Dendritic Cell Vaccination in Combination with Anti-CD25 Monoclonal Antibody Treatment: A Phase I/II Study in Metastatic Melanoma Patients

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Abstract

Purpose: The success of cancer immunotherapy depends on the balance between effector T cells and suppressive immune regulatory mechanisms within the tumor microenvironment. In this study we investigated whether transient monoclonal antibody-mediated depletion of CD25high regulatory T cells (Treg) is capable of enhancing the immunostimulatory efficacy of dendritic cell vaccines.

Experimental Design: Thirty HLA-A2.1+ metastatic melanoma patients were vaccinated with mature dendritic cells pulsed with tumor peptide and keyhole limpet hemocyanin (KLH). Half of the patients were pretreated with daclizumab, a humanized antibody against the interleukin-2 (IL-2) receptor α-chain (CD25), either four or eight days before dendritic cell vaccinations. Clinical and immunologic parameters were determined.

Results: Daclizumab efficiently depleted all CD25high immune cells, including CD4+FoxP3+CD25high cells, from the peripheral blood within four days of administration. Thirty days after administration, daclizumab was cleared from the circulation and all CD25+ cells reappeared. The presence of daclizumab during dendritic cell vaccinations prevented the induction of specific antibodies in vivo but not the presence of antigen-specific T cells. Daclizumab, however, did prevent these CD25+ T cells from acquiring effector functions. Consequently, significantly less patients pretreated with daclizumab developed functional, vaccine-specific effector T cells and antibodies compared with controls. Daclizumab pretreatment had no significant effect on progression-free survival compared with the control group.

Conclusions: Although daclizumab depleted the CD4+FoxP3+CD25high Tregs from the peripheral circulation, it did not enhance the efficacy of the dendritic cell vaccine. Residual daclizumab functionally suppressed de novo induced CD25+ effector cells during dendritic cell vaccinations. Our results indicate that for immunotherapeutic benefit of transient Treg depletion, timing and dosing as well as Treg specificity are extremely important.

Melanoma is considered one of the most immunogenic types of cancers. This is based on the following arguments: (a) several melanoma-specific antigens have been identified (1, 2); (b) functional lymphocytes specific for melanoma antigens are increased in melanoma patients (3); (c) immune-stimulating agents can have a positive effect on disease outcome (4, 5); and (d) spontaneous melanoma regressions with simultaneous onset of vitiligo have been reported (6).

Immunotherapeutic clinical trials have succeeded in expanding melanoma-specific effector T cells in vivo, but favorable outcomes are still limited because tumor-induced mechanisms of immune evasion may render the host tolerant for melanoma antigens (7, 8). Immunosuppression at the tumor microenvironment mediated by regulatory T cells (Treg) is one of the most critical mechanisms of tumor-immune escape and a major hurdle for successful immunotherapy (9–11).

In melanoma patients, selective accumulation of Tregs in the primary tumor, tumor-infiltrated lymph nodes, and in metastases has been observed (12–14). The functional importance of these Tregs is shown in mouse melanoma models in which transient Treg depletion induces antitumor immunity (15–19). These studies show that CD25 depletion effectively eliminates the CD25high...
In the present study we investigated whether treatment with a single low dose of daclizumab resulted in transient depletion of Tregs in human melanoma patients and enhancement of the immunostimulatory efficacy of subsequent dendritic cell vaccinations. We therefore vaccinated 30 HLA-A2.1 metastatic melanoma patients with dendritic cells pulsed with tumor peptide and keyhole limpet hemocyanin (KLH). Half of the patients were pretreated with daclizumab.

Materials and Methods

Patient inclusion

Inclusion criteria were metastatic melanoma with measurable disease parameters, expression of gp100 and tyrosinase in at least one metastasis, HLA-A2.1 phenotype, WHO performance status 0 or 1, no second malignancy, no serious concomitant disease, no concomitant treatment with immunosuppressive drugs, and normal hepatic and renal function. Patients with central nervous system metastases were excluded. Prior treatment related to metastatic disease was allowed, provided a treatment-free period of at least four months was observed. Patients with distant nonvisceral metastases were categorized according to the American Joint Committee on Cancer (AJCC) as M1a, patients with metastases to the lungs were categorized as M1b, and patients with metastases to any other visceral site or with elevated serum lactate dehydrogenase were categorized as M1c (31). All patients gave written informed consent prior to inclusion in the study. Approval from the local regulatory committee was obtained.

Clinical protocol and immunization schedule

In eligible patients, a leukapheresis was done from which dendritic cells were generated (32). Antigen-pulsed mature dendritic cells were administered i.v. and i.d. in close proximity to the inguinal lymph nodes three times biweekly. Patients in the daclizumab group were pretreated with 0.5 mg/kg daclizumab i.v. either 4 days (first cohort of 7 patients) or 8 days (second cohort of 8 patients) before the first dendritic cell vaccination. Patients were treated in a nonrandomized fashion. First the control group was included and treated, then the daclizumab group.

A clinical response was defined as stable disease for ≥4 months or any partial or complete response. Response was measured by Response Evaluation Criteria in Solid Tumors (33). Observation of progressive disease led to cessation of vaccinations. Progression-free survival (PFS) was calculated from the day of the first vaccination. Vaccine-specific immune response was the primary end point. Clinical response was the secondary end point.

Soluble IL-2Rx measurement

Soluble IL-2Rx (sIL-2Rx) levels were determined in urine samples (U/mmol creatinine) using a commercially available immunometric assay (Immulite).
Antibodies and immunostaining
To characterize the phenotype of the ex vivo generated dendritic cells and the immune-cell subpopulations in the peripheral blood, flow cytometry was done using either FITC-, phycoerythrin (PE)- or allophycocyanin-conjugated mAbs. The following mAbs were used: anti-HLA class I (W6/32), anti-HLA DR/DP (Q5/13), anti-CD80 (Becton Dickinson.), anti-CD14, anti-CD83 (both Beckman Coulter), anti-CD8, anti-CD25 (clone M-A251, no interference with daclizumab binding to CD25), anti-CD36, anti-CD86 (BD PharMingen.), anti-Foxp3 (eBioscences), anti-gp100 (Dako), anti-tyrosinase (Novocastra), and anti-CX3CR1 (MBL). Daclizumab antibody was covalently labeled to Alexa-488 fluor using the Alexa-488 protein labeling kit according to manufacturer’s procedure (Invitrogen).

Dendritic cells: preparation, characterization, and route of administration
Dendritic cells were generated as described previously (32). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Pharmacia, 30 minutes, 4°C, 2,100 rpm) after leukapheresis. PBMCs were washed and monocytes were isolated by adherence to plastic. Monocytes were cultured at 15 × 10^6 per 75-cm² tissue culture flasks (Costar) in 20 mL of IL-4 (500 U/mL; Schering-Plough International), granulocyte-monocyte-colony stimulating factor (800 U/mL; Schering-Plough International), granulocyte macrophage-conditioned medium enriched with 10 μg/mL prostaglandin E2 (Dr. G.R. Adolf, Bender & Co.) and 10 ng/mL tumor necrosis factor-α (TNF-α; kindly provided by Dr. Adolf Bender, Vienna, Austria). Cells were harvested on day 9. This procedure gave rise to mature dendritic cells as determined by high expression levels of MHC class I and II, CD80, CD86, and the absence of CD14 (data not shown).

Cryopreservation of PBMCs and dendritic cells
All dendritic cells were cultured directly after leukapheresis and frozen as immature or mature dendritic cells for multiple vaccinations. Dendritic cells and PBMCs were frozen using a cryo 1°C freezing container (Nalgene), in freezing medium consisting of 50% X-VIVO-15 (5% HS), 40% HS albumin, and 10% DMSO. Cells were thawed in a 37°C water bath, after which the cells were washed once in 0.9% sodium chloride and suspended in 100 μL (5 × 10^6 dendritic cells) for i.d. injection and in 200 μL (10 × 10^6 dendritic cells) for i.v. injection.

Delayed-type hypersensitivity
One week after the last of three dendritic cell vaccinations, a delayed-type hypersensitivity (DTH) skin test was done as previously described (34). Briefly, unpulsed dendritic cells, dendritic cells pulsed with gp100, dendritic cells pulsed with KLH and gp100, dendritic cells pulsed with tyrosinase, and dendritic cells pulsed with tyrosinase and KLH (10 × 10^5 dendritic cells each) were injected i.d. in the skin of the back of the patients at four different sites. The diameter (in millimeters) of induration was measured by a dermatologist after 48 hours, and an induration of >2 mm was considered positive. From DTH sites, punch biopsies (6 mm) were obtained under local anesthesia. Biopsies were cut in half; one part was for immunohistochemistry, whereas the other part was cut in small pieces and cultured in RPMI/7% HS supplemented with IL-2 (100 units/mL). Every 7 days, half of the medium was replaced by fresh IL-2 containing RPMI/7% HS. After 2 to 4 weeks of culturing, T cells were tested for tetramer binding, antigen recognition, and cytokine secretion.

Humoral response to KLH
Before each vaccination, 80 mL of blood were collected for immunologic monitoring. Antibodies against KLH were measured in the serum of vaccinated patients by enzyme-linked immunosorbent assay as previously described (35). Briefly, 96-well plates were coated overnight at 4°C with the protein KLH (25 μg/mL) in PBS (0.1 mL/well). After washing the plates different concentrations of patient serum were added for 1 hour at room temperature. After extensive washing, specific antibodies (against IgM, total IgG, IgG1, IgG2, and IgG4) labeled with horse-radish peroxidase were allowed to bind for 1 hour at room temperature. Peroxidase activity was revealed using 3,3′,5,5-tetramethyl-benzide as substrate and measured in a microtiter plate reader at 450 mm.

Proliferative response and cytokine production to KLH
Cellular responses against KLH were measured in a proliferation assay. Briefly, 1 × 10^5 PBMCs, isolated from blood samples taken before each vaccination, were plated per well of a 96-well tissue culture microplate either in the presence of KLH or without. After 16 hours of culture, supernatants (50 μL) were taken, and the levels of IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were measured by a cytometric bead array (Th1/Th2 Cytokine CBA 1, BD PharMingen) according to the manufacturer’s instructions. After 4 days of culture, 1 μCi/well of tritiated thymidine was added. Incorporation of tritiated thymidine was measured in a β-counter.

MHC tetramer staining
Tetrameric-MHC complexes were kindly provided by Dr. Rosalie Luiten and Dr. Hergen Spits from the Netherlands.
Cancer Institute, Amsterdam, the Netherlands. Each tetramer was validated by staining against a CTL line specific for HLA-A2 in association with the peptide of interest. PBMCs (1 × 10^5 cells in 10 μL) were incubated with PE-labeled tetrameric-MHC complexes for 1 hour at room temperature. After washing, the samples were analyzed by flow cytometry.

**Cytokine-secretion assay**

The ability of DTH-site infiltrating lymphocytes to recognize vaccine-specific antigens and produce cytokines was measured in response to T2 cells pulsed with vaccine-specific peptides or the irrelevant HLA-A2.1 binding peptide G250. To test for recognition of endogenously processed antigens, DTH-infiltrating lymphocytes were incubated with transfected HLA-A2.1-positive BLM target cells, expressing either control antigen G250 or the antigens of study, gp100 or tyrosinase. Cytokine production was measured in supernatants after 16 hours by cytometric bead array as described above.

**Autoantibody screening**

Sera were screened for antinuclear antibodies by indirect immunofluorescence on Hep-2000 cells in a serum dilution of 1:40, according to the manufacturer’s protocol (Biomedical Diagnostics). Sera were measured prior to start of the vaccinations and after the third vaccination.

**Statistical analysis**

Kaplan-Meier probability estimates of overall survival were calculated. The log rank test was used to determine statistical significance. Paired t-tests were done to test for treatment-related fluctuation of immune cells in the peripheral blood. Differences in KLH-specific proliferation and KLH-specific antibodies were analyzed by ANOVA. All statistical tests were two-sided, and significance was determined as \( P < 0.05 \).

**Results**

**Patient characteristics**

Thirty HLA-A^*0201*-positive patients with progressive metastatic melanoma received either daclizumab combined with dendritic cell vaccine therapy (daclizumab group; \( n = 15 \)) or dendritic cell vaccine therapy alone (controls; \( n = 15 \)). Patient characteristics are shown in Table 1. Patients in the daclizumab group were on average 50.9 years old (range, 25-69), which did not significantly differ from the control group (mean age, 51.7; range 22-73). The two groups were also not significantly different concerning male to female ratio, AJCC stage of the melanoma, and prior therapies.

**Toxicity and side effects**

No severe toxicity (common toxicity criteria grade 3-4) occurred. Vaccine-related side effects seen in patients in both groups were mild fatigue, anorexia, fever, and erythema at the intradermal injection site. None of the side effects lasted >2 days after a vaccination. No daclizumab-related toxicity was observed. During follow-up, we did not observe any clinical signs of autoimmune disease. Because daclizumab-induced transient Treg depletion may increase the risk of inducing autoreactive antibodies, we specifically tested for the presence of antinuclear antibodies before and after vaccination. In both groups there was no significant increase of antinuclear antibodies observed (Supplementary Table S1). During follow-up, one patient in each group developed vitiligo.

**Binding of daclizumab to different blood subsets**

To test for the capacity of daclizumab to bind to different blood subsets, we incubated directly labeled daclizumab to erythrocyte-lysed whole blood cells of healthy controls (\( n = 3 \)). Supplementary Fig. S1A shows that daclizumab does not bind to granulocytes (gate 1) or monocytes (gate 2) in the peripheral blood. Daclizumab binds to approximately 9% of the lymphocytes (gate 3). Daclizumab does not bind to CD20^+ B cells, but binds to a small fraction of CD56^+ natural killer (NK) cells, CD4^+ Th cells, and CD8^+ T cells, and to a large fraction of CD4^+FoxP3^+ Tregs. Incubation of lymphocytes with both daclizumab-PE and non–cross-reactive anti-CD25-PE showed only a double-positive and a double-negative population, which shows that daclizumab specifically and exclusively binds to CD25^+ cells (Supplementary Fig. S1B).

**Daclizumab pharmacokinetics**

The pharmacokinetics of daclizumab administered to patients with metastatic melanoma is not known. Because renal clearance of sIL-2Rα bound to daclizumab is blocked, measurement of sIL-2Rα concentrations in the urine is an excellent predictor for daclizumab concentration in the serum (36). Analysis of urine samples showed that the sIL-2Rα concentration decreased to undetectable levels immediately after daclizumab administration in all 15 patients (\( P < 0.001 \), paired t-test). Concentrations of sIL-2Rα were restored in approximately 4 weeks (Fig. 1A). Daclizumab administration caused a complete and rapid depletion of all CD25^high cells from the peripheral blood (\( P < 0.001 \), paired t-test). Repopulation of CD25^high cells occurred approximately 4 weeks after daclizumab administration (Fig. 1B), as shown by using non-cross-reactive anti-CD25-PE.

**Depletion of Tregs in vivo**

Daclizumab treatment transiently depleted CD4^+ CD25^highFoxP3^+ T cells from the circulation (Fig. 1C and D; \( P < 0.001 \), paired t-test). The percentage of FoxP3^+ cells was already significantly decreased 4 days after daclizumab administration (Fig. 1E; 44% reduction after 4 days, \( P = 0.027 \), paired t-test). The CD25^highFoxP3^+ Tregs were especially depleted. Daclizumab did not effectively deplete the FoxP3^+ cells with low or intermediate CD25 expression, although we cannot exclude that they were functionally impaired by bound daclizumab. Dendritic cell vaccinations without daclizumab had no effect on the
percentage of circulating CD4+FoxP3+CD25<sup>high</sup> Tregs (Fig. 1C-E). Daclizumab administration did not significantly affect the percentage of peripheral blood CD4+ Th-cells, CD8+ CTLs, CD56+ NK-cells, and CD20+ B cells (data not shown). Daclizumab did, however, transiently increase the regulatory fraction of the NK cells (CD56<sup>bright</sup>CX3CR1<sup>low</sup> cells; Supplementary Fig. S2). This is consistent with previous reports showing daclizumab-induced regulatory NK-cell expansion in patients suffering from autoimmune disease (37, 38).

**Humoral responses against KLH in serum**

Humoral responses against KLH were detected in serum of patients not pretreated with daclizumab. Total IgG titers were detectable in most patients already after a single vaccination with KLH-pulsed dendritic cells. After subsequent

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient*</th>
<th>Age/sex</th>
<th>Sites of metastases</th>
<th>AJCC stage</th>
<th>Prior therapies</th>
<th>Prestudy LDH (U/L)</th>
<th>Anti-KLH response†</th>
<th>Tumor-specific T cells</th>
<th>PFS (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dac-1.</td>
<td>61/M</td>
<td>LN, skin, s.c.</td>
<td>M1&lt;sub&gt;a&lt;/sub&gt;</td>
<td>S</td>
<td>364</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Dac-2.</td>
<td>56/M</td>
<td>Lungs</td>
<td>M1&lt;sub&gt;b&lt;/sub&gt;</td>
<td>S, CTx, I</td>
<td>357</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
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<td>Dac-3.</td>
<td>60/M</td>
<td>Liver</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S, CTx</td>
<td>472</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>Dac-4.</td>
<td>28/F</td>
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<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S</td>
<td>567</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
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<td>Lung, skin</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S, CTx</td>
<td>451</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>Dac-6.</td>
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<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S</td>
<td>375</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>Dac-7.</td>
<td>25/M</td>
<td>Liver, lung, LN, skin</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S</td>
<td>649</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Dac-8.</td>
<td>53/F</td>
<td>Liver, lung, LN, skin</td>
<td>M1&lt;sub&gt;b&lt;/sub&gt;</td>
<td>S, RTx</td>
<td>318</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>Lung, LN, skin</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S</td>
<td>518</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>Dac-10.</td>
<td>59/M</td>
<td>Liver, lung, LN</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S, RTx, RF</td>
<td>352</td>
<td>+</td>
<td>-</td>
<td>n.t.</td>
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<tr>
<td>Dac-11.</td>
<td>46/M</td>
<td>LN</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S, I</td>
<td>560</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dac-12.</td>
<td>56/M</td>
<td>Liver</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S, RF, CTx</td>
<td>434</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Dac-13.</td>
<td>69/M</td>
<td>Lung, skin, brain</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S, RTx</td>
<td>735</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t</td>
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<td>54/M</td>
<td>Liver</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S</td>
<td>277</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>Liver</td>
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<td>S</td>
<td>1,289</td>
<td>+</td>
<td>-</td>
<td>n.t.</td>
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<td>Lung, cut</td>
<td>M1&lt;sub&gt;b&lt;/sub&gt;</td>
<td>S, RTx</td>
<td>355</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>Co-2.</td>
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<td>Lung</td>
<td>M1&lt;sub&gt;b&lt;/sub&gt;</td>
<td>S, I</td>
<td>302</td>
<td>+</td>
<td>+</td>
<td>n.t.</td>
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<tr>
<td>Co-3.</td>
<td>50/F</td>
<td>Intestine, s.c.</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S, I</td>
<td>366</td>
<td>+</td>
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<td>n.t.</td>
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<td>Co-4.</td>
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<td>LN</td>
<td>M1a</td>
<td>S</td>
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<td>Co-5.</td>
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<td>Kidney, LN, spleen</td>
<td>M1c</td>
<td>S</td>
<td>911</td>
<td>+</td>
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<td>Intestine, s.c.</td>
<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S, I</td>
<td>389</td>
<td>+</td>
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<td>-</td>
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<td>Co-7.</td>
<td>22/M</td>
<td>Sc, testicle</td>
<td>M1c</td>
<td>S</td>
<td>341</td>
<td>-</td>
<td>+</td>
<td>n.t.</td>
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<td>M1&lt;sub&gt;c&lt;/sub&gt;</td>
<td>S, I</td>
<td>551</td>
<td>-</td>
<td>+</td>
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<td>LN</td>
<td>M1a</td>
<td>S</td>
<td>484</td>
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<td>+</td>
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<td>S</td>
<td>389</td>
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<td>M1c</td>
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<td>531</td>
<td>+</td>
<td>-</td>
<td>n.t.</td>
</tr>
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</table>

Abbreviations: LDH, lactate dehydrogenase; CTx, chemotherapy; cut, cutaneous; I, IFN-α; LN, lymph node; RF, radiofrequency ablation; RTx, radiotherapy; S, surgery; n.t., not tested.

*Dacizumab patients 1 to 7 received daclizumab 4 days before the first dendritic cell vaccination and patients 8 to 15 received daclizumab 8 days before the first dendritic vaccination.

†KLH-specific cellular proliferation and antibodies (total IgG) after the first vaccination.

‡Presence of tetramer-positive T cells in DTH is marked as +, presence of functional tetramer-positive T cells in DTH is marked as +++.

§Tetramer-specific T cells in the blood were also detected before vaccination.

∥Ongoing complete remission.
vaccinations, the IgG antibody titers further increased (Fig. 2; ANOVA $p_{\text{after 2 vacc.}} = 0.011$; $p_{\text{after 3 vacc.}} = 0.002$). With subtype analysis of IgG antibodies we observed only IgG1 and IgG2, indicating the presence of IFN-$\gamma$ and hence a Th1 response (data not shown; ref. 39). In contrast, in serum of patients pretreated with daclizumab, IgG antibodies reactive with KLH remained absent after the first dendritic cell vaccination. After additional vaccinations, a nonsignificant increase in KLH-specific antibodies could be observed in these patients (Fig. 2). The effect of the anti-CD25 antibody, which is still present in the serum of patients during dendritic cell vaccinations (Fig. 1), on antibody production could possibly be explained by effects on the CD25$^{\text{high}}$CD4$^{+}$ Th cells, the CD25-expressing B cells themselves, or both.

Fig. 1. Daclizumab-mediated immune effects in vivo. A, soluble IL-2R$\alpha$ levels in urine decreased to undetectable levels in all patients immediately after daclizumab administration. Restoration of soluble IL-2R$\alpha$ levels started three weeks after daclizumab administration. B, kinetics of CD25$^{\text{high}}$ cells (% of total lymphocyte fraction) in the blood after daclizumab administration. C, representative dot plots on the expression of CD25 (Y-axis) and FoxP3 (X-axis) on CD4$^{+}$-gated cells in PBMC during immunotherapeutic treatment. D, longitudinal measurement of the CD25$^{\text{high}}$:CD25$^{\text{intermediate}}$ ratio of CD4$^{+}$FoxP3$^{+}$ cells. $\triangle$, time of daclizumab infusion; $\blacktriangle$, dendritic cell vaccinations. Dendritic cell vaccination alone ($\bullet$; $n = 6$) does not affect the percentage of CD4$^{+}$FoxP3$^{+}$ CD25$^{\text{high}}$ cells; daclizumab pretreatment ($\circ$; $n = 6$) depletes CD4$^{+}$FoxP3$^{+}$CD25$^{\text{high}}$ cells. E, longitudinal measurements of the percentage of CD4$^{+}$FoxP3$^{+}$ cells in both control patients and patients pretreated with daclizumab.
Proliferation of PBMCs upon KLH stimulation

Irrespective of daclizumab treatment, all patients developed strong proliferative responses against KLH already after a single dendritic cell vaccination (Fig. 3A). During KLH-specific proliferation of PBMCs high levels of TNF-α and IFN-γ, but not IL-4, could be measured, indicating the Th1 nature of the immune response (Fig. 3B). These data show that daclizumab does not affect the generation of KLH-reactive T cells in vivo and these T cells are functional upon stimulation in vitro. To study whether the KLH-specific T-cell activation also occurs in vivo in the presence of daclizumab, the KLH-specific proliferative capacity of day-33 PBMCs was tested in the presence of autologous serum taken at different time points after in vivo daclizumab administration. Figure 3C shows that the proliferative capacity of day-33 PBMCs was almost completely abrogated when serum was added with high concentrations of daclizumab (i.e., a few days after daclizumab administration). Even after 5 weeks the daclizumab concentration in the serum of this patient was high enough to reduce proliferation with 40% compared with serum without daclizumab (i.e., day −4). No inhibition of KLH-specific proliferation was observed with serum from control patients (data not shown). These data suggest that in patients pretreated with daclizumab, vaccine-induced KLH-specific CD4+ Th cells are generated but are functionally impaired as long as daclizumab is present.

Detection of tumor-specific T cells in vivo

To determine the effect of daclizumab on the presence of tumor-associated antigen-specific CD8+ T cells, tetramer stainings were done after three vaccinations (Fig. 4A and Supplementary Fig. S3A). In peripheral blood, tetramer-positive cells could be seen in only two patients (Dac-4 and Co-4). In patient Dac-4 the tetramer-positive T cells were also detectable in the blood prior to dendritic cell vaccinations. In both groups, significantly more antigen-specific CD8+ T cells were found in short-term T-cell vaccinations.
cultures (2-4 weeks) from biopsies of positive DTH reactions. In 2 of 10 tested control patients antigen-specific T cells were detected. The antigen-specific CD8+ T cells in these two patients were fully functional and produced high levels of IL-2 and IFN-γ upon antigen-specific stimulation (Supplementary Fig. S3B). In patients pretreated with daclizumab, we detected antigen-specific T cells in 5 of 13 evaluable patients. Strikingly, in 4 of 5 patients these tetramer-positive CD8+ T cells were completely non-functional and did not produce any cytokines upon antigen-specific stimulation. The gp100154-tetramer-specific cells of patient Dac-8 produced low amounts of IL-2, IL-5, and IFN-γ upon stimulation with the gp100154 peptide but not upon stimulation with the endogenously expressed gp100 protein (Fig. 4B and C). In conclusion, vaccine-induced CD8+ T cells in the daclizumab group were present after the dendritic cell vaccinations. In contrast to the CD4+ Th cells, however, these CD8+ T cells were functionally impaired in vitro even in the absence of daclizumab.

Clinical outcome

No significant difference was seen in PFS between patients that received daclizumab at day -4 or day -8 (Table 1). Analyzed as one group, patients that received daclizumab prior to dendritic cell vaccinations had the same PFS compared with patients that received only dendritic cell vaccinations (Fig. 5 and Table 1). Both the immunomonitoring data and the clinical follow-up indicate that daclizumab administration prior to dendritic cell vaccination does not enhance the efficacy of the vaccine. Survival in both the daclizumab group and the control group was comparable with what has been reported in the literature for a population of patients with metastatic melanoma (40). Although the number of patients in this study with specific T cells was limited and did not allow statistical analysis, the data are in accordance with our previous work (34) showing that those patients with functional antigen-specific T cells after vaccination have a prolonged PFS (Table 1). Despite the presence of specific T cells in five patients within the daclizumab group, their inability to respond to tumor cells most likely explains the lack of impact on PFS in these patients.

Discussion

Immune-suppressive CD4+FoxP3+CD25high Tregs accumulate in primary and metastatic melanomas, which undermines spontaneous and/or vaccine-induced immune responses against melanomas (12-14). The aim of this
Fig. 4. Tumor-specific T cells in vivo in patients pretreated with daclizumab. A, flow cytometric tetramer analysis of T cells derived after three dendritic cell vaccinations from biopsies of positive DTH reactions to dendritic cells pulsed with gp100 peptides. Percentage of tetramer-specific CD8 cells is indicated in the dot plot. IFN-γ (black bars) and IL-2 (white bars) production of T cells derived from DTH reactions from patient Dac-8 (this is one representative patient out of four; B) and patient dac-9 (C) after stimulation with nonspecific and specific stimuli.
These observations have raised the question of whether Treg depletion provokes tumor-specific immune responses (15, 18). In human melanoma models it has been shown that transient depletion of Tregs following organ transplantation (41) and additionally third-party antigens that are essential for thymic development, peripheral survival, and functional fitness of Tregs (22-24). Beside activated lymphocytes, Tregs especially express high levels of CD25 and are highly dependent on IL-2 (25). IL-2 delivers signals that are essential for thymic development, peripheral survival, and functional fitness of Tregs (22-24). In mouse melanoma models it has been shown that transient depletion of Tregs using anti-CD25 monoclonal antibodies provokes tumor-specific immune responses (15, 18, 19). These observations have raised the question of whether interference with IL-2 signaling via daclizumab might decrease the number of functional Tregs (43).

We show that daclizumab efficiently depleted CD4+FoxP3+CD25high Tregs from the peripheral blood within 4 days after administration. Approximately 30 days after the administration, daclizumab was cleared from the circulation and CD4+FoxP3+CD25high Tregs reappeared. Immune response analysis revealed that significantly less patients pretreated with daclizumab developed functional vaccine-specific effector T cells and antibodies compared with controls. No antibodies against the "de novo" antigen KLH were present in the serum of daclizumab-treated patients. Subsequent in vitro analysis showed that in patients pretreated with daclizumab, vaccine-induced KLH-specific CD4+Th cells are generated and functional but only in the absence of daclizumab. Vaccine-related tumor-associated antigen-specific CD8+ T cells were detected in DTH biopsies from daclizumab-treated patients. However, these cells failed to acquire effector function upon target cell recognition even in the absence of daclizumab in vitro. These immunomonitoring data suggest that in daclizumab-treated patients the lack of proper Th-cell activation may have prevented effective generation of KLH reactive antibodies and vaccine-induced CD8+ T-cell responses, both of which depend on effective T-cell help. The direct impact of daclizumab on CD25+ B cells remains unclear.

Multiple explanations for why daclizumab did not enhance or might even inhibit the immunostimulatory efficacy of subsequent dendritic cell vaccines can be envisaged. Firstly, daclizumab does not deplete all FoxP3+ cells. The residual CD4+FoxP3+CD25intermediate cells likely represent a functional (precursor) pool of Tregs. In addition, we cannot exclude that daclizumab-mediated CD4+FoxP3+CD25high-Treg depletion was less effective at the side of the tumor and tumor draining lymph nodes. Secondly, daclizumab recognizes CD25 that is not exclusively expressed on Tregs (44). CD25 is known to be expressed on activated CD4 and CD8 T cells, but melanoma cells themselves can also express low levels of CD25 (45, 46). Moreover, a single dose of daclizumab administered to melanoma patients transiently increases the number of regulatory NK cells that may have further compromised the efficacy of the subsequent dendritic cell vaccinations. Our in vitro data show that daclizumab in the serum of patients disarms effector T cells. Aside from inhibiting the de novo induction of effector T cells, daclizumab may also affect existing antitumor immune responses already present in the patients. Previously, it had been reported that the number of antigen-experienced CD25+ lymphocytes is greatly elevated in tumors and their draining lymph nodes (13). Because we show that daclizumab depletes all CD25high lymphocytes in vivo, daclizumab may partly neutralize this pre-existing antitumor immune response. A similar hypothesis was raised by Curtin et al. to explain why anti-CD25 mAb in a mouse glioblastoma model can eliminate newly induced tumors but cannot inhibit tumor progression in established tumors (47). Thirdly, in all patients that received a single dose of 0.5 mg/kg daclizumab, the drug was still present during subsequent dendritic cell vaccinations. Residual daclizumab titers may have prevented the induction of functional CD25+ effector cells by antigen-loaded dendritic cells. This may also explain why depletion of Tregs by Ontak can enhance T-cell responses upon vaccination in certain cancer patients (48). The half-life of this compound is very short, therefore the drug is no longer present at the time that vaccine-induced effector T-cell expansion occurs. In this context, it might be interesting to explore the original mouse mAb anti-Tac on which humanized daclizumab is based (49), as it will have a much shorter half-life than daclizumab in humans.

Our results indicate that immunotherapeutic trials in which antibodies are used to target CD25, or related Treg-depleting strategies such as CD25-directed toxins...
(27–29, 50), must carefully monitor the effects on all CD25+, not Tregs only, and adjust timing and dosing schedules accordingly. A Treg-specific cell surface marker that could be targeted with antibodies to specifically deplete/inactivate Tregs would overcome the daclizumab-related problems encountered in this clinical trial.

We conclude that, although daclizumab depleted the CD4+FoxP3+CD25high Tregs from the peripheral circulation, it did not enhance the efficacy of the dendritic cell vaccine. We show that residual daclizumab titers suppressed de novo induction of CD25+ effector cells during dendritic cell vaccinations. Our results indicate that for immunotherapeutic benefit of the transient Treg depletion, both timing and dosing of daclizumab administration are extremely important. Alternatively, to overcome the immunoregulatory influence of Tregs, novel approaches to specifically target and comprehensively eliminate or abrogate the function of Tregs in vivo are required.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Dr. Strijf for doing the ultrasound guided, intranodal dendritic cell vaccinations and Dr. Gerritsen for the biopsies of the DTH site.

Grant Support
The Dutch Cancer Society (grant# KUN2003-2833), the Netherlands Organization for Scientific Research (NWO-grant# 917-76363), the EU-project Cancerimmunotherapy (LSHC-CT-2006-518234), and the EU-project DCTHERA (LSHB-CT-2004-512074).

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Received 07/08/2010; accepted 08/09/2010; published OnlineFirst 08/24/2010.

References


Clinical Cancer Research

Dendritic Cell Vaccination in Combination with Anti-CD25 Monoclonal Antibody Treatment: A Phase I/II Study in Metastatic Melanoma Patients


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