

Lack of IL-2 in PPAR- α -deficient mice triggers allergic contact dermatitis by affecting regulatory T cells

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The aim of the present work was to decipher the cellular basis of the immunoregulatory role of peroxisome proliferator-activated receptor (PPAR)- α in cutaneous hypersensitivity reactions. After challenge with a contact allergen, we observed augmented hypersensitivity reactions with increased numbers of activated T lymphocytes in the skin of PPAR- α ^{-/-} mice. Furthermore, following antigen challenge, the percentages of Tregs in the blood, draining lymph nodes and skin were decreased in these mice. PPAR- α deficiency impaired the production of IL-2 in lymph nodes, whereas TGF- β levels remained unchanged. Injection of IL-2 into PPAR- α ^{-/-} mice restored the Treg population in the skin-draining lymph nodes of allergen-challenged mice. In vivo induction of Tregs from WT CD4⁺CD25⁻ T cells was impaired when adoptively transferred into PPAR- α ^{-/-} mice as compared with transfer into WT mice, and reversed by injection of IL-2 into PPAR- α ^{-/-} mice. Furthermore, the suppressive capacity of PPAR- α ^{-/-} Tregs was impaired when compared to WT Tregs in vitro and in co-adoptive transfer experiments. Finally, injection of IL-2 to PPAR- α ^{-/-} mice decreased skin inflammation to a level similar to WT mice. In conclusion, the pro-inflammatory skin phenotype of PPAR- α ^{-/-} mice is due to lack of IL-2-mediated Treg induction in these mice.

Key words: Inflammation • Nuclear hormone receptors • Skin



Supporting Information available online

Introduction

Peroxisome proliferator-activated receptor (PPAR)- α is a nuclear hormone receptor that, upon heterodimerization with the retinoid X receptor (RXR), functions as a transcriptional regulator of glucose and lipid metabolism [1]. Fatty acid, fibrates and eicosanoids are ligands of PPAR- α [2–4]. PPAR- α is highly expressed in the liver, heart, muscle, kidney and arterial blood vessels. Furthermore, it has been shown that PPAR- α is expressed in cells of the immune system,

including B and T lymphocytes, monocytes/macrophages, DCs and epidermal Langerhans cells [5–7].

Since PPAR- α is expressed in all key cells of the adaptive immune system and since specific PPAR- α ligands have been identified, the immune-regulatory role of PPAR- α can now be explored in depth. Previous work has shown that PPAR- α is strongly linked to inflammatory reactions. For example, inflammatory stimuli such as lipopolysaccharide and TNF- α activate NF- κ B, a major transcription factor mediating inflammation by promoting the secretion of inflammatory cytokines. PPAR- α ligands can block the NF- κ B pathway, and thereby modulate the immune response [7, 8].

Since PPAR- α activation exerts anti-inflammatory effects [8–10], it can be hypothesized that PPAR- α deficiency leads to a

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pro-inflammatory phenotype. Indeed, after induction of experimental autoimmune encephalomyelitis, PPAR- α -deficient mice develop more severe clinical symptoms than control mice in a gender-dependent manner [11]. In these experiments, the authors also found a higher capacity of PPAR- α -deficient T lymphocytes to proliferate and to produce IFN- γ and TNF- α in vitro because of de-inhibition of NF- κ B and c-jun [11]. These findings confirmed prior work showing increased production of IFN- γ by PPAR- α -deficient CD4⁺ T lymphocytes, presumably due to an early up-regulation of T-bet [12]. Moreover, it has recently been reported that PPAR- α -deficient mice exhibit increased extent and severity of carrageenan-induced-paw oedema and pleurisy. In these experiments carrageenan-treated PPAR- α -deficient mice exhibited enhanced immunostaining for FAS ligand in the paw and in the lung and a higher expression of TNF- α and IL-1 β in the lungs [13]. In addition, PPAR- α is involved in B-cell development [14].

In contrast, the consequences of PPAR- α deficiency in Tregs is unknown, although they are key cells of the adaptive immune response and peripheral tolerance [15], and despite it being shown that PPAR- γ -deficient Tregs are unable to prevent effector T-cell-induced colitis in a co-transfer study [16]. Because Tregs are crucial in maintaining peripheral tolerance and are actively involved in the contraction phase of the immune response, the aim of our present work was to decipher the role of PPAR- α in Tregs in order to understand the pro-inflammatory effects of PPAR- α deficiency in skin.

Results

PPAR- α deficiency worsens and prolongs allergic contact dermatitis

First, we studied the amplitude of skin inflammation in PPAR- α -deficient mice and their littermate controls after skin challenge with a contact allergen. As it was reported in the literature that PPAR- α deficiency preferentially leads to the production of Th1 cytokines by T lymphocytes [11, 12], we used a well-established Th1 contact hypersensitivity (CHS) mouse model [17]. Following challenge of PPAR- α -deficient and littermate controls mouse ears with 2,4,6-trinitro-1-chlorobenzene picryl chloride (TNCB) [17], PPAR- α -deficient mice exhibited increased ear thickness compared to littermate controls in response to antigen challenge (Fig. 1A). Increased ear swelling in PPAR- α -deficient mice was accompanied by enhanced dermal inflammatory cell infiltration (Fig. 1A and B). The inflammatory infiltrates were composed of T lymphocytes, the majority expressing CD4 (Fig. 1B). Numbers (Fig. 1C) and percentages (Fig. 1D) of activated CD4⁺ T lymphocytes were higher in PPAR- α -deficient mouse skin as compared to littermate control mice and were associated with higher expression of the activation marker CD25 (Fig. 1D). These results show that PPAR- α deficiency exacerbates allergic CHS and increases numbers of activated T lymphocytes in the skin. We next studied the effects of PPAR- α deficiency on the duration of skin inflammation after challenge with TNCB. PPAR- α -deficient mice exhibited a prolonged ear swelling

response after challenge (Fig. 1E). In addition, histological analyses revealed a slight increase in inflammatory infiltrates in PPAR- α -deficient skin when compared with littermate controls and a moderate increase in the percentage of CD4⁺ T lymphocytes (data not shown). These results indicate that PPAR- α deficiency not only exacerbates but also prolongs allergic CHS.

PPAR- α deficiency decreases the population of Tregs after topical application of a hapten

Since the effects of PPAR- α deficiency on Tregs were unknown, we explored possible consequences of PPAR- α deficiency on the size of the Treg population in vivo under basal conditions, after skin sensitization and upon challenge with an antigen. We found that the relative quantity of Tregs gradually increased over baseline after skin sensitization and challenge in the skin-draining lymph nodes of WT mice (Fig. 2A). Interestingly, this increase did not occur in PPAR- α -deficient mice (Fig. 2A). Numbers of total cells in skin-draining lymph nodes were comparable in PPAR- α -deficient and littermate control mice after skin sensitization and challenge (Supporting Information Fig. 1A). We also observed decreased percentages of circulating Tregs in the blood of PPAR- α -deficient mice when compared with littermate controls after antigen challenge (Fig. 2B). Furthermore, the percentage of Tregs was decreased in antigen-challenged skin isolated from PPAR- α -deficient mice when compared with controls (Fig. 2C). Thus, PPAR- α deficiency prevents the increase in Treg populations, which normally occurs after skin sensitization and antigen challenge, suggesting that PPAR- α deficiency reduces Foxp3 up-regulation or Treg proliferation.

Impaired in vivo production of IL-2 is responsible for the reduced Treg population in PPAR- α -deficient mice

Prior studies showed that Foxp3 can be induced in CD4⁺Foxp3⁺ cells in the presence of TGF- β and IL-2 [18]. Since it has been shown that production of IL-2 was impaired in PPAR- α -deficient T lymphocytes at least in vitro [12], we next examined the in vivo production of TGF- β and IL-2 in PPAR- α -deficient mice. We found that production of TGF- β 1 was not altered (Fig. 3A) while the production of IL-2 was dramatically reduced in PPAR- α -deficient lymph node cells after skin sensitization when compared with cells from WT controls (Fig. 3B). Flow cytometry analysis showed that PPAR- α -deficient T lymphocytes produced less IL-2 (Supporting Information Fig. 1B and C). Therefore, we next investigated if injection of IL-2 into PPAR- α -deficient mice, when administrated during the sensitization phase, could restore the size of the Treg population in the skin-draining lymph nodes after antigen challenge. Figure 3C shows that IL-2 injection restored the size of the Treg population in the PPAR- α -deficient mice at a level similar to that found in vehicle-treated WT mice and that injection of IL-2 into WT mice also increased the percentages of Tregs (Supporting Information Fig. 2A). In contrast, injection of IL-2 did not

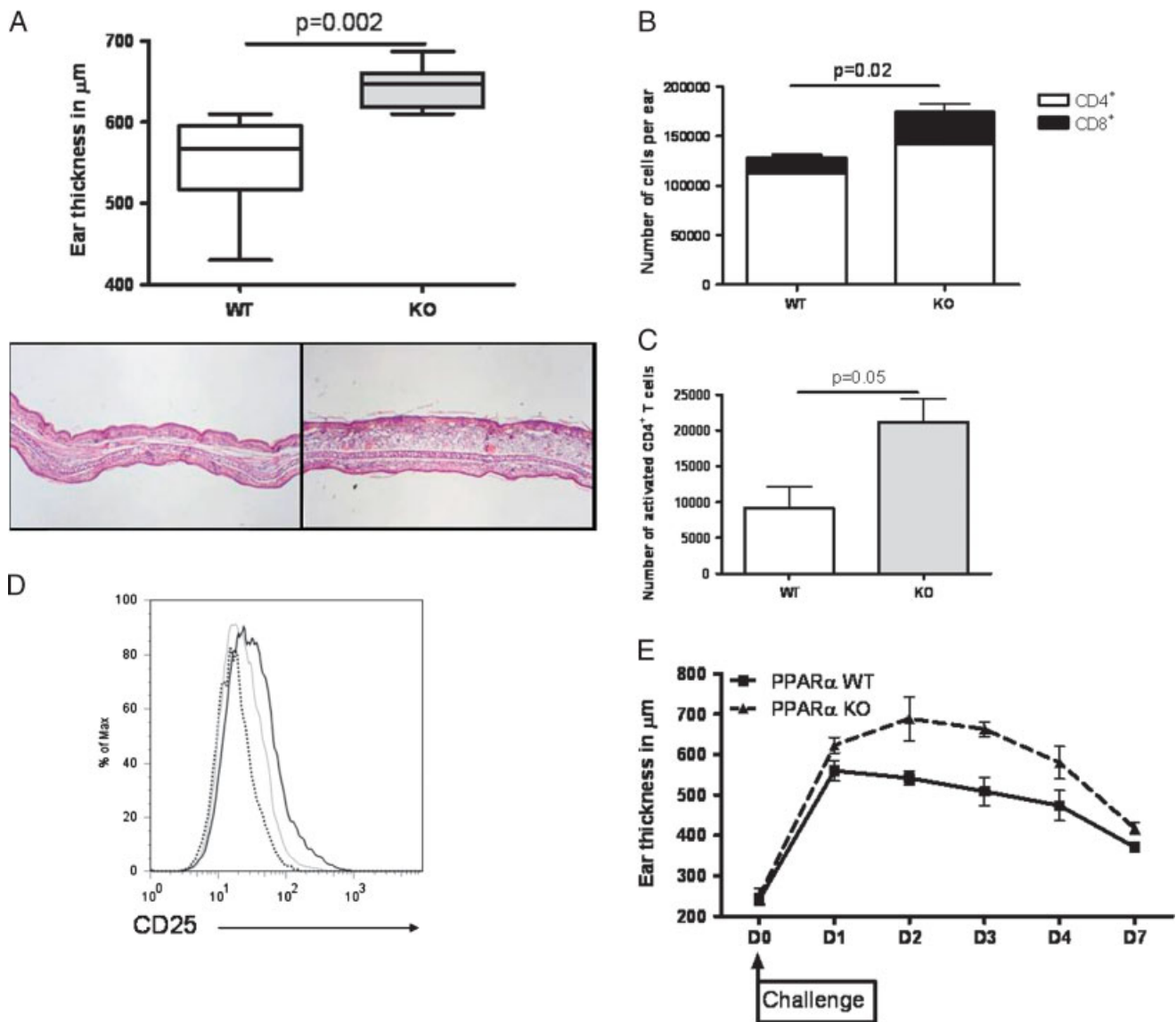


Figure 1. PPAR- α deficiency exacerbates and prolongs allergic contact dermatitis: Mouse ears were challenged with TNCB 5 days after sensitization with TNCB as described in Materials and methods. (A) Ear thickness 48 h after antigen challenge and representative H&E staining ($4\times$ magnification) showing dermal inflammatory infiltrates. (B) Numbers of CD4⁺ and CD8⁺ T lymphocytes in challenged skin determined by flow cytometry analysis and cell count numbers. (C) Numbers of activated CD4⁺ T cells (CD4⁺CD25⁺Foxp3⁻ cells) in challenged skin determined by flow cytometry and cell count numbers. (D) Representative histograms show fluorescence intensity for CD25 in PPAR- α -deficient (plain black histogram) versus littermate control (plain grey histogram) skin CD4⁺ T lymphocytes versus isotype-matched control (dotted black histogram). Alive cells were gated as CD4⁺ and Foxp3⁻. (E) Time course of ear thickness after skin challenge. Results are expressed as mean $\pm$ SEM, $n = 3$ –8. Data were analysed with the Student's t -test.

significantly modify the numbers of skin-draining lymph node cells (Supporting Information Fig. 2B). These results suggest that impaired *in vivo* production of IL-2 is responsible for the reduced Treg population after antigen challenge in PPAR- α -deficient mice.

Lack of IL-2 in PPAR- α -deficient mice is responsible for the decreased induction of Tregs *in vivo*

To investigate whether PPAR- α per se or the lack of IL-2 production was responsible for the reduced size of Tregs after stimulation in

PPAR- α -deficient mice, we generated *in vitro* inducible Tregs (iTregs) from CD4⁺CD25⁻ T lymphocytes isolated from naïve WT and PPAR- α -deficient mice in the presence of various amounts of IL-2. We found that in the presence of sufficient amounts of IL-2, CD4⁺CD25⁻ T lymphocytes isolated from naïve PPAR- α -deficient mice were able to up-regulate Foxp3, yet to a lower extent than in WT cells (Supporting Information Fig. 3A and B). This difference was abrogated with lower amounts of IL-2 (Supporting Information Fig. 3B). To verify if these results were reproducible *in vivo*, we adoptively transferred CD4⁺CD25⁻ T lymphocytes isolated from naïve WT and PPAR- α -deficient mice

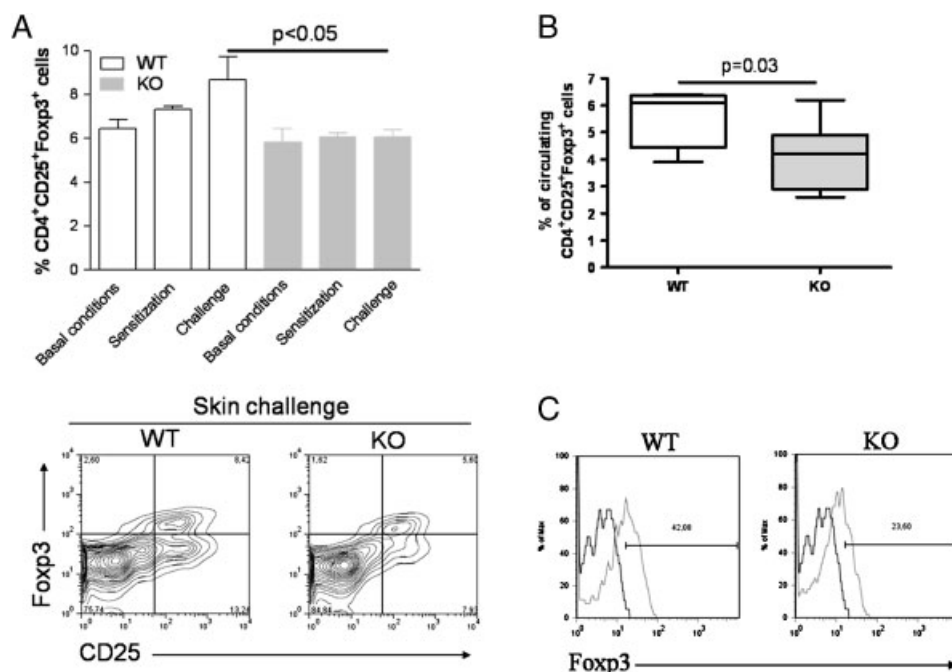


Figure 2. PPAR- α deficiency decreases the population of Tregs after topical application of a hapten: (A) Percentage of CD4⁺CD25⁺Foxp3⁺ Tregs isolated from auricular lymph nodes under basal conditions or 5 days after ear sensitization or 24 h after ear challenge of PPAR- α -deficient and littermate control mice. Sensitization was done by a single topical application of TNCB onto mouse ears while challenge occurred via a topical application of TNCB on ears 5 days after sensitization with TNCB. Density plots show percentages of Foxp3⁺ and CD25⁺ cells. (B) Percentages of CD4⁺CD25⁺Foxp3⁺ Tregs in the blood of PPAR- α -deficient and littermate control mice after skin challenge. (C) Representative FACS plots show percentages of CD4⁺CD25⁺Foxp3⁺ Tregs in challenged skin of PPAR- α -deficient and littermate control mice. Plain grey histograms show Foxp3 expression while plain black histograms show isotype controls. Cells were gated on CD4 for flow cytometry analysis. Results show the mean $\pm$ SEM, $n = 3$ –10 and representative FACS plots. Data were analysed with a Student's *t*-test (B) or a two-way ANOVA followed by Bonferroni post hoc test (A).

into Ly 5.1 recipient mice or CD4⁺CD25[−] T lymphocytes isolated from naïve Ly 5.1 mice into naïve WT and PPAR- α -deficient recipient mice further sensitized with TNCB on ears. We found that PPAR- α deficiency did not significantly alter percentages of iTregs in Ly5.1 recipients (Supporting Information Fig. 3C). However, we found reduced percentages of iTregs after transfer of Ly 5.1 cells into PPAR- α -deficient recipient mice, which was corrected by injection of IL-2 (Fig. 4).

PPAR- α deficiency has little effect on in vivo naturally occurring Treg proliferation

We next investigated the effects of PPAR- α deficiency on the proliferation and the viability of naturally occurring Tregs (nTregs) in vitro. We found that PPAR- α deficiency did not significantly modify viability (Supporting Information Fig. 4A) or proliferation (Supporting Information Fig. 4B) of nTregs in vitro. We next investigated the effects of PPAR- α deficiency on the proliferation of nTregs in vivo. CD4⁺CD25⁺ nTregs isolated from naïve WT and PPAR- α -deficient mice were labelled with CFSE and adoptively transferred into recipient mice further sensitized with TNCB on their ears. We found that PPAR- α deficiency moderately decreased proliferation of nTregs in vivo (Fig. 5).

PPAR- α deficiency decreases the suppressive capacity of Tregs in vitro

We next investigated if PPAR- α deficiency could modify the suppressive effect of Tregs. We co-cultured allogenic mature epidermal DCs with WT CD4⁺CD25[−] T lymphocytes in the presence of various amounts of Tregs isolated from naïve PPAR- α -deficient and littermate control mice. We found that PPAR- α deficiency largely compromised the suppressive capacity of Tregs (Fig. 6A). These results demonstrate a direct effect of PPAR- α deficiency on Treg function.

PPAR- α deficient Tregs lack suppressive capacity in vivo

To test the capacity of PPAR- α -deficient Tregs to suppress the immune response when compared with WT controls, we either transferred WT CD4⁺CD25[−] T lymphocytes alone or we co-transferred WT CD4⁺CD25[−] T lymphocytes together with Tregs isolated from PPAR- α -deficient or littermate control mice 5 days after sensitization with TNCB into WT recipients. Five days after sensitization, most of the CD4⁺CD25⁺ T cells were Foxp3⁺, mimicking the situation that was seen in naïve mice (Supporting Information Fig. 5).

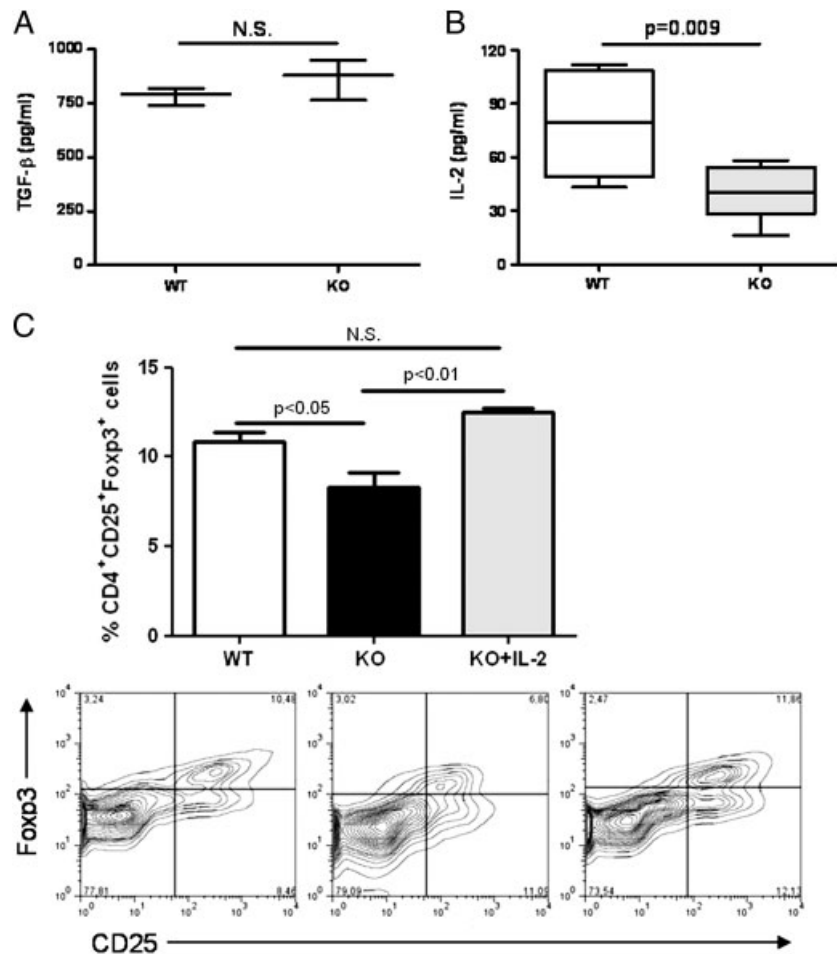


Figure 3. Impaired in vivo production of IL-2 is responsible for the decrease in Treg population after skin sensitization and challenge in PPAR- α -deficient mice: Production of (A) TGF- β and (B) IL-2 by auricular lymph node cells as determined by ELISA in PPAR- α -deficient and littermate control mice sensitized with TNCB on ears. (C) Percentages of CD4⁺CD25⁺Foxp3⁺ Tregs isolated from auricular lymph nodes after ear challenge of PPAR- α -deficient and littermate control mice. Challenge was done by a topical application of TNCB on ears 5 days after sensitization with TNCB. Vehicle or recombinant murine IL-2 was injected once a day throughout the experiment. Density plots show percentages of Foxp3⁺ and CD25⁺ cells gated on CD4. Results show the mean $\pm$ SEM, $n = 3$ –6 and representative FACS plots. Data were analysed with a Student's t -test (A and B) or a one-way ANOVA followed by a Bonferroni post hoc test (C).

Subsequently, recipient mice were challenged on the right ear with TNCB or on the left ear with vehicle. We found that TNCB-activated WT CD4⁺CD25⁺ T lymphocytes alone induced ear swelling (Fig. 6B and C). This effect was diminished when WT Tregs were co-transferred (Fig. 6B and C). However, PPAR- α -deficient Tregs failed to reduce the WT CD4⁺CD25⁺ T-cell-induced ear swelling (Fig. 6B and C). Moreover, histological analyses (Fig. 6C) and immunophenotyping of the inflammatory dermal infiltrates showed that the number of CD4⁺ T lymphocytes increased in the skin of co-transferred recipient mice (WT CD4⁺ T cells+WT Treg: 63 902, pool of five mice; WT CD4⁺ T cells+KO Treg: 67 410, pool of three mice) when compared to recipient mice injected with WT CD4⁺ T cells only (41 486, pool of seven mice). Similar results were found for the number of skin Tregs (WT CD4⁺ T cells only: 2892; WT CD4⁺ T cells+WT Tregs: 5879; WT CD4⁺ T cells+KO Tregs: 7213, pool of three to seven mice). These results demonstrate

that the observed effects are not due to a migratory defect. Instead, the data suggest a lack of suppressive capacity of PPAR- α -deficient Tregs in vivo.

PPAR- α deficient Tregs produce less IL-10 and express less perforin/granzyme B after Ag challenge

In an effort to better understand the reason for the reduced suppressive capacity of PPAR- α -deficient Tregs, we measured the in vivo production of IL-10 in circulating Tregs after antigen challenge. We found that PPAR- α -deficient blood Tregs produced slightly, but consistently, less IL-10 than WT controls (Fig. 7A). Production of IL-10 by lymph node Tregs was below detection levels (data not shown). Moreover, the expression of perforin (Fig. 7B) and granzyme B (Fig. 7C) was decreased. Interestingly, injection of IL-2

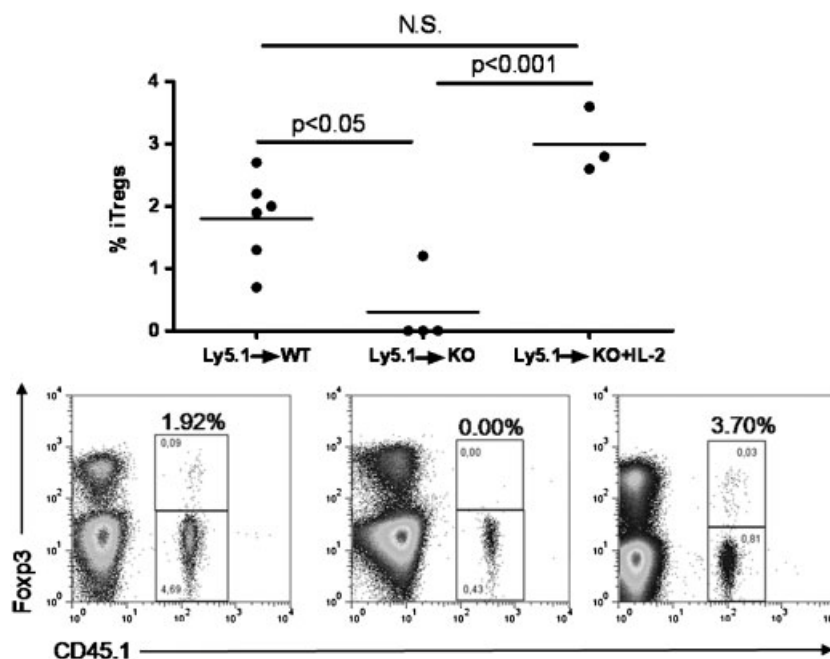


Figure 4. Lack of IL-2 in PPAR- α -deficient mice is responsible for decreased induction of Tregs in vivo: Ly 5.1 CD4⁺CD25⁻ cells were injected i.v. into PPAR- α and littermate control recipient mice further sensitized with TNCB on ears as described in Materials and methods. After 5 days, peripheral blood iTregs (CD45.1⁺CD4⁺Foxp3⁺) were analysed. FACS plots show CD45.1⁺Foxp3⁺ cells gated on CD4. Alternatively, PPAR- α -recipient mice were injected with recombinant murine IL-2 for 5 days. Histograms and numbers show the relative percentages of iTregs. Results are given as mean $\pm$ SEM, $n = 3$ –6 and representative FACS plots are shown. Data were analysed with a one-way ANOVA followed by a Bonferroni post hoc test.

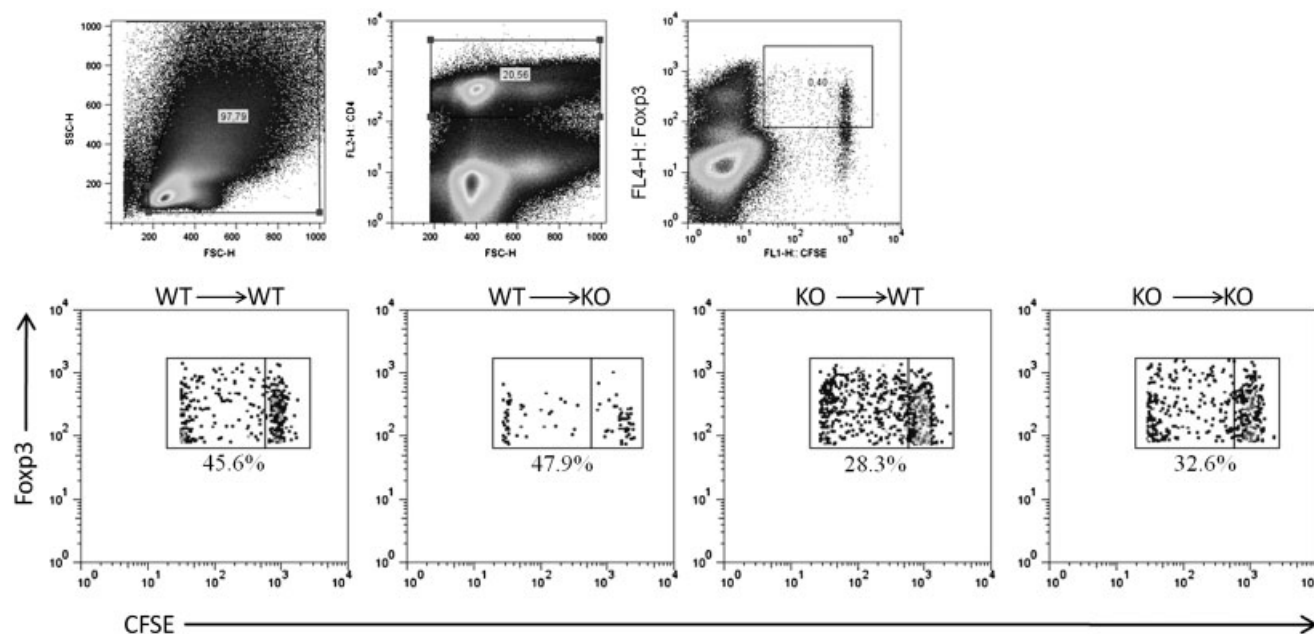


Figure 5. PPAR- α deficiency has little effects on in vivo nTreg proliferation: CD4⁺CD25⁺ nTregs were labelled with CFSE and adoptively transferred into recipient mice further sensitized with TNCB on both ears. After 3 days, auricular lymph nodes were analysed by flow cytometry. FACS plots show CFSE⁺Foxp3⁺ cells gated on CD4. Percentages show proliferating cells. Two independent experiments were done.

during the sensitization phase fully restored the level of perforin expression by Tregs (Fig. 7B) but not of granzyme B (Fig. 7C). We therefore conclude that decreased production of IL-10, perforin and granzyme B impairs the suppressive capacity of Tregs.

IL-2 injection normalizes allergic contact dermatitis in PPAR- α -deficient mice

Finally, we quantified the effects of IL-2 injection on ear inflammation in PPAR- α -deficient mice after antigen challenge.

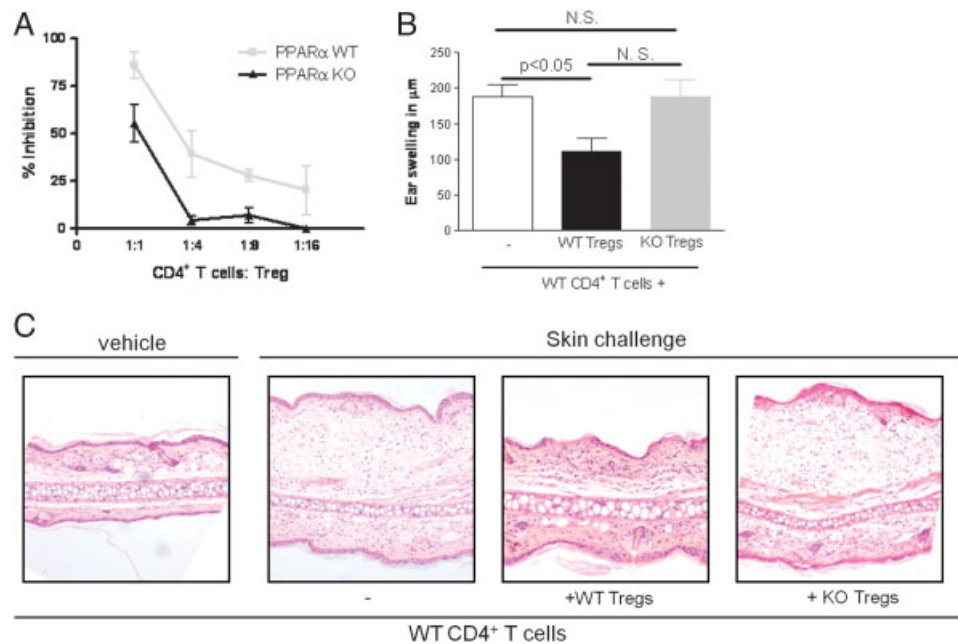


Figure 6. PPAR- α deficiency decreases the suppressive capacity of Tregs: (A) Mixed leukocyte reactions showing the suppressive capacity of nTregs isolated from PPAR- α -deficient and littermate control mice. CD4⁺CD25⁻ T cells were activated with allogenic mature epidermal DCs and co-cultured with various concentrations of nTregs as described in Materials and methods. The assay was carried out in triplicate and three independent experiments were done. Results show mean $\pm$ SEM. (B) Ear swelling (TNB-induced ear thickness–vehicle-induced ear thickness) and (C) H&E staining (10 $\times$ magnification) showing dermal inflammatory infiltrates after adoptive transfer of WT CD4⁺ T lymphocytes only or after co-transfer with WT or PPAR- α -deficient Tregs. Results show mean $\pm$ SEM, n = 5–10. Data were analysed using a one-way ANOVA followed by a Bonferroni post hoc test (B).

Injection of IL-2 during the sensitization phase decreased skin inflammation in PPAR- α -deficient mice to the level of WT controls 48 h after antigen challenge (Fig. 7D) but not after 24 h (Supporting Information Fig. 6). In contrast, injection of IL-2 did not alter skin inflammation in WT mice 24 and 48 h after challenge (Fig. 7D and Supporting Information Fig. 6). Thus, lack of IL-2 in PPAR- α -deficient mice is largely responsible for the augmented inflammatory response to antigen challenge in PPAR- α -deficient mice.

Discussion

PPAR- α regulates inflammatory reactions after activation by locally generated eicosanoids through the MAPK and NF- κ B pathways. The consequences of PPAR- α deficiency on the phenotype of T lymphocytes, and its pathophysiologic role in experimental autoimmune encephalomyelitis, as well as on carrageenan-induced-paw oedema and pleurisy were previously explored [11–13]. In these disease models, lack of PPAR- α uniformly promoted inflammation. Similarly, in models of cutaneous inflammation, lack of PPAR- α is pro-inflammatory [6, 19]. Although Tregs are key cells of the adaptive immune response and peripheral tolerance, the effect of PPAR- α on Tregs is unknown. Here, we link PPAR- α to the induction and activity of Tregs in skin inflammation.

PPAR- α deficiency worsens and prolongs allergic contact dermatitis

Our results show that PPAR- α deficiency promotes and prolongs allergic contact dermatitis. Increased skin inflammation was accompanied by pronounced T-lymphocyte infiltration. The inflammatory cells consisted primarily of CD4⁺ T lymphocytes, exhibiting increased expression of the activation marker CD25. These results are consistent with prior observations of PPAR- α -deficient mice displaying prolonged leukotriene B₄-induced ear swelling [6]. In addition, Staumont-Salle et al. showed that PPAR- α -deficient mice exhibited exacerbated skin inflammation in a mouse model of chronic inflammation, which was associated with an increase in both Th1 and Th2 cytokines [19]. In summary, PPAR- α deficiency has pro-inflammatory effects in the skin by activating T lymphocytes.

PPAR- α deficiency affects the size of the Treg population in skin inflammation

We found that PPAR- α deficiency prevents the expansion of the Treg population in skin-draining lymph nodes of mice following skin sensitization or challenge. Although the number of Tregs was similar in WT and PPAR- α -deficient mice under baseline

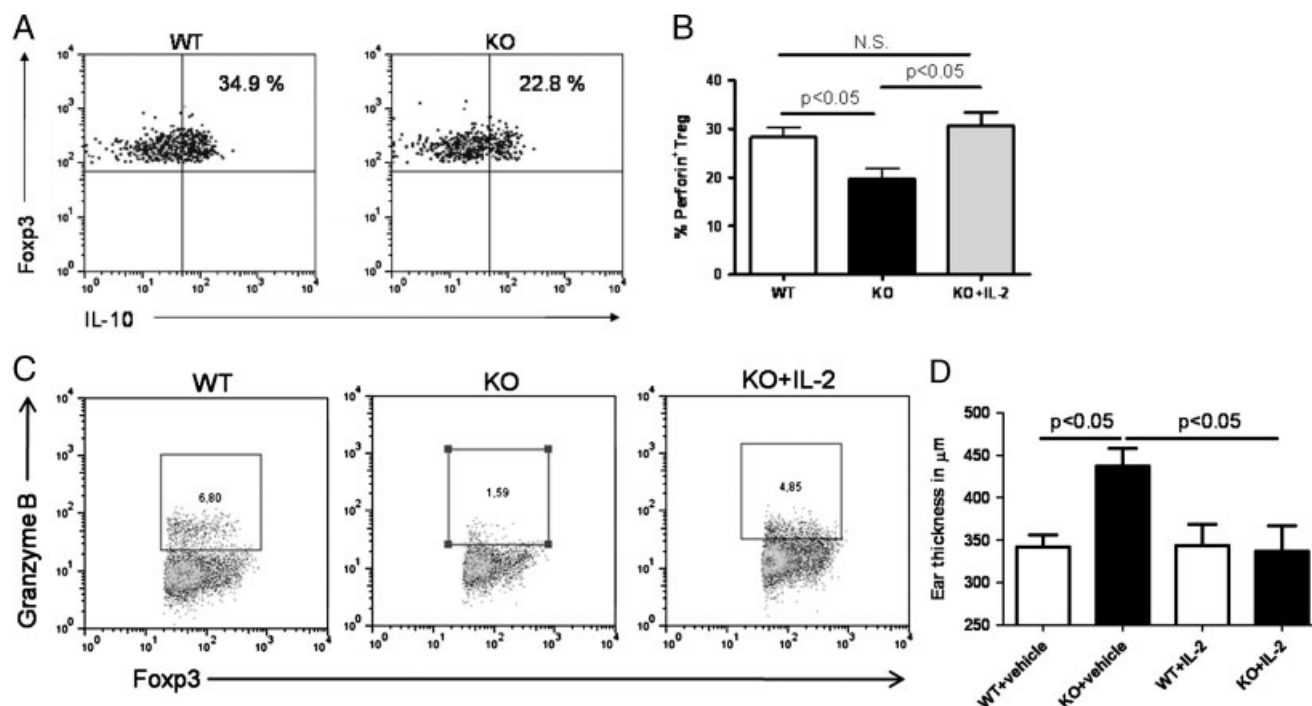


Figure 7. PPAR- α deficiency decreases IL10, perforin and granzyme B in Tregs: (A) Representative flow cytometry plots showing the production of IL-10 by Tregs in the blood of challenged mice. (B) Percentages of perforin⁺ Tregs (CD4⁺ Fxp3⁺) in auricular lymph nodes of mice sensitized with TNCB on ears and injected with IL-2. Cells were gated on CD4. (C) Representative FACS plots showing granzyme B⁺ Tregs (CD4⁺ Fxp3⁺) in auricular lymph nodes of mice sensitized with TNCB on ears and injected with IL-2. Cells were gated on CD4. (D) Ear thickness measured 48 h after TNCB challenge onto mouse ears and injection with IL-2. Results show mean $\pm$ SEM, $n = 3$ –9. Data were analysed using a one-way ANOVA followed by a Bonferroni post hoc test.

conditions, the percentages of Tregs after antigen challenge were moderately but consistently reduced in the blood, the skin-draining lymph nodes and in the skin of PPAR- α -deficient mice when compared to control mice. We found that lack of IL-2 production in skin-draining lymph nodes was responsible for the decreased numbers of Tregs in PPAR- α -deficient mice. Injection of IL-2 restored the Treg population when administered to PPAR- α -deficient mice. The principal physiological source of IL-2 for the maintenance of Tregs appears to be other T cells [20, 21]. These results are consistent with previous work showing that IL-2 is essential for Fxp3 expression. Accordingly, it has been shown that the Fxp3 promoter contains five STAT5B consensus sequences and that IL-2 induces Fxp3 expression through STAT5 signalling [22, 23]. More recently, Visekruna et al. showed that c-Rel-deficient CD4⁺CD25⁺ T lymphocytes failed to up-regulate Fxp3 because of a lack of endogenous IL-2 [24]. Therefore, the size of the Treg population might depend on the amount of endogenous IL-2 in PPAR- α -deficient mice. Indeed, IL-2-deficient mice exhibit reduced Tregs, showing that IL-2 signalling is critically required for maintaining homeostasis and competitive fitness of Tregs in vivo [25]. Furthermore, the number of Tregs can be decreased by the neutralization of circulating IL-2 by anti-IL-2 antibody treatment, which provokes autoimmune disease [20]. These results emphasize the key role of IL-2 in the homeostasis of Tregs in PPAR- α -deficient mice.

Lack of IL-2 in PPAR- α -deficient mice impairs the induction of Tregs in vivo

The reduced Treg population could either result from diminished proliferation, an increase in cell death or a defect in Treg induction. We found that in vitro PPAR- α -deficient CD4⁺CD25⁺ T cells up-regulated Fxp3 to a lesser extent than WT cells. Interestingly, this difference was not seen after adoptive transfer into WT recipient mice while and it was not the case for WT CD4⁺CD25⁺ T cells when adoptively transferred into PPAR- α -deficient recipient mice. Moreover, injection of IL-2 to PPAR- α -deficient mice abolished the difference. Therefore, lack of IL-2 production is mainly responsible for the limited induction of Tregs in PPAR- α -deficient mice whereas PPAR- α -deficient CD4⁺ T lymphocytes may exhibit an intrinsic defect leading to a reduced Fxp3 expression after appropriate stimulation. This intrinsic defect potentially activates a signal transduction pathway that can be bypassed in vivo by other pathways. Together, these results demonstrate that induction of Tregs is prevented by insufficient amounts of IL-2 in PPAR- α -deficient mice. Our present results are supported by recent data showing that extrathymic differentiation of Tregs requires IL-2 and phosphatidylinositol-3-kinase [26]. Mechanistically, PPAR- α down-regulates IL-2 production in lymphocytes via p38 MAPK [12]. Decreased IL-2 reduces the amount of active STAT5, which in turn controls Fxp3

expression [22, 23]. We also observed slightly decreased *in vivo* proliferation of PPAR- α -deficient nTregs after adoptive transfer to WT and PPAR- α -deficient recipients. Thus, changes in the Treg population after antigen challenge may result from impaired induction of Tregs in PPAR- α -deficient mice, yet moderate impairment of nTreg proliferation cannot totally be excluded.

Lack of suppressive capacity of PPAR- α -deficient Tregs

PPAR- α deficiency largely compromised the suppressive effects of Tregs in mixed leukocyte reactions. We found that adoptive transfer of hapten-activated WT CD4⁺CD25⁻ T lymphocytes alone into recipient mice induced ear swelling that is markedly reduced when these cells are co-transferred with WT Tregs. However, PPAR- α -deficient Tregs failed to reduce the ear swelling induced by WT CD4⁺CD25⁻ T cells, independent of the capacity of transferred cells to migrate to the site of the inflammatory challenge. These results support a role for PPAR- α in Treg function. While our experiments did not discriminate between the function of PPAR- α -deficient nTregs versus iTregs, iTregs seem to play a crucial role in the regulation of antigen-specific allergic reactions [27]. The mechanism by which PPAR- α compromises the suppressive capacity of Tregs remains to be elucidated. A possible explanation relates to our observation of reduced IL-10, perforin and granzyme B in PPAR- α -deficient Tregs after antigen challenge. In this regard, the suppressive capacity of Tregs was previously shown to be mediated by the release of the inhibitory cytokines IL-10 and TGF- β 1 [28–30]. Indeed, Ring et al. have shown that the release of cytokines by Tregs rather than cell–cell contacts is involved in the suppression of CHS reactions by Tregs *in vivo* [30]. However, others have shown that neutralization of either IL-10 or TGF- β did not abrogate the suppressive activity of Tregs *in vitro* [29, 31]. While the release of perforin and granzyme B by Tregs was involved in the killing of target cells, there is no evidence that the release of these molecules by Tregs is involved in the compression phase of CHS [31–34]. However, it was previously shown that perforin and granzyme B were decreased in Tregs from mice lacking IL-2, and that administration of recombinant IL-2 restored their expression to the level in WT Tregs [25]. In the present work, IL-2 injection restored perforin and granzyme B in Tregs isolated from PPAR- α mice. Notably, at low IL-2 concentration, the cytotoxic capacity of iTregs is dependent on perforin and granzyme B [31]. Moreover, Kish et al. reported increased and prolonged CHS in mice after neutralization of IL-2 with an IL-2 antibody, thus corroborating our findings [21]. We here report that injection of IL-2 into PPAR- α -deficient mice decreased skin inflammation to a level similar to WT mice. Thus, the pro-inflammatory skin phenotype of PPAR- α -deficient mice is due to lack of IL-2-mediated Treg activation.

Our findings highlight a new role of PPAR- α in the adaptive immune system. We report that PPAR- α -mediated control of Tregs *in vivo* depends on sufficient amounts of IL-2. Moreover,

PPAR- α controls the suppressive capacity of Tregs by affecting multiple pathways including production of IL-10, perforin and granzyme B. These findings should stimulate the development of new strategies for anti-inflammatory therapy based on drugs targeting PPAR- α .

Materials and methods

Animals

Males of the inbred strain C57BL/6, BALB/c or Ly 5.1 were purchased from Charles River Laboratories (Sulzfeld, Germany). PPAR- α -deficient and littermate control males were bred on a C57BL6/129S background as described previously [34]. Animal experiments were conducted under a protocol approved by the Institutional Animal Care Committee.

FACS analysis

Directly labelled primary mAbs specific for CD4, CD3, CD8, CD25, IL-2, IL-10, CD45.1 and CD45.2 were purchased from BD-Pharmingen (San Diego, CA) and for perforin and granzyme B from eBioscience (San Diego, CA). Tregs were detected with an anti-Foxp3 antibody according to the manufacturer's direction (clone FJK-16s, eBioscience). Specimens were analyzed on a FACScalibur (BD Immunocytometry Systems, Becton Dickinson, San Jose, CA) using FlowJo Software (Ashland, OR). Specificity of staining was confirmed using isotype-matched control mAbs.

CHS responses

Allergic contact dermatitis was induced by topical application of 20 μ L of 1% TNCB (Kodak Eastman, Rochester, NY) to the inner and outer surfaces of ears of PPAR- α -deficient and littermate control mice 5 days following primary sensitization with 1% TNCB. Ear thickness was measured with a digital calliper (Kroeplin, Schluechtern, Germany). Inflammatory skin infiltrates were analysed by flow cytometry. Paraffin-embedded sections were stained with H&E for histological analysis.

Cell preparation from skin and skin-draining lymph nodes

Mouse ears were incubated in 0.8% trypsin (Merck, Darmstadt, Germany) for 25–45 min at 37°C. Epidermis and dermis were separated, cut into small pieces and further incubated for 45 min in R5 and DNase I (200 U/mL) at 37°C under continuous shaking [7, 35]. Alternatively, cells from auricular lymph nodes were prepared by digestion with 0.2 mg/mL collagenase P (Roche Applied Science, Mannheim, Germany) for 30 min [7, 35].

Cell isolation

CD4⁺CD25[−] T cells and CD4⁺CD25⁺ Tregs were isolated from spleens and lymph nodes using MACS (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Cells were isolated from naïve mice 5 days after skin sensitization with 1% TNCB (Kodak Eastman).

Suppression assay

Epidermal cells isolated from BALB/c mouse epidermis were cultured for 72 h as described previously [7]. Epidermal DC-containing interphases (10 000 cells) were co-cultured with allogenic WT CD4⁺CD25[−] T lymphocytes (3×10^5 per well) and with PPAR- α -deficient or littermate control CD4⁺CD25⁺ Tregs isolated from naïve mice at various ratios for 4 days. Proliferation was measured in flat-bottomed 96-well microtiter plates as ³H-thymidine incorporation for the last 16 h (activity 4 μ Ci/mL = 148 KBq/mL; New England Nuclear/PerkinElmer, Boston, MA).

Co-adoptive transfer

PPAR- α -deficient or WT mice were sensitized on their shaved backs and on both ears with 1% TNCB (Kodak Eastman). After 5 days, CD4⁺CD25[−] T cells and CD4⁺CD25⁺ Tregs were purified from spleens and skin-draining lymph nodes as described above. WT mice were adoptively transferred with 7.5×10^6 purified CD4⁺CD25[−] T cells isolated from WT mice or with 2.5×10^6 CD4⁺CD25⁺ Tregs isolated from PPAR- α -deficient and littermate control mice. Recipient mice were challenged with 1% TNCB (right ear) or vehicle (left ear) 1 h after adoptive transfer via injection into tail vein. Ear thickness was measured with a digital calliper (Kroeplin) 24 h after challenge. Ear swelling was calculated as the difference between thickness of TNCB-treated ear and thickness of vehicle-treated ear in the same mouse. Inflammatory skin infiltrates were analysed by flow cytometry. Paraffin-embedded sections were stained with H&E for histologic analysis.

In vivo induction of Tregs

CD4⁺CD25[−] cells (10^7) were isolated from spleens and lymph nodes of naïve PPAR- α (CD45.2), WT (CD45.2) or Ly 5.1 (CD45.1) mice by positive selection using MACS (Miltenyi Biotec) according to the manufacturer's instructions and adoptively transferred into recipient mice further sensitized with 1% TNCB (Kodak Eastman) on both ears. After 5 days, blood was collected and cells were analysed by flow cytometry.

Injection of IL-2

PPAR- α -deficient or WT mice were sensitized with 1% TNCB (Kodak Eastman) on their left ears. In vivo IL-2 stimulation was

performed by injecting 20 000 IU recombinant murine IL-2 (Peprotech, Rocky Hill, NJ) i.p. at days 0–5 of sensitization. IL-2 was diluted in 0.2% BSA to avoid loss of cytokine by adherence to plastics. Mice were challenged on right ears with 1% TNCB 5 days after sensitization. Skin-draining lymph nodes from right ears were collected, digested and stained as described above. Alternatively, PPAR- α -deficient mice were adoptively transferred with Ly 5.1 CD4⁺CD25[−] cells, sensitized with 1% TNCB on both ears and injected with IL-2 as described above. After 5 days, blood was collected and cells were analysed by flow cytometry.

Production of cytokines by lymphocytes and Tregs

Mouse ear skin was topically treated with 1% TNCB (Kodak Eastman) at day 0. At day 5, cells were isolated from auricular lymph nodes by collagenase P digestion as described above. Production of IL-2 by lymphocytes was detected by intracellular staining after culture with 1 μ g/mL Brefeldin A (BD-Pharmingen) for 4.5 h to prevent cytokine secretion. Cells were first fixed and permeabilized with a cell permeabilization kit (Fix&PermTM, An der Grub Bio-Research, Kaumberg, Austria) for 15 min at room temperature, washed and then incubated with directly labelled primary mAbs for 30 min at room temperature. Alternatively, cells isolated from skin-draining lymph nodes after skin sensitization were further cultured for 18 h and supernatants were collected to measure the concentration of TGF- β 1 (R&D Systems, Minneapolis, MN) and IL-2 (ebiosciences, San Diego, CA) by ELISA. Mice were challenged on ears with TNCB 5 days after sensitization. Twenty-four hours after antigen challenge, peripheral blood was collected and red blood cells were eliminated by erylyse. The remaining cells were cultured for 4.5 h with 1 μ g/mL Brefeldin A as described above. Then cells were first stained with anti-mouse CD4 mAb followed by fixation and permeabilization by using the Foxp3 staining kit as described above. Further staining was simultaneously done with anti-mouse Foxp3 and IL-10 mAb.

Viability and proliferation of nTregs

CD4⁺CD25⁺ nTregs were isolated from naïve mouse spleens by positive selection using MACS (Miltenyi Biotec) according to the manufacturer's instructions. Cells were stimulated using a combination of plate-bound anti-CD3 mAb (2 μ g/mL) and anti-CD28 mAb (2 μ g/mL) for 48 h. In vitro proliferation of nTregs was measured in flat-bottomed 96-well microtitre plates by ³H-thymidine incorporation for 48 h (activity 4 μ Ci/mL = 148 KBq/mL; New England Nuclear/PerkinElmer, Boston, MA). Viability of nTregs was assessed by trypan blue staining. Alternatively, 2×10^6 CD4⁺CD25⁺ nTregs were labelled with CFSE and adoptively transferred into recipient mice further sensitized with 1% TNCB onto both ears. After 3 days, auricular lymph nodes were collected, cells prepared, labelled with anti-CD4 and anti-Foxp3 mAb and analysed by flow cytometry as described above.

Statistical analysis

The results are expressed as mean $\pm$ SEM and analysed for statistical significance as appropriate (see figure legends).

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References

- Mangelsdorf, D. J. and Evans, R. M., The RXR heterodimers and orphan receptors. *Cell* 1995. 1583: 841–850.
- Desvergne, B., Michalik, L. and Wahli, W., Be fit or be sick: peroxisome proliferator-activated receptors are down the road. *Mol. Endocrinol.* 2004. 18: 1321–1332.
- Chakravarthy, M. V., Lodhi, I. J., Yin, L., Malapaka, R. R., Xu, H. E., Turk, J. and Semenkovich, C. F., Identification of a physiologically relevant endogenous ligand for PPAR- α in liver. *Cell* 2009. 138: 476–488.
- Narala, V. R., Adapala, R. K., Suresh, M. V., Brock, T. G., Peters-Golden, M. and Reddy, R. C., Leukotriene B₄ is a physiologically relevant endogenous peroxisome proliferator-activated receptor- α agonist. *J. Biol. Chem.* 2010. 285: 22067–22074.
- Glass, C. K. and Ogawa, S., Combinatorial roles of nuclear receptors in inflammation and immunity. *Nature* 2006. 6: 44–55.
- Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J. and Wahli, W., The PPAR α -leukotriene B₄ pathway to inflammation control. *Nature* 1996. 384: 39–43.
- Dubrac, S., Stoitzner, P., Pirkebner, D., Elentener, A., Shoonjans, K., Auwerx, J., Saeland, S. et al., Peroxisome proliferator-activated receptor- α activation inhibits Langerhans cell function. *J. Immunol.* 2007. 178: 4362–4372.
- Daynes, R. and Jones, D. C., Emerging roles of PPARs in inflammation and immunity. *Nat. Rev. Immunol.* 2002. 2: 748–759.
- Kippenberger, S., Loitsch, S. M., Grundmann-Kollmann, M., Simon, S., Dang, T., Hardt-Weinelt, K., Kaufmann, R. and Bernd, A., Activators of peroxisome proliferator-activated receptors protect human skin from ultraviolet-B-light-induced inflammation. *J. Invest. Dermatol.* 2001. 117: 1430–1436.
- Sheu, M. Y., Fowler, A. J., Kao, J., Schmuth, M., Schoonjans, K., Auwerx, J., Fluhr, J. W. et al., Topical peroxisome proliferator activated receptor- α activators reduce inflammation in irritant and allergic contact dermatitis models. *J. Invest. Dermatol.* 2002. 118: 94–101.
- Dunn, S. E., Ousman, S. S., Sobel, R. A., Zuniga, L., Baranzini, S. E., Youssef, S., Crowell, A. et al., Peroxisome proliferator-activated receptor (PPAR)- α expression in T cells mediates gender differences in development of T cell-mediated autoimmunity. *J. Exp. Med.* 2004. 204: 321–330.
- Jones, D. C., Ding, X., Zhang, T. Y. and Daynes, R. A., Peroxisome proliferator-activated receptor- α negatively regulates T-bet transcription through suppression of p38 mitogen-activated protein kinase activation. *J. Immunol.* 2003. 171: 196–203.
- Cuzzocrea, S., Mazzon, E., Di Paola, R., Peli, A., Bonato, A., Britti, D., Genovese, T. et al., The role of the peroxisome proliferator-activated receptor- α (PPAR- α) in the regulation of acute inflammation. *J. Leukoc. Biol.* 2006. 79: 999–1010.
- Yang, Q. and Gonzalez, F. J., Peroxisome proliferator-activated receptor α regulates B lymphocyte development via an indirect pathway in mice. *Biochem. Pharmacol.* 2004. 68: 2143–2150.
- Izcue, A., Coombes, J. L. and Powrie, F., Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol. Rev.* 2006. 212: 256–271.
- Hontecillas, R. and Bassaganya-Riera, J., Peroxisome proliferator-activated receptor gamma is required for regulatory CD4⁺ T cell-mediated protection against colitis. *J. Immunol.* 2007. 178: 2940–2949.
- Hayashi, M., Higashi, K., Kato, H. and Kaneko, H., Assessment of preferential Th1 or Th2 induction by low-molecular-weight compounds using a reverse transcription-polymerase chain reaction method: comparison of two mouse strains, C57BL/6 and Balb/c. *Toxicol. Appl. Pharmacol.* 2001. 177: 38–45.
- Zheng, S. G., The critical role of TGF- β 1 in the development of induced Foxp3⁺ regulatory T Cells. *Int. J. Clin. Exp. Med.* 2008. 1: 192–202.
- Staumont-Sallé, D., Abboud, G., Brénuchon, C., Kanda, A., Roumier, T., Lavogiez, C., Fleury, S. et al., Peroxisome proliferator-activated receptor α regulates skin inflammation and humoral response in atopic dermatitis. *J. Allergy Clin. Immunol.* 2008. 121: 962–968.
- Setoguchi, R., Hori, S., Takahashi, T. and Sakaguchi, S., Homeostatic maintenance of natural Foxp3⁺CD25⁺CD4⁺ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 2005. 201: 723–735.
- Kish, D. D., Gorbachev, A. V. and Fairchild, R. L., CD8⁺ T cells produce IL-2, which is required for CD4⁺CD25⁺ T cell regulation of effector CD8⁺ T cell development for contact hypersensitivity responses. *J. Leukoc. Biol.* 2005. 78:725–735.
- Murawski, M. R., Litherland, S. A., Clare-Salzler, M. J. and Davoodi-Semiromi, A., Upregulation of Foxp3 expression in mouse and human Treg is IL-2/STAT5 dependent: implications for the NOD STAT5B mutation in diabetes pathogenesis. *Ann. N. Y. Acad. Sci.* 2006. 1079: 198–204.
- Zorn, E., Nelson, E. A., Mohseni, M., Porcheray, F., Kim, H., Litsa, D., Bellucci, R. et al., IL-2 regulates Foxp3 expression in human CD4⁺CD25⁺ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood* 2006. 108: 1571–1579.
- Visekruna, A., Huber, M., Hellhund, A., Bothur, E., Reinhard, K., Bollig, N., Schmidt, N. et al., c-Rel is crucial for the induction of Foxp3⁺ regulatory CD4⁺ T cells but not T(H)17 cells. *Eur. J. Immunol.* 2010. 40: 671–676.
- Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. and Rudensky, A. Y., A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 2005. 6: 1142–1151.
- Schallenberg, S., Tsai, P. Y., Riewaldt, J. and Kretschmer, K., Identification of an immediate Foxp3⁺ precursor to Foxp3⁺ regulatory T cells in peripheral lymphoid organs of nonmanipulated mice. *J. Exp. Med.* 2010. 207: 1393–1407.

- 27 Saito, M., Nagasawa, M., Tadaka, H., Hara, T., Tsuchiya, S., Agematsu, K., Yamada, M. et al., Defective IL-10 signaling in hyper-IgE syndrome results in impaired generation of tolerogenic dendritic cells and induced regulatory T cells. *J. Exp. Med.* 2011. **208**: 235–249.
- 28 Strauss, L., Bergmann, C., Szczepanski, M., Gooding, W., Johnson, J. T. and Whiteside, T. L., A unique subset of CD4⁺CD25^{high}Foxp3⁺ T cells secreting IL-10 and TGF-beta1 mediates suppression in the tumor microenvironment. *Clin. Cancer Res.* 2007. **13**: 4345–4354.
- 29 Miyara, M. and Sakaguchi, S., Natural regulatory T cells: mechanisms of suppression. *Trends Mol. Med.* 2007. **13**: 108–116.
- 30 Ring, S., Schäfer, S. C., Mahnke, K., Lehr, H. A. and Enk, A. H., CD4⁺CD25⁺ regulatory T cells suppress contact hypersensitivity reactions by blocking influx of effector T cells into inflamed tissue. *Eur. J. Immunol.* 2006. **36**: 2981–2992.
- 31 Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J. and Sakaguchi, S., Immunologic self-tolerance maintained by CD25⁺CD4⁺ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* 1998. **10**: 1969–1980.
- 32 Thornton, A. M. and Shevach, E. M., CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J. Exp. Med.* 1998. **188**: 287–296.
- 33 Grossman, W. J., Verbsky, J. W., Barchet, W., Colonna, M., Atkinson, J. P. and Ley, T. J., Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 2004. **21**: 589–601.
- 34 Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S. and Noelle, R. J., Cutting edge: contact-mediated suppression by CD4⁺CD25⁺ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J. Immunol.* 2005. **174**: 1783–1786.
- 35 Cao, X., Cai, S. F., Fehniger, T. A., Song, J., Collins, L. I., Piwnicka-Worms, D. R. and Ley, T. J., Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 2007. **27**: 635–646.
- 36 Zhao, D. M., Thornton, A. M., DiPaolo, R. J. and Shevach, E. M., Activated CD4⁺CD25⁺ T cells selectively kill B lymphocytes. *Blood* 2006. **107**: 3925–3932.
- 37 Schmuth, M., Schoonjans, K., Yu, Q. C., Fluhr, J. W., Crumrine, D., Hachem, J. P., Lau, P. et al., Role of peroxisome proliferator-activated receptor alpha in epidermal development in utero. *J. Invest. Dermatol.* 2002. **119**: 1298–1303.
- 38 Elentner, A., Finke, D., Schmuth, M., Chappaz, S., Ebner, S., Malissen, B., Kissenpfennig, A. et al., Langerhans cells are critical in the development of atopic dermatitis-like inflammation and symptoms in mice. *J. Cell Mol. Med.* 2009. **13**: 2658–2672.

Abbreviations: CHS: contact hypersensitivity · iTreg: inducible Treg · nTreg: naturally occurring Treg · PPAR: peroxisome proliferator-activated receptor · TNCB: 2,4,6-trinitro-1-chlorobenzene picryl chloride

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Supporting Information

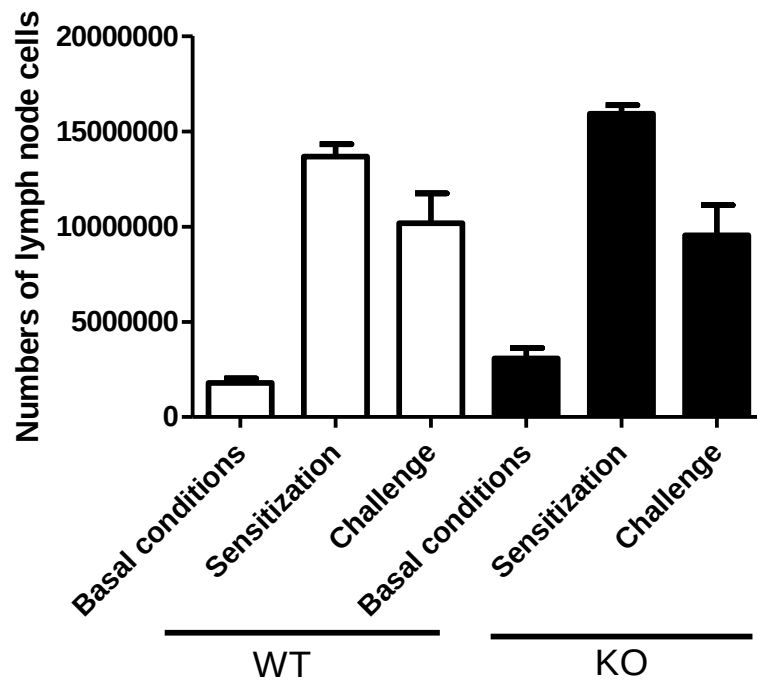
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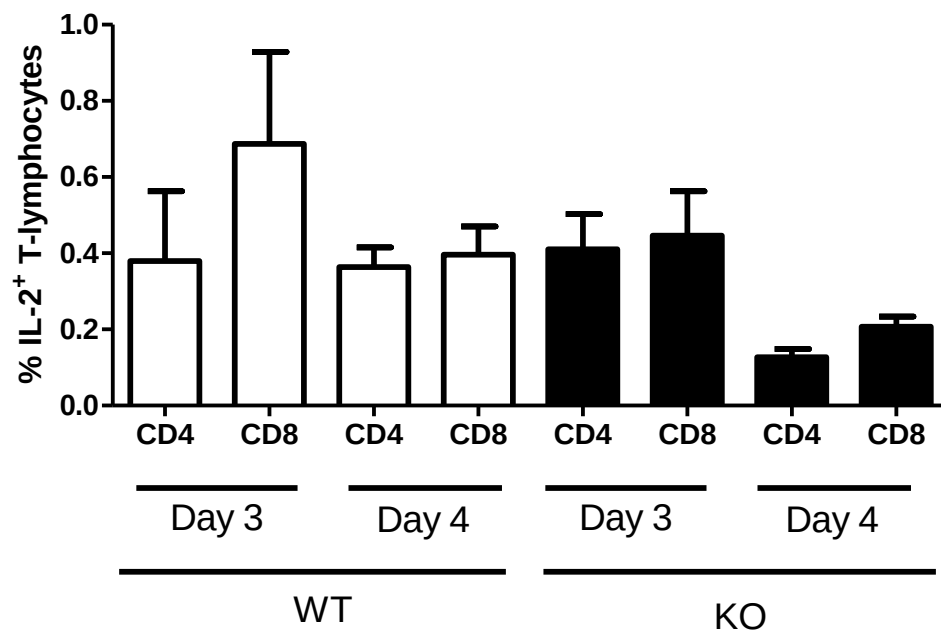
Lack of IL-2 in PPAR- α -deficient mice triggers allergic contact dermatitis by affecting regulatory T cells

Sandrine Dubrac, Andreas Elentner, Kristina Schoonjans, Johan Auwerx
and Matthias Schmith

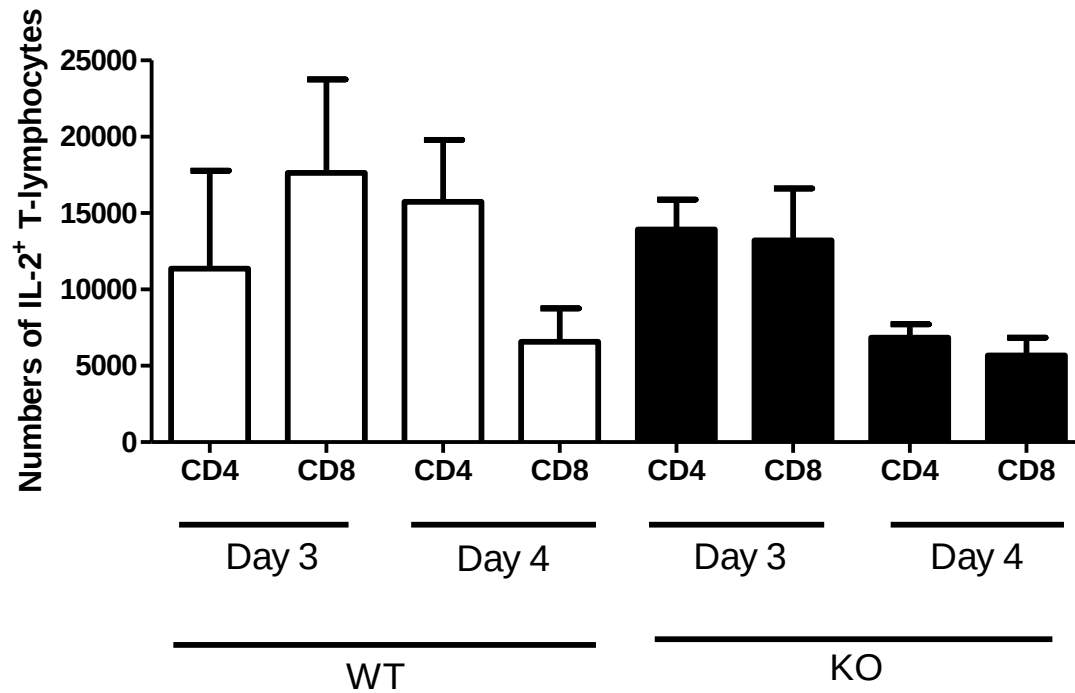
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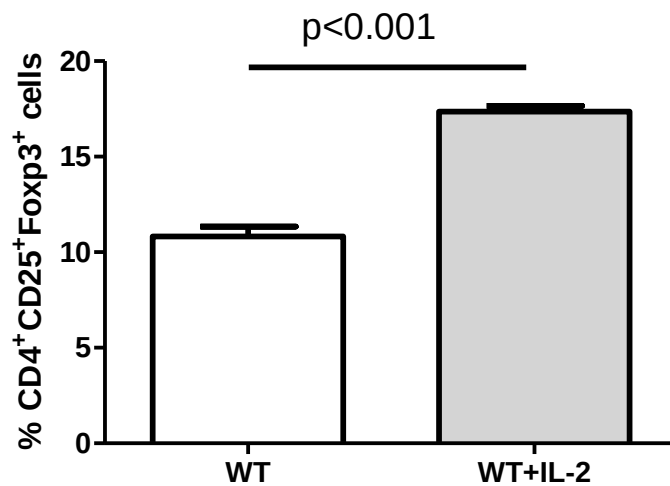


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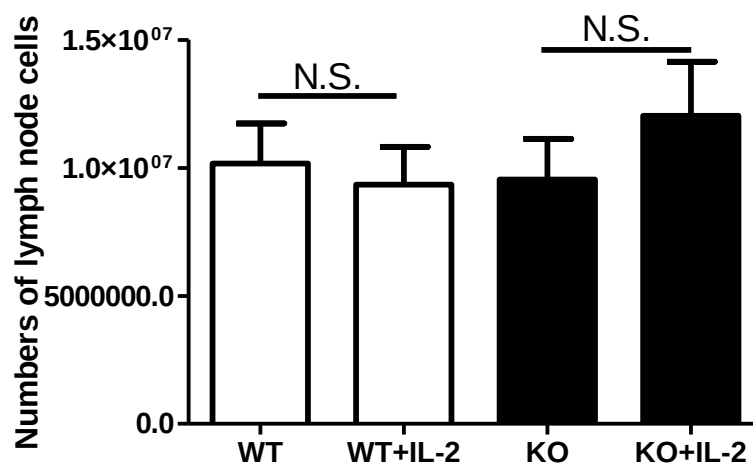


Supplemental Fig. 1: A. Numbers of lymph node cells isolated from skin draining lymph nodes under basal conditions or 5 days after skin sensitization or 24h after skin challenge of PPAR-alpha deficient and littermate control mice. **(B)** Percentages and **(C)** numbers of IL-2⁺ T-lymphocytes in the skin draining lymph nodes 3 and 4 days after skin sensitization of PPAR-alpha deficient and littermate control mice. Results show the mean $\pm$ SEM, n=3-9.

A.

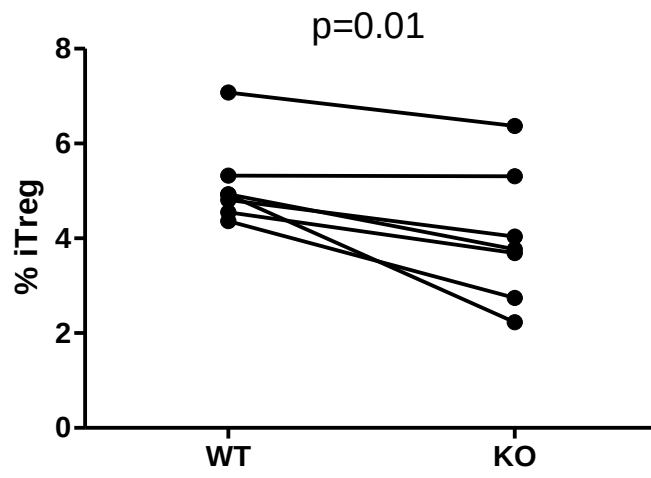


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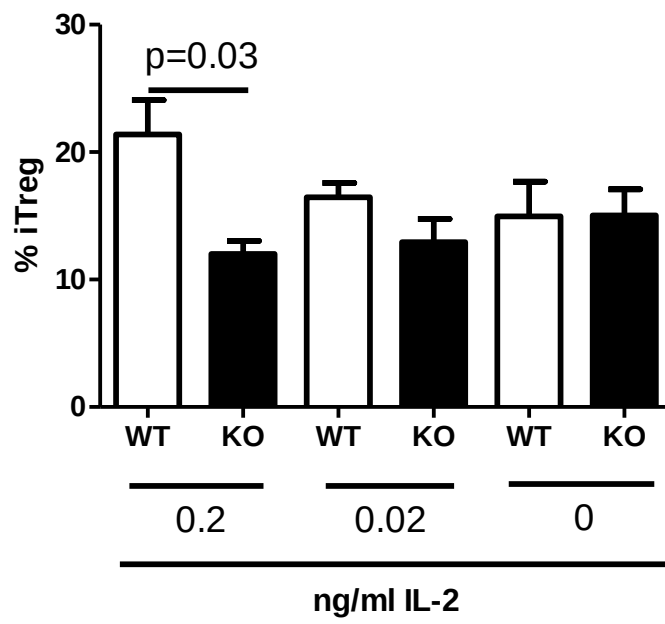


Supplemental Fig. 2: Right mouse ears were challenged by topical application of TNCB five days after sensitization. Vehicle or recombinant murine IL-2 was injected once daily throughout the experiment. **A.** Percentages of CD4⁺CD25⁺Foxp3⁺ Tregs isolated from skin draining lymph nodes of wild type mice. Cells were gated on CD4. **B.** Numbers of lymph node cells isolated from skin draining lymph nodes of PPAR- α deficient and littermate control mice. Results show the mean $\pm$ SEM, n=3-6. Data were analysed with a Student-t-test (N.S.: not significant).

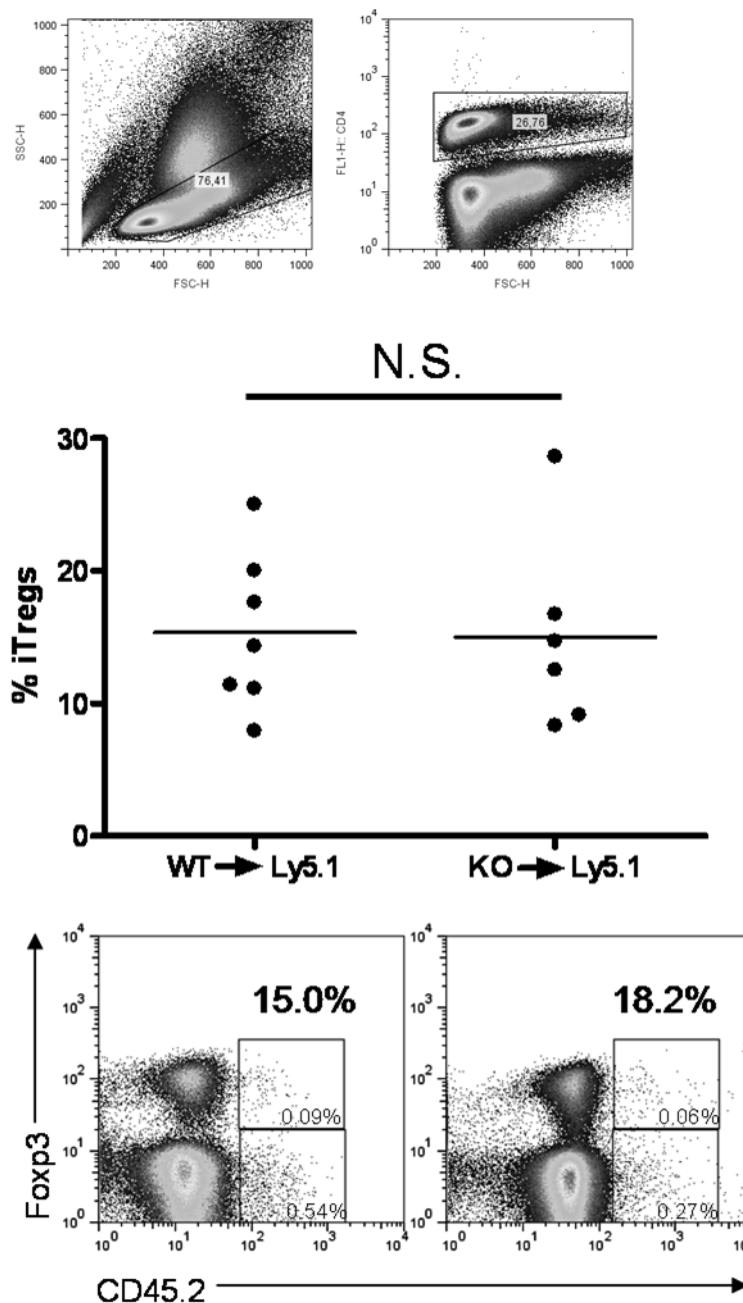
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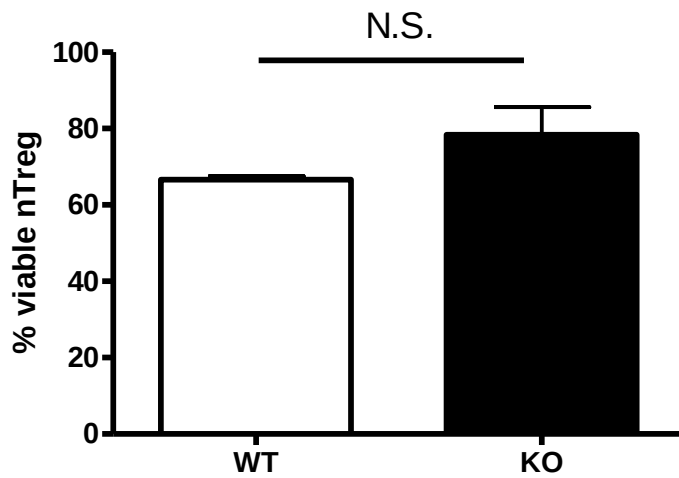
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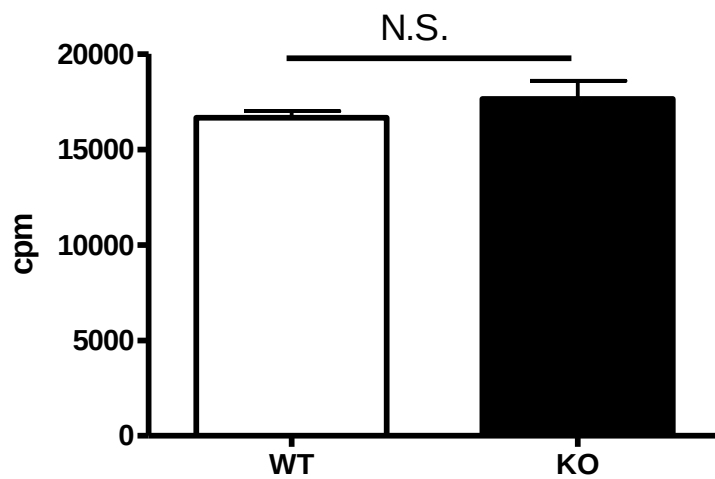
Supplemental Fig. 3: PPAR- α or littermate control $CD4^+CD25^-$ cells isolated from mouse spleens were stimulated with coated anti-CD3 and soluble anti-CD28 antibodies for 3 days in the presence of (A) 2 or (B) 4 ng/ml TGF- β and (A, B) 0.2, 0.02 or (B) 0 ng/ml IL-2. Inducible Tregs (iTregs) were analyzed by flow cytometry. Viable cells (live-dead negative) were stained with anti-CD25 and anti-Foxp3 mab. Figures either show results from (A) 6 independent experiments or from (B) one experiment done in triplicate. C. PPAR- α or littermate control $CD4^+CD25^-$ cells were injected into Ly 5.1 recipient mice further sensitized with TNCB and $CD45.2^+CD4^+Foxp3^+$ Tregs were analysed in the blood of recipient mice. Relative percentages of iTregs are given. Representative FACS plots show percentages of

CD45.2⁺ Foxp3⁺ cells gated on CD4, n=6-7. Data were analysed with a Student-t-test (N.S.: not significant).

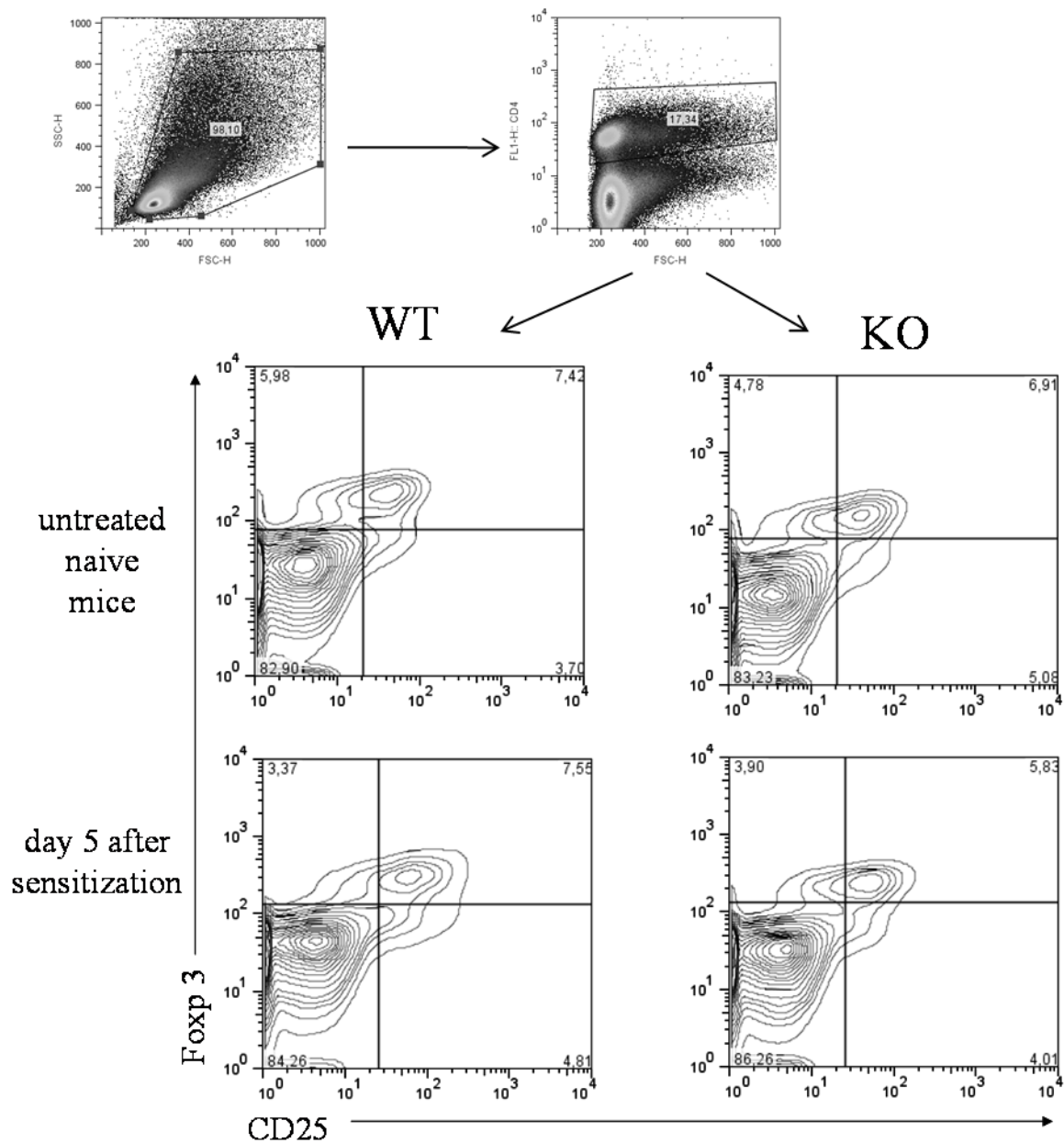
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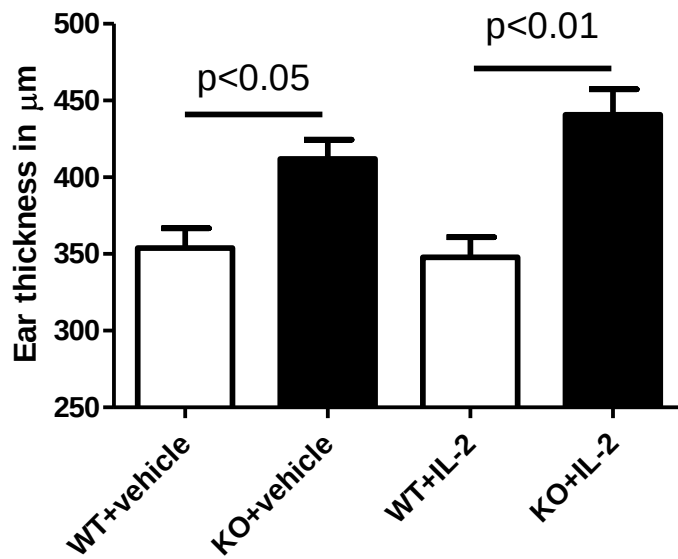
B.



Supplemental Fig. 4: CD4⁺CD25⁺ nTregs were isolated from spleens by positive selection. Cells were stimulated through the TCR pathway (anti CD3 + anti CD28) for 48 hours as described in Materials and Methods. **A.** Viability of nTregs was assessed by trypan blue staining. **B.** In vitro proliferation of nTregs was measured by ³H-thymidine incorporation for 48 hours. Results show the mean $\pm$ SEM, n=3-5. Data were analysed with a Student-t-test (N.S.: not significant).



Supplemental Fig. 5: Representative FACS plots showing percentages of Tregs ($CD4^+CD25^+Foxp3^+$) and activated $CD4^+$ T-cells ($CD4^+CD25^+Foxp3^-$) in auricular draining lymph nodes of PPAR-alpha deficient and littermate control mice under basal conditions (untreated naive mice) or 5 days after skin sensitization with TNCB. Cells were gated on CD4.



Supplemental Fig. 6: Right mouse ears were challenged by topical application of TNCB five days after sensitization. Vehicle or recombinant murine IL-2 was injected once daily throughout the experiment. Results show ear thickness measured 24h after challenge. Results show the mean $\pm$ SEM, $n=4-9$. Data were analysed with a one-way ANOVA followed by a Bonferroni post-hoc test.