

# Influence of Metronomic Cyclophosphamide and Interleukine-2 alone or Combined on Blood Regulatory T Cells in Patients with Advanced Malignant Melanoma Treated with Dendritic Cell Vaccines

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## Abstract

**Background:** Interleukine-2 and Cyclophosphamide are known to influence the circulating T cells and are used for immune manipulation in cancer patients. We analyzed the influence on the Treg population in three different dendritic cell (DC) vaccine trials including either concurrent interleukine-2 (IL-2), combined IL-2 and metronomic Cyclophosphamide (MCy), or only MCy.

**Methods:** Melanoma patients received treatment with autologous DCs. IL-2 was applied in trial I and II and MCy was applied in trial I and III. Flowcytometry analysis was performed on fresh drawn blood-samples measuring CD4, CD25 and CD127.

**Results:** In the trials where IL-2 was applied a marked increase in the proportion of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Tregs from baseline to the 4th vaccine was observed. This was followed by a decrease, although not to baseline values. Additional analysis showed that the inhibitory function was predominant in the CD49d<sup>-</sup> subpopulation of Tregs which only increased slightly during treatment. The absolute lymphocyte count (ALC) and the number of CD4<sup>+</sup> T cells also increased in these trials. In the trial where only MCy was applied the Tregs remained stable throughout the trial whereas a decrease in the ALC and CD4<sup>+</sup> T cells were observed.

**Conclusion:** We found that adjuvant treatment with low doses of IL-2 during DC vaccination therapy causes a significant increase in blood level of classically defined, CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Tregs but far less in the CD49d<sup>-</sup> subpopulation and that the use of MCy was unable to reduce Treg blood level and unable to counteract IL-2 dependent increase in the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cells.

These findings have implications for the clinical use of IL-2 as well as MCy as immune modulators.

**Keywords:** Regulatory T cells; Interleukine-2; Cyclophosphamide; Dendritic cell vaccines; Malignant melanoma; CD49d

**Abbreviations:** IL-2: Interleukine-2; Treg: Regulatory T cell; Ce: Celecoxib; Cy: Cyclophosphamide; MCy: Metronomic Cyclophosphamide; TMZ: Temozolomide; DC: Dendritic Cell; SD: Stable Disease; PD: Progressive Disease; DC-MCy-IL2: DC vaccine with IL-2 + MCy (trial I); DC-IL2: DC vaccine with IL-2 (trial II); DC-MCy: DC vaccine with MCy (trial III); TMZ150: Temozolomide 150 mg/m<sup>2</sup>/day for a week followed by one week break

## Introduction

Malignant melanoma is a cancer with increasing incidence and a poor prognosis. Once metastasized there remains no standard curable treatment although IL-2, Dacarbazine and Temozolamide are often used. The first phase III trial ever to show improved overall survival in melanoma patients has been published recently. This trial was performed in pre-treated melanoma patients treated with Ipilimumab [1]. Because of limited options of treatment, various experimental treatments have been applied. As spontaneous remission has been described in melanoma and regression-zones are often seen within the primary tumor, melanoma is defined as an immunogenic disease. Therefore, melanoma has been the target of intensive research especially from an immunological aspect. Dendritic cell vaccination is one of the immune-therapeutic strategies that have been tested in a number of trials in metastatic melanoma patients [2]. In some of these studies a fraction of patients achieved clinical responses, but even more patients

achieved immunological responses. The reason for this difference is still unclear, but one possible explanation could be that intrinsic mechanisms in the immune system down-regulate vaccine induced immune response. During the past decade focus has been on the role of regulatory T cells (Tregs) and on possible ways to down-regulate the number and function of Tregs [3,4]. Recently, Ghiringhelli et al. [5] published that metronomic Cyclophosphamide (MCy) selectively decrease the number of Tregs and impair their function. Therefore, we investigated the effect of MCy as an immune modulator either alone or in combination with Interleukine-2 (IL-2) as adjuvant treatment in our dendritic cell (DC) vaccination trials. Here we describe the treatment induced changes in absolute lymphocyte count (ALC), CD4<sup>+</sup> T cells and Tregs.

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## Methods and Materials

### Patients and blood samples

Peripheral blood-samples were collected from patients with advanced malignant melanoma treated with DC vaccination according to three consecutive protocols. Blood samples were drawn before treatment initiation, at the 4<sup>th</sup>, 6<sup>th</sup> and 10<sup>th</sup> vaccination and every third month thereafter, until progression.

All clinical protocols were approved by the local ethics committee and the Danish Medicines Agency. Written informed consent from each patient was obtained prior to study entry. ClinicalTrials.gov identifier: NCT00197912 and NCT00978913.

**Trial resumes: (i) Trial I, DC-MCy-IL2:** Patients (n=28) with advanced melanoma stage IV were included in a non-randomized Phase II study at University Hospital Herlev from October 2008 to February 2010 (manuscript in preparation, E. Ellebaek et al). The patients received treatment with autologous monocyte-derived mature dendritic cells (DC) pulsed with p53, survivin and telomerase derived peptides (HLA-A2<sup>+</sup> patients) or tumor lysate (HLA-A2<sup>-</sup> patients). DCs were generated as previously described in details [6]. Vaccination number 1-4 was given once a week and vaccination number 5-10 every second week.

The patients received adjuvant treatment with IL-2 2 MIU day 1-5 following each vaccine, cyclophosphamide (Cy) 50 mg × 2 daily for a week every second week (MCy) and Celecoxib (Ce) 200 mg daily.

In this trial the panel for whole blood FACS analysis was expanded to include CD49d in 11 patients.

**(ii) Trial II, DC-IL2:** Results from this phase I/II trial was recently published [6,7]. In short 46 patients with advanced melanoma were treated with the same vaccine in the same vaccination-schedule as in trial I (DC-MCy-IL2). In this trial IL-2 was administered as in DC-MCy-IL2, but together with interferon- $\alpha$  2b and without the use of MCy.

In this cohort, fresh blood FACS analyses did not include CD127 and FoxP3, thus this was performed on frozen samples from 11 patients.

**(iii) Trial III, DC-MCy:** In this ongoing phase I trial until now 11 patients with progressive advanced malignant melanoma and 2 patients with breast cancer have been treated with autologous monocyte-derived mature DCs transfected with mRNA for survivin, hTERT and p53. DC vaccinations were given six times every second week, hereafter every four weeks until progression. Concomitant with DC vaccinations the patients were treated with MCy as described in trial I. Immunological response was measured in peripheral blood-samples at baseline, at the 4<sup>th</sup> and 6<sup>th</sup> vaccines and, if the patient achieved SD, every 3<sup>rd</sup> month in 11 patients.

### Whole blood flow cytometry

Fresh samples of 100  $\mu$ l of whole blood were aliquoted into staining tubes and lysed with 4 ml of x10 Ortho-Mune Lysing Solution. After centrifugation and removal of the supernatant samples were incubated with antibodies for 30 min at 4°C in the dark and subsequently washed and resuspended in cold buffer (PBS, 0.5% BSA, 0.1% NaN<sub>3</sub>). The following monoclonal antibodies (mAbs) were used for surface staining; CD25 APC, CD127 FITC (eBioscience.com) and CD3 APC-Cy7, CD4 PerCP, CD49d PE and IgG isotype matched control antibodies (BD Bioscience, San José, CA). All mAbs were used in concentrations based on manufacturers' recommendations.

Multicolor flow cytometry was performed on a FACSCanto flowcytometer (BD Bioscience). Day-to-day consistency of measurements was checked by CST beads (BD Bioscience) and data were acquired using FACSDiva software (BD Bioscience). 50000 leucocytes were collected using a forward scatter (FSC) threshold to exclude debris. FSC versus SSC gating of lymphocytes and SSC versus CD4 gating of CD4 positive cells was employed in data analysis. These cells were expressed in a configuration of CD127 versus CD25 and a population seen as CD25 positive and CD127 negative appeared and was defined as the Treg cells (Figure 1).

### Analysis of Foxp3 expression in CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cells

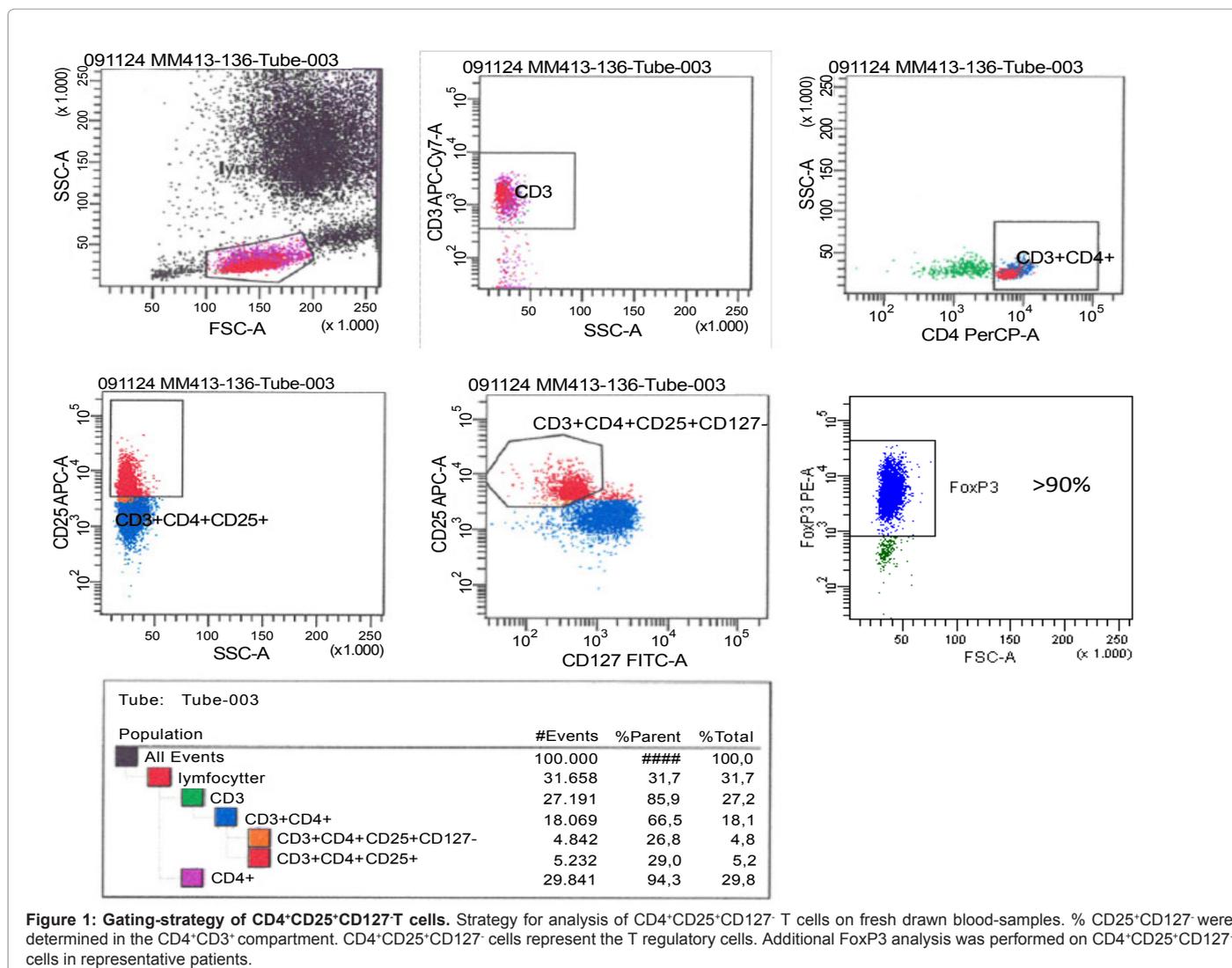
Frozen PBMCs were thawed and washed twice in cold PBS + 0.5 % BSA. Cells were incubated with mouse serum for 10 minutes and stained with anti-CD4-PerCP (BD Bioscience), anti-CD25-APC (eBioscience) and anti-CD127-FITC (eBioscience) at 4°C. After surface antigen staining cells were washed twice and permeabilized using a standard Fix/Perm kit from eBioscience following the manufacturers' instructions. Following washing with permeabilization buffer, cells were incubated for 15 minutes in normal rat serum (eBioscience), and then intracellular staining with anti-FoxP3-PE (eBioscience) was performed for 30 minutes at 4°C. All antibodies were used in concentrations recommended by the manufacturer. After washing in cold permeabilization buffer and PBS + 0.5 % BSA, cells were resuspended in PBS + 0.5 % BSA. Four colour flowcytometry were performed on a FACSCalibur (BD Biosciences) and data analyses were done using Cell Quest Pro software.

### T cell proliferation assay (inhibitory assay)

Blood samples drawn at the 4<sup>th</sup> vaccination were used. To analyse the inhibitory capacity of cells expanded in the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> gate a PKH based proliferation assay was performed. Round bottomed 96 well microtitre plates were coated overnight at 37°C with anti-CD3 antibodies (0.5  $\mu$ g/ml in PBS) (OKT3 from eBioscience). Prior to the assay, autologous MNCs (responder cells) were stained with PKH26 (Sigma, Saint Louis, Missouri, USA) following the protocol from the manufacturer. Fifty thousand PKH26 responder cells were mixed with 5×10<sup>4</sup>, 2.5×10<sup>4</sup> or 1.25×10<sup>4</sup> CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cells or CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup> T cells (control cells) in a final volume of 200  $\mu$ l RPMI-1640 + GlutaMAX<sup>TM</sup> + 25 mM HEPES (Gibco), supplemented with 10% FBS (Gibco) + penicillium-streptomycin (100  $\mu$ g/ml). The plates were incubated for 6 days at 37°C in humidified 5% CO<sub>2</sub> atmosphere. Then, cells were stained with APC anti-human CD4 (BD Biosciences) and FITC anti-human CD8 (BD Biosciences) and analyzed for proliferation (dilution of the PKH26 dye). PKH26 labeled MNCs cultured with anti-CD3 antibody and without anti-CD3 antibody were included as positive and negative controls, respectively. Percent inhibition was calculated as percent proliferation of CD4<sup>+</sup>/CD8<sup>+</sup> responder T cells added anti-CD3 antibodies – percent proliferation of CD4<sup>+</sup>/CD8<sup>+</sup> responder T cells added anti-CD3 antibodies and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cells, divided by percent proliferation of CD4<sup>+</sup>/CD8<sup>+</sup> responder T cells added anti-CD3 antibodies.

### Statistical analysis

Data are expressed as a mean and standard deviation for percentage. Parameters were compared by the use of student's t-test as they were regarded normally distributed. Statistical analysis was performed using R statistical software version 2.9.2 (R Foundation for Statistical Software, Vienna, Austria).



## Results

Changes in blood T cell subtypes, including Tregs, were measured in trial I (DC-MCy-IL2) and compared with results from trial II (DC-IL2) and trial III (DC-MCy), to investigate the influence of MCy and IL-2 on the ALC, CD4<sup>+</sup> T cells and Tregs.

### Treatment-induced changes in absolute lymphocyte and CD4 T cell count

In trial I (DC-MCy-IL2) MCy and IL-2 were used in combination. At baseline and by the time of 4<sup>th</sup> and 6<sup>th</sup> vaccine ALC was measured and the T cells were further evaluated by flow cytometry on freshly drawn blood-samples. If the patient achieved SD, we performed the same analysis by the time of the 10<sup>th</sup>, 13<sup>th</sup> and 16<sup>th</sup> vaccine.

ALC increased significantly from baseline to the time of the 4<sup>th</sup> vaccine from 1.69 to 1.93 × 10<sup>9</sup>/L (p = 0.04) followed by a significant decrease to 1.6 at the 6<sup>th</sup> vaccine (p = 0.001) which was not significantly different from baseline values (Figure 2A). A continued decreasing tendency through the 10<sup>th</sup> and 13<sup>th</sup> vaccine was seen, although none of the patients experienced lymphopenia (< 0.8 × 10<sup>9</sup>/L). The absolute CD4<sup>+</sup> T cell count showed a temporary increase from baseline to the 4<sup>th</sup>

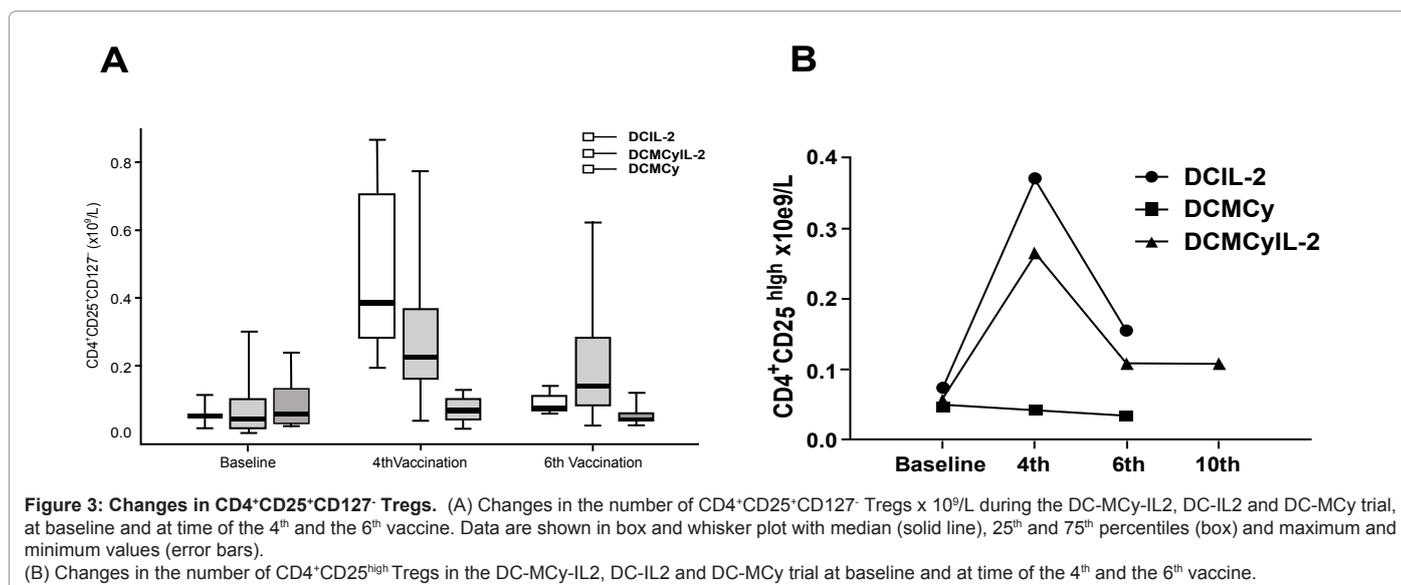
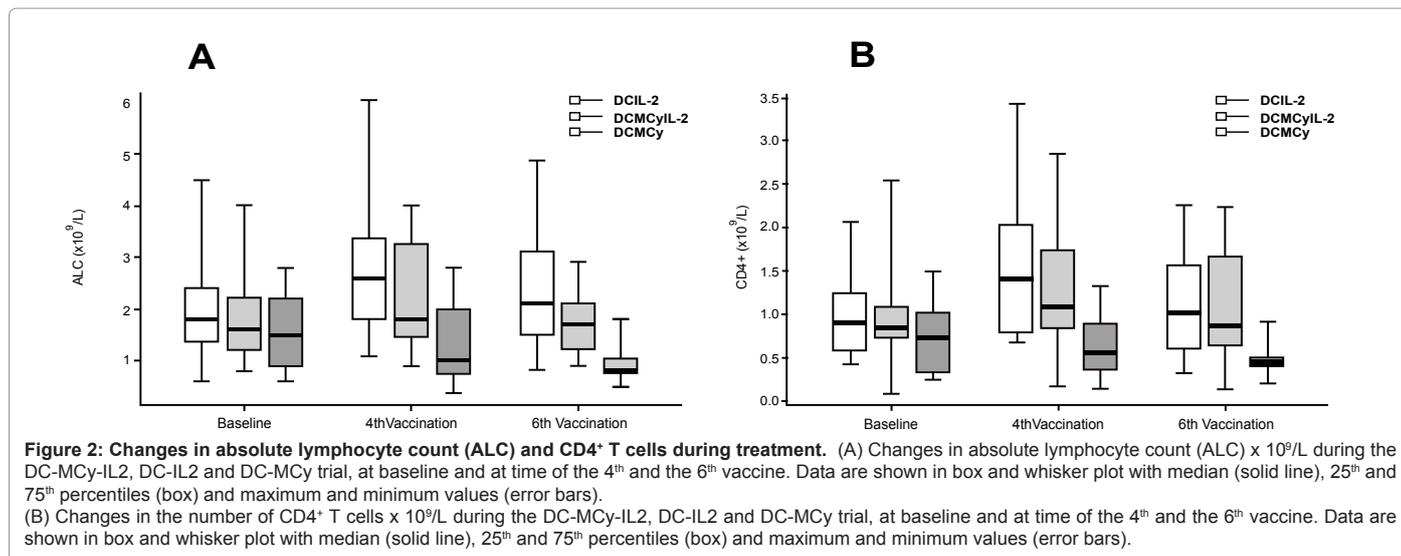
vaccine from 0.9 to 1.2 × 10<sup>9</sup>/L (p < 0.0001). Again, this was followed by a significant decrease (p = 0.0002) towards baseline values at time of the 6<sup>th</sup> vaccine (Figure 2B). Thus, there was no difference in the CD4<sup>+</sup> T cell count at baseline and the 6<sup>th</sup> vaccine (p = 0.8), however, a further decreasing tendency was seen at the time of the 10<sup>th</sup> vaccine.

A more pronounced increase in the ALC was observed in trial II (DC-IL2) where only IL-2 was applied. Thus, the increase in ALC from baseline to the 4<sup>th</sup> vaccine was even higher and only followed by a slight decrease in ALC at the time of the 6<sup>th</sup> vaccine. Thereafter the ALC remained stable, at an elevated level. The same pattern was seen for CD4<sup>+</sup> T cells.

Trial III (DC-MCy) including only MCy showed diverting changes. In contrast to the initial increase in ALC and CD4<sup>+</sup> T cell count observed in trial I + II, a significant decrease over time was observed in both cell types during treatment (p=0.003 and p=0.008 respectively).

### Treatment-induced changes in Tregs

In trial I (DC-MCy-IL2) the level of blood Tregs (defined as CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>) showed a pronounced increase (x 4.5) from baseline to the 4<sup>th</sup> vaccine; from 0.04 to 0.23 × 10<sup>9</sup>/L (p < 0.0001)



(Figure 3A). Again, this initial increase was followed by a decrease, but remained at higher levels than the baseline values ( $0.14 \times 10^9/L$ ,  $p < 0.0001$ ). From this time-point Tregs decreased slowly throughout the vaccination-course, but at the time of the 13<sup>th</sup> vaccine it was still significantly higher than the baseline value.

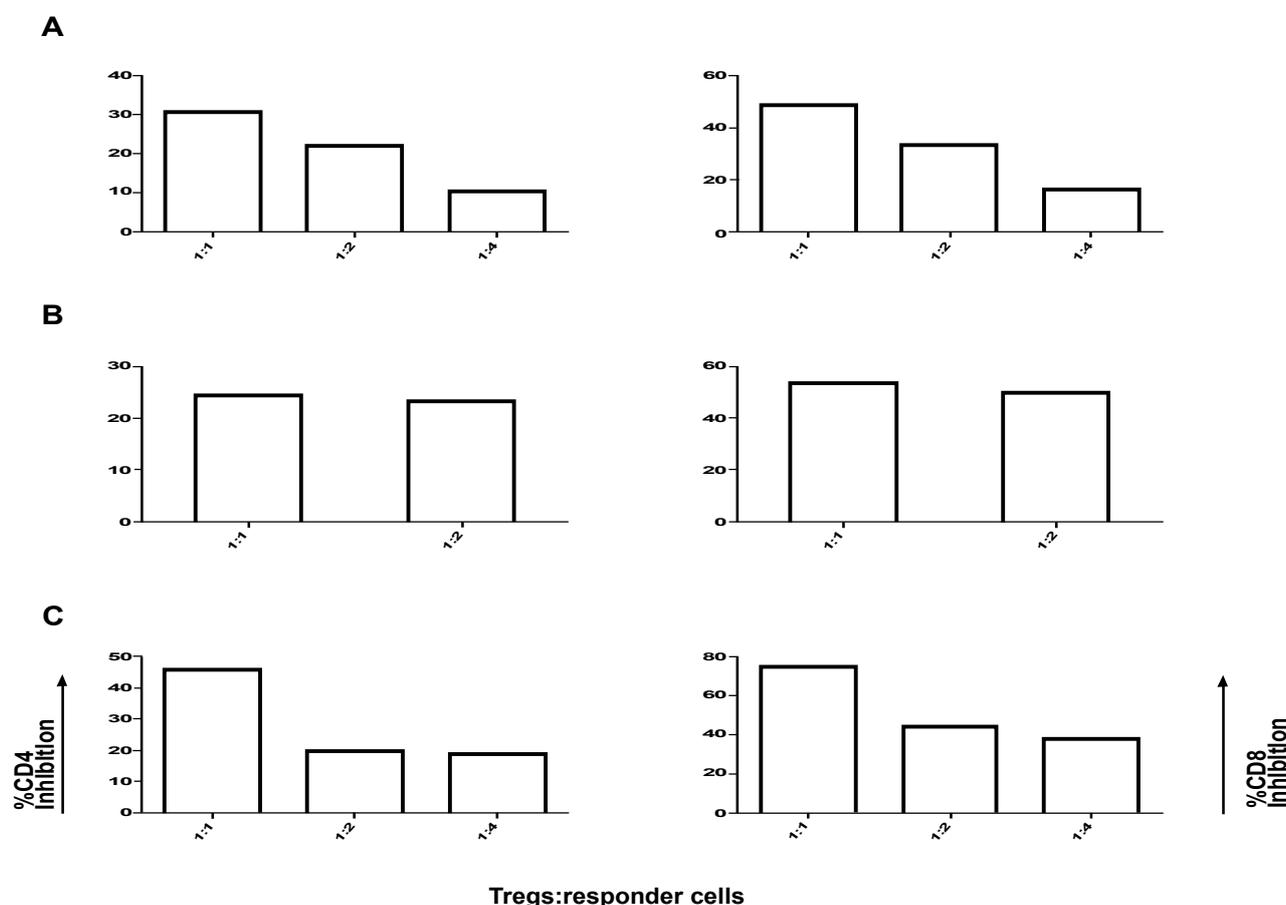
An even more pronounced increase in the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Tregs at the 4<sup>th</sup> vaccine was seen in trial II (DC-IL2) where IL-2 was applied without MCy. Again this was followed by a decrease, but without reaching baseline values. In contrast, in trial III (DC-MCy) employing only MCy the number of Tregs did not increase after treatment initiation but showed constant values during therapy ( $p=0.09$ ).

Additional analysis of Foxp3 expression was performed on samples from 5 random selected patients from trial I, 11 patients from trial II and 3 patients from trial III. These analyses showed no differences between the trials as more than 90 % of the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cells co-expressed Foxp3 (Figure 1).

In the literature Tregs are frequently defined as CD4<sup>+</sup>CD25<sup>high</sup> T cells [5,8-13]. For that reason we have also evaluated the changes in the number of Tregs according to this definition. Overall, treatment associated changes in the CD25<sup>high</sup> CD4<sup>+</sup> T cells were comparable to the observed changes in CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Tregs. Thus, in trial I and II (DC-MCy-IL2 and DC-IL2) the CD25<sup>high</sup> cells increased from baseline to the 4<sup>th</sup> vaccine, followed by a decrease although not to baseline levels. Whereas trial III (DC-MCy) showed stable or slightly decreasing numbers of CD25<sup>high</sup> cells (Figure 3B).

### Functional capacity of Tregs

It has been demonstrated by others that activated T cells may express CD25 as well as Foxp3 [14]. Therefore, to confirm the regulatory potential of the Tregs as defined in our setting, we performed a functional analysis of Tregs from 3 randomly selected patients from trial I and 5 patients from trial II. In patients from both trials we found that Tregs, to some extent, were capable of inhibiting both CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation and that the inhibition was dependent on the



**Figure 4: Functional properties of Treg cells in three representative patients at 4<sup>th</sup> vaccination.** For analysis of inhibitory capacity of Treg cells, CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>-</sup> T cells or CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup> T cells (control cells) were added to anti-CD3 stimulated autologous PBMCs in a ratio of 1:1, 1:2 and 1:4 (Tregs:responder cells). The cultures were incubated for 6 days and the percentage of CD4 and CD8 T cell proliferation was analysed. (A) % inhibition of CD4 and CD8 T cell proliferation in patient 120. (B) % inhibition of CD4 and CD8 T cell proliferation in patient 129. (C) % inhibition of CD4 and CD8 T cell proliferation in patient 130.

effector-target ratio (Figure 4). Thus, the total CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cell population did possess regulatory potential, even though complete inhibition was not seen.

It was recently published that the surface marker CD49d can be used to discriminate true suppressive Tregs (CD49d<sup>-</sup>) from CD4<sup>+</sup> effector cells (CD49d<sup>+</sup>) [15]. To further explore our Treg population we therefore analyzed CD49d expression on CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Treg cells from 11 patients in trial I and from 11 patients in trial III. The results from trial I, DC-MCy-IL2, is depicted in Figure 5A and show that the CD49d<sup>-</sup> subpopulation of Tregs constituted approximately 5 % of the CD4<sup>+</sup> T cells with only a slight increase by the time of the 4<sup>th</sup> vaccine. This is in contrast to the marked increase seen in the total Treg population and suggests that IL-2 treatment mainly increased the CD49d<sup>+</sup> cells and only to a lesser extent the CD49d<sup>-</sup> Tregs. Results from trial III (DC-MCy) using MCy alone, showed that the fraction of CD49d<sup>-</sup> Tregs was unchanged over time, as was the total population of Tregs (Figure 5B). The absolute numbers of CD49d<sup>-</sup> Treg cells are shown in Figure 5C.

Additional inhibition-assays were performed to analyze the function of CD49d<sup>+</sup>/<sup>-</sup> gated CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cells using CD4<sup>+</sup>CD25<sup>-</sup>CD127<sup>+</sup> T cells as negative control (Figure 5D). The results demonstrate that the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>CD49d<sup>-</sup> sub-population, had a

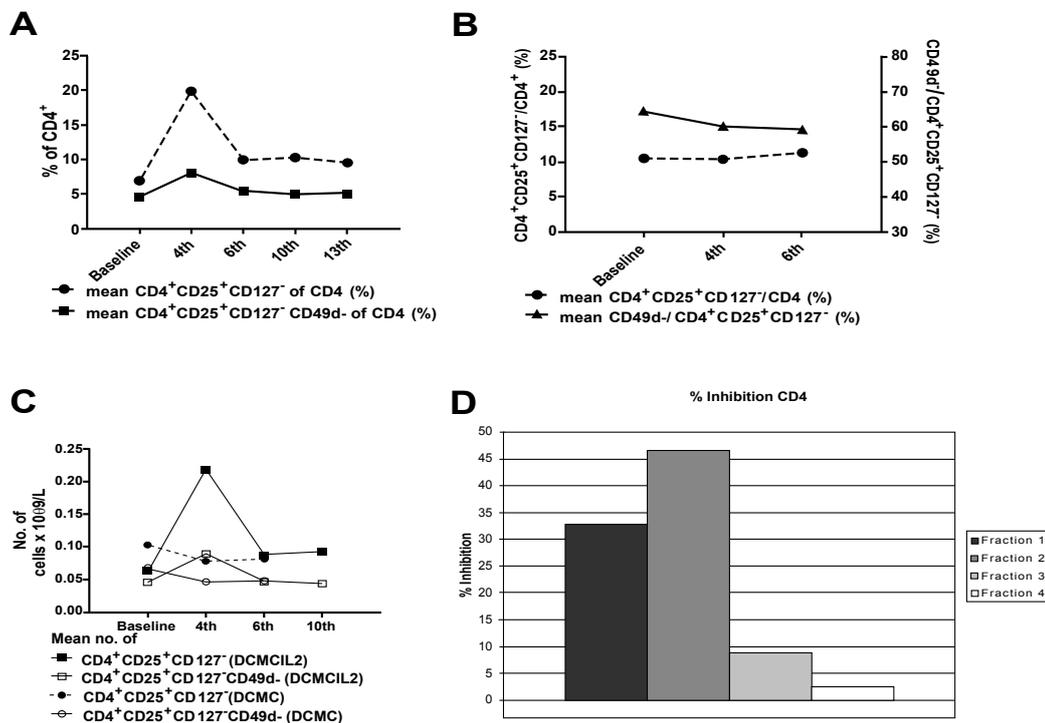
more pronounced inhibitory effect than the total (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>) Treg population, while the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>CD49d<sup>+</sup> T cells exhibited a very low inhibitory potential. This indicates that the main suppressive function of the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Tregs is exhibited by the CD49d<sup>-</sup> subpopulation.

#### Adjuvant IL-2 and CD8<sup>+</sup> T cells

In trial I (DC-MCy-IL2) and trial II (DC-IL2) low-dose IL-2 was administered. As shown, the use of even low doses of IL-2 as adjuvant therapy in our vaccine trials was found to be associated with the induction of Tregs. To further examine the relation between Tregs and CD8<sup>+</sup> T cells we measured the CD8<sup>+</sup> T cell levels during treatment in patients from the DC-MCy-IL2 trial and found that the number of CD8<sup>+</sup> T cells showed great inter-patient variation as well as variation over time in the individual patients. Overall, we found no significant treatment induced changes in the CD8<sup>+</sup> T cell levels (p = 0.42)(data not shown).

#### Influence of previous treatment with high-dose IL-2

Most of the patients (21 out of 28) in the DC-MCy-IL2 trial were treated with high-dose IL-2 (decreasing regimen) for their metastatic disease prior to enrollment. As high-dose IL-2 is described to increase the number of Tregs even in a period after treatment [8], we divided



**Figure 5: Changes in CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Treg subsets according to CD49d expression.**

(A) Changes in the percentage of CD25<sup>+</sup>CD127<sup>-</sup> and CD25<sup>+</sup>CD127<sup>-</sup>CD49d<sup>-</sup> Tregs during treatment in the DC-MCy-IL2 trial, at baseline and at time of the 4<sup>th</sup> and the 6<sup>th</sup> vaccine.

(B) Changes in the percentage of CD25<sup>+</sup>CD127<sup>-</sup> and CD25<sup>+</sup>CD127<sup>-</sup>CD49d<sup>-</sup> Tregs during treatment in the DC-MCy trial, at baseline and at time of the 4<sup>th</sup> and the 6<sup>th</sup>.

(C) Changes in the number of CD25<sup>+</sup>CD127<sup>-</sup> and CD25<sup>+</sup>CD127<sup>-</sup>CD49d<sup>-</sup> Tregs during treatment in the DC-MCy-IL2 trial and in the DC-MCy trial, at baseline and at time of the 4<sup>th</sup> and the 6<sup>th</sup> vaccine.

(D) Functional properties of Treg subsets: For analysis of inhibitory capacity of different Treg cell subsets, the CD25<sup>+</sup>CD127<sup>-</sup> (fraction 1), the CD25<sup>+</sup>CD127<sup>-</sup>CD49d<sup>-</sup> (fraction 2), the CD25<sup>+</sup>CD127<sup>-</sup>CD49d<sup>+</sup> (fraction 3) or the CD25<sup>+</sup>CD127<sup>-</sup> (control cells) (fraction 4) T cells were added to anti-CD3 stimulated autologous PBMCs from a buffy coat in a ratio of 1:1 (Tregs:responder cells). The cultures were incubated for 5 days and the percentage of CD4 T cell proliferation was analysed. % inhibition of CD4 is depicted.

the patients into two groups depending on the time interval from high-dose treatment with IL-2. These results are pictured in Figure 6A and show a significant higher number of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Tregs in the patients who were treated with IL-2 < 2 months before inclusion compared to patients treated with IL-2 ≥10 months prior to inclusion (p= 0.03), three patients were treated >2, but <10 months prior to inclusion and were not included in this analysis. This difference in Treg levels were equalized at the time of the 4<sup>th</sup> and the 6<sup>th</sup> vaccine (p= 0.5 and 0.5) indicating a transient effect of IL-2.

### Influence of previous treatment with Temozolomide

Some of the patients (10 out of 28) included in the DC-MCy-IL2 trial, were treated with Temozolomide (TMZ) prior to enrollment in the trial. As TMZ is known to specifically decrease the level of ALC and CD4<sup>+</sup> cells [16,17] we compared patients +/- TMZ pretreatment. We found that the levels of Tregs, CD4<sup>+</sup> T cells and ALC were all lower (p=0.05, p=0.05, p=0.01) in the TMZ pre-treated group at baseline, while CD8<sup>+</sup> T cells were unchanged. Reduced number of Tregs in the TMZ pre-treated group was only significant at baseline as the difference declined by the time of the 4<sup>th</sup> and 6<sup>th</sup> vaccine although a tendency of a lower number of Tregs in the pre-treated patients was seen (Figure 6B).

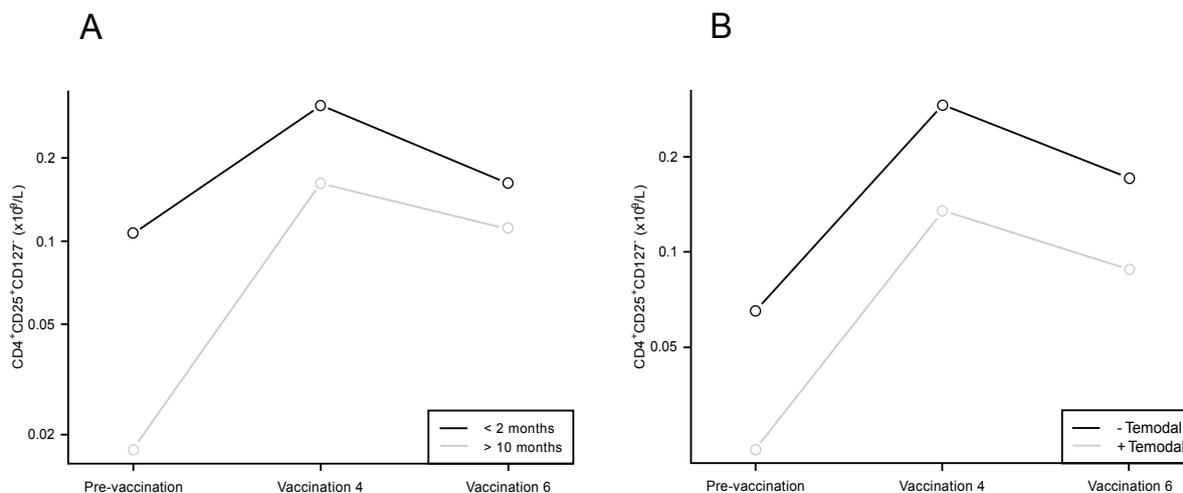
### Discussion

The role of Treg in cancer immunotherapeutic settings has

gathered much attention lately, and different methods to decrease Treg induction or increase Treg elimination are sought. We have performed three consecutive phase I/II DC vaccination trials using different additional immune modulating strategies; one trial where adjuvant treatment with low-dose IL-2 was applied (DC-IL2), one trial where MCy was applied (DC-MCy), and one trial where MCy and IL-2 were combined (DC-MCy-IL2). Blood samples from these patients have now enabled us to scrutinize the influence of IL-2 and MCy, alone and in combination, on blood Tregs and thereby contribute with important information to the uncovering of Treg manipulation strategies.

When we compared blood levels of T cell subsets from these three DC vaccine trials we found an increase in ALC, CD4<sup>+</sup> T cells, and Tregs defined as CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> when IL-2 was applied. In contrast we found a decrease in the number of ALC and CD4<sup>+</sup> T cells and stable numbers of Tregs when only MCy was applied. In an attempt to decrease the number of Tregs as described by Ghiringhelli et al. [5] we added MCy to our treatment schedule in the DC-MCy-IL2 and the DC-MCy trials. However, we could not confirm the findings of Ghiringhelli as our data showed that MCy was unable to reduce the Treg level when used in combination with low doses of IL-2 as well as alone.

A decrease in ALC (and other hematologic parameters) could be expected during continuous treatment with MCy due to the myelo-suppressive function of Cy. However, we only observed a slight decrease in ALC and no CTC grade 1 or more lymphopenia (< 0.8 × 10<sup>9</sup>/l).



**Figure 6: Changes in Tregs according to previous treatment.**

(A) Changes in Tregs according to treatment with high-dose IL-2 prior to inclusion. Patients recently treated (< 2 months) was compared with patients treated with high-dose IL-2 >10 months ago.

(B) Changes in the Tregs according to the treatment with Temozolomide (TMZ) prior to inclusion. Patients previously treated with TMZ were compared with TMZ untreated patients.

A beneficial effect of adding Cy to DC vaccination has been proposed by Höltl et al. [18] who found an improvement in median survival of patients with renal cell carcinoma when pretreated with 300 mg/m<sup>2</sup> Cy before DC vaccination. Whether this difference in survival correlated with changes in population of Tregs was not reported.

As described, an increase in ALC, CD4<sup>+</sup> T cells and Tregs was only observed in trial I and II where adjuvant treatment with IL-2 was applied. This is in accordance with the findings in our previous trials in patients with breast cancer and renal-cell carcinoma [12,19], where low-dose IL-2 were also applied. It is therefore obvious to speculate that IL-2 is responsible for these changes. In line with that, no influence on Treg frequencies was observed in a phase I/II trial where AML patients were treated with mRNA transfected DCs [20], every second week without the use of IL-2.

CD25 is an IL-2 receptor and therefore treatment with IL-2 could theoretically lead to an increased number of Tregs through an up-regulation of the CD25 receptor due to increased ligation. Accordingly, we found a higher number of Tregs in patients recently (< 2 months) treated with high-dose IL-2 as compared to patients treated >10 months ago. Van der Vliet et al. [13] also found that high-dose IL-2 did increase the CD4<sup>+</sup>CD25<sup>+</sup> T cells in melanoma and RCC patients. However, they could not demonstrate any correlation between clinical outcome and pre-treatment frequency of Tregs or treatment induced increases in Tregs. Furthermore, Zou [21] describe that IL-2 administration increases the number of peripheral Tregs in various cancer-patients and that IL-2 stimulates chemokine-receptor expression on Tregs and promotes their migration towards tumor micro-environment. Thus, several groups have demonstrated that administration of high-dose IL-2 alters the homeostasis of regulatory T cells. Here we show that even low doses of IL-2 are associated with a significant increase in CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Treg.

FoxP3 is generally considered the best marker to distinguish Tregs from other T cells [22], although other and more readily applicable Treg phenotypes have been suggested. Here we defined Tregs as CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> and supported our data by assessment of FoxP3

expression as well as Treg inhibitory function. We found that more than 90% of the gated CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Tregs were in fact FoxP3 positive. Furthermore, cells in the Treg gate (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>) possessed some regulatory capacity with the ability to inhibit both CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation in a dose-dependent matter.

Recently, Kleinewietfeld et al. [15] published that a new marker; CD49d, could distinguish between CD4<sup>+</sup> effector cells (CD49d<sup>+</sup>) and Tregs (CD49d<sup>-</sup>). Interestingly, when we applied CD49d analysis on Tregs from patients treated in the DC-MCy-IL2 trial, we found only a temporary minor increase in the CD49d cells. This is in contrast to the significant increase in the whole Treg population. Thus the increase in CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> T cells were mainly due to an increase in CD49d<sup>+</sup> cells which is described to represent CD4<sup>+</sup> effector cells. This indicates that IL-2 did only increase the true regulatory cells to a minor degree. Supportive to this, we found the most potent inhibitory potential in the CD49d<sup>-</sup> Tregs while the inhibitory potential of the CD49<sup>+</sup> subpopulation was very low. The results of the CD49d differentiate the results of the CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> and in part contradict the interpretation of these results. However as CD49d is a newly described marker, more knowledge on the application and interpretation is needed before firm conclusions can be made.

As mentioned, MCy was unable to reduce the Treg level in our setting so the question of how to eliminate Tregs efficiently during DC vaccination therapy is still open although different other strategies have been investigated. Mahnke et al. [11] managed to decrease the number of Tregs in melanoma patients by treatment with ONTAK. This decrease was associated with an increase of peptide specific T cells after Melan A + GP 100 vaccines and an increase in the reaction to a contact allergen, indicating a suppressed function of Tregs. Also, Dannull et al. [9] found that it was possible to reduce peripheral blood Treg and Foxp3 level by use of ONTAK during DC vaccination, and that it was associated with higher frequencies of tumor-specific CD8<sup>+</sup> T cells. Another approach to reduce Tregs was described by Jacobs et al. [10]. They found a complete and rapid depletion of all CD25<sup>high</sup> cells from peripheral blood after Daclizumab administration in combination with DC vaccination. However de Vries et al. [23] just published data

indicating that Daclizumab blunt the anti-vaccine T cell response in patients treated with DC vaccines and Daclizumab. In the future, we will probably see an increasing number of trials where vaccines are combined with chemotherapeutics or other Treg suppressing agents. One of the challenges will be to optimize timing of these therapies and vaccines. However, fluctuation of the Tregs during different therapeutic regimens is investigated [24] but not yet fully described. Therefore, frequent analyses of Treg parameters during the different treatment schedules are exceedingly important to obtain essential knowledge on how to perform optimal immune modulation.

In summary, adjuvant treatment with low-dose IL-2 during DC vaccination therapy causes a significant increase in blood level of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup> Tregs. However a newly described marker, CD49d, applied on the Treg population indicates that only a minor part of this increase was due to an increase in 'true suppressive' Tregs and that the increase was mostly due to the increased number of CD49d<sup>+</sup> positive cells i.e. the cytokine-secreting cells. Furthermore, the use of metronomic Cyclophosphamide was unable to reduce Treg blood level and unable to counteract IL-2 dependent Treg increase.

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#### References

- Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, et al. (2010) Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363: 711-723.
- Engell-Noerregaard L, Hansen TH, Andersen MH, Thor Straten P, Svane IM (2009) Review of clinical studies on dendritic cell-based vaccination of patients with malignant melanoma: assessment of correlation between clinical response and vaccine parameters. *Cancer Immunol Immunother* 58: 1-14.
- Akbar AN, Vukmanovic-Stejic M, Taams LS, Macallan DC (2007) The dynamic co-evolution of memory and regulatory CD4<sup>+</sup> T cells in the periphery. *Nat Rev Immunol* 7: 231-237.
- Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC (2010) Regulatory T cells in cancer. *Adv Cancer Res* 107: 57-117.
- Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, et al. (2007) Metronomic cyclophosphamide regimen selectively depletes CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol Immunother* 56: 641-648.
- Trepiakas R, Berntsen A, Hadrup SR, Bjorn J, Geertsen PF, et al. (2010) Vaccination with autologous dendritic cells pulsed with multiple tumor antigens for treatment of patients with malignant melanoma: results from a phase I/II trial. *Cytotherapy* 12: 721-34.
- Bjoern J, Brimnes MK, Andersen MH, Thor Straten P, Svane IM, et al. (2010) Changes in peripheral blood level of regulatory T cells in patients with malignant melanoma during treatment with dendritic cell vaccination and low-dose IL-2. *Scand J Immunol* 73: 222-233.
- Ahmadzadeh M, Rosenberg SA (2006) IL-2 administration increases CD4<sup>+</sup>CD25<sup>(hi)</sup> Foxp3<sup>+</sup> regulatory T cells in cancer patients. *Blood* 107: 2409-14.
- Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, et al. (2005) Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* 115: 3623-33.
- Jacobs JF, Punt CJ, Lesterhuis WJ, Suttmuller RP, Brouwer HM, et al. (2010) Dendritic cell vaccination in combination with anti-CD25 monoclonal antibody treatment: a phase I/II study in metastatic melanoma patients. *Clin Cancer Res* 16: 5067-78.
- Mahnke K, Schonfeld K, Fondel S, Ring S, Karakhanova S, et al. (2007) Depletion of CD4<sup>+</sup>CD25<sup>+</sup> human regulatory T cells in vivo: kinetics of Treg depletion and alterations in immune functions in vivo and in vitro. *Int J Cancer* 120: 2723-33.
- Svane IM, Pedersen AE, Nikolajsen K, Zocca MB (2008) Alterations in p53-specific T cells and other lymphocyte subsets in breast cancer patients during vaccination with p53-peptide loaded dendritic cells and low-dose interleukin-2. *Vaccine* 26: 4716-24.
- van der Vliet HJ, Koon HB, Yue SC, Uzunpapak B, Seery V, et al. (2007) Effects of the administration of high-dose interleukin-2 on immunoregulatory cell subsets in patients with advanced melanoma and renal cell cancer. *Clin Cancer Res* 13: 2100-8.
- Pillai V, Ortega SB, Wang CK, Karandikar NJ (2007) Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin Immunol* 123: 18-29.
- Kleinewietfeld M, Starke M, Di Mitri D, Borsellino G, Battistini L, et al. (2009) CD49d provides access to "untouched" human Foxp3<sup>+</sup> Treg free of contaminating effector cells. *Blood* 113: 827-36.
- Rietschel P, Wolchok JD, Krown S, Gerst S, Jungbluth AA, et al. (2008) Phase II study of extended-dose temozolomide in patients with melanoma. *J Clin Oncol* 26: 2299-304.
- Su YB, Sohn S, Krown SE, Livingston PO, Wolchok JD, et al. (2004) Selective CD4<sup>+</sup> lymphopenia in melanoma patients treated with temozolomide: a toxicity with therapeutic implications. *J Clin Oncol* 22: 610-6.
- Holt L, Ramoner R, Zelle-Rieser C, Gander H, Putz T, et al. (2005) Allogeneic dendritic cell vaccination against metastatic renal cell carcinoma with or without cyclophosphamide. *Cancer Immunol Immunother* 54: 663-70.
- Berntsen A, Brimnes MK, Thor Straten P, Svane IM (2010) Increase of circulating CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup> regulatory T cells in patients with metastatic renal cell carcinoma during treatment with dendritic cell vaccination and low-dose interleukin-2. *J Immunother* 33: 425-34.
- Van Tendeloo VF, Van de Velde A, Van Driessche A, Cools N, Anguille S, et al. (2010) Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. *Proc Natl Acad Sci U S A* 107: 13824-9.
- Zou W (2006) Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 6: 295-307.
- Golovina TN, Vonderheide RH (2010) Regulatory T cells: overcoming suppression of T-cell immunity. *Cancer J* 16: 342-7.
- de Vries IJ, Castelli C, Huygens C, Jacobs JF, Stockis J, et al. (2011) Frequency of circulating Tregs with demethylated FOXP3 intron 1 in melanoma patients receiving tumor vaccines and potentially Treg-depleting agents. *Clin Cancer Res* 17: 841-8.
- Emens LA (2010) Chemoimmunotherapy. *Cancer J* 16: 295-303.

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