Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment, a phase I/II study in metastatic melanoma patients

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Running title: Treg depletion prior to DC-vaccination in melanoma patients

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Translational relevance

Immunity against malignant cells depends on the balance between the activity of immune effector cells and immune regulatory mechanisms. CD25^+Foxp3^+ regulatory T cells (Tregs) prevent immune attack by directly suppressing the activation of immune effector cells. The detrimental effect of Tregs in cancer has been demonstrated in multiple murine models in which transient depletion of Tregs enhances anti-tumor immunity.

In this study, we treated metastatic melanoma patients with the anti-CD25 antibody daclizumab prior to dendritic-cell based vaccination. We investigated whether the anti-CD25 antibody transiently depleted CD4^+FoxP3^+CD25^{high}-Tregs and whether this enhanced the immunostimulatory efficacy of dendritic cell vaccines. Our results stress that despite transient Treg depletion using anti-CD25 in patients, a blunted rather than enhanced T cell response to the vaccine was observed. The data imply that for immunotherapeutic benefit, timing and dosing and especially Treg specificity of the depleting compound are extremely important.
Abstract

Purpose. 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 mAb-mediated depletion of CD25\textsuperscript{high} regulatory T-cells (Tregs) is capable of enhancing the immunostimulatory efficacy of dendritic cell (DC) vaccines.

Experimental Design. Thirty HLA-A2.1\textsuperscript{+}, metastatic melanoma patients were vaccinated with tumor-peptide- and keyhole limpet hemocyanin (KLH)-pulsed mature DCs. Half of the patients were pre-treated with daclizumab, a humanized antibody against the IL-2 receptor α-chain (CD25), either 4 or 8 days before DC vaccinations. Clinical and immunological parameters were determined.

Results. Daclizumab efficiently depleted all CD25\textsuperscript{high} immune cells, including CD4\textsuperscript{+}FoxP3\textsuperscript{+}CD25\textsuperscript{high}-cells, from the peripheral blood within 4 days of administration. Thirty days after administration, daclizumab was cleared from the circulation and all CD25\textsuperscript{+} cells reappeared. The presence of daclizumab during DC vaccinations prevented the induction of specific antibodies \textit{in-vivo} but not the presence of antigen-specific T-cells. Daclizumab however did prevent these CD25\textsuperscript{+} T-cells to acquire effector functions. Consequently, significantly less patients pre-treated with daclizumab developed functional, vaccine specific effector T-cells and antibodies compared to controls. Daclizumab pre-treatment had no significant effect on progression free survival compared to the control group.

Conclusions. Although daclizumab depleted the CD4\textsuperscript{+}FoxP3\textsuperscript{+}CD25\textsuperscript{high}-Tregs from the peripheral circulation, it did not enhance the efficacy of the DC vaccine. Residual daclizumab functionally suppressed de-novo induced CD25\textsuperscript{+} effector cells during DC vaccinations. Our results stress that for immunotherapeutic benefit of transient Treg depletion, timing and dosing as well as Treg specificity are extremely important.
**Introduction**

Melanoma is considered one of the most immunogenic types of cancers. This is based on the following arguments: (i) several melanoma-specific antigens have been identified (1, 2); (ii) functional lymphocytes specific for melanoma-antigens are increased in melanoma patients (3); (iii) immune-stimulating agents can have a positive effect on disease outcome (4, 5); (iv) 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*, however favourable 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 Tregs 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 demonstrated in mouse melanoma-models in which transient Treg depletion induces anti-tumor immunity (15-19). These studies show that CD25 depletion effectively eliminates the CD25<sup>high</sup> Tregs, although an effect on CD25<sup>+</sup> effector lymphocytes has also been reported. Apparently, CD25 depletion temporarily resets the T-effector/Treg balance in favour of the T-effector subset. In mice several successful methodologies of Treg depletion have been described (20). This led to great interest in the depletion of Tregs as part of a multi-faceted immunotherapeutic treatment of melanoma patients. However, the ability to effectively eliminate human Tregs *in-vivo* has been limited by the lack of an exclusive cell surface marker. Tregs constitutively express high levels of the IL-2 receptor (IL-2R) (21). IL-2
delivers signals that are essential for thymic development, peripheral survival and functional fitness of Tregs (22-25). These observations have raised the question whether drugs that interfere with IL-2 signaling, might decrease the number of functional Tregs.

Denileukin diftitox (Ontak) is a recombinant IL-2/diphtheria toxin conjugate that is designed to direct the cytotoxic action of diphtheria toxin to cells that express the IL-2R (26). Thus far, clinical studies in which Ontak was administered to melanoma patients demonstrated that it may reduce the number of circulating Tregs and cause regression of melanoma metastases (27-29).

Compared to Ontak, monoclonal antibodies against the IL-2R α-chain (CD25) are more effective to reduce Tregs in melanoma bearing mice (20). In addition, anti-CD25 antibodies had a synergistic effect when combined with DC-based immunotherapy in mice (20). Daclizumab (F. Hoffmann-La Roche, Basel, Switzerland) is a humanized antibody directed against CD25 (30).

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 DC-vaccinations. We therefore vaccinated 30 HLA-A2.1+, metastatic melanoma patients with tumor-peptide- and KLH-pulsed mature DCs. Half of the patients were pre-treated with daclizumab.
Materials and methods

**Patient inclusion.** Inclusion criteria were: metastatic melanoma with measureable 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; 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 4 months was observed. Patients with distant non-visceral metastases were categorized according to the 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 LDH 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 performed from which DCs were generated (32). Antigen-pulsed mature DCs were administered intravenously and intradermally in close proximity to the inguinal lymph nodes three times biweekly. Patients in the daclizumab-group, were pre-treated with 0.5 mg/kg daclizumab intravenously either 4 days (first cohort of 7 patients) or 8 days (second cohort of 8 patients) before the first DC-vaccination. Patients were treated in a non-randomized 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 (RECIST) (33). Observation of progressive disease (PD) led to cessation of vaccinations. Progression-free survival was calculated from the day of the first vaccination. Vaccine-specific immune response was the primary endpoint. Clinical response was the secondary endpoint.
**Soluble IL-2Ra measurement.** Soluble IL-2Ra levels were determined in urine samples (U/mmol creatinine) using a commercially available immunometric assay (Immulite, Los Angeles, CA).

**Antibodies and Immunostaining.** To characterize the phenotype of the ex-vivo generated DCs and the immune-cell subpopulations in the peripheral blood, flow cytometry was performed using either FITC-, PE- or APC-conjugated mAbs. The following mAbs were used: anti-HLA class I (W6/32), anti-HLA DR/DQ (Q5/13), anti-CD80 (Becton Dickinson, Mountain View, CA), anti-CD14, anti-CD83 (both Beckman Coulter, Mijdrecht, the Netherlands), anti-CD8, anti-CD25 (clone M-A251, no interference with daclizumab binding to CD25), anti-CD56, anti-CD86 (BD PharMingen, San Diego, CA), anti-Foxp3 (eBiosciences, San Diego, CA), anti-gp100 (Dako, Glostrup, Denmark), anti-tyrosinase (Novocastra, Newcastle, UK) and anti-CX3CR1 (MBL, Woburn, MA). Daclizumab antibody was covalently labeled to Alexa-488 fluor using the Alexa-488 protein labeling kit according to manufacturer's procedure (Invitrogen, Carlsbad, CA).

**DCs: Preparation, Characterization, and Route of Administration.** DCs were generated as described previously (32). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Pharmacia, 30 min, 4°C, 2100 rpm) after leukapheresis. PBMC were washed and monocytes were isolated by adherence to plastic. Monocytes were cultured at 15x10^6 per 75 cm^2 tissue culture flasks (Costar, Badhoevedorp, The Netherlands) in 20 ml of interleukin (IL)-4 (500 U/ml Schering-Plough International, Kenilworth, USA), granulocyte-monocyte colony stimulating factor (GM-CSF 800 U/ml, Schering-Plough International, Kenilworth, USA) and 2% human serum (HS; bloodbank Rivierenland, Nijmegen, The Netherlands) containing X-VIVO 15 medium (BioWhittaker, Walkersville, MD). On day 6 half of the medium was replaced by autologous MCM enriched with 10 µg/ml PGE2 (Pharmacia & Upjohn, Puurs, Belgium) and 10 ng/ml TNF-α (kindly...
provided by Dr. Adolf Bender, Vienna, Austria). Cells were harvested on day 9. This procedure gave rise to mature DCs as demonstrated by high expression levels of MHC class I and II, CD80, CD83, and CD86, and absence of CD14 (data not shown).

**Cryopreservation of PBMCs and DCs.** All DCs were cultured directly after leukapheresis and frozen as immature or mature DCs for multiple vaccinations. DCs and PBMCs were frozen using a cryo 1°C freezing container (Nalgene, Rochester, NY), 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 cold medium and once in medium of room temperature before use.

**Peptide Pulsing.** DCs were pulsed with wild-type peptides, gp100\textsubscript{154-167}, gp100\textsubscript{280-288} and tyrosinase\textsubscript{369-376}. Pulsing was done directly after harvesting or after thawing. On the day of vaccination we added peptides (50 µg/ml) for 90 min and kept DC at 37 °C/5% CO\textsubscript{2}. Thereafter, fresh peptides (25 µg/ml) were added, and DCs were kept at room temperature for another 60 min. After peptide loading, DCs were washed once in 0.9% sodium chloride and resuspended in 100 µl (5 x 10\textsuperscript{6} DCs) for i.d. and in 200 µl (10 x 10\textsuperscript{6} DCs) for i.v. injection.

**DTH.** One week after the last of three DC vaccinations, a DTH skin test was performed as previously described (34). Briefly, unpulsed DCs, DCs pulsed with gp100, DCs pulsed with KLH and gp100, DCs pulsed with tyrosinase, and DCs pulsed with tyrosinase and KLH (10 x 10\textsuperscript{5} DCs 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 h, 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 for immunohistochemistry, and 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 2-4 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 was collected for immunological monitoring. Antibodies against KLH were measured in the serum of vaccinated patients by ELISA as previously described (35). Briefly, 96-well plates were coated overnight at 4º 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 h at room temperature. After extensive washing, specific Abs (against IgM, total IgG, IgG1, IgG2 and IgG4) labeled with horseradish peroxidase were allowed to bind for 1 h 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 nm.

**Proliferative Response and Cytokine Production to KLH.** Cellular responses against KLH were measured in a proliferation assay. Briefly, 1 x 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 h 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 x 10^5 cells in 10 µl) were incubated with PE-labeled tetrameric-MHC complexes for 1 h 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.

**Auto-antibody screening.** Sera were screened for anti-nuclear antibodies by indirect immunofluorescence on HEp-2000 cells in a serum dilution of 1:40, according to the manufacturer’s protocol (Biomedical Diagnostics, Antwerp, Belgium). 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 performed to test for treatment related fluctuation of immune cells in the peripheral blood. Differences in KLH-specific proliferation and KLH-specific antibodies were analysed by ANOVA. All statistical tests were two-sided, 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 DC-vaccine therapy (‘daclizumab-group’ n=15) or DC-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 to 69) which did not significantly differ from the control group (mean age 51.7; range 22 to 73). The two groups were also not significantly different concerning male/female-ratio, AJCC-stage of the melanoma and prior therapies.

Toxicity and side effects. No severe toxicity (common toxicity criteria grade III-IV) 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 longer than 2 days after a vaccination. No daclizumab-related toxicity was observed. During follow-up, we did not observe any clinical signs of auto-immune disease. Since daclizumab-induced transient Treg depletion may increase the risk of inducing auto-reactive antibodies, we specifically tested for the presence of anti-nuclear antibodies (ANAs) before and after vaccination. In both groups there was no significant increase of ANAs observed (supplementary table 1). During follow up, 1 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 figure 1a 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⁺ NK-cells, CD4⁺ Th-cells, and
CD8\(^+\) CTLs, and to a large fraction of CD4\(^+\)FoxP3\(^+\) Tregs. Incubation of lymphocytes with both daclizumab-alexa\(^{488}\) and non-cross-reactive anti-CD25-PE showed only a double positive and a double negative population, which demonstrates that daclizumab specifically and exclusively binds to CD25\(^+\) cells (Supplementary figure 1b).

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

**Depletion of Tregs in-vivo.** Daclizumab treatment transiently depleted CD4\(^-\)CD25\(^{\text{high}}\)FoxP3\(^+\) cells from the circulation (figure 1c,d; \(p<0.001\) paired t-test). The percentage of FoxP3\(^+\) cells was already significantly decreased 4 days after daclizumab administration (figure 1e; 44% reduction after 4 days, \(p=0.027\), paired t-test). Especially the CD25\(^{\text{high}}\)FoxP3\(^+\) Tregs were depleted. Daclizumab did not effectively deplete the FoxP3\(^+\) cells with low or intermediate CD25-expression, although we cannot exclude that they are functionally impaired by bound daclizumab. DC-vaccinations without daclizumab had no effect on the percentage of circulating CD4\(^-\)FoxP3\(^-\)CD25\(^{\text{high}}\) Tregs (figure 1c,d,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$^{\text{bright}}$CX3XR1$^{\text{low}}$-cells) (supplementary figure 2). This is consistent with previous reports showing daclizumab-induced regulatory NK-cell expansion in patients suffering from auto-immune disease (37, 38).

**Humoral responses against KLH in serum.** Humoral responses against KLH were detected in serum of patients not pre-treated with daclizumab. Total IgG titers were detectable in most patients already after a single vaccination with KLH-pulsed DCs. After subsequent vaccinations, the IgG antibody titers further increased (figure 2; ANOVA $p_{\text{after 2 vac}}=0.011$; $p_{\text{after 3 vac}}=0.002$). 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) (39). In contrast, in serum of patients pre-treated with daclizumab, IgG antibodies reactive with KLH remained absent after the first DC-vaccination. After additional vaccinations, a non-significant increase in KLH-specific antibodies could be observed in these patients (figure 2). The effect of the anti-CD25 antibody, which is still present in the serum of patients during DC-vaccinations (figure 1), on antibody production could possibly be explained by effects on the CD25$^+$CD4$^+$ Th-cells, the CD25-expressing B-cells themselves, or both.

**Proliferation of PBMCs upon KLH stimulation.** Irrespective of daclizumab treatment, all patients developed strong proliferative responses against KLH already after a single DC-vaccination (figure 3a). During KLH-specific proliferation of PBMCs high levels of TNF-$\alpha$ and IFN-$\gamma$, but not IL-4 could be measured, indicating the Th1-nature of the immune response (figure 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 is almost completely abrogated when serum is 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 is high enough to reduce proliferation with 40% compared to 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 pre-treated 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 performed after 3 vaccinations (Figure 4a and supplementary figure 3a). In peripheral blood, tetramer positive cells could be demonstrated in only 2 patients (Dac-4 and Co-4). In patient Dac-4 the tetramer positive T-cells were also detectable in the blood prior to DC-vaccinations. In both groups, significantly more antigen-specific CD8⁺ T-cells were found in short term T-cell cultures (2-4 weeks) from biopsies of positive DTH reactions. In 2 out 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 figure 3b). In patients pretreated with daclizumab, we detected antigen-specific T-cells in 5 out of 13 evaluable patients. Strikingly, in 4 out 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 gp100\textsubscript{154} peptide but not upon stimulation with the endogenously expressed gp100 protein (Figure 4b,c). In conclusion, vaccine-induced CD\textsuperscript{8}\textsuperscript{+} T-cells in the daclizumab–group are present after the DC-vaccinations. In contrast to the CD\textsuperscript{4}\textsuperscript{+} Th-cells, however, these CD\textsuperscript{8}\textsuperscript{+} T-cells are functionally impaired \textit{in-vitro} even in the absence of daclizumab.

\textbf{Clinical outcome.} No significant difference was seen in progression-free survival (PFS) between patients that received daclizumab at day -4 or day -8 (table 1). Analyzed as one group, patients that received daclizumab prior to DC-vaccinations had the same PFS compared to patients that received only DC-vaccinations (figure 5 and table 1). Both the immunomonitoring data and the clinical follow-up indicate that daclizumab administration prior to DC-vaccination does not enhance the efficacy of the vaccine. Survival in both the daclizumab-group and the control-group is comparable to what has been reported in literature for a population of patients with metastatic melanoma (40). Although the number of patients in this study with specific T-cells is limited and does 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 study was to investigate whether a single low dose of daclizumab (0.5 mg/kg) transiently depletes Tregs in melanoma patients and to assess possible effects of this drug on immunity following DC-vaccinations. In this proof-of-principle clinical trial we vaccinated 30 metastatic melanoma patients with peptide- and KLH-pulsed mature DCs. Half of the patients were pre-treated with daclizumab, a humanized antibody against CD25. High dose daclizumab (1 mg/kg biweekly) was designed to target effector lymphocytes to prevent acute graft rejection following organ transplantation (41) and additionally proved effective for the treatment of patients with autoimmune diseases or T-cell leukemia (42). Beside activated lymphocytes, especially Tregs 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 demonstrated that transient depletion of Tregs using anti-CD25 monoclonal antibodies provokes tumor-specific immune responses (15, 18, 19). These observations have raised the question whether interference with IL-2 signaling via daclizumab, might decrease the number of functional Tregs (43).

We show that daclizumab efficiently depletes 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 pre-treated with daclizumab developed functional vaccine-specific effector T-cells and antibodies compared to 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 pre-treated
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 DC-vaccines can be envisaged. Firstly, daclizumab does not deplete all FoxP3+-cells. The residual CD4+FoxP3+CD25\text{intermediate}-cells likely represent a functional (precursor) pool of Tregs. In addition, we cannot exclude that daclizumab-mediated CD4+FoxP3+CD25\text{high}-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 also melanoma cells themselves can 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 which may have further compromised the efficacy of the subsequent DC-vaccinations. Our in-vitro data show that daclizumab in the serum of patients disarms effector T-cells. Aside inhibiting the ‘de novo’ induction of effector T-cells, daclizumab may also affect existing anti-tumor immune-responses already present in the patients. Previously, it has been reported that the number of antigen experienced CD25+ lymphocytes is greatly elevated in tumors and their draining lymph nodes (13). Since we demonstrate that daclizumab depletes all CD25\text{high} lymphocytes in-vivo, daclizumab may partly neutralize this pre-existing anti-tumor 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 DC-vaccinations. Residual daclizumab titers may have prevented the induction of functional CD25⁺ effector cells by antigen loaded DCs. 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 DC-vaccine. We show that residual daclizumab titers suppressed de-novo induction of CD25⁺ effector cells during DC-vaccinations. Our results stress that for immunotherapeutic benefit of the transient Treg depletion, both timing and dosing of daclizumab administration is 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.
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Legends

Figure 1. Daclizumab mediated immune-effects in-vivo (a) Soluble IL-2Rα levels in urine dropped to undetectable levels in all patients immediately after daclizumab administration. Restoration of soluble IL-2Rα levels started three weeks after daclizumab administration. (b) Kinetics of CD25\textsuperscript{high} cells (% of total lymphocyte fraction) in the blood after daclizumab administration (c) Representative dot plots show the expression of CD25 (y-axis) and FoxP3 (x-axis) on CD4\textsuperscript{+}-gated cells in PBMC during immunotherapeutic treatment. (d) Longitudinal measurement of the CD25\textsuperscript{high}:CD25\textsuperscript{intermediate} ratio of CD4\textsuperscript{+}FoxP3\textsuperscript{+} cells. Open triangle indicates time of daclizumab infusion, closed triangles represent DC vaccinations. DC-vaccination only (closed circles, n=6) does not affect the percentage of CD4\textsuperscript{+}FoxP3\textsuperscript{+}CD25\textsuperscript{high}-cells; daclizumab pre-treatment (open circles, n=6) depletes CD4\textsuperscript{+}FoxP3\textsuperscript{+}CD25\textsuperscript{high}-cells (e) Longitudinal measurements of the percentage of CD4\textsuperscript{+}FoxP3\textsuperscript{+} cells in both control patients and patients pre-treated with daclizumab.

Figure 2. Antibodies against KLH in-vivo. (a) Representative total IgG antibodies against KLH in the serum of a control patient (DC-vaccination alone), a patient pre-treated with daclizumab at day -4 and a patient pre-treated with daclizumab at day -8. (b) Results of total IgG against KLH of all patients vaccinated with either DCs alone (black bars), DCs with daclizumab pre-treatment day -4 (white bars) and DCs with daclizumab pre-treatment day -8 (grey bars). A significant increase in total IgG titers was detected only in control patients (ANOVA $p_{\text{after 2 vac}}=0.011$; $p_{\text{after 3 vac}}=0.002$).

Figure 3. KLH-specific proliferation of PBMCs before and after vaccination. (a) Both controls (open circles) and daclizumab patient-derived PBMCs (closed circles) show a strong proliferative response against KLH that could already be detected after a single DC-
vaccination. (b) Cytokine production by PBMCs of representative patients after three DC-vaccinations. (c) KLH-specific proliferation of PBMCs after three DC-vaccinations (day 33) in the presence of autologous serum obtained at various time points (patient Dac-1).

**Figure 4. Tumor specific T-cells in-vivo in patients pre-treated with daclizumab.** (a) Flow cytometric tetramer analysis of T-cells derived after three DC vaccinations from biopsies of positive DTH reactions to DC 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 non-specific and specific stimuli.

**Figure 5. Progression-free survival.** Kaplan-Meier plot comparing the PFS of metastatic melanoma patients that were vaccinated with DCs alone (closed circles) and those that received DC-vaccinations and pre-treatment with daclizumab (open circles). No significant difference between both groups was detected (p=0.24, Log rank test).
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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>Pre-study LDH (u/l)</th>
<th>Anti-KLH responsea</th>
<th>Tumor specific T cells</th>
<th>Progression free Survival (months)</th>
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<tbody>
<tr>
<td>Dac-1.</td>
<td>61/M</td>
<td>LN, skin, sc</td>
<td>M1a</td>
<td>S</td>
<td>364</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>Dac-2.</td>
<td>56/M</td>
<td>Lungs</td>
<td>M1b</td>
<td>S, CTx, I</td>
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<td>-</td>
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<td>Dac-3.</td>
<td>60/M</td>
<td>Liver</td>
<td>M1c</td>
<td>S, CTx</td>
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<td>-</td>
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<tr>
<td>Dac-4.</td>
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<td>S</td>
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<td>-</td>
<td>4d</td>
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<tr>
<td>Dac-5.</td>
<td>38/M</td>
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<td>-</td>
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<td>Dac-6.</td>
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<td>Lung, LN, skin</td>
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<td>S</td>
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<td>Dac-7.</td>
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<tr>
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<tr>
<td>Dac-10.</td>
<td>59/M</td>
<td>Liver, lung, LN</td>
<td>M1c</td>
<td>S, RTx, RF</td>
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<tr>
<td>Dac-11.</td>
<td>46/M</td>
<td>LN</td>
<td>M1c</td>
<td>S, I</td>
<td>560</td>
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<td>-</td>
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<td>Dac-12.</td>
<td>56/M</td>
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<td>M1c</td>
<td>S, RF, C</td>
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<td>69/M</td>
<td>Lung, skin, brain</td>
<td>M1c</td>
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<td>S</td>
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<td>1289</td>
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<td>Co-1.</td>
<td>73/M</td>
<td>Lung, cut</td>
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<td>S, RTx</td>
<td>355</td>
<td>+</td>
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<td>-</td>
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<tr>
<td>Co-2.</td>
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<td>Lung</td>
<td>M1b</td>
<td>S, I</td>
<td>302</td>
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<td>+</td>
<td>-</td>
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<td>Co-3.</td>
<td>50/F</td>
<td>Intestine, sc</td>
<td>M1c</td>
<td>S, I</td>
<td>366</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Co-4.</td>
<td>66/M</td>
<td>LN</td>
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<td>S</td>
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<td>+</td>
<td>+</td>
<td>+++</td>
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<td>911</td>
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<td>Co-6.</td>
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<tr>
<td>Co-7.</td>
<td>22/M</td>
<td>Sc, testicle</td>
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<tr>
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<td>551</td>
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<td>LN</td>
<td>M1a</td>
<td>S</td>
<td>484</td>
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<td>Sc</td>
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<tr>
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<td>34/M</td>
<td>LN, lung</td>
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<td>531</td>
<td>+</td>
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</tbody>
</table>

*a Daclizumab-patients 1-7 received daclizumab 4 days before the first DC vaccination and patients 8-15 received daclizumab 8 days before the first DC vaccination. bKLH-specific cellular proliferation and antibodies (total IgG) after the first vaccination. cPresence of tetramer-positive T cells in DTH is marked as +, presence of functional tetramer-positive T cells in DTH is marked as +++. dTetramer-specific T cells in the blood were also detected before vaccination. eOngoing complete remission. CTx, chemotherapy; cut, cutaneous; I, IFN-α; LN, lymph node; RF radiofrequency ablation; RTx, radiotherapy; S, surgery; sc, subcutaneous.
Figure 1

A. sCD25/creatinine

B. CD25<sup>high</sup> cells (%)

C.

D. CD25<sup>high:CD25<sup>int</sup></sup>-ratio

E. Dynamics FoxP3<sup>+</sup> cells (apheresis = 1)
Figure 2.

A.

Control

Before
After 1 vacc
After 2 vacc
After 3 vacc

Serum dilution (x100)

OD 450 nm

Daclizumab -4

Daclizumab -8

OD 450 nm

Serum dilution (x100)

B.

KLH specific total IgG

(before vacc = 1)

Before vacc
After 1 vacc
After 2 vacc
After 3 vacc

Daclizumab -4
Daclizumab -8
Controls

* **
Figure 3.

A. Controls
Daclizumab

KLH-specific proliferation (index)

before after 1 after 2 after 3 vacc. vacc. vacc. vacc.

B. pg/ml pg/ml

Co-1

IL-2 0 7
IL-4 0 0
IL-5 0 0
IL-10 69 158 393
TNF-α 0 0
INF-γ 0 0

DC

DC + KLH

IL-2 0 0
IL-4 0 0
IL-5 0 0
IL-10 600 1099
TNF-α 4 1005
INF-γ 0 0

Dac-1

DC + KLH

IL-2 0 0
IL-4 0 0
IL-5 0 0
IL-10 2 4
TNF-α 600 1099
INF-γ 4 1005

C.

Proliferation-index PBMCs day 33 (autologous serum day -4 = 1)

day -4 day 0 day 4 day 14 day 28 day 33 none

[anti-CD25 in serum]
Figure 4.

A. 

B. 

C. 

- IFN-γ
- IL-2
Figure 5.
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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