# Lack of Foxp3$^+$ macrophages in both untreated and B16 melanoma-bearing mice

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Foxp3$^+$ Tregs are essential for maintaining immune tolerance in mice and men. Except for expression in a minor population of CD8$^+$ T cells[1], Foxp3 is currently believed to be restricted to CD4$^+$ Tregs in mice, since widespread non-hematopoietic Foxp3 expression and its putative implication in tolerance have been refuted[2,3]. Thus, the employment of Foxp3 reporter mice, including Foxp3$^{DTR\text{-}eGFP}$ (DEREG) mice[4], has been valuable for the investigation of Foxp3$^+$ Treg biology. Recently, we demonstrated that the depletion of Foxp3$^+$ Tregs unleashes potent therapeutic tumor-specific immunity[5,6], unlike less selective agents e.g. targeting CD25. The recent postulation of immunoregulatory Foxp3$^+$ macrophages[7] implies that macrophage deletion in DEREG mice could contribute to the striking effects of Foxp3$^+$ cell ablation as cancer immunotherapy. We thus specifically investigated this issue. Foxp3-PE stainings of WT spleens revealed a CD11b$^-$Foxp3$^+$ Treg population as expected, whereas the CD11b$^+$ fraction contained a clearly weaker PE signal (Fig. 1A). The latter was identified as autofluorescence since it persisted in isotype stainings (Fig. 1A) or unstained samples (not shown), in contrast to CD11b$^-$Foxp3$^+$ Tregs. To further assess if the CD11b$^+$PE$^{low}$ population actively transcribes the *foxp3* locus, we analyzed spleens of DT-treated DEREG mice. Strikingly, CD11b$^+$PE$^{low}$ cells persisted upon DT administration, whereas CD11b$^-$Foxp3$^+$ Tregs were depleted (Fig. 1A), suggesting that the autofluorescent CD11b$^+$ population does not express Foxp3$^{DTR\text{-}eGFP}$. Similar results were obtained with BM (not shown). In line with these findings, CD11b$^+$PE$^{low}$ cells were present at rather increased percentages in the BM of scurfy mice harbouring a mutated non-detectable Foxp3 protein[8], when compared to Foxp3-sufficient litters (Fig. 1B). In contrast, CD11b$^-$ Foxp3$^+$ Tregs were absent from scurfy mice as expected, demonstrating a lack of Foxp3 staining in CD11b$^+$ cells (Fig. 1B). Since Manrique et al. used the FITC/GFP channel for revealing Foxp3 in their study[7], which is more prone to autofluorescence than the PE channel, we next assessed the deletion of Foxp3$^{DTR\text{-}eGFP+}$ cells in DT-treated DEREG mice. About 4% of T cell-excluded BM cells in WT mice displayed strong autofluorescence (Fig. 1C),

comparable to the reported frequency[7]. DEREG mice did not harbour increased frequencies of GFP/autofluorescence$^+$ cells within the T cell-excluded CD11b$^+$ BM fraction (Fig. 1C), which would be expected from Foxp3-reporting cells. Similarly, DT administration to DEREG mice did not result in depletion of the GFP/autofluorescence$^+$ population (Fig. 1C). DT efficiently accessed the BM because GFP$^+$ Tregs were eliminated (not shown). Of note, autofluorescent CD11b$^+$ cells are FSC$^{hi}$SSC$^{hi}$ compared to non-autofluorescent CD11b$^+$ cells and T cells (not shown). Consistent with Manrique et al.[7], we readily detected CD11b$^+$ autofluorescent cells in spleens and BM of RagKO mice, but those similarly lacked Foxp3 expression (not shown). We next generated BM-derived macrophages (BMM) from WT or DEREG mice in presence or absence of DT during the culture period. Again, we could not detect Foxp3 staining in CD11b$^+$ BMM, and no increased rate of cell death was observed after culture in presence of DT (Fig. 1D and not shown). LPS stimulation - which was implicated in *de novo* Foxp3 expression by Foxp3$^-$ macrophages[7] - did not induce Foxp3 expression in BMM (not shown). The authors demonstrated a regulatory role for putative Foxp3$^+$ macrophages in B16 melanoma[7] . To exclude that Foxp3 is induced in macrophages by melanoma-dependent mechanisms, we analyzed B16-OVA melanoma-bearing mice. Spleens and BM lacked CD11b$^+$Foxp3$^+$ cells (not shown), as did tumor-free mice (Fig. 1A-C). Furthermore, single cell suspensions of tumors raised in WT mice revealed no specific Foxp3 expression in CD11b$^+$ cells, whereas CD11b$^-$Foxp3$^+$ Tregs were present (Fig. 1E), in line with previous results[5]. Similarly, immunofluorescence analyses of tumor sections revealed that Foxp3$^+$ cells were exclusively CD3$^+$, but did not co-express F4/80 or CD11b, whereas Foxp3$^+$ cells were completely absent from tumors elicited in RagKO mice (Fig. 1F, not shown).

Concluding, we found no evidence for Foxp3 expression in macrophages by combining flow cytometry, immunofluorescence microscopy and genetic approaches. This is fully in line with the previously noted lack of Foxp3 protein expression in RagKO spleens [2]. Thus, macrophage subpopulations are not targeted in genetically engineered DEREG mice and do not contribute

to the onset of self/tumor-specific immunity upon Foxp3$^{+}$ Treg ablation. These results are of immediate relevance for the use of Foxp3 reporter mice and for the translational design of novel cancer immunotherapies.

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**Authorship and conflict of interest statements**

C.T.M. designed and performed experiments and wrote the manuscript. A.A.K. and C.L. performed and evaluated immunfluorescence stainings. T.S. supervised the study, designed experiments and wrote the manuscript.

The authors declare no competing financial interests.

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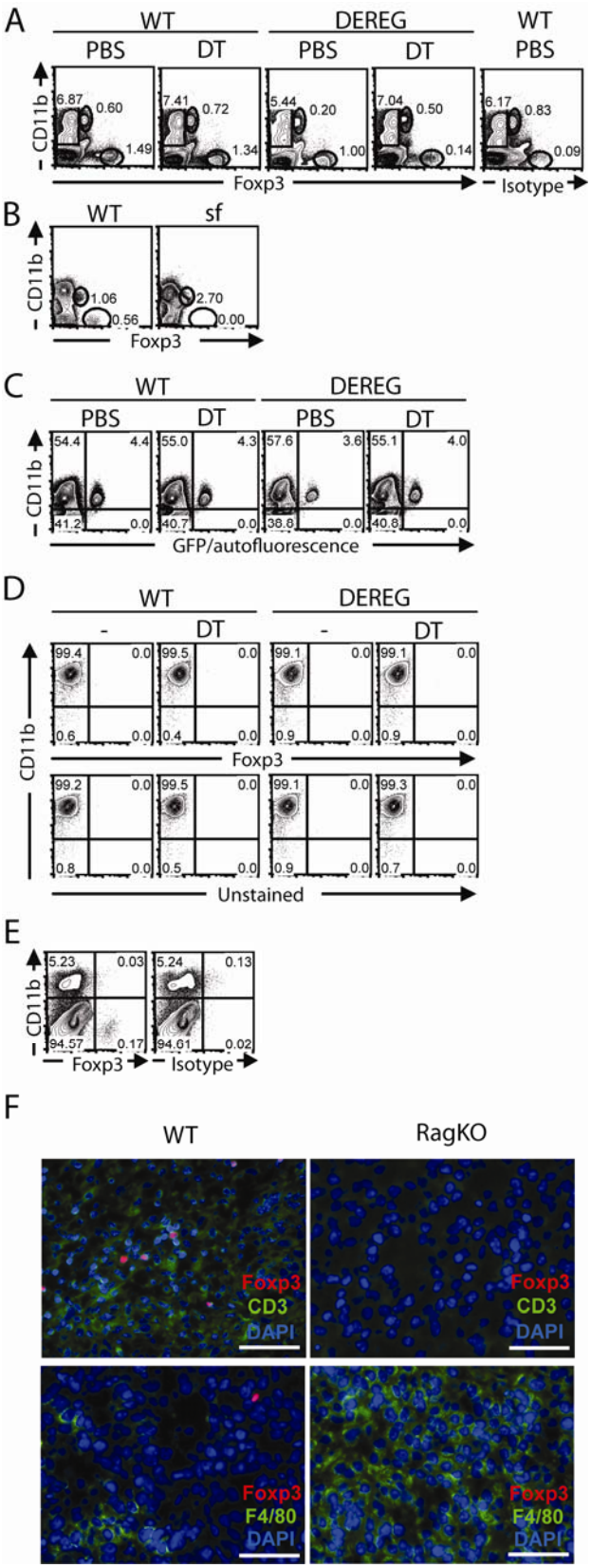
**Figure legend**

**Figure 1. Lack of Foxp3 expression by macrophages**

(A) WT and DEREG mice were treated on two consecutive days with 1µg DT i.p. or PBS. One day later, collagenase/DNase-digested splenocytes were analyzed by flow cytometry. CD11b (M1/70), Foxp3 (FJK-16s) or rat IgG2a/κ isotype staining is shown. (B) BM was isolated from 2-3 weeks old WT or scurfy males and analyzed by flow cytometry. CD11b and Foxp3 expression is shown. (C) BM of PBS- or DT-treated WT and DEREG mice was analyzed by flow cytometry. CD11b expression of CD3ε/TCRβ/TCRγδ$^-$ cells is plotted against GFP/autofluorescence. (D) BMM were generated as described[9] from WT or DEREG mice except 100ng/ml DT was added on d0 and d3 of culture where indicated. BMM were harvested using accutase and analyzed by flow cytometry. CD11b vs. Foxp3 expression is shown in the upper panel. The lower panel is unstained for Foxp3. (E) B16-OVA tumors were established in WT mice[5], harvested, collagenase/DNase-digested and analyzed by flow cytometry. CD11b, Foxp3 or isotype staining is displayed. (F) B16-OVA tumors were established in WT or RagKO mice, resected and cryopreserved. 5µm sections were analyzed by immunofluorescence microscopy after staining for Foxp3 (FJK-16s), F4/80 (BM8), CD3 (N1580) and AlexaFluor488- or AlexaFluor555-labeled secondary antibodies. AxioImager Z1, Axiovision software and AxioCam MRm were applied for image acquisition and analysis. Foxp3 (red), CD3 (green) and DAPI (blue) signals are displayed in the upper panel; the lower panel shows Foxp3 (red), F4/80 (green) and DAPI (blue) stainings. All images were acquired with 400x magnification and a 50µm white scale bar is displayed.

(A-E) All flow cytometry analyses were performed after Fc receptor blocking with anti-CD16/32 (2.4G2). Cells were acquired on LSRII (BD), analyzed by FlowJo (Tristar) and dead cells were excluded by ethidium monoazide (EMA) photolysis.

(A-F) All experiments are representative for two to three independent experiments.


A
WT
DEREG
WT
PBS
DT
PBS
DT
PBS
CD11b
6.87
0.60
1.49
7.41
0.72
1.34
5.44
0.20
1.00
7.04
0.50
0.14
6.17
0.83
0.09
Foxp3
Isotype
B
WT
sf
CD11b
1.06
0.56
2.70
0.00
Foxp3
C
WT
DEREG
PBS
DT
PBS
DT
CD11b
54.4
4.4
41.2
0.0
55.0
4.3
40.7
0.0
57.6
3.6
38.6
0.0
55.1
4.0
40.6
0.0
GFP/autofluorescence
D
WT
DEREG
-
DT
-
DT
CD11b
99.4
0.0
0.6
0.0
99.5
0.0
0.4
0.0
99.1
0.0
0.9
0.0
99.1
0.0
0.9
0.0
Foxp3
99.2
0.0
0.8
0.0
99.5
0.0
0.5
0.0
99.1
0.0
0.9
0.0
99.3
0.0
0.7
0.0
Unstained
E
CD11b
5.23
0.03
94.57
0.17
5.24
0.13
94.61
0.02
Foxp3
Isotype
F
WT
RagKO
Foxp3
CD3
DAPI
Foxp3
CD3
DAPI
Foxp3
F4/80
DAPI
Foxp3
F4/80
DAPI