

Frequency of Circulating Tregs with Demethylated *FOXP3* Intron 1 in Melanoma Patients Receiving Tumor Vaccines and Potentially Treg-Depleting Agents

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Abstract

Purpose: Regulatory T cells (Tregs) are thought to inhibit antitumor immune responses, and their depletion could therefore have a synergistic effect with therapeutic cancer vaccines. We investigated the impact of three medications on blood Treg frequency in vaccinated cancer patients.

Experimental Design: To date, the most specific marker for human Tregs is demethylation in the DNA that encodes the transcription factor *FOXP3*. Thus, we used a *FOXP3* methylation-specific quantitative PCR assay (MS-qPCR) to measure Treg frequencies in the peripheral blood mononuclear cells (PBMCs) of melanoma patients. The patients participated in three clinical trials that combined tumor vaccines with potential Treg-depleting agents: low-dose cyclophosphamide, anti-CD25 monoclonal antibody daclizumab, and the IL-2/diphtheria toxin fusion protein denileukin diftitox.

Results: In the nine control patients, blood Treg frequencies varied over time; there was a 46% reduction in one patient. In treated patients, a more than 2-fold decrease in Tregs was observed in one out of 11 patients receiving cyclophosphamide and in four out of 13 receiving daclizumab, but there was no such Treg decrease in any of the six patients who received denileukin diftitox. As a positive control, a more than 2-fold increase in blood Tregs was detected in four out of nine patients who were treated with interleukin-2.

Conclusions: We used a MS-qPCR method that detects Tregs but not other activated T lymphocytes; however, none of the Treg-depleting strategies that we tested led, in the majority of patients, to a conservative 50% reduction in blood Tregs. *Clin Cancer Res*; 17(4); 841–8. ©2010 AACR.

Introduction

Antigens on human tumor cells that are recognized by T lymphocytes can be used to develop tumor-specific active immunotherapy. Several small clinical trials have investigated the therapeutic and immunologic efficacy of vaccinating advanced-stage cancer patients with defined tumor antigens. In previously published studies of patients with metastatic melanoma, about 5% of vaccinated patients showed a complete or partial clinical response, whereas

an additional 10% displayed some evidence of tumor regression without clear clinical benefit (1, 2). Because most antitumor vaccination modalities are not associated with serious adverse effects, therapeutic vaccination with defined antigens remains a promising approach, provided that its clinical efficacy increases substantially.

Improvements in clinical efficacy could be made by vaccinating earlier, at a stage in which there is minimal residual disease after treatment of the primary tumor; from the use of improved vaccines that induce stronger and broader T cell responses; or from combining vaccines with immunomodulation. Indeed, detailed studies of T cell responses in melanoma patients suggest that the major factor limiting the efficacy of cancer vaccines may be an immunosuppressive environment within tumors (1, 3–5). Candidate immunosuppressive mechanisms include production by tumor cells of soluble immunosuppressive molecules such as TGF- β , galectins, or indoleamine 2,3-dioxygenase (IDO), or suppressive cells such as myeloid-derived suppressive cells or regulatory T cells (Tregs; ref. 1).

Tregs are a subset of CD4⁺CD25⁺ T cells that specialize in the inhibition of immune responses (6). Their development and function depends on the transcription factor *FOXP3*, as illustrated by the severe Treg deficiency and autoimmune disease observed in patients with *FOXP3*

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Translational Relevance

Regulatory T cells (Tregs) are thought to inhibit antitumor immune responses. Agents that deplete Tregs in cancer patients could therefore improve the efficacy of therapeutic cancer vaccines. We compared the efficiency of 3 potentially Treg-depleting agents administered to vaccinated melanoma patients by measuring blood Treg frequencies with an epigenetic assay that is not confounded by the presence of activated non-Tregs. Our results show that neither daclizumab, nor low-dose cyclophosphamide or denileukin diftitox induced a more than 50% decrease in blood Tregs in a majority of the patients. This study indicates that different therapeutic strategies are required to further evaluate whether Treg depletion can improve cancer vaccine response.

mutations (7). In mice, there is ample evidence that Tregs inhibit spontaneous or vaccine-induced antitumor immune responses (8). In cancer patients, however, this has remained difficult to prove, because of the lack of a good Treg marker. CD25, the alpha chain of the IL-2 receptor, is upregulated in all activated T cells. Moreover, while in mice, FOXP3 is restricted to the Treg lineage, in humans it is also detected in activated CD4⁺ T cells that are not suppressive (9–11). Recently, it was shown that in humans, the demethylation of a conserved region in the *FOXP3* intron 1 is strictly specific to Tregs (12). This region, which we call "*FOXP3i1*" but is also referred to as TSDR (12–14) or CNS2 (15), is demethylated in CD4⁺CD25⁺ T cells and in suppressive CD4⁺ T cell clones. It is completely methylated in other human T cells and clones, even after TCR activation (11, 12). Zheng and colleagues recently reported that demethylation of this regulatory region is required for the maintenance of stable *Foxp3* expression in murine Tregs (15). We and others developed methylation-specific quantitative PCR (MS-qPCR) assays to quantify demethylated *FOXP3i1* sequences (11, 14).

One way to assess the role of Tregs in cancer immunity is to observe the effects of Treg depletion over the course of therapeutic anticancer vaccination. Low-dose cyclophosphamide, recombinant diphtheria toxin fused to IL-2 (denileukin diftitox), and monoclonal antibodies that target cells expressing CD25 have been proposed to deplete Tregs (8, 16, 17). However, their efficacy as Treg-depleting agents is subject to debate (18–25), which is not surprising, as Tregs were enumerated in previous studies by their expression of CD25 or FOXP3, methods that detect both Tregs and other activated CD4⁺ T cells.

In the present study, we used MS-qPCR for *FOXP3i1* to measure Treg frequency in melanoma patients enrolled in 4 independent clinical trials. Three of the trials combined cancer vaccines with either denileukin diftitox, cyclophosphamide, or the anti-CD25 humanized monoclonal antibody daclizumab. To the best of our knowledge, this is the first comparison of these compounds using a truly

Treg-specific parameter. The fourth trial combined vaccination with IL-2, which increases Tregs.

Materials and Methods

Patients

Vaccination with peptide pulsed DCs combined to daclizumab. Inclusion criteria comprised AJCC stage IV melanoma with measureable disease parameters; additional inclusion criteria are described in (26). DCs were generated from leukapheresis samples as previously described (27). Autologous mature DCs pulsed with gp100 and tyrosinase peptides and keyhole limpet hemocyanin (KLH) were administered intravenously (i.v.) and intradermally 3 times biweekly in close proximity to the inguinal lymph nodes that were clinically free of metastases and at which site no surgery had been performed. Patients received injections of daclizumab (0.5 mg/kg, i.v.) either 4 days (first cohort of 7 patients) or 8 days (second cohort of 8 patients) before the first dendritic cell (DC) vaccination. Patients were treated in a nonrandomized fashion. Analyses of clinical and immune responses are described elsewhere (26). PBMC samples from 13 patients were available for this retrospective study.

Vaccination with peptides in Montanide combined to cyclophosphamide. This phase II study included 22 HLA-A*0201 patients with histologically confirmed AJCC stage IIB/C or III melanoma. Patients received 2 cycles of vaccination with multiple peptides including Melan-A/MART-1_[26-35/27L], gp100_[209-217/210M], NY-ESO-1_[157-165/165V], and Survivin_[96-104/97M], emulsified in Montanide ISA-51. The first cycle consisted of 2 peptide injections at a 2 week-interval, and was preceded 4 to 7 days before by administration of 1 low dose of cyclophosphamide (300 mg/m²). The second cycle consisted of 4 biweekly peptide injections. Low-dose cyclophosphamide was administered before the first and third vaccines of the second cycle, and 3 daily injections of low-dose IL-2 (3 MIU, s.c.) were performed after the third and fourth vaccines. The study included an observation arm in which 21 patients received no other treatment beside surgery. Analyses of clinical and immune responses will be described elsewhere.¹ PBMC samples from 16 patients were available for this retrospective study.

Vaccination with peptide pulsed DC combined to denileukin diftitox. Stage III and IV metastatic melanoma patients were vaccinated with autologous DC pulsed with class I and class II peptides. Separate batches of DCs, prepared as previously described (28), were loaded with different class I peptides (Influenza NP, Influenza P B1, MAGE-1, MAGE-3, and 2 different Tyrosinase peptides for HLA-A1 patients, Influenza MP, Influenza B NP, MAGE-10, NY-Eso1, MelanA, and gp100 for HLA-A2 patients) and additionally with class II peptides (Tyrosinase.DR4 peptides, gp100.DR4, MAGE3.DP4, NY-ESO-1.DP4, MAGE3.DR11, and MAGE3.DR13) then frozen in aliquots. Half of the cells were also loaded with CD40L (1 µg/mL) during

¹Filipazzi et al., manuscript in preparation.

the peptide pulse. Batches of 12 million peptide loaded-DC per MHC class I peptide were injected intracutaneously on days 0, 14, 42 and 70. For the first vaccination only, a single additional batch of DC was pulsed with KLH (10 µg/mL). On days -3, -2, and -1 before the first DC injection, 18 patients received infusions of denileukin diftiox (Ontak, 5 µg/kg, i.v.). Analyses of clinical and immune responses will be described elsewhere.² PBMC samples from 6 patients were available for this retrospective study.

Vaccination with peptide pulsed DCs combined to IL-2. Patients with AJCC stage III melanoma were enrolled in this study on the basis of the same additional inclusion criteria described for the DC + daclizumab study. Vaccines consisted of autologous mature DCs pulsed with gp100 and tyrosinase peptides and KLH injected into an inguinal clinically tumor-free lymph node under ultrasound guidance. A total of 22 patients received 1 cycle of 4 vaccinations every 2 weeks. In addition to the vaccines, 10 of the patients received daily low-dose IL-2 (9 MIU, s.c.) during 1 week. Analyses of clinical and immune responses will be described elsewhere.³

For all studies, approval from the local regulatory committee was obtained and patients gave written informed consent prior to inclusion.

MS-qPCR for FOXP3i1

Genomic DNA (gDNA) was prepared from frozen pellets containing 4×10^5 to 10^6 total PBMCs with the PureLink Genomic DNA Mini Kit (Invitrogen). One to 2 µg of EcoRI-digested gDNA was treated with sodium bisulfite using the MethylCode Bisulfite Conversion kit (Invitrogen). Real-time PCR amplification of methylated and demethylated *FOXP3i1* sequences were performed on 30 to 125 ng of bisulfite converted DNA in a final volume of 25 µL with 0.6 U of HotGoldStar DNA polymerase (Eurogentec), 300 nM of each primer, 100 nM of probe, 200 µM of dNTP, and 5 mM of MgCl₂ in an ABI 7300 Real Time PCR system (Applied Biosystems) under standard conditions: 94°C for 10 minutes, 45 cycles of 94°C for 15 seconds and 64°C for 1 minute. Sequences of primers and probes are indicated in Supplemental Table S1, and their positions in the *FOXP3* gene are schematized in Supplemental Figure S1. Cloned PCR products served to demonstrate the complete specificity of the assay (Supplemental Figure S1), and were further used as standards for quantification of methylated and demethylated sequences. Proportion of cells with demethylated *FOXP3i1* is calculated as follows: [number of demethylated *FOXP3i1* sequences / (number of demethylated *FOXP3i1* sequences + number of methylated *FOXP3i1* sequences)] × number of X chromosomes per cell. Gene *FOXP3* is located on the X chromosome. In female patients, 1 of the 2 copies of the X chromosome is inactivated by methylation.

²Schuler-Thurner et al., manuscript in preparation.

³Lesterhuis et al., manuscript in preparation.

Magnetic sorting of PBMC subsets

Total PBMCs from a hemochromatosis donor were purified on a LymphoprepTM gradient, and separated into different subsets on an autoMacs[®] Separator instrument after labeling with CD4, CD19, CD56, or CD14 microbeads, with the CD8 T cell Isolation Kit, or with the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (all from Miltenyi Biotec).

FACS analysis

Cells were stained for surface antigens with anti-CD4/FITC, or anti-CD4/APC and anti-CD3/PerCP antibodies (BD Biosciences), then fixed and permeabilized overnight prior to staining with anti-FOXP3/PE antibody (clone 236A/E7, eBioscience) or an isotype control. Acquisition was performed on a FACSCalibur instrument (BD Biosciences), and data analyzed with the FlowJo software (Treestar).

Statistical analysis

Mean variations in Treg frequencies by comparison to the first day of observation or to pretreatment levels were calculated for 5 groups of patients (Supplemental Table S2). The control group comprised the 5 patients in the observation arm of the cyclophosphamide trial, as well as the 4 patients in the "DC vaccine only" arm of the "DC vaccine + IL-2" trial. Mean variations in the other groups were compared to mean variation in the control group by performing a Student's *t*-test (*P* values are indicated in Supplemental Table S2).

Results and Discussion

FOXP3i1 MS-qPCR assay to measure frequency of human Tregs

We previously developed a MS-qPCR assay to quantify cells with demethylated *FOXP3i1*, and used it on a large panel of human T cell clones to show that demethylated *FOXP3i1* is found in CD4⁺ Treg clones with suppressive activity, but not in others (11). In this study, we used the assay on blood mononuclear cells. Cells with demethylated *FOXP3i1* were enriched in the CD4⁺ subset, and present in the other subsets as a proportion of the contaminating CD4⁺ lymphocytes (Fig. 1A). These results are in line with those of Wiczorek and colleagues, who showed that demethylated *FOXP3i1* sequences are found only in the CD4⁺CD25^{hi} fraction (14). It is important to note that the frequencies of cells with demethylated *FOXP3i1* were not increased in purified CD4⁺CD25⁻ cells that were activated *in vitro*, even in the presence of TGF-β (Fig. 1B). This is in sharp contrast with the proportion of FOXP3-expressing cells, which increased after activation, especially in the presence of TGF-β (Fig. 1B). In human CD4⁺ T cells, this transient FOXP3 expression after activation with TGF-β is not associated with the acquisition of suppressive functions (10, 29). Taken together, these results confirmed that detecting demethylated *FOXP3i1* by MS-qPCR is a reliable method for measuring Tregs in human blood.

A	Ex vivo analysis of total PBMCs and purified subsets			B	CD4 ⁺ CD25 ⁻ purified cells analysed ex vivo and after in vitro culture			
	MS-qPCR	FACS			Cytokines in culture	MS-qPCR	FACS	
	Cells with demethylated FOXP3i1	CD3 ⁺ CD4 ⁺ lymphocytes	CD4 ⁺ FOXP3 ⁺ cells			Cells with demethylated FOXP3i1	CD25 ^{hi} cells	FOXP3 ⁺ cells
Total PBMCs	5.4%	50.2%	3.8%	Ex vivo	n.a.	4.2%	0.6%	6.4%
CD4 ⁺ cells	11.8%	97.7%	8.8%	Day 5 postactivation with plastic coated >CD3 and soluble >CD28	IL-2	1.9%	98%	10.5%
CD8 ⁺ cells	0.8%	2.6%	0.2%		IL-2 + TGF-β	2.4%	98%	66.3%
CD19 ⁺ cells	0.1%	0.9%	0.1%	Day 5 postactivation with >CD3/>CD28 coated beads	IL-2	1.2%	95%	18.0%
CD14 ⁺ cells	0.1%	0.7%	0.3%		IL-2 + TGF-β	1.4%	93%	62.7%
CD56 ⁺ cells	0.4%	1.3%	0.2%					

Figure 1. MS-qPCR assay to quantify human Tregs: (A) The indicated subsets of PBMCs were purified with magnetic beads. Total PBMCs and purified subsets were analyzed by MS-qPCR to quantify cells with demethylated *FOXP3i1*, and by flow cytometry for surface CD3 and CD4 and intracellular FOXP3 protein expression. (B) CD4⁺CD25⁻ cells purified from PBMCs with magnetic beads were analyzed by MS-qPCR and by FACS, either *ex vivo* or after 5 days of *in vitro* stimulation with anti-CD3 and anti-CD28 antibodies in the presence of IL-2 ± TGF-β, as indicated.

Frequency of Tregs in metastatic melanoma patients not receiving Treg-depleting agents

In this retrospective study, we measured the blood Tregs of patients with melanoma who were participating in 3 independent clinical trials that combined tumor vaccines with potential Treg-depleting agents, and in a fourth trial combining vaccines with IL-2. The numbers of included patients were small, and multiple samples were available for only 9 patients who received neither a Treg-depleting agent nor IL-2. In these control patients, Treg frequency varied over time, with a maximum increase of 260% and decrease of 46% (Supplemental Table S2). We therefore chose a conservative reduction of 50% to qualify patients as responders to the Treg-depleting agents.

Frequency of Tregs in patients receiving tumor vaccines and a Treg-depleting agent

In the first trial, 15 patients with stage IV melanoma received daclizumab (0.5 mg/kg, intravenous [i.v.]) 4 or 8 days before vaccination with mature dendritic cells (DCs) pulsed with keyhole limpet hemocyanin and the melanoma-associated peptides gp100₁₅₄₋₁₆₇, gp100₂₈₀₋₂₈₈, and tyrosinase₃₆₉₋₃₇₆ (26). Daclizumab caused a rapid and complete depletion of all blood cells expressing high levels of CD25 (including CD4⁺CD25⁺⁺FOXP3⁺CD127⁻ cells), but did not affect FOXP3⁺ cells expressing low levels or no CD25 (26). Because in humans, expression of CD25 or FOXP3 is not restricted to Tregs, these observations do not prove that daclizumab depleted Tregs. We therefore measured the frequency of Tregs with demethylated *FOXP3i1* in PBMCs from 13 of the patients for whom samples collected at ≥3 time points were available (Fig. 2A and Supplemental Table S2). Compared to predaclizumab levels, a more than 50% decrease in Treg frequencies was observed in 2 out of 7 and in 2 out of 6 patients who received the drug 8 and 4

days, respectively, before the first vaccine. These 55% to 96% decreases were transient: Treg frequencies returned to baseline levels after 1 month.

In the second trial,⁴ 22 patients with stage II or III melanoma received low-dose cyclophosphamide (300 mg/m², i.v.) 1 week before and 7 and 11 weeks after the initiation of vaccination with subcutaneous (s.c.) injections of 4 melanoma peptides emulsified in the adjuvant Montanide ISA 51. Later, patients received 3 daily injections of IL-2 (3 million international units [MIU]/day) to boost vaccine-induced immune responses. In a control arm, 21 patients received no treatment after surgery (observation only). After the second cyclophosphamide injection (day 56), a 94% decrease in Treg frequency was observed in 1 out of 11 patients analyzed (Fig. 2B and Supplemental Table S2). Small decreases (42%) were observed in 2 patients, but also (36%) in 1 of the 5 untreated patients who were available to be analyzed. For the patients who received IL-2, Treg frequencies increased more than 2-fold in 4 out of 9 evaluated cases.

The third trial explored the effect of denileukin diftitox (5 μg/kg) administered 3, 2, and 1 days before vaccination with mature, monocyte-derived DCs pulsed with tumor peptides.⁵ As shown in Figure 2C, none of the 6 evaluated patients had a more than 50% decrease in Treg frequency.

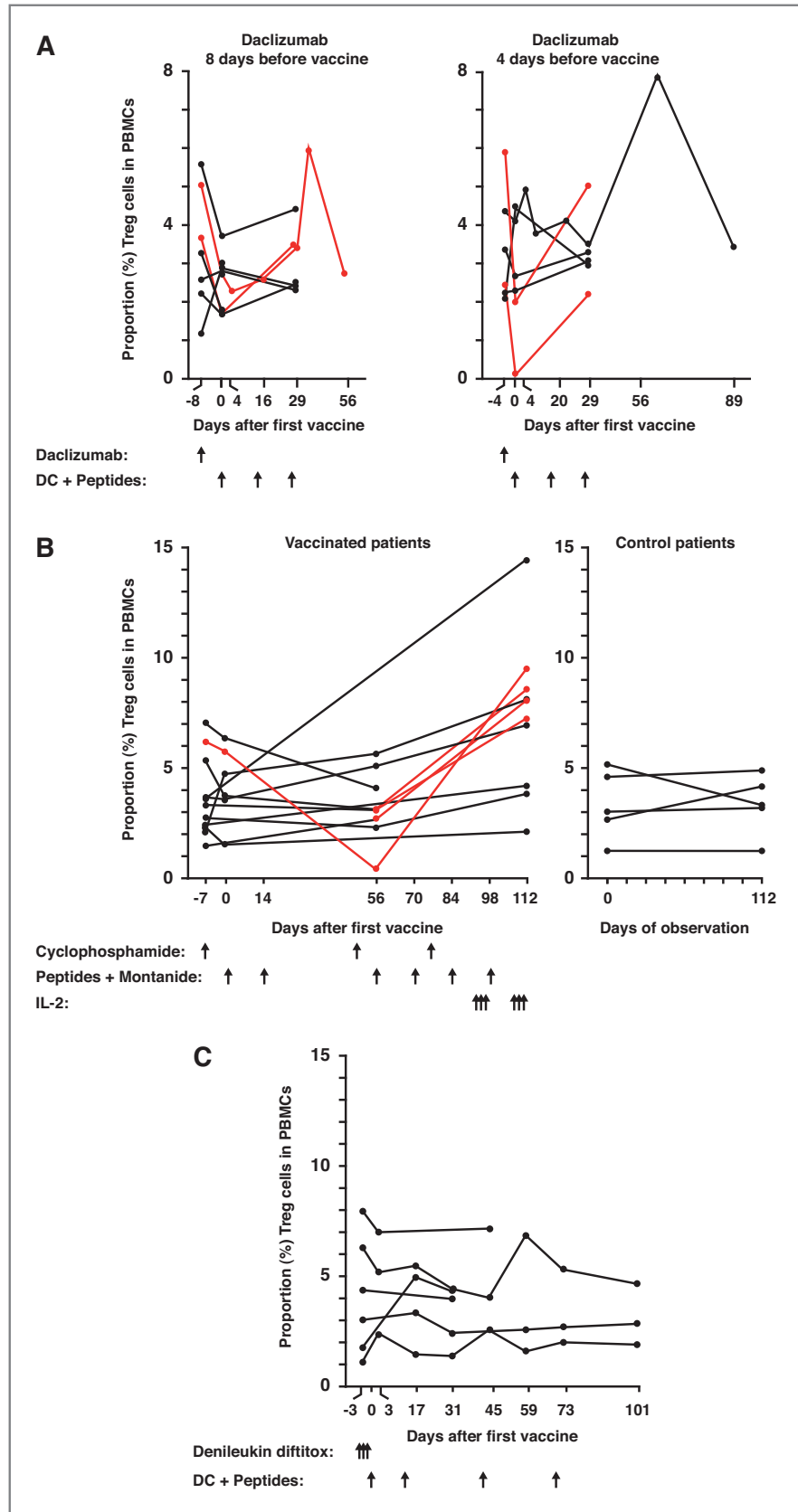
Frequency of Tregs in patients receiving a tumor vaccine and IL-2

IL-2 therapy can increase Tregs, as suggested by increases in FOXP3⁺ or CD25⁺CD4⁺ T cells in cancer patients receiving IL-2 (30–33). However, firm conclusions cannot be drawn from these studies, because FOXP3 and CD25

⁴Filippazzi et al., manuscript in preparation.

⁵Schuler-Thurner et al., manuscript in preparation.

Figure 2. Treg frequencies in patients receiving tumor vaccines combined to potentially Treg-depleting agents. PBMC samples were analyzed by MS-qPCR for *FOXP3/1*. Each line represents Treg frequencies in 1 patient. (A) Samples collected from patients who received daclizumab injections before vaccination with KLH and melanoma peptide-pulsed DCs (injection time points indicated by arrows below the graphs). Patients with more than 2-fold Treg decrease compared to baseline levels are indicated by red lines. (B) Samples collected from patients who received injections of low dose cyclophosphamide, melanoma peptides emulsified in Montanide and IL-2 (injection time points indicated by arrows below the graphs). Patients with more than 2-fold Treg decrease compared to prevaccine levels (day 7) or with more than 2-fold increase compared to pre-IL-2 levels (day 56) are indicated by red lines. (C) Samples collected from patients who received denileukin diftotox infusions 3, 2, and 1 days prior to vaccination with melanoma peptide pulsed DCs (injection time points indicated by arrows below the graphs).



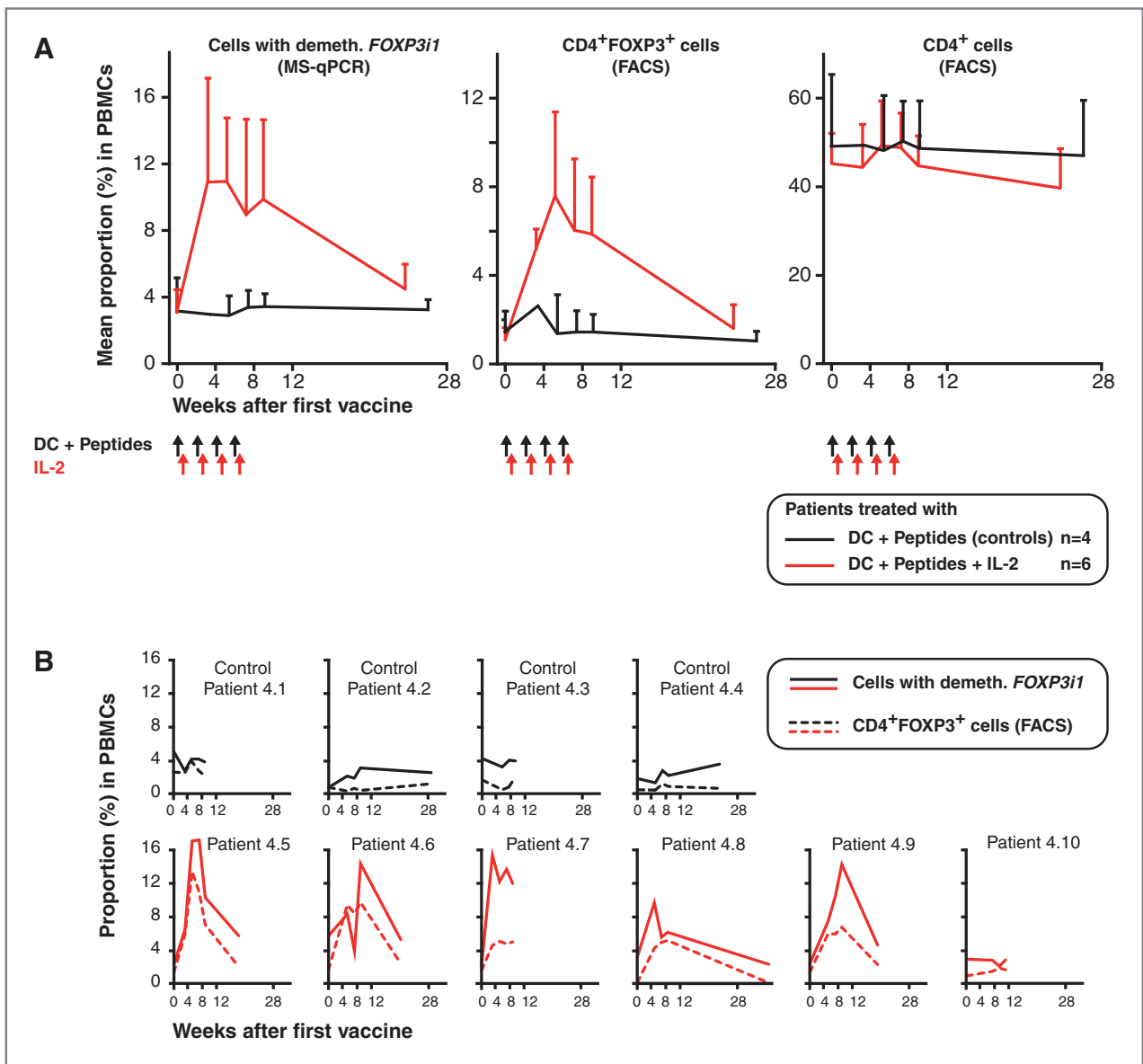


Figure 3. Treg frequencies in patients vaccinated with DCs pulsed with peptides combined or not to IL-2 injections: (A) Frequencies of cells with demethylated *FOXP3i1* were measured by MS-qPCR (graph on the left). Frequencies of $CD4^+FOXP3^+$ (middle graph) and of $CD4^+$ lymphocytes (graph on the right) were determined by flow cytometry. All analyses were performed on PBMC samples collected at the indicated time points from patients vaccinated either with DC + peptides (black lines), or with DC + peptides + IL-2 (red lines). Schedules of DC + peptide injections (black arrows) and IL-2 injections if any (red arrows) are indicated below the X-axis. Data represent mean values for the indicated number of patients + SD. (B) Same as in (A), but data is shown for each individual patient (graphs on bottom: patients receiving DC + peptides + IL-2; graphs on top: patients receiving DC + peptides only), with MS-qPCR results illustrated as plain lines, and FACS results as dotted lines.

expression is induced in non-Treg T cells by antigenic activation or by IL-2 itself. Wiezcorek and colleagues recently reported increases in the frequency of Tregs with demethylated *FOXP3i1* in melanoma patients treated with IL-2 (14). Thus, the 2-fold Treg increase that we observed after IL-2 administration in half of the patients who received cyclophosphamide combined with a vaccine is in line with this previous observation (Fig. 2B). We wished to confirm this effect of IL-2, and to compare the frequen-

cies of Tregs with demethylated *FOXP3i1* with those of $FOXP3^+CD4^+$ cells. To this end, we used both MS-qPCR and flow cytometry on PBMCs from patients in a fourth clinical trial, who received a vaccine with or without IL-2.⁶ Stage III melanoma patients were injected 4 times at biweekly intervals with DCs pulsed with gp100 and tyr-

⁶Lesterhuis et al., manuscript in preparation.

osinase peptides and KLH, as in the daclizumab trial. Two days after each injection, some patients received low-dose IL-2 (9 MIU, s.c. daily for 1 week). Using MS-qPCR for *FOXP3i1*, the mean Treg frequencies were shown to increase 3-fold after IL-2 was administered (Fig. 3A, left panel). Individual analyses showed this increase in all but 1 out of the 6 patients receiving IL-2, and in none out of the 4 patients who received the vaccine alone (Fig. 3B). Treg increases were detectable 3 weeks after the first IL-2 injection, and persisted for at least 3 weeks after the last injection. Using flow cytometry, we also found increases in the frequencies of CD4⁺ cells expressing FOXP3 (Fig. 3A, middle panel, and Fig. 3B). The increases observed with MS-qPCR were slightly higher than those observed with flow cytometry, notably for patient 4.7 (Fig. 3B). We have no definitive explanation for these differences. The mean frequencies of total CD4⁺ cell did not vary between the different time points or groups of patients (Fig. 3A, right panel). The results clearly confirmed that an increase in blood Tregs occurred after administration of IL-2, and provided a positive control for our *FOXP3i1* MS-qPCR method.

In conclusion, our quantification of Tregs using a method that is not confounded by the presence of activated CD4⁺ T cells indicated that none of the Treg-depleting regimens tested here reduced the frequency of blood Tregs efficiently (i.e., by a factor of 2 or more) in most of the treated patients. However, of the 3 regimens tested, daclizumab was the most promising. Unfortunately, its administration had no significant effect on the progression-free survival of the vaccinated melanoma patients included in the trial (26). Furthermore, it appeared to blunt the antivaccine T cell response, because functional antivaccine CD8⁺ T cells were not detected in

any of the daclizumab-treated patients. This effect is probably a consequence of the transient expression of CD25 on the surface of all activated T cells, and of the persistence of the depleting anti-CD25 antibody in the serum for at least 1 month (26). At present, it is unclear whether it will be possible to deplete Tregs with anti-CD25 reagents while sparing activated T cells. New strategies, possibly based on a better understanding of human Treg immunosuppressive mechanisms, are required to assess the impact of depletion or functional invalidation of these cells during cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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