Frequency of circulating Tregs with demethylated FOXP3 intron 1 in melanoma patients receiving tumor vaccines and potentially Treg-depleting agents

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Running title: Melanoma vaccination and Treg depleting agents
Keywords: cancer vaccine, regulatory T cells, melanoma, FOXP3, human Treg marker
Statement of 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 three 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 regulatory T cells. 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.
Abstract

Purpose: Regulatory T cells (Tregs) are thought to inhibit anti-tumor 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.
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 immunological 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 IDO, or suppressive cells such as myeloid-derived suppressive cells or regulatory T cells (Tregs) (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 mutations (7). In mice, there is ample evidence that Tregs inhibit spontaneous or vaccine-induced anti-tumor 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).
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 et al. 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 anti-cancer 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 four 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 three 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 DC vaccination. Patients were treated in a non-randomized 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 two 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 two peptide injections at a two week-interval, and was preceded 4 to 7 days before by administration of one 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 elsewhere1. PBMC samples from 16 patients were available for this retrospective study.

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1 Filipazzi et al, manuscript in preparation.

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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 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 diftitox (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 one cycle of four vaccinations every 2 weeks. In addition to the vaccines, 10 of the patients received daily low-dose IL-2 (9 MIU, s.c.) during one 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.

2 Schuler-Thurner et al, manuscript in preparation.
3 Lesterhuis et al, manuscript in preparation.
MS-qPCR for **FOXP3**

Genomic DNA (gDNA) was prepared from frozen pellets containing 4x10^5 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 **FOXP3** 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 **FOXP3** is calculated as follows: [number of demethylated **FOXP3** sequences / (number of demethylated **FOXP3** sequences + number of methylated **FOXP3** sequences)] x number of X chromosomes per cell. Gene **FOXP3** is located on the X chromosome. In female patients, one of the two copies of the X chromosome is inactivated by methylation.

**Magnetic sorting of PBMC subsets**

Total PBMCs from a hemochromatosis donor were purified on a Lymphoprep™ 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 Biotech).

**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 pre-treatment 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 the present 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 (Figure 1A). These results are in line with those of Wieczorek et al., who showed that demethylated FOXP3i1 sequences are found only in the CD4+CD25hi 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 (Figure 1B). This is in sharp contrast with the proportion of FOXP3-expressing cells, which increased after activation, especially in the presence of TGF-β (Figure 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.

**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 three 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 nine 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 (Figure 2A and Supplemental table S2). Compared to pre-daclizumab levels, a more than 50% decrease in Treg frequencies was observed in two out of seven and in two out of six patients who received the drug 8 and 4 days, respectively, before the first vaccine. These 55%–96% decreases were transient: Treg frequencies returned to baseline levels after one month.

In the second trial, 22 patients with stage II or III melanoma received low-dose cyclophosphamide (300 mg/m^2, i.v.) 1 week before and 7 and 11 weeks after the initiation of vaccination with subcutaneous (s.c.) injections of four melanoma peptides emulsified in the adjuvant Montanide ISA 51. Later, patients received three 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 one out of 11 patients analyzed (Figure 2B and Supplemental table S2). Small decreases (42%) were observed in two patients, but also (36%) in one of the five untreated patients who were available to be analyzed. For the patients who received IL-2, Treg frequencies increased more than 2-fold in four out of nine 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

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4 Filipazzi et al., manuscript in preparation

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tumor peptides\textsuperscript{5}. As shown in Figure 2C, none of the six 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\textsuperscript{+} or CD25\textsuperscript{+}CD4\textsuperscript{+} T cells in cancer patients receiving IL-2 (30-33). However, firm conclusions cannot be drawn from these studies, because FOXP3 and CD25 expression is induced in non-Treg T cells by antigenic activation or by IL-2 itself. Wiezcorek et al. recently reported increases in the frequency of Tregs with demethylated FOXP3\textsuperscript{i1} 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 (Figure 2B). We wished to confirm this effect of IL-2, and to compare the frequencies of Tregs with demethylated FOXP3\textsuperscript{i1} with those of FOXP3\textsuperscript{+}CD4\textsuperscript{+} 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\textsuperscript{6}. Stage III melanoma patients were injected four times at biweekly intervals with DCs pulsed with gp100 and tyrosinase peptides and KLH, as in the daclizumab trial. Two days after each injection, some patients received low-dose IL-2 (9 MIU, \textit{s.c.} daily for one week). Using MS-qPCR for FOXP3\textsuperscript{i1}, the mean Treg frequencies were shown to increase 3-fold after IL-2 was administered (Figure 3A, left panel). Individual analyses showed this increase in all but one out of the six patients receiving IL-2, and in none out of the four patients who received the vaccine alone (Figure 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\textsuperscript{+} cells expressing FOXP3 (Figure 3A, middle panel, and Figure 3B). The increases observed with MS-qPCR were slightly higher than those observed with flow cytometry, notably for patient 4.7 (Figure 3B). We have no definitive explanation for these differences. The mean frequencies of total CD4\textsuperscript{+} cell did not vary between the different time points or groups of patients (Figure 3A, right panel). The results clearly confirmed

\textsuperscript{5} Schuler-Thurner \textit{et al.}, manuscript in preparation
\textsuperscript{6} Lesterhuis \textit{et al.}, manuscript in preparation
that an increase in blood Tregs occurred after administration of IL-2, and provided a positive control for our *FOXP3* MSqPCR 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 two or more) in most of the treated patients. However, of the three 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 anti-vaccine T cell response, because functional anti-vaccine 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.
Figure legends

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 $FOXP3^i1$, 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.

Figure 2. Treg frequencies in patients receiving tumor vaccines combined to potentially Treg-depleting agents
PBMC samples were analyzed by MS-qPCR for $FOXP3^i1$. Each line represents Treg frequencies in one 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 pre-vaccine 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 diftitox 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 $FOXP3^i1$ 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.
References


### Figure 1

**A**  
*Ex vivo* analysis of total PBMCs and purified subsets

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<td>Total PBMCs</td>
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<tr>
<td>CD56⁺ cells</td>
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**B**  
CD4⁺CD25⁺ purified cells analysed *ex vivo* and after *in vitro* culture

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<td><em>Ex vivo</em></td>
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<td><strong>Day 5 post-activation with plastic coated &gt;CD3 and soluble &gt;CD28</strong></td>
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<td><strong>Day 5 post-activation with &gt;CD3/CD28 coated beads</strong></td>
<td>IL-2 + TGF-β</td>
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<td><strong>IL-2</strong></td>
<td>1.2%</td>
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Figure 3

**A**

Cells with demeth. *FOXP3* (MS-qPCR)

CD4*FOXP3* cells (FACS)

CD4* cells (FACS)

Mean proportion (%) in PBMCs

Weeks after first vaccine

DC + Peptides

IL-2

Patients treated with

- DC + Peptides (controls) n=4
- DC + Peptides + IL-2 n=6

**B**

Cells with demeth. *FOXP3* (FACS)

CD4*FOXP3* cells (FACS)

Proportion (%) in PBMCs

Weeks after first vaccine

Control

Patients 4.1 to 4.10
## Clinical Cancer Research

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