Dendritic Cell Vaccination Combined with CTLA4 Blockade in Patients with Metastatic Melanoma

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Abstract

Purpose: Tumor antigen–loaded dendritic cells (DC) are believed to activate antitumor immunity by stimulating T cells, and CTL-associated antigen 4 (CTLA4)–blocking antibodies should release a key negative regulatory pathway on T cells. The combination was tested in a phase I clinical trial in patients with advanced melanoma.

Experimental Design: Autologous DC were pulsed with MART-126-35 peptide and administered with a dose escalation of the CTLA4-blocking antibody tremelimumab. Sixteen patients were accrued to five dose levels. Primary end points were safety and immune effects; clinical efficacy was a secondary end point.

Results: Dose-limiting toxicities of grade 3 diarrhea and grade 2 hypophysitis developed in two of three patients receiving tremelimumab at 10 mg/kg monthly. Four patients had an objective tumor response, two partial responses and two complete responses, all melanoma free between 2 and 4 years after study initiation. There was no difference in immune monitoring results between patients with an objective tumor response and those without a response. Exploratory gene expression analysis suggested that immune-related gene signatures, in particular for B-cell function, may be important in predicting response.

Conclusion: The combination of MART-1 peptide–pulsed DC and tremelimumab results in objective and durable tumor responses at the higher range of the expected response rate with either agent alone. (Clin Cancer Res 2009;15(19):6267–76)

The CTL-associated antigen 4 (CTLA4) is a main negative regulator of the immune system, which inhibits costimulatory signaling provided by dendritic cells (DC) to activate T lymphocytes. Antibodies to CTLA4 block this negative signaling, which allows a dominant positive signaling provided by costimulatory molecules on DCs recognized by CD28 on T cells (1). In animal models, administration of CTLA4-blocking antibodies alone induced rejection of established tumors, provided that these were immunogenic tumor models (1, 2). However, in poorly immunogenic tumor models, which may more closely resemble human disease, prior immunization with tumor vaccines, including DC-based vaccines, was required for CTLA4 blockade to exert robust antitumor effects (1).

Tremelimumab (formerly CP-675,206) is a fully human CTLA4-blocking monoclonal antibody being developed for the treatment of cancer (3). In phase I testing, plasma levels of antibody of 30 μg/mL, which corresponds to levels predicted to result in continuous CTLA4 blockade in vitro, were achieved for at least 1 month at doses beyond 6 mg/kg (4). Cumulative clinical data suggests that single agent tremelimumab has antitumor activity in ~10% of patients with advanced melanoma, and these responses tend to be long lasting.

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Note: Supplementary data for this article are available at Clinical Cancer Research online (http://clincancerres.aacrjournals.org/).

Supplementary Materials

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Translational Relevance

Antitumor responses induced by the activation of the immune system to tumor antigens results in a low percentage of clinical responses, but most of these are durable. A major limitation of tumor immunotherapy is immune cell intrinsic-negative regulatory networks. In a phase I clinical trial, we provided immune activation signals derived by tumor antigen presented by dendritic cells together with the blockade of a major negative regulator of the immune system, the CTL-associated antigen 4. We describe the safety, immune effects, and clinical antitumor activity of this combination in patients with metastatic melanoma. Our results provide evidence that the combination is safe and results in clinically relevant and durable tumor regressions, which should warrant the continued clinical testing of this combination.

Dosing (3, 5). Dose-limiting inflammatory and autoimmune toxicities such as colitis, hypophysitis, and thyroiditis support the notion that this antibody can break peripheral tolerance to self-tissues (4). When administered as single agent, there is no evidence of expansion of T cells specific for tumor antigens in the majority of experiences, as assessed using immune monitoring assays in peripheral blood (6, 7). However, analysis of tumor biopsies showed that its antitumor activity is associated by large intratumoral infiltrates with CD8+ T cells, with variable infiltration with CD4+ T cells (8). Given the low frequency of clinical tumor responses as a single agent, the data from animal models suggesting the beneficial effects of adding a vaccine approach to CTLA4 blocking strategies and the validation of the mechanism of action with immune infiltration in regressing lesions in humans, we reasoned that a combination of tremelimumab with tumor antigen-loaded DC vaccines may increase the expansion of melanoma antigen-specific T cells leading to an increased frequency of long lasting objective tumor responses.

Among patients with malignant melanoma and the MHC class I haplotype HLA-A*0201 (the most common in the general population), T cells reactive to the melanoma-associated antigen recognized by T cells (MART-1) can be readily recognized in peripheral blood and tumors of some patients at relative high frequencies (9–11). Therefore, MART-1 represents a reasonable target for melanoma tumor immunotherapy. MART-126-35 peptide immunization, either pulsed onto autologous DC or emulsified with incomplete Freund’s adjuvant, resulted in the activation of epitope-specific T cells and occasional clinical responses in patients with metastatic melanoma (12, 13). In our prior experience in a phase I/II dose-escalation clinical trial (14, 15), HLA-A*0201–positive patients with MART-1–expressing melanoma received autologous DC pulsed with the MART-127-35 Peptide. DCs were generated from autologous leukapheresis products by a 1-week culture in granulocyte-macrophage colony stimulating factor and interleukin (IL)-4 under good manufacturing practice (GMP) standards, pulsed with the MART-127-35 peptide, and administered at cell doses between 10^5 to 10^7 through two routes, i.e. or intradermal (i.d.). Immunologic testing provided evidence that the i.d. route resulted in higher frequencies of MART-127-35–specific T cells compared with the i.v. route, and that the DC dose was not critical for this T-cell expansion (14). Two of 18 patients with measurable metastatic melanoma in this phase I/II clinical trial had durable complete responses (CR) after MART-126-35 peptide–pulsed DC vaccine (MART-126-35/DC) administration. One patient had regression of over 90 skin metastasis after receiving the three i.d. administration of 10^7 MART-1 peptide–pulsed DC vaccines, and is currently disease-free for 9 years (14). This is the only therapy for metastatic melanoma that this patient has ever received, demonstrating that this mode of therapy can induce occasional responses as single agent. A second patient with bulky lung metastasis and s.c. metastasis is currently disease free for 8 years (15). This patient sequentially received DC vaccines followed 1 month later with the CTLA4-blocking monoclonal antibody ipilimumab (formerly MDX010, Medarex, Inc.) administered within the first-in-human clinical trial of this antibody (15). Immunologic analysis in this patient revealed that the DC vaccines increased the number of circulating MART-127-35–specific cells and further generated determinant (or epitope) spreading to other melanoma antigens (16). The anti-CTLA4 antibody resulted in an enhancement in the frequency of precursor cells in peripheral blood reactive to antigens to which this patient had been exposed in the past, including infectious disease antigens and antigens derived from her melanoma, but not to antigens to which the patient had not been in contact with (15). The phenomenon of determinant spreading has been observed with high frequency in patients with tumor responses to single epitope-based vaccination strategies (17), which suggests that breaking tolerance to cancer may follow similar pathways as breaking self-tolerance in autoimmune diseases (18).

Based on these preliminary experiences, we planned to address the hypothesis that CTLA4 blockade may enhance T-cell responses stimulated by tumor antigen peptide–pulsed DC within a phase I clinical trial in patients with advanced melanoma. Our results suggest that this combination is feasible and can be safely administered to patients resulting in clinically meaningful and durable objective responses at a frequency that may be at the high end of what should be expected with either agent alone.

Patients and Methods

Clinical trial identification and conduct. This clinical trial was registered as NCT00090896. The protocol and consent forms, and all modifications, were approved by the University of California at Los Angeles (UCLA) Institutional Review Board (IRB; #03-12-023), under the investigator new drug 11579 filed with the Food and Drug Administration. Collected data were prospectively reviewed by an independent monitor, and the study also underwent periodic ad hoc reviews by the compliance officers from the Jonsson Comprehensive Cancer Center Quality Assurance Committee. The clinical trial was activated on June 2004, and the last study visit was in July 2007. Consent patients with an ongoing response at that time were offered enrollment in a rollover protocol allowing maintenance dosing with tremelimumab (IRB #06-11-026). The median follow up at the time of reporting was 40 mo (range, 59+ to 28+ mo).

Study endpoints and assessments. The primary objectives were safety, feasibility, and biological activity. Dose-limiting toxicities (DLT) were defined as treatment-related toxicities equal or greater than
grade 3 according to the National Cancer Institute common toxicity criteria version 2.0 excluding skin toxicity, or clinical evidence of grade 2 or higher autoimmune toxicities in critical organs (heart, lung, kidney, bowel, bone marrow, musculoskeletal, central nervous system, and the eye). All patients underwent baseline and follow-up eye exams every 2 to 4 mo throughout study participation following our previously published eye surveillance protocol (19, 20). Secondary end point was efficacy as measured by objective clinical response criteria following a slightly modified Response Evaluation Criteria in Solid Tumors (21) to include the evaluation of lesions detectable by physical exam only that had been recorded at baseline using a photographic camera with a measuring tape or ruler. The tertiary study end point was the determination of the lowest dose of tremelimumab necessary to significantly increase the frequency of MART-1 26–35-specific CD8+ T cells detectable using immune monitoring assays in peripheral blood. A baseline leukapheresis provided peripheral blood mononuclear cells (PBMC) for both the DC manufacture and for predosing immune monitoring assays. Peripheral blood samples (40 mL) were collected every 2 wk for 3 mo and at the end of the initial 120 study period. After a protocol amendment, patients enrolled in the last two cohorts underwent a postdosing leukapheresis scheduled between study days 42 and 60.

**Clinical trial design.** This was a phase I open-label, single-site clinical trial with a fixed dose of 1 × 10^7 MART-1 26–35/DC administered i.d. in the lower abdominal region above the groin in three biweekly vaccinations (study days 1, 14, and 28), concomitantly with a dose escalation of tremelimumab at 3, 6, and 10 mg/kg i.v. initially administered at monthly intervals starting on day 1. After reaching DLTs in the third study cohort, the protocol was amended to administer tremelimumab at 10 and 15 mg/kg every 3 mo. If a patient at any cohort had not received the planned three doses of MART-1 26–35/DC, additional patients would be accrued to that cohort to have at least three patients that had completed the combined planned therapy. Dose escalation was allowed when three subjects in a cohort had completed 90 d of follow-up with no DLTs. Restaging exams were conducted at study day 120, and patients without disease progression were eligible for continued dosing with tremelimumab.

**Study eligibility.** Eligible patients had histologically proven malignant melanoma stages IIIC or IV, measurable disease by computed tomography (CT) scans or by physical examination (requiring photographic documentation), adequate performance, hematopoietic, hepatic and renal function, be HLA-A*0201-positive by DNA typing, and express MART-1 in the tumor. Major exclusion criteria were history of chronic inflammatory or autoimmune disease, congenital or acquired immunodeficiency, requirement of ongoing immunosuppressive therapy, and presence of active brain metastases.

**Tremelimumab.** Tremelimumab (compound code CP-675,206) is a fully human IgG2 monoclonal antibody with high binding affinity for human CTLA4 and a plasma half-life of 22.1 d (3, 4). It was supplied by Pfizer, Inc., and administered i.v. at a rate of 100 mL/h.

**MART-1 26–35 peptide–pulsed DCs.** A single unmobilized leukapheresis processing two plasma volumes was done to obtain PBMC, which were isolated by Ficoll-Hypaque (Amersham-Pharmacia) gradient centrifugation and cryopreserved in clinical grade RPMI 1640 (Life Technologies Bethesda Research Laboratories) plus 20% heat-inactivated autologous serum, 800 U/mL of granulocyte-macrophage colony stimulating factor (Berlex), and 500 U/mL of IL-4 (CellGenix) as we have previously described (14, 17). All culture reagents and in-process samples were tested for bacterial and fungal sterility. On the day of immunization, DC were pulsed with 10 pg/mL MART-1 26–35 peptide (A27L peptide analogue, sequence ELAGIGILTV, ClinAlfa; refs. 22, 23) in serum-free RPMI 1640 for 1 to 2 h, and 1 × 10^6 DC were then collected in 0.2 mL of saline. The sterility of the final product after all manipulations was confirmed by Gram stain, a rapid Mycoplasma assay (Mycoplasma PCR-ELISA, Roche Applied Science), and an endotoxin assay (Limulus Amebocyte Lysate, BioWhit-taker) read by an automated automatic endotoxin detection system (Associates of Cape Cod, Inc.), before being administered to patients. DC culture and all manipulations were done following good manufacturing practice at the Jonsson Comprehensive Cancer Center/UCLA Cell and Gene Therapy good manufacturing practice suite.

**DC phenotype and cytokine profile analysis.** Cellular aliquots taken on the day of DC harvesting were stained with preconjugated antibodies specific for DC cell surface markers including CD80 (B7.1), CD86 (B7-2; Invitrogen), and HLA-DR (Beckman-Coulter); markers to study the degree of DC maturation including CCR6 and CCR7 (BD Bio-sciences), CD40, CD80, and CD83 (Invitrogen); viability assessed by Annexin V and propidium iodine (BD Biosciences); and markers to quantify the percentage of other cell subsets included in the preparation, including CD3 for T cells, CD14 for monocytes/macrophages, and CD19 for B cells (Caltag). Supernatant from DC preparations was collected and stored frozen. After thawing, supernatants were analyzed for IL12p70 cytokine (Bio-Rad) following the package insert.

**Immunologic assays.** PBMC samples from peripheral blood or leukapheresis were cryopreserved and analyzed by MHC tetramer assay as previously described (6, 7) All HLA-A*0201 tetramers were purchased from Beckman Coulter, Inc. IFN-γ ELISPOT assays were also done as we have previously described (6, 7) using peptide-pulsed HLA-A*0201-transfected K562 (K562/A*0201) as stimulators with cells seeded directly into anti–IFN-γ antibody coated ELISPOT plates and cultured for 20 h.

**Immunohistochemistry staining and evaluation.** Paraffin sections from tumors or from 4-mm punch biopsies of the skin were deparaffinized and stained with H&E or subjected to heat-induced epitope retrieval and stained with antibodies to CD3, CD4, CD8, CD68, granzyme B, MART-1, tyrosinase, and HMB45 as previously described (8).

**Gene expression profiling in PBMCs.** Total RNA was isolated from cryopreserved PBMC collected before and following therapy using Qiagen RNeasy mini kit according to manufacturer’s instructions and quantified using Agilent Bioanalyzer 2000 (Agilent Technologies). RNA amplification and labeling were done as previously described (24, 25). 36K whole genome arrays were fabricated at Division of International Services, NIH. Hybridization was carried at 42°C for 18 to 24 h and analyzed using BRB array software (24, 25).

**Statistical analysis.** The previously defined 99% reference change value (RCV; ref. 6) was applied to detect statistically significant changes in values above the lower limit of detection for the tetramer and ELISPOT assays as we have previously described (6). For gene expression analysis, data were uploaded to the mAbdb database and 4 raw intensity data were collated (26). Owing to differences in sample collection, gene expression in PBMC obtained from leukapheresis and from standard peripheral blood draws were compared; 846 genes were differentially expressed at a cutoff P value of <0.001 and were removed from the analysis. In addition, 4,929 genes observed to vary according to batch assessment (sample collection, shipment, and experimental batches) were removed from further analysis based on quality control assessment of reference concordance as previously discussed (27). Significant difference were defined based on a two-tailed P value of <0.05 for class comparison due to small sample size (Student’s test). Global and univariate permutation significance level was defined as P value of <0.05 based on 1,000 and 10,000 permutations, respectively. Data were visualized using Cluster and TreeView software (28). Venn Diagrams were based on gene selection and display using mAbdb webtools.

**Results**

**Patient characteristics.** Sixteen HLA-A*0201-positive patients were enrolled between June 2004 and March 2007, with...
a mean age of 53 years (Table 1). Half of the patients had visceral metastasis or an elevated lactate dehydrogenase (LDH; stage IV, M1c). Among the remaining patients, two had in-transit melanoma (stage IIIc), three had skin, s.c. or nodal metastasis (stage IV, M1a), and three had lung metastasis (stage IV, M1b). Four patients had received prior immunotherapy, three chemotherapy and two biochemotherapy, whereas seven patients were treated as first line therapy for metastatic melanoma.

**DC preparation and phenotype.** Table 2 provides the characteristics of the DC preparations. All final MART126-35 peptide-pulsed DC preparations had high viability and met the lot release criteria defined in the study investigator new drug, including being free of endotoxin and *Mycoplasma* contamination. The DC surface markers CD40, CD86, and HLA-DR, were expressed by all DC preparations, but CD80 was infrequently expressed. The cells were partially mature DC, with only 50% of the cultures staining positive for the DC maturation marker CD83 as expected following our protocol without a dedicated maturation step. Nearly all preparations had contaminant CD3+ T cells as expected, because we harvested loosely adherent and nonadherent cells at culture day 8 without a further purification step. The level of contamination with monocytes/macrophages and B cells was low. Further confirmation that these were immature DC was that the culture supernatants did not have detectable IL12p70 (data not shown). Overall, these DC preparations were phenotypically similar to our prior experience following the same protocol (14, 15).

**Does escalation and toxicities.** Patients were enrolled sequentially to five dose cohorts (Table 1) with tremelimumab administered at monthly (cohorts A-C) and then at quarterly intervals (cohorts D and E). One patient in cohort B did not receive the three planned DC administrations due to early progression, and an additional patient was enrolled to that cohort. There were no significant toxicities attributed to the study agents in the seven patients enrolled in the first two cohorts. Protocol-specified DLTs were noted in two of three patients in cohort C with a monthly dose of 10 mg/kg of tremelimumab. One patient (NRA10) developed grade 3 diarrhea and biopsy-proven colitis requiring hospital admission, improved without requiring corticosteroids. Another patient (NRA8) developed grade 2 hypophysitis requiring supplemental dosing with hydrocortisone. Therefore, dose escalation was halted and the protocol amended to decrease the frequency of tremelimumab dosing. Two new cohorts of patients were then enrolled at tremelimumab doses of 10 and 15 mg/kg administered every 3 months without further DLTs.

**Table 1. Patient characteristics, toxicities, and response to therapy**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>M/DC dose</th>
<th>Treme dose</th>
<th>Pt study #</th>
<th>Sex (M/F)</th>
<th>Age</th>
<th>Prior treatments</th>
<th>Active metastasis sites</th>
<th>LDH Stage</th>
<th>DLT</th>
<th>ORR</th>
<th>Current status</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>3 mg/kg qmo</td>
<td>NRA1</td>
<td>M</td>
<td>43</td>
<td>None</td>
<td>S.C.</td>
<td>N IIIc</td>
<td>SD</td>
<td>NED 59+ mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NRA2</td>
<td>F</td>
<td>48</td>
<td>None</td>
<td>Lung</td>
<td>N M1b</td>
<td>PR</td>
<td>NED 58+ mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NRA3</td>
<td>F</td>
<td>53</td>
<td>None</td>
<td>Lung</td>
<td>N M1b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>6 mg/kg qmo</td>
<td>NRA4</td>
<td>F</td>
<td>62</td>
<td>None</td>
<td>LN, S.C.</td>
<td>N M1a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NRA5</td>
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<td>28</td>
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<td>Lung</td>
<td>H M1c</td>
<td></td>
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<td></td>
<td></td>
<td>NRA6</td>
<td>M</td>
<td>38</td>
<td>None</td>
<td>S.C., Liver, Spleen Bowel, Lung, LN Adrenal</td>
<td>H M1c</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>C</td>
<td>1×10^7 10 mg/kg qmo</td>
<td>NRA7</td>
<td>M</td>
<td>56</td>
<td>Chemotherapy</td>
<td>Lung</td>
<td>M1b</td>
<td>G2 hypophysitis</td>
<td>CR</td>
<td>NED 44+ mo</td>
<td></td>
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<td></td>
<td></td>
<td>NRA8</td>
<td>M</td>
<td>67</td>
<td>Biochemotherapy Chemotherapy</td>
<td>Biochemotherapy</td>
<td>Lung, LN</td>
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<td>NRA9</td>
<td>M</td>
<td>51</td>
<td>None</td>
<td>S.C.</td>
<td>N IIIc</td>
<td>CR</td>
<td>NED 28+ mo</td>
<td></td>
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<td></td>
<td></td>
<td>NRA10</td>
<td>M</td>
<td>54</td>
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<td>N M1c</td>
<td>G3 diarrhea</td>
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<td>D</td>
<td>10 mg/kg q3mo</td>
<td>NRA11</td>
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<td>LN, Muscle, Lung Liver</td>
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<td></td>
<td>NRA12</td>
<td>M</td>
<td>55</td>
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<td></td>
<td></td>
<td>NRA13</td>
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<td>34</td>
<td>HD-IL2</td>
<td>S.C., LN, Muscle, Breast</td>
<td>H M1c</td>
<td></td>
<td></td>
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<td></td>
<td>NRA14</td>
<td>M</td>
<td>57</td>
<td>MART-1 + gp100 peptide vaccines with IL-12</td>
<td>S.C.</td>
<td>N IIIc</td>
<td>CR</td>
<td>NED 28+ mo</td>
<td></td>
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<td>NRA15</td>
<td>M</td>
<td>48</td>
<td>None</td>
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<td>N M1a</td>
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<td>NRA16</td>
<td>F</td>
<td>61</td>
<td>Anti-CD137 HD-IL2</td>
<td>S.C.</td>
<td>N M1a</td>
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Abbreviations: M/DC, MART-1 peptide–pulsed DCs; Treme, tremelimumab; M, male; F, female; N, normal LDH level; H, LDH above the upper limit of normal; L.N., lymph nodes; S.C., s.c.; G, grade; ORR, objective response rate; HD IFN, high-dose IFN; SD, stable disease; NED, no evidence of disease.
Clinical outcome. Four patients showed an objective response (Table 1). One patient (NRA3) in the first cohort receiving tremelimumab at 3 mg/kg monthly had a partial response (PR) of bulky lung metastasis. This was the first therapy for metastatic melanoma for this patient. Four lung metastases completely regressed within 4 months, and a large paraspinal mass initially measuring over 16 cm in maximum diameter regressed 55% (Fig. 1A). His only toxicity during this period was grade 2 pruritic skin rash, which at biopsy revealed a multifocal acantholytic dyskeratosis consistent with Grover’s disease of the skin (Fig. 1B and C), a condition we have previously seen with tremelimumab but not with DC vaccines alone.11 Nine months after starting the combined therapy, a CT scan suggested that the residual paraspinal mass had increased 1 cm in maximum diameter. A decision was made to surgically resect this lesion. The patient is currently disease free 58+ months later.

One patient in cohort C, who had lung metastases progressing after multiagent chemotherapy, had a complete response that is ongoing at 44+ months (NRA9; Fig. 1D). This patient developed recurrent grade 2 diarrhea while on monthly doses of tremelimumab for 1.5 years. The diarrhea improved when switched to maintenance dosing at 15 mg/kg every 3 months. The patient developed grade 2 skin rash clinically consistent with Grover’s disease during the first year of dosing. During the third year on maintenance dosing, the patient developed a new pruritic skin rash (maculopapular lesions coalescing to form thick plaques with some scaling). Most affected areas were the proximal extensor surfaces of the arms and legs followed by the trunk. Skin biopsy showed a mixed granulomatous inflammation (Fig. 1E and F).

Two patients in cohort E also had objective responses to therapy. One (NRA14) had a single in-transit s.c. lesion of 1 cm that appeared after three prior resections of other similar lesions in the same leg. This patient had previously received a vaccine consisting of gp100209-217, MART-126-35, and tyrosinase368-376 peptides together with IL-12 (29) 2 years before and then progressed. This in-transit lesion completely regressed within 1 month of initiating study participation and the patient continues tumor free 28+ months later. His only toxicity was grade 1 pruritic skin rash, which at biopsy was consistent with lichenoid dermatitis. The other patient (NRA15) had CT and positron emission tomography–positive metastatic lymph nodes up to 2 cm in maximum diameter, and was treatment naïve at the time of starting participation in this protocol. The lymph node metastasis has nearly completely regressed and the patient continues to be tumor free 28+ months later while on maintenance dosing with tremelimumab. He has not had any evidence of side effects to this combined therapy.

Pathologic analysis. The partially regressed paraspinal mass from patient NRA3 was analyzed for the presence of immune infiltrating cells upon surgical resection. There were large areas of infiltration by CD3+ cells, with a higher ratio of CD8+ cells than CD4+ cells (Fig. 2). These cells were consistent with CTLs because there was a diffuse cytoplasmic staining with granzyme B, a granule that mediates cytotoxic signals delivered by CD8+ CTLs. There were also frequent macrophages, stained with the CD68 marker. When stained with the melanoma markers MART-1, HMB45 (gp100), tyrosinase, and S-100, a large portion of the mass was negative for MART-1. However, the same areas negative for MART-1 were positive for the other melanosomal antigens. This finding suggests that an area of this resected mass included antigen variant melanoma cells having lost MART-1 expression by immunohistochemistry (IHC).

Immunomonitoring for melanoma-specific T-cell responses. T-cell responses to the immunizing MART-126-35 peptide were assessed using the MHC tetramer and the ELISPOT assays. Changes from baseline were scored following our algorithm to detect statistically significant antigen-specific T-cell expansions (6, 7). Six patients, including two of the four patients with an objective response to therapy, had increases in MART-126-35–specific CD8-positive T cells by the MHC tetramer assay, and five patients, including two of the four patients with an objective response, using the ELISPOT assay (Table 3). There was no trend of differential expansion of MART-126-35–specific T cells among study cohorts, suggesting that tremelimumab does not directly increase the number of CD8+ T cells specific for this melanosomal antigen in peripheral blood in a dose-response fashion when combined with DC-based vaccination. Also, there was no clear increasing trend in the number of peripheral blood cells specific for other melanosomal antigens (tyrosinase368-376 and gp100209-217), nor to control infectious disease antigens (CMV pp65,195-503 and EBV BMLF1259-267).

Gene expression profiling in peripheral blood. Because the antigen-specific immune monitoring assays did not allow a clear definition of effects in blood that would differentiate clinical responders from nonresponders, we undertook an exploratory whole genome profiling in PBMC obtained at baseline and 2 to 3 months after the initial dose. Unsupervised cluster analysis did not identify clear segregation between pretreatment and posttreatment samples, suggesting that a relatively small proportion of genes in peripheral blood cells were affected at the transcriptional level by the treatment. We then compared pretreatment with posttreatment PBMC samples independently of the clinical outcome. This class comparison identified 170 differentially expressed genes (P < 0.005; pp < 0.022). Among them, 115 genes were downregulated and 55 upregulated (Fig. 3A). To stratify genes potentially responsible for tumor rejection, we grouped samples according to clinical outcome and evaluated treatment-induced gene expression in samples from patients experiencing progressive disease (PD) or those who experienced PR or CR. Comparison of

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<table>
<thead>
<tr>
<th>Category</th>
<th>Marker</th>
<th>% DC vaccines with positive markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC surface</td>
<td>CD80 (B7.1)</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>CD86 (B7.2)</td>
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</tr>
<tr>
<td></td>
<td>HLA-DR (MHC class II)</td>
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<td>CCR6/CCR7</td>
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</tr>
<tr>
<td></td>
<td>CD40</td>
<td>100%</td>
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<tr>
<td></td>
<td>CD83</td>
<td>53%</td>
</tr>
<tr>
<td>Other cell subsets</td>
<td>CD3 (T lymphocytes)</td>
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</tr>
<tr>
<td></td>
<td>CD14 (macrophages)</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>CD19 (B cells)</td>
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</tr>
<tr>
<td>Cell viability</td>
<td>Annexin-V/PI</td>
<td>0%</td>
</tr>
</tbody>
</table>

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11 A. Ribas, unpublished.
pretreatment with posttreatment samples in patients with an objective response identified 48 genes differentially expressed (P < 0.005, permutation not significant). 10 of which were found to be in common with the 124 genes significantly altered (P < 0.005; pp = 0.062) in patients with PD (Supplementary Table S1; Fig. 3C). We finally compared pretreatment samples from patients experiencing PR or CR versus PD to explore if we could define baseline gene expression profiles that...
could later identify patients with a response or resistance. Out of baseline upregulated genes, genes associated with immune function were highly enriched (14 of 55 in CR and 3 of 12 in PR; Supplementary Table S2). Seven genes were B-cell signal transduction and function-related genes, including CD24, helicase (DNA) B (HELB), and B-cell linker (Supplementary Table S2).

**Discussion**

The combination of tumor antigen–pulsed DC and the CTLA4-blocking monoclonal antibody tremelimumab can be safely administered to humans and results in antitumor activity. The maximum tolerated dose of this combination was 10^7 MART-1/DC with concomitant tremelimumab at 6 mg/kg when administered monthly, but in this clinical trial, we could not determine an maximum tolerated dose when tremelimumab was administered every 3 months at doses up to 15 mg/kg. Neither one of the two patients that developed DLTs had an objective response to therapy, suggesting that worse autoimmune or inflammatory toxicity is not directly associated with response to this combined therapy. In addition, tumor responses in this study did not follow the proposed immune-related response criteria (30), although the late increase in size of a lung lesion in patient NRA3 without further progression may reflect a transient inflammatory response. This pilot clinical trial cannot discern between responses to the DC vaccines alone or to tremelimumab alone from responses to the combination. One of the four patients with an objective response received dosing of tremelimumab at 3 mg/kg, a dose that achieves plasma levels of 30 μg/mL for <2 weeks. This concentration is

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**Fig. 2.** Pathologic analysis of a partially regressed mass from patient NRA3. A, high power (×40) images of H&E and immune subset IHC stains, including CD3 staining for T cells, CD4 staining for T helper subset, CD8 staining for CTL subset, CD68 for macrophages, and granzyme B for CTL-associated cytotoxic granules. B, low (×4) and high power (×40) images of IHC staining of the resected mass for the melanosomal antigen tyrosinase (left) and gp100 (stained with the HMB45 antibody; middle) showing uniform staining, and the melanosomal antigen MART-1 (right) showing large areas without brown IHC staining.
below the target plasma level set up prospectively in the first-in-human testing of this antibody (3, 4), suggesting that this response may not be explained solely by single-agent tremelimumab. The other three responding patients received doses of tremelimumab at 10 or 15 mg/kg, which are known to induce tumor response in approximately 7% to 10% of patients in single-agent studies (5, 31, 32). Therefore, these other responses may be explained by tremelimumab alone, although they seem to be at the higher end of what can be reasonably expected with this agent as single therapy.

We could not detect a dose-response effect of tremelimumab on the expansion of MART-126-35-specific T cells. It is possible that the immune-stimulating effect of CTLA4-blocking monoclonal antibodies has a more marked effect on CD4+ T helper cells than on CD8+ CTLs, as described in preclinical models (33), which would explain the lack of expansion of MART-126-35-specific CD8+ T cells with increasing doses of tremelimumab. In addition, we could not confirm our prior observations that determinant spreading is an immunologic feature of patients with a clinical response after single tumor antigen peptide–specific immunization (14, 15), although there were expansions of non-MART-1-specific T cells beyond the assay variability. In the absence of a detectable MART-1 driver clone, it is difficult to consider expansion of other tumor antigen–specific cells as subdominant clones triggered by determinant spreading. Overall, the level of MART-126-35-specific T-cell expansion in peripheral blood with this combination was low, which is a common finding in other tumor antigen peptide–specific clinical trials in patients with metastatic disease despite objective clinical responses (34) in particular when given with high-dose IL-2 (13, 35). One possibility is that the vaccine-primed effector cells accumulate in draining lymph nodes and tumor lesions at much higher frequencies than in peripheral blood as has been shown for MAGE-3 vaccination (34). Supporting this concept, we have previously described an increased frequency of melanoma-specific T cells in tumor samples of patients responding to single-agent tremelimumab (8), although we do not detect expansions in peripheral blood with this treatment (7). In fact, it has been reported that CTLA4 may have a key role in establishing durable interactions between antigen-specific T cells and their targets (36), suggesting that vaccine-primed lymphocytes should accumulate in tumors as opposed to circulate in blood. An indirect evidence of this possibility in the current clinical trial is the development of large areas of MART-1-negative tumor escape variant cells in a responding patient with a slowly regressing large lung mass.

In this study, we used DC generated by granulocyte-macrophage colony stimulating factor and IL-4 in vitro differentiation from monocytes without an additional maturation step. A large body of preclinical data suggests that DC that have been matured with monocyte-conditioned media or a cytokine cocktail are superior to nonmatured cells in their ability to stimulate T-cell responses (37, 38). However, it is still unclear what the optimal DC maturation conditions are for clinical use. Initial reports suggested favorable outcomes with matured DC (39), but a larger clinical testing of matured DC resulted in very low melanoma tumor responses (40). Furthermore, a clinical trial directly comparing matured and nonmatured DC vaccination failed to show improved immunologic or clinical potency of the matured DC (41). For this study, we chose to use the same conditions for DC generation that had resulted in long lived objective responses in occasional patients with metastatic melanoma in our prior experience (14, 15), with the goal of testing if the concomitant administration of a CTLA4-blocking monoclonal antibody would result in improvement of these DC-based vaccines.

Changes in gene expression profiling in blood samples obtained from patients receiving tumor immunotherapy strategies have been seldomly described, although we believe they are of key importance to understanding how the immunotherapeutic approach impacts on the recipient’s immune system (42, 43). In our limited set of patients, we explored basic variations in transcription induced by the combined treatment and, at the same time, tested the possibility that pretreatment signatures may provide insights into future biomarker discovery (44). Of interest to us were genes consistently upregulated in pretreatment samples from patients who experienced an objective response, which if validated could serve as prognostic factors in future studies. The finding of immune-related genes, in particular related to B-cell function, were upregulated at baseline in patients who went onto an objective response may correlate with the detection

<table>
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<th>Assay</th>
<th>Epitope</th>
<th>No objective tumor response</th>
<th>Objective tumor response</th>
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<tr>
<td></td>
<td></td>
<td>&lt;99% RCV</td>
<td>&gt;99% RCV</td>
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<td>MHC tetramer</td>
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<td>NRA 4, 7, 8, 10, 12, 13, 16</td>
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<td>NRA 2, 11, 13</td>
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NOTE: Patient codes are listed based on at least once point with MHC Tetramer or ELISPOT assay results beyond the 99% RCV for each peptide epitope and immune monitoring assay.
of increased humoral responses in patients responding to CTLA4-blocking antibodies described by others (45, 46). We fully acknowledge that the low number of samples from clinical responders in our series demands further validation studies in larger cohorts, where gene expression profiling in blood should be compared with gene expression profiling in tumors, where it should be most relevant.

In conclusion, the combination of MART-1/DC with concomitant tremelimumab is feasible in patients with metastatic melanoma, especially when tremelimumab is administered every 3 months, and results in durable objective clinical responses at the higher range of the expected objective tumor response rates with either therapy alone. Therefore, this combination warrants further study in patients with advanced malignant melanoma.

### Disclosure of Potential Conflicts of Interest

A. Ribas has received commercial research grants and honoraria from Pfizer, Inc.; J. Glaspy has received honoraria from and is a member of the speaker’s bureau of Pfizer, Inc.; J. Gomez-Navarro is employed by Pfizer, Inc.

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![Fig. 3. Heat map describing tremelimumab-induced PBMC gene expression; blue and red bars, pretreatment posttreatment samples, respectively. A, all pretreatment and posttreatment samples are displayed showing 170 transcripts whose expression was significantly (P < 0.005) altered by treatment (paired two sample t test, permutation P < 0.022). B, samples for patients with PD were compared between pretreatment and posttreatment collections (P < 0.005, 124 genes). C, clinical responder’s (PR+CR) pretreatment versus posttreatment analysis (P < 0.005, 48 genes). D, Venn diagram displaying overlap of genes within the three groups (all patients, PD and CR+PR).](image_url)


Dendritic Cell Vaccination Combined with CTLA4 Blockade in Patients with Metastatic Melanoma

Antoni Ribas, Begoña Comín-Anduix, Bartosz Chmielowski, et al.

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