

# CCR4 – a potential marker for effector-type regulatory T cells

## CCR4 – a potential marker for the selective removal of effector-type FOXP3<sup>+</sup>CD4<sup>+</sup> regulatory T cells in cancer immunotherapies

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### Background

CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (Treg) cells expressing the transcription factor forkhead box P3 (FOXP3) play an important role in suppressing antitumor immune responses. Some clinical studies have shown the potential of depleting CD25-expressing lymphocytes to augment antitumor immune responses<sup>1,2</sup>. Yet other similar studies did not support this claim<sup>3-5</sup>. The depletion of CD25<sup>+</sup> cells is debatable, because activated effector T cells also express CD25 and promote the expansion of CD8<sup>+</sup> cytotoxic lymphocytes, for example. Their depletion may abrogate the effect of Treg cell depletion, i.e., counteract the augmentation of antitumor immunity<sup>3</sup>. Moreover, based on studies with animal models it has been suggested that depletion of Treg cells can result in autoimmunity<sup>6-8</sup>. Therefore, a current challenge is to determine more specific markers to control the immunosuppressive effect of Treg cells without compromising effector T cells or eliciting deleterious autoimmunity.

Compared to peripheral blood T cells, tumor-infiltrating T cells contain a larger percentage of effector Treg (eTreg) cells, which are defined as FOXP3<sup>hi</sup> and CD45RA<sup>-</sup>, terminally differentiating, and most suppressive. We could show that eTreg cells, but not FOXP3<sup>lo</sup> and CD45RA<sup>+</sup> naive Treg cells, predominantly express C-C chemokine receptor 4 (CCR4) in both melanoma tissues and peripheral blood. *In vivo* and *in vitro* anti-CCR4 mAb treatment selectively depleted eTreg cells and efficiently induced tumor-antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells.<sup>9</sup>

This note exemplifies the use of the gentleMACS™ Dissociator and the autoMACS® Pro Separator for an automated experimental setup, providing reproducible results from sample preparation to cell separation, in our search for more specific cell markers in cancer immunotherapies.

### Material

- gentleMACS Dissociator
- gentleMACS C Tubes
- autoMACS Pro Separator
- Biotin-anti-CD25 monoclonal antibody (BC96)
- Biotin-anti-CCR4 monoclonal antibody (1G1)
- Anti-Biotin MicroBeads (Miltenyi Biotec)

### Methods

- Peripheral blood mononuclear cells (PBMCs) were prepared from peripheral blood of healthy donors and melanoma patients.
- Primary human melanomas were resected and the surrounding healthy tissue was removed. Single-cell suspensions were prepared using the gentleMACS Dissociator and C Tubes.
- CD25<sup>-</sup> or CCR4<sup>-</sup> cells were prepared from PBMCs and melanoma-derived single-cell suspensions using biotin-anti-CD25 or biotin-anti-CCR4 antibodies (0.01 mg/mL), respectively. Cells were incubated with antibodies for 15 min at 4 °C. Subsequently, Anti-Biotin MicroBeads were added as described in the manufacturer's protocol. Then cells were washed using PBS containing 2% (v/v) FCS. CD25<sup>-</sup> or CCR4<sup>-</sup> cells were separated using an autoMACS Pro Separator.
- Original fractions before separation and fractions depleted of CD25<sup>+</sup> or CCR4<sup>+</sup> cells were analyzed by flow cytometry using CD4, CD25, CD45RA, CD194 (CCR4), and Anti-FOXP3 antibodies.

## Results

### Depletion of CCR4<sup>+</sup> cells removes eTreg cells from PBMCs selectively

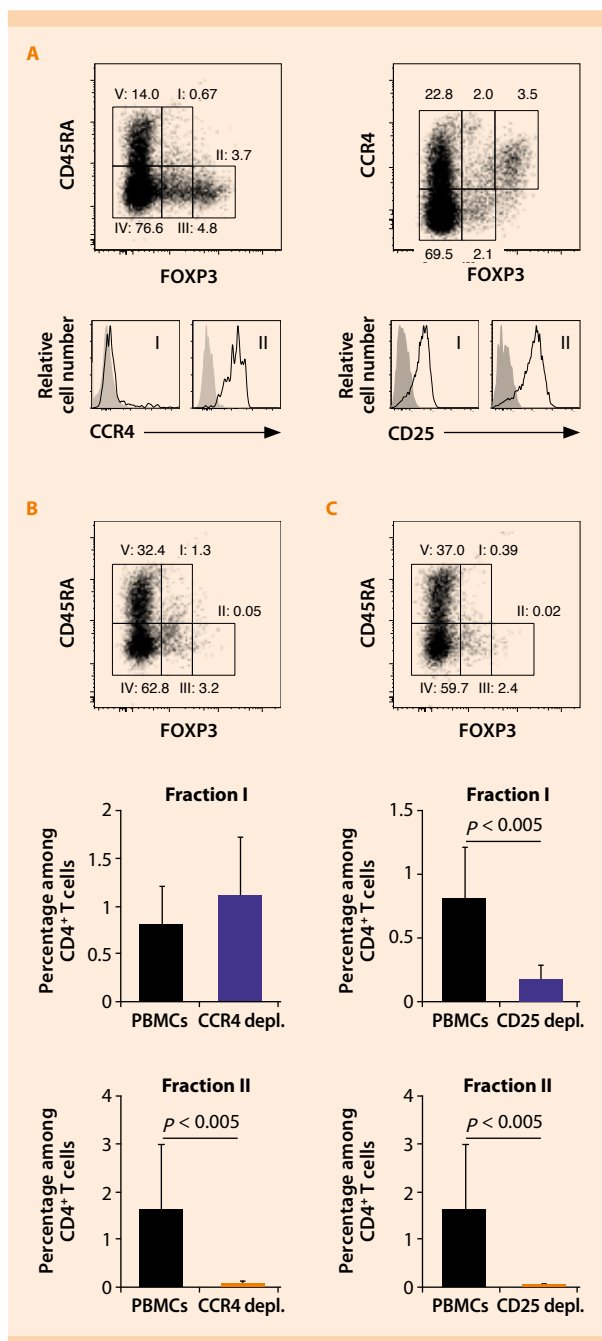
When PBMCs from melanoma patients were stained with various antibodies and analyzed by gating on CD4<sup>+</sup> T cells, CCR4 expression was found to be particularly high in fractions of eTreg cells and almost non-existent in naive Treg (nTreg) cell fractions (fig. 1A). This tendency was similar in PBMCs from healthy individuals<sup>9</sup>. Depletion of CCR4<sup>+</sup> cells led to a remarkable reduction of eTreg cells whereas nTreg cells remained unaffected (fig. 1B). However, both eTreg cells and nTreg cells were greatly reduced after CD25<sup>+</sup> cell depletion (fig. 1C).

### CCR4<sup>+</sup> eTreg cells infiltrate into melanoma tissue predominantly and can be efficiently depleted

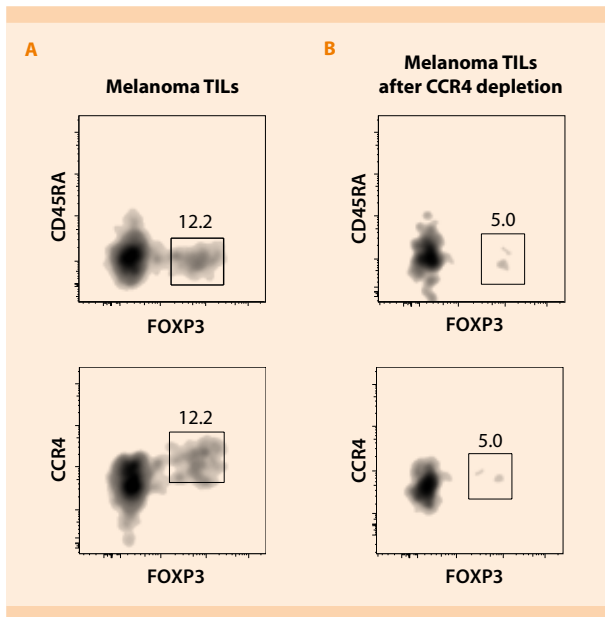
It is known that FOXP3<sup>+</sup> T cells invade tumors locally, but their detailed phenotype is not well defined. Therefore, tumor-infiltrating lymphocytes (TILs) from melanoma tissue were prepared using the gentleMACS Dissociator and the same flow cytometric analysis was performed as in figure 1A. A high percentage of eTreg cells infiltrated into the melanoma tissue (fig. 2A) resulting in higher eTreg cell frequencies compared to the PBMC samples. Nevertheless, we could significantly reduce the number of melanoma tissue-infiltrating eTreg cells by selective depletion of CCR4<sup>+</sup> T cells (fig. 2B).

## Conclusions

Screening new marker candidates for effective cancer immunotherapies is challenging and labor intensive. The combination of gentleMACS Dissociator and autoMACS Pro Separator enabled us to automate our workflow and to use CCR4 as a selective marker to remove eTreg cells – the most immunosuppressive Treg cell type – in a standardized manner. Depletion of eTreg cells based on CCR4 did not affect other Treg cell subpopulations. In contrast, depletion based on CD25 resulted in the removal of various Treg cell subsets.



**Figure 1:** *In vitro* CCR4<sup>+</sup> cell depletion reduces effector Treg cells effectively. Expression of FOXP3, CD45RA, and CCR4 in CD4<sup>+</sup> T cells from PBMCs obtained from a melanoma patient was analyzed by flow cytometry (A). Numbers in the dot plots show the percentage of cells in each fraction with CD4<sup>+</sup> T cells representing 100%. The various fractions indicate nTreg cells (I), eTreg cells (II), FOXP3<sup>lo</sup> non-Treg cells (III), memory and activated T<sub>H</sub> (IV), and naive T<sub>H</sub> cells (V). Expression of CCR4 and CD25 in fractions I and II is shown in the histograms (A; lower panel). CCR4<sup>+</sup> cells (B) or CD25<sup>+</sup> cells (C) were depleted from PBMCs using the autoMACS Pro Separator and the CCR4<sup>-</sup> and CD25<sup>-</sup> cells were analyzed for FOXP3 and CD45RA expression (B, C; upper panel). The percentage of nTreg cells (fraction I) and eTreg cells (fraction II) in PBMCs prior to depletion and after CCR4<sup>+</sup> (B) or CD25<sup>+</sup> (C) cell depletion is shown for six different donors. CD4<sup>+</sup> cells represent 100%.



**Figure 2:** CCR4<sup>+</sup> eTreg cells invade into melanoma tissue predominantly. (A) Melanoma tissue-infiltrating lymphocytes were prepared with the gentleMACS Dissociator and analyzed by flow cytometry. (B) After depletion of CCR4<sup>+</sup> cells using the autoMACS Pro Separator the frequency of FOXP3<sup>hi</sup> eTreg cells was analyzed. Numbers indicate the percentage of FOXP3<sup>hi</sup> CCR4<sup>+</sup> cells among CD4<sup>+</sup> T cells.

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