Left upper lobe lung small metastasis	DC + KLH	Left upper lobe lung small metastasis regression (PR)	(-)
MT28	Clark NA, Breslow 1.5	Vulva	Right inguinal lymph nodes, lung	DC + ALOH	No	(+)
MT29	Clark IV, Breslow 3.3	Peri umbilical abdomen	Transient metastasis under left nipple, single lung metastasis	DC + ALOH	No	(-)
MT30	Clark IV, Breslow 2.2	Ventral left knee	Regional inguinal and popliteal lymph nodes; single liver metastasis	DC + ALOH	No	(+)
MT31	Not apply	Right eyelid	Local orbit metastasis and cervical lymph nodes	DC + ALOH + KLH	No	(+)
MT32	Clark III, Breslow 2.5	Ventral left forearm	Compromised armpit lymph nodes 7/10 and soft tissues	DC + KLH + ALOH	No	NT
MT33	Clark IV, Breslow 2.3	Right calf	Right inguinal lymph node chain (1 of 10) and local tissue compromised	DC + KLH	No	(+)
MT34	Acral melanoma	Right big toe	Scalp transient metastasis. Cervical lymph nodes(3/23), lung metastasis	First cycle DC + KLH; second cycle rDC + KLH	No	(-)

(continued on following page)

Prolonged Survival of DC-Treated Melanoma Patients

Table A1. Characteristics of Patients and Objective Clinical Response to Therapy (continued)

Code	Clark/Breslow (mm)	Primary Tumor Location	Metastasis	Vaccine Type	Objective Clinical Response	HLA-A2
MT35	Clark IV, Breslow 0.88	Left dorsal calf	Transient metastasis	DC+KLH	No	(+)
MT36	Clark IV, Breslow 4,5	Right dorsal calf	Right leg transient metastasis, left cervical lymph nodes, lung metastasis	DC+KLH	No	NT
MT37	NA/NA	Right anterior neck	Left cervical lymph nodes, transient metastasis in face and cervical area	DC+KLH	No	(-)
MT38	Breslow 5.3, Clark NA	Right dorsal shoulder	Cervical, lung, gall bladder, brain, subcutaneous	DC+KLH	No	(+)
MT39	Clark III, Breslow 4	Ventral right foot	Right inguinal lymph nodes, extensive local compromise and in transit metastasis	DC+KLH	No	(-)
MT40	Clark NA, Breslow 6.2	Left dorsal thorax	Multiple lung, brain and cranial vault metastasis	DC+KLH	No	(+)
MT41	NA	Right retro auricular	Cervical lymph node, lung	DC+KLH	No	(-)
MT42	Clark II, Breslow NA	Right groin	Right inguinal lymph nodes, lung single metastasis, multiple brain metastases	rDC+KLH	No	(-)
MT43	Clark IV, Breslow 3.8	Right heel	Right inguinal lymph node, soft tissues compromised and muscle of inguinal area, transient metastasis	rDC+KLH	Partial regression of skin metastases (PR)	NT
MT44	Clark IV, Breslow 5.7	Left shoulder	Right cervical armpit, and left inguinal lymph nodes; local cervical area soft tissues and muscle extensive compromised	rDC+KLH	Local tumor regression (PR)	(+)
MT45	Primary unknown	Not determined	Local extend compromised sternocleidomastoid muscle	rDC+KLH	No	NT
MT46	Clark IV, Breslow 6	Back	Lung multiple metastasis	rDC+KLH	No	(+)
MT47	NA	Left ankle	Multiple transient metastasis in left lower limb and brain metastasis	rDC+KLH	No	(+)
MT48	Clark IV ulcerated, Breslow 2	Left forearm	Extensive transient metastasis with compromised ipsi lateral mammary gland	rDC+KLH	No	(+)
MT49	NA	Back	Left armpit lymph node metastasis with extensive extra nodular compromise	rDC+KLH	No	NT
MT50	NA	Left foot sole	Left inguinal lymph nodes and transient metastasis	rDC+KLH	No	NT

Abbreviations: NA, not available; DC, dendritic cells; KLH, keyhole limpet hemocyanin; ALOH, aluminum hydroxide; rDC, short-time produced DCs; PR, partial response; IL-2, interleukin-2; NT, not tested.

López et al

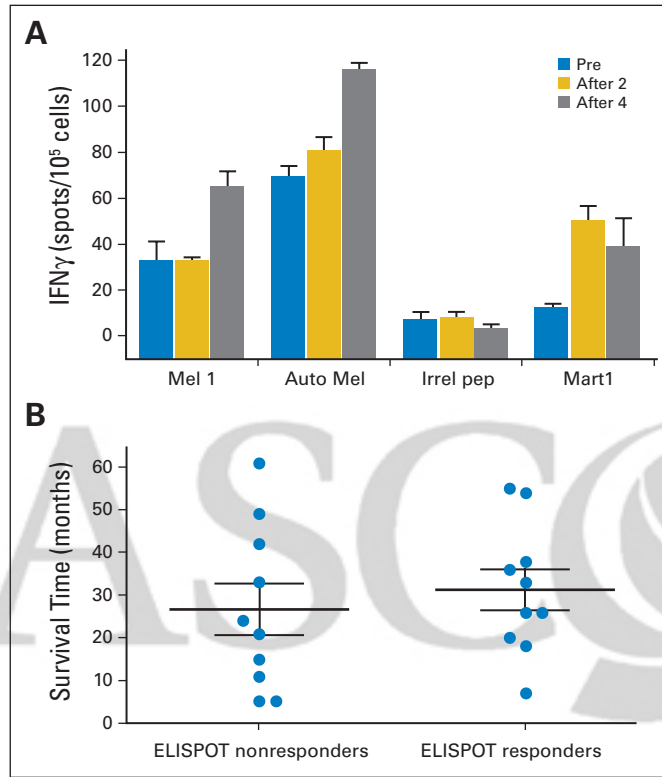


Fig A1. Melanoma-specific interferon (IFN)-γ production by peripheral blood mononuclear cells (PBMC) and correlation to improved survival of malignant melanoma (MM) patients. (A) IFN-γ production against an allogeneic melanoma, autologous melanoma cells, and MART1/MelanA antigen by pre- and postimmunization PBMC obtained from patient MT38. (B) Correlation between post vaccination IFN-γ response and median of survival of MM patients. Elispot responder refers to patients recognizing at least two different allogeneic melanomas. Elispot nonresponder refers to patients no recognizing any of three different allogeneic melanomas. Differences of survival between groups are not statistically significant.

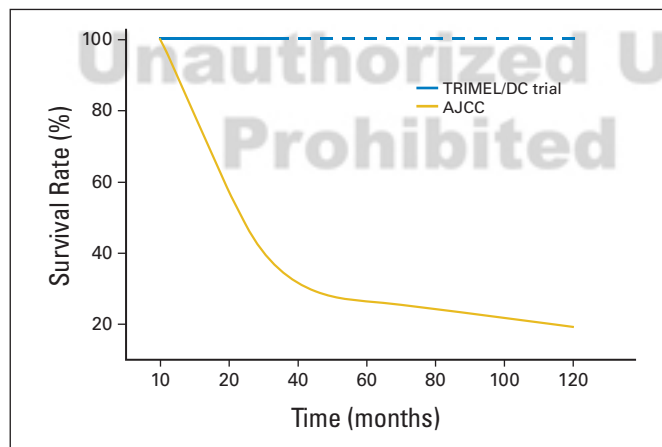


Fig A2. Survival rate of stage III malignant melanoma (MM) patients treated with TRIMEL/dendritic cells (DC) compared with American Joint Committee on Cancer expected survival for this stage. Seven stage III patients were treated with TRIMEL/DCs according the described protocol. All patients remained alive and tumor-free for a median follow-up period of 48 months (range, 33 to 64 months). The dotted line shows projected survival. American Joint Committee on Cancer data were obtained from Balch et al (Balch CM, Soong SJ, Gershenwald JE, et al: J Clin Oncol 19:3622-3634, 2001).