



# Activation of the aryl hydrocarbon receptor reduces the number of precursor and effector T cells, but preserves thymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells

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## HIGHLIGHTS

- ▶ Thymic Treg cells are preserved from reducing effects of AhR on precursor T cells.
- ▶ AhR activation does not increase the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in the thymus and MLN, but increases the number of these Treg cells in the spleen.
- ▶ AhR activation decreases the number of precursor and effector T cells.
- ▶ Effects of AhR activation on T cells are partly independent of sensitization.

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## ABSTRACT

Aryl hydrocarbon receptor (AhR) activation suppresses immune responses, including allergic sensitization, by increasing the percentage of regulatory (Treg) cells. Furthermore, AhR activation is known to affect thymic precursor T cells. However, the effect of AhR activation on intrathymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells is unknown. Therefore, we investigated the effect of AhR activation on the percentage and number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells during allergic sensitization in relevant immunological organs.

C3H/HeOuj mice were treated on day 0 with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and subsequently sensitized to peanut. On day 8, mice were sacrificed and thymus, spleen and mesenteric lymph nodes (MLN) were isolated. TCDD treatment decreased the number of CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> precursor T cells, but not the number of thymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells. TCDD treatment increased the number of splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and decreased Th1, Th2 and cytotoxic T cells in the spleen. This appeared to be independent of allergic sensitization. In MLN, TCDD treatment suppressed the increase of the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, Th1, Th2 and cytotoxic T cells induced by peanut sensitization.

Together, TCDD treatment preserves thymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and decreases peripheral T helper and cytotoxic T cells. This effect of TCDD may contribute to the increased influence of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells on immune mediated responses and to the understanding of how AhR activation modulates immune mediated diseases, including food allergy.

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## 1. Introduction

The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix protein and upon ligation the AhR acts as a transcription factor by

**Abbreviations:** AhR, aryl hydrocarbon receptor; BW, body weight; CT, cholera toxin; DRE, dioxin-responsive-element; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveoretinitis; Treg, regulatory T cells; FACS, fluorescence activated cell sorting; GvHD, graft versus host disease; ITE, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester; MLN, mesenteric lymph nodes; PE, peanut extract; SEM, standard error of the mean; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCR, T cell receptor.

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binding to dioxin-responsive-elements (DRE). This results in gene transcription followed by a range of species- and tissue-specific biological and toxic effects. One of the most potent AhR ligands is the halogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Denison and Nagy, 2003). AhR ligation by TCDD, VAG539 or 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), has been shown to suppress experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveoretinitis (EAU), graft versus host disease (GvHD) and viral immunopathology by increasing the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory (Treg) cells (Hauben et al., 2008; Quintana et al., 2008, 2010; Zhang et al., 2008). However, a numerical increase of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells after AhR activation has not been demonstrated in these studies. Possibly, the increase of the percentage of

Treg cells observed after AhR activation results from a decrease of other cell subsets (Stockinger et al., 2011). Nevertheless, in vitro treatment of naïve CD4<sup>+</sup> T cells with TCDD or the endogenous AhR ligand 6-formylindolo[3,2-b]carbazole (FICZ) induces CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells from naïve T cells in the presence, but not in the absence of TGF- $\beta$  (Kimura et al., 2008). Currently, the question whether AhR activation induces, expands or preserves Treg cells, in particular CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, is not solved yet.

For a long time TCDD is known to induce immune suppression, to affect the differentiation of thymic precursor T cells and to induce thymic atrophy (Fine et al., 1990; Hundeiker et al., 1999; Kremer et al., 1994; Lai et al., 1997; Laiosa et al., 2003; Lundberg et al., 1990; Lundberg, 1991; Silverstone et al., 1994). A single dose of 0.1  $\mu$ g/kg BW TCDD is already effective in decreasing thymocyte numbers in mice (Kamath et al., 1999). Discussion is ongoing how TCDD induces apoptosis of thymocytes resulting in thymic atrophy. Both direct as well as indirect effects of TCDD on thymocytes via stromal cells have been reported (Chopra and Schrenk, 2011). Notably, the effect of AhR activation on Foxp3<sup>+</sup> Treg cells in the thymus has not been described in any of the previously mentioned in vivo studies. Quintana et al. (2010) only demonstrated that the frequency of Foxp3<sup>+</sup> Treg cells was unchanged in mice with reduced AhR ligand binding capacity. The lack of studies into the effects of

AhR ligands on thymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells is remarkable given the known alterations induced by AhR activation on T cell precursors in the thymus and the substantial contribution of thymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells to the peripheral CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg population (Fontenot et al., 2003; Gavin et al., 2007).

Activation of the AhR has been shown to suppress Th2-type immune responses by decreasing antigen-specific antibody levels and Th2-related cytokines (Fujimaki et al., 2002; Inouye et al., 2005; Ito et al., 2002; Kato et al., 2003; Luebke et al., 2001; Negishi et al., 2005; Nohara et al., 2002). In this Th2-mediated immune response, including allergic sensitization, Treg cells play an important role (Torgerson and Ochs, 2007; Van Wijk et al., 2007). Recently, we have shown AhR activation suppresses allergic sensitization at least in part by increasing the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells (Schulz et al., 2011).

In light of the prominent thymocyte depleting effect of TCDD, we here investigated the effect of TCDD on the intrathymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cell population and its relation to other T cell subsets in the thymus, spleen and mesenteric lymph nodes (MLN). This was done in the context of a T cell dependent disease using an established peanut allergy model with cholera toxin as an adjuvant to induce Type 2 (and in part Type 1) T cell activation (Van Wijk et al., 2007).

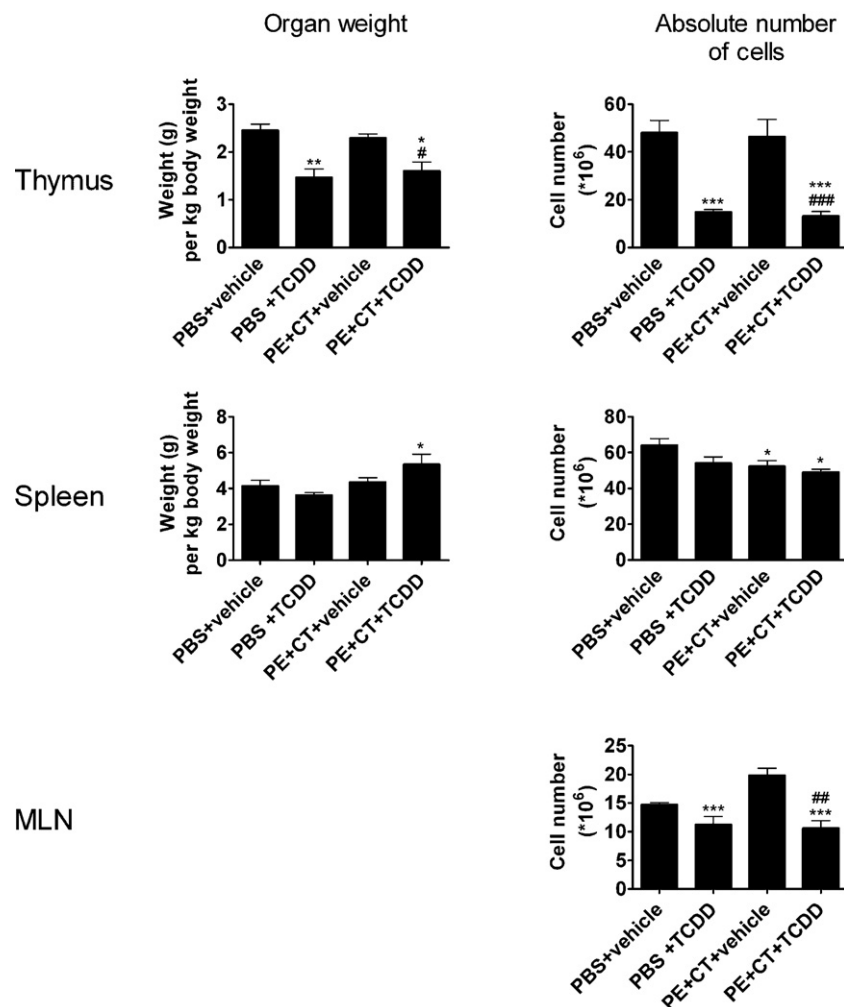


**Fig. 1.** Effect of AhR activation by TCDD on thymus and spleen weight and cell number in spleen, thymus and MLN. Mice were treated with vehicle or TCDD (15  $\mu$ g/kg BW) on day 0 and sensitized to PE (PE + CT) on days 3–5 or treated with PBS. On day 8, mice were sacrificed and thymus, spleen and MLNs were isolated. Relative weights of thymus and spleen and cellularity of thymus, spleen and MLN were determined. Values are presented as mean  $\pm$  SEM ( $n=4$ ). \* $p < 0.05$  compared with PBS, \*\* $p < 0.01$  compared with PBS, \*\*\* $p < 0.001$  compared with PBS, # $p < 0.05$  compared with PE + CT, ## $p < 0.01$  compared with PE + CT, and ### $p < 0.001$  compared with PE + CT.

## 2. Materials and methods

### 2.1. Mice and reagents

4–5 weeks old female C3H/HeOuj mice (AhR<sup>b-2</sup>), purchased from Charles River (France), were maintained under controlled conditions (relative humidity of 50–55%, 12 h light/dark cycle, temperature of 23 ± 2 °C) in filter-topped macrolon cages with wood chip bedding. Food pellets and drinking water were available ad libitum and prior to the start of the experiments, mice were acclimatized. All experiments were approved by the animal experiments committee of Utrecht University. 2,3,7,8-TCDD (Cambridge Isotope Lab, USA) was dissolved in anisole (Sigma–Aldrich, The Netherlands) at 20.5 µg/ml and diluted in corn oil (Sigma–Aldrich, The Netherlands) to the final exposure concentration (0.07%, v/v, anisole). Anisole diluted in corn oil (0.07%, v/v) was used as vehicle-control. Peanut extract (PE) was prepared from raw peanuts (Intersnack Nederland BV, The Netherlands) as described previously (van Wijk et al., 2005). PE was prepared according to standard procedures and checked for protein content by BCA analysis (Pierce, USA). Cholera toxin (CT) was purchased from List Biological Laboratories (USA).

### 2.2. Experimental design

C3H/HeOuj mice ( $n = 4$  per group) were treated with PBS or sensitized to PE (6 mg PE, 200 µl/mouse) mixed with CT (15 µg/mouse) on three consecutive days (days 3–5) by gavage. On day 0 mice were treated with TCDD (15 µg/kg BW) or vehicle-control by oral gavage. The dose of TCDD used was based on previous research (Funatake et al., 2005; Schulz et al., 2011). On day 8, mice were sacrificed by cervical dislocation and thymus, spleen and MLNs were isolated. The number of mice per group was calculated by using a power analysis (power > 0.9) and was approved by the animal welfare committee. Experiments were performed in duplicate, with similar results.

### 2.3. Flow cytometry analysis

Single cell suspensions of thymus, spleen (after red blood cell lysis) and MLN ( $2.5 \times 10^6$  cells for extracellular stainings,  $1 \times 10^7$  cells/ml for intracellular stainings) were incubated for 15 min with anti-mouse CD16/32 (clone 93, eBioscience, Austria) in fluorescence activated cell sorting (FACS) buffer (PBS containing 0.25% BSA, 0.05% NaN<sub>3</sub>, 0.5 mM EDTA) and subsequently stained extracellularly in FACS-buffer for 30 min at 4 °C using the following FACS-antibodies: anti-CD19-APC (clone 1D3, BD Biosciences, USA), anti-CD3e-FITC (clone 145-2C11, eBioscience, Austria), anti-CD8a-PE (clone 53-6.7, eBioscience, Austria), anti-CD4-PerCP (clone RM 4-5, BD Biosciences, USA), anti-CD62L-APC (clone MEL-14, eBioscience, Austria), anti-CD44-PE (clone Pgp-1, Ly-24, eBioscience, Austria), anti-CD25-FITC (clone 3C7, BD Biosciences, USA). Next, cells were washed with FACS-buffer and fixed with 1% formaline in FACS buffer or subsequently stained intracellularly for Foxp3 (Foxp3-APC, clone FJK-16s, eBioscience, Austria) according to the manufacturer's instructions. For intracellular cytokine staining (IL-4 PE [clone 11B11], IFN-γ FITC [clone XMG1.2])  $1 \times 10^7$  cells/ml were stimulated for 4 h at 37 °C with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 10 µg/ml Brefeldin A (all Sigma–Aldrich, The Netherlands). Next, cells were washed and stained according to manufacturer's instructions. Analysis of all stainings was performed on a BD FACSCanto II using BD FACS Diva software (BD Biosciences, USA).

### 2.4. Statistical analysis

Results are presented as the mean ± standard error of the mean (SEM) of 4 mice per group. All data were logarithmically transformed to achieve normal distribution and were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. A value of  $p < 0.05$  was considered as statistically significant. All statistical analyses were performed using Graphpad Prism software.

## 3. Results

### 3.1. Effect of AhR activation by TCDD on thymus and spleen weight and cell numbers in spleen, thymus and MLN

First, thymus and spleen weight and total cell numbers in thymus, spleen and MLN were examined 8 days after TCDD treatment. TCDD treatment decreased thymus weight and thymocyte number independent of PE-sensitization. In the spleen, the number of spleen cells was not affected by TCDD treatment, however, TCDD treatment increased the spleen weight of PE-sensitized mice compared to vehicle-control non-sensitized mice (Fig. 1). In the MLN, TCDD treatment decreased cell numbers independent of PE-sensitization (Fig. 1). Together, these results show that, also during

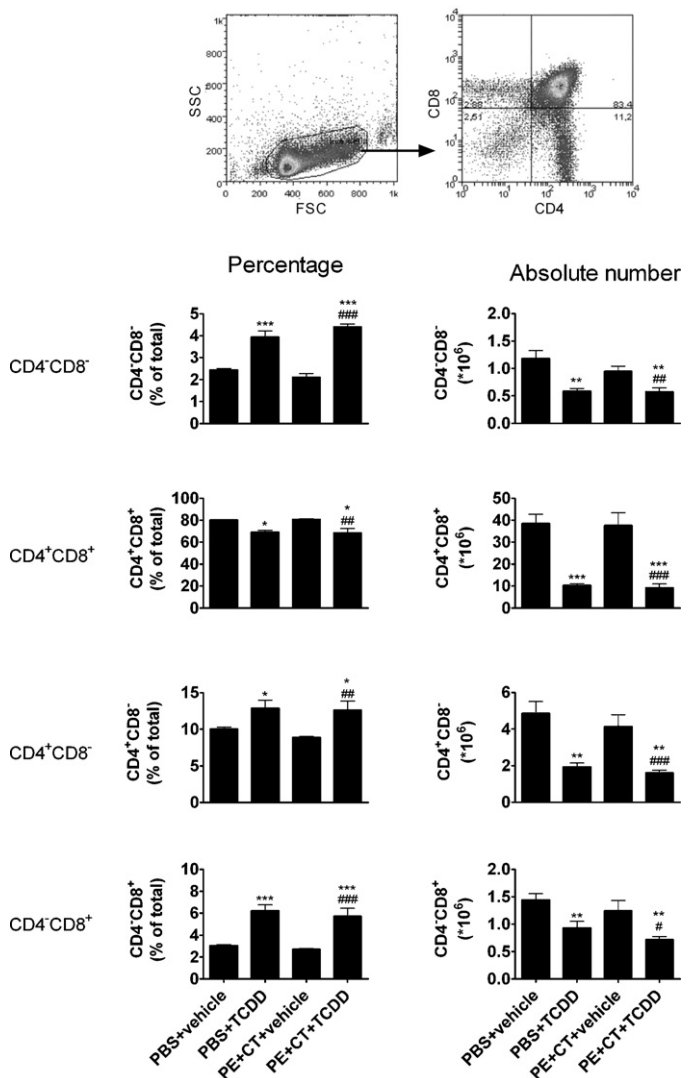


**Fig. 2.** Effect of AhR activation by TCDD on thymic T cell precursors. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE + CT) on days 3–5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>+</sup> cells in the thymus were examined by flow cytometry analysis. Representative dot-plots of thymocytes stained with antibodies to CD4 and CD8 are shown. Values are presented as mean ± SEM ( $n = 4$ ). \* $p < 0.05$  compared with PBS, \*\* $p < 0.01$  compared with PBS, \*\*\* $p < 0.001$  compared with PBS, # $p < 0.05$  compared with PE + CT, ## $p < 0.01$  compared with PE + CT, and ### $p < 0.001$  compared with PE + CT.

PE-sensitization, TCDD treatment affects the cellularity in the thymus and the MLN, but not in the spleen.

### 3.2. Effect of AhR activation by TCDD on thymic T cell precursors and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells during allergic sensitization

Next, we studied the effect of AhR activation by TCDD on the frequency and absolute number of major thymocyte T cell subsets and thymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells. In both non-sensitized and PE-sensitized mice, TCDD treatment decreased the absolute number of CD4<sup>+</sup>CD8<sup>+</sup> cells, followed by CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>+</sup> cells, respectively (Fig. 2). As a consequence of the strong decrease of the number of CD4<sup>+</sup>CD8<sup>+</sup> cells, the frequencies of the other cell populations, in particular CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>+</sup> cells were increased. TCDD treatment did not affect the absolute number of thymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells compared to their appropriate controls, but increased the percentage

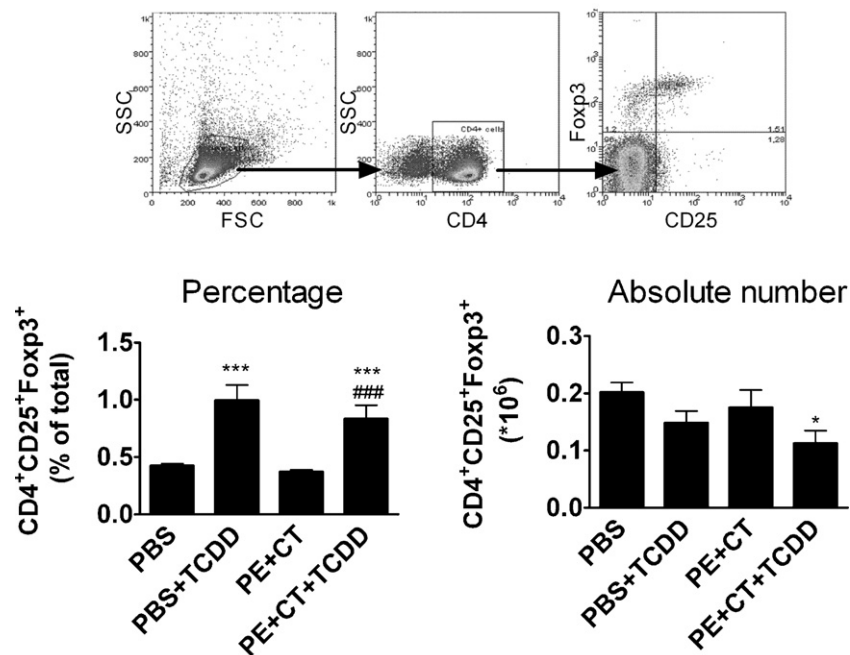


**Fig. 3.** Effect of AhR activation by TCDD on CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in the thymus during allergic sensitization. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE + CT) on days 3–5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in the thymus was examined by flow cytometry analysis. Representative dot-plots of thymus cells stained with antibodies to CD4, CD25 and Fopx3 are shown. Values are presented as mean ± SEM (n = 4). \*p < 0.05 compared with PBS, \*\*\*p < 0.001 compared with PBS, #p < 0.05 compared with PE + CT, and ###p < 0.001 compared with PE + CT.

of these cells independent of PE-sensitization (Fig. 3). Combined, these data show that TCDD treatment decreased the absolute number of major thymocyte subsets but did not affect the absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> cells. This appeared to be independent of allergic sensitization.

### 3.3. Effect of AhR activation by TCDD during allergic sensitization on B cells, CD8<sup>+</sup> and CD4<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in the spleen and MLN

To investigate the effects of AhR activation during sensitization in the periphery, we analyzed the effect of TCDD treatment on B cells, T cells and CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in the spleen and MLN after 8 days. PE-sensitization decreased the absolute number of B cells and CD8<sup>+</sup> T cells in the spleen and increased the absolute number of CD8<sup>+</sup> T cells in the MLN. The absolute number of CD4<sup>+</sup> T cells in the spleen and MLN was not affected by PE-sensitization (Fig. 4). In PE-sensitized mice, TCDD treatment increased the percentage of CD19<sup>+</sup> B cells in the spleen, but not in the MLN (Fig. 4). No effect of TCDD treatment was observed on the absolute number of CD19<sup>+</sup> B cells in the spleen and MLN (Fig. 4). Furthermore, TCDD treatment did not affect the percentage of CD8<sup>+</sup> and CD4<sup>+</sup> T cells in the spleen and MLN. However, the absolute numbers of these cells in the MLN, but not in the spleen, decreased by TCDD treatment. This appeared to be independent of PE-sensitization (Fig. 4). The percentage and absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in the spleen and MLN were not affected by PE-sensitization (Fig. 5). In the spleen, TCDD treatment increased both the percentage and absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> cells, independent of PE-sensitization (Fig. 5). In the MLN, no effect of TCDD was observed on the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> cells, whereas the absolute numbers were decreased by TCDD treatment in PE-sensitized mice (Fig. 5). Together, these data show that on day 8 TCDD treatment increased the absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in

the spleen independent of PE-sensitization and decreased the absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells compared to PBS or PE + CT controls in the MLN.

### 3.4. Effect of AhR activation by TCDD on peripheral naïve, central memory, effector memory, Th1, Th2 and cytotoxic T cells during allergic sensitization

Thereafter, the effect of TCDD treatment during sensitization on naïve (CD4<sup>+</sup>CD62L<sup>+</sup>CD44<sup>-</sup>), central memory (CD4<sup>+</sup>CD62L<sup>+</sup>CD44<sup>+</sup>) and effector memory (CD4<sup>+</sup>CD62L<sup>-</sup>CD44<sup>+</sup>) T cells in the spleen and MLN was studied (according to Lanzavecchia and Sallusto, 2005; Sallusto et al., 2004).

In the spleen, PE-sensitization decreased the absolute number of naïve CD4<sup>+</sup> T cells but did not affect central memory and effector memory CD4<sup>+</sup> T cells, whereas in the MLN PE-sensitization increased the absolute number of naïve and central memory CD4<sup>+</sup> T cells (Fig. 6). Compared to PE-sensitized mice, TCDD treatment did not affect the absolute number of naïve, central memory and effector memory CD4<sup>+</sup> T cells in the spleen (Fig. 6). In the MLN, the increase of naïve and central memory CD4<sup>+</sup> T cells induced by PE-sensitization was decreased by TCDD treatment (Fig. 6). Finally, the effect of TCDD treatment on Th1 (CD4<sup>+</sup>IFN-γ<sup>+</sup>), Th2 (CD4<sup>+</sup>IL-4<sup>+</sup>) and cytotoxic T cells (CD8<sup>+</sup>IFN-γ<sup>+</sup>) in the spleen and MLN was analyzed. PE-sensitization increased the percentage of Th2 cells, but not Th1 and cytotoxic T cells, in the spleen and increased the percentage and absolute number of Th2, Th1 and cytotoxic T cells in the MLN. TCDD treatment decreased both the percentage and absolute number of Th2, Th1 and cytotoxic T cells in the spleen (Fig. 7). In the MLN, TCDD treatment prevented the increase of PE-sensitization induced Th2, Th1 and cytotoxic T cells (Fig. 7). Combined, these results show that TCDD treatment mainly affects naïve and central memory T cells and suppresses Th1, Th2 and cytotoxic T cells in the spleen and MLN.

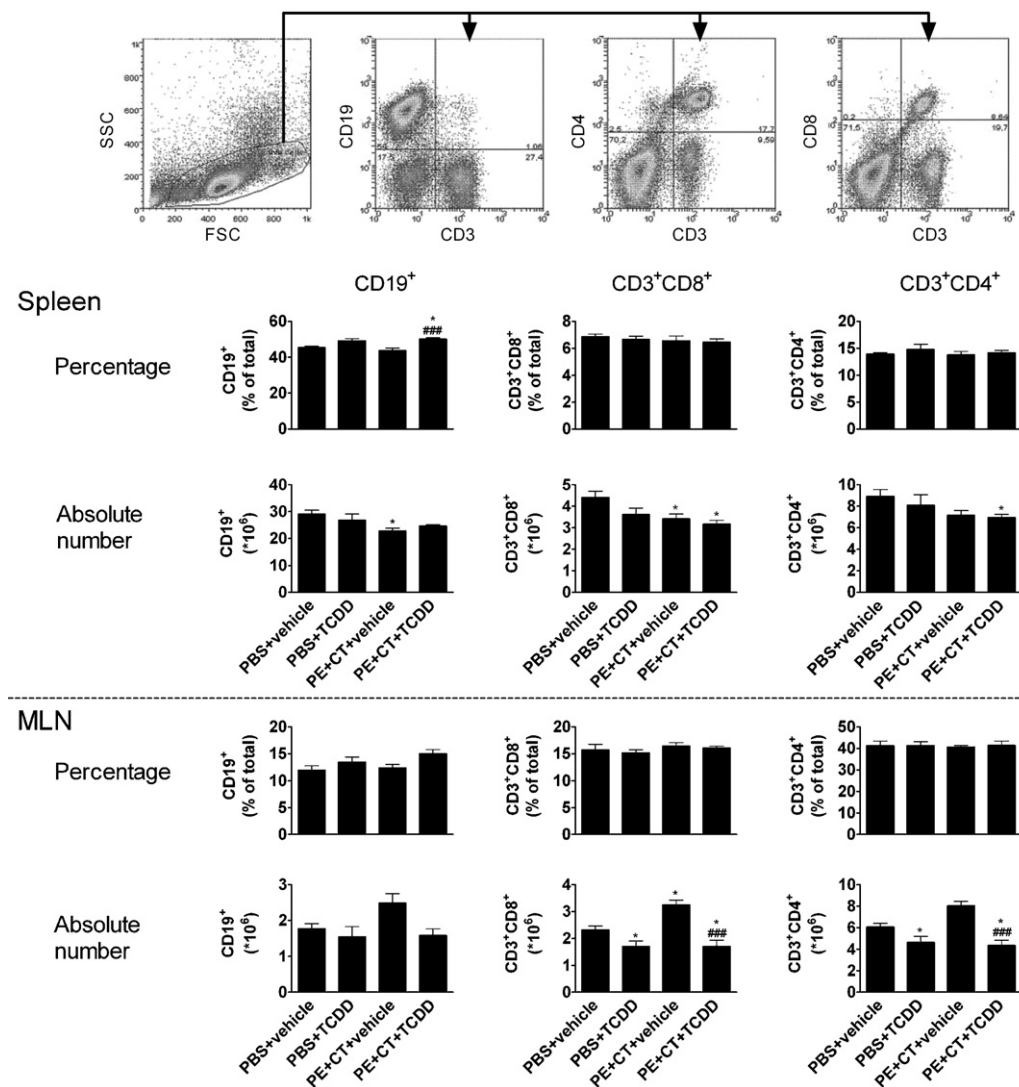


**Fig. 4.** Effect of AhR activation by TCDD during allergic sensitization on B cells, CD8<sup>+</sup> and CD4<sup>+</sup> T cells in the spleen and MLN. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE+CT) on days 3–5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of CD19<sup>+</sup> B cells and CD8<sup>+</sup> and CD4<sup>+</sup> T cells in the spleen and MLN were examined by flow cytometry analysis. Representative dot-plots of splenocytes stained with antibodies to CD3, CD19, CD4 and CD8 are shown. MLN cells were analyzed similarly. Values are presented as mean ± SEM (*n* = 4). \**p* < 0.05 compared with PBS, and \*\*\**p* < 0.001 compared with PE + CT.

#### 4. Discussion

AhR activation suppresses a variety of immune responses accompanied by an increase in the percentage of Foxp3<sup>+</sup> Treg cells (Hauben et al., 2008; Quintana et al., 2008, 2010; Zhang et al., 2008) and regulates the generation of type 1 regulatory T cells together with c-MAF (Apetoh et al., 2010). Foxp3, transforming growth factor (TGF)-β and IL-10, all important for Treg induction, contain DREs in their promoter regions (Kerkvliet, 2009; Sun et al., 2004) and AhR activation of naïve T cells has been shown to increase the percentage of Foxp3<sup>+</sup> Treg cells in vitro (Gandhi et al., 2010; Kimura et al., 2008; Quintana et al., 2010). Furthermore, tolerogenic dendritic cells producing retinoic acid or indoleamine 2,3-dioxygenase after AhR activation have been shown to increase the percentage of splenic Foxp3<sup>+</sup> Treg cells in vivo and Foxp3<sup>+</sup> gene expression in the spleen, respectively (Quintana et al., 2010; Vogel et al., 2008).

Recently, we have shown that AhR-mediated suppression of T cell dependent allergic sensitization was at least to a substantial extent due to an increase of the percentage, but not of the absolute number, of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in spleen and MLN (Schulz et al., 2011). In line with this,

others also suggested that the increased percentage of Foxp3<sup>+</sup> Treg cells observed after AhR activation results from a decrease of other cell subsets (Stockinger et al., 2011). So, we proposed that a shift of the T cell population as a whole to a more regulatory phenotype is responsible for the disease suppressing effect of AhR activation. Interestingly, TCDD inhibits the development of thymic T cell precursors (Chopra and Schrenk, 2011). In addition, the thymus contributes substantially to the peripheral CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg population (Fontenot et al., 2003; Gavin et al., 2007). However, the effect of AhR activation on the intrathymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cell population has not been investigated until now. Therefore, we wondered whether TCDD increases or rather preserves this population during allergic sensitization.

The observed increased percentages of CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> cells and the decreased percentage of CD4<sup>+</sup>CD8<sup>+</sup> cells in the thymus after TCDD treatment are in line with findings of others (Nohara et al., 2005; Temchura et al., 2005). However, the present study shows for the first time that the absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in the thymus is not changed shortly after TCDD treatment. Moreover, we show that the percentage of

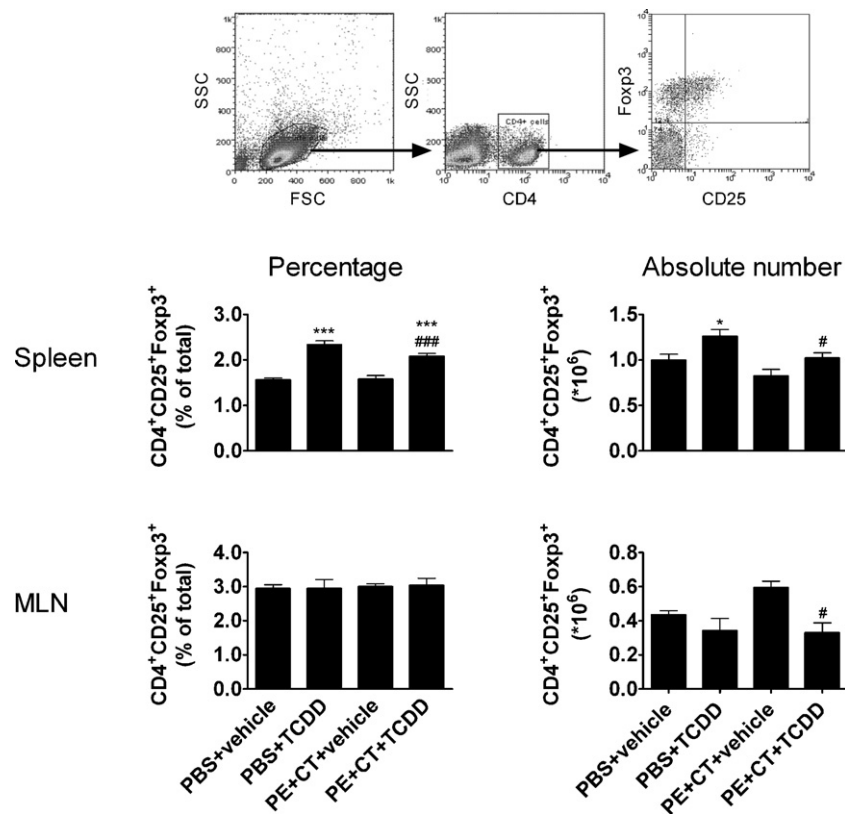


**Fig. 5.** Effect of AhR activation by TCDD on CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in the spleen and MLN during allergic sensitization. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE + CT) on days 3–5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in the spleen and MLN was examined by flow cytometry analysis. Representative dot-plots of MLN cells stained with antibodies to CD4, CD25 and Fopx3 are shown. Values are presented as mean ± SEM (n = 4). \*p < 0.05 compared with PBS, \*\*\*p < 0.001 compared with PBS, #p < 0.05 compared with PE + CT, and ###p < 0.001 compared with PE + CT.

these cells strongly increases, indicative of a shift to a more regulatory T cell phenotype in the thymus. It also indicates that AhR ligation does not cause generation of Fopx3<sup>+</sup> Treg cells in the thymus, but rather preserves these cells from AhR-mediated depletion. Importantly, the effect of AhR activation on thymic T cells appears to be independent of T cell activation during allergic sensitization.

In the present study, focused on short-term (8 days) rather than previously reported long term (34 days) effects of TCDD, AhR activation increased both the percentage and absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in the spleen, but in the MLN the percentage of these cells was not affected and the absolute number even decreased. Remarkably, Veiga-Parga et al. (2011), using a viral infection model, have shown that a single intraperitoneal TCDD treatment increases the percentage of splenic CD4<sup>+</sup>Fopx3<sup>+</sup> Treg cells, without affecting the number of splenic CD4<sup>+</sup>Fopx3<sup>+</sup> Treg cells. Possibly, factors such as route and dose of TCDD administration, kinetics of effects after TCDD exposure, mouse strain and mode of T cell activation used are crucial for the ultimate effect on CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells.

Temchura and colleagues have shown that TCDD treatment decreases the absolute number of CD4<sup>+</sup> recent thymic emigrants (RTE) in the spleen and MLN about two-fold, but they did not analyze CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells within these RTE (Temchura et al., 2005). Our present data suggest that during allergic sensitization and AhR activation by TCDD, the increase in the absolute number of splenic CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells may indeed result from the enhanced presence of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells within the diminished population of RTE. This would imply that increased occurrence of the naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup>

Treg population in the thymus contributes to expansion of this population in the periphery after TCDD exposure. Of note, this does not exclude formation of new adaptive CD4<sup>+</sup>CD25<sup>+</sup> Treg cells by TCDD (Funatake et al., 2005), for instance by increased production of TGF-β (Kimura et al., 2008; Quintana et al., 2008). Intriguingly, not all CD4<sup>+</sup>CD25<sup>+</sup> Treg cells identified by Funatake and colleagues were Fopx3<sup>+</sup>, suggesting that TCDD stimulates other regulatory T cells as well. However, the absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> Treg cells in the MLN is apparently less influenced by the imbalance in RTE and more by the general decrease of cell numbers in the MLN after TCDD treatment. This is in line with the fact that the number of thymic Fopx3<sup>+</sup> Treg cell emigrants in the spleen is approximately three times higher in the spleen than in the MLN (Lee et al., 2007).

Previously, others have described the suppressive effects of AhR activation on CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the periphery (Dearstynne and Kerkvliet, 2002; Funatake et al., 2004; Mitchell and Lawrence, 2003; Shepherd et al., 2000). Our present data add to this by showing that the suppressive effect of TCDD is organ-specifically influenced by sensitization, i.e. TCDD induced decreases of CD4<sup>+</sup> and CD8<sup>+</sup> T cell numbers (central memory and/or effector memory T cells) in the spleen appeared independent of allergic sensitization whereas in the MLN decreases of cell numbers were dependent of allergic sensitization. This dependence of MLN T cells on sensitization is possibly linked to the unique gut-associated location of MLN and their role in the local induction of allergic sensitization (Pabst and Mowat, 2012). The suppressive effect of TCDD on T cells in the MLN suggests that AhR activation interferes specifically with the induction of allergic sensitization by increasing the relative proportion of Treg cells, or by direct immunosuppressive effects on effector

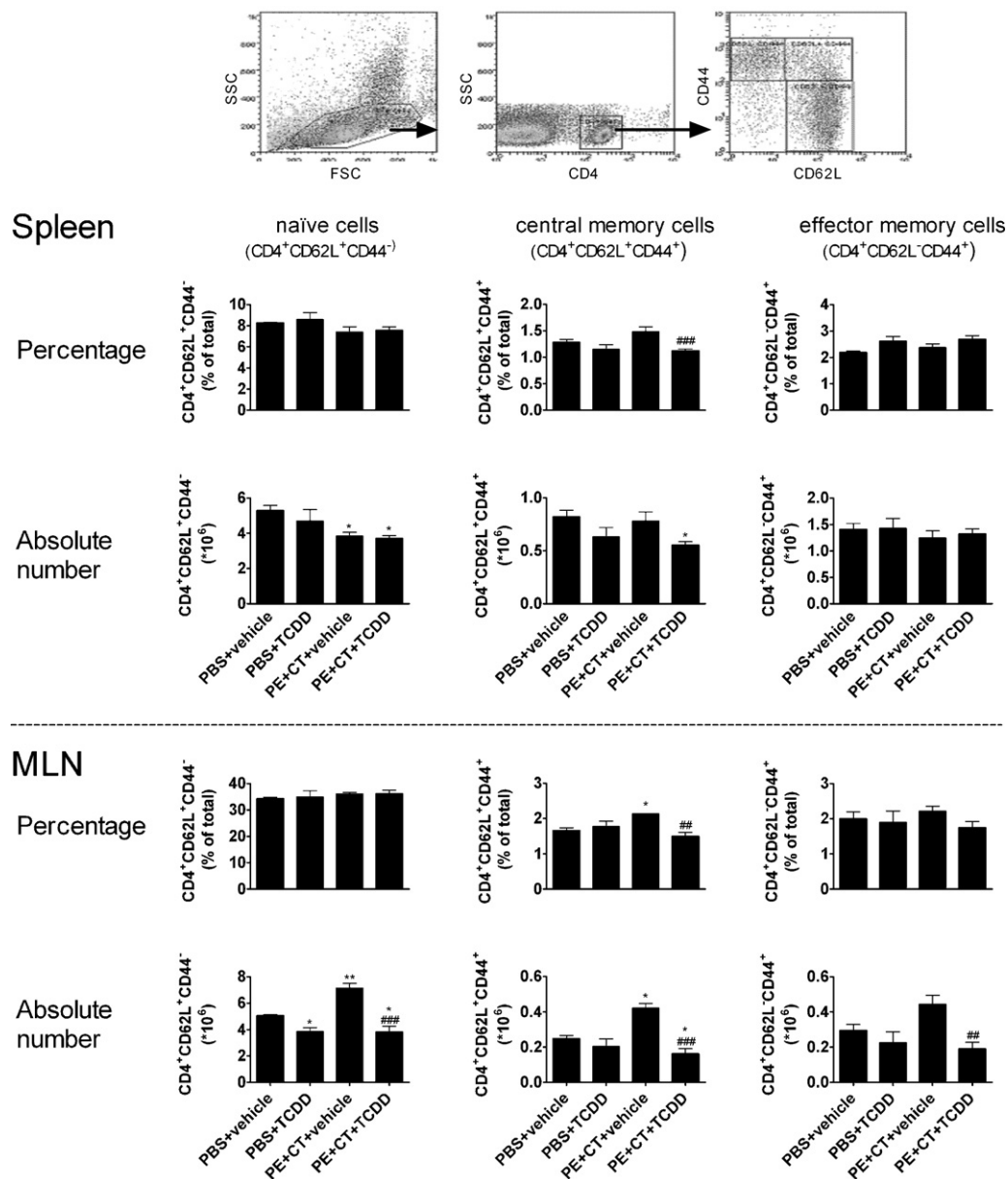


**Fig. 6.** Effect of AhR activation by TCDD on peripheral naïve, central memory, effector memory, Th1, Th2 and cytotoxic T cells during allergic sensitization. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE+CT) on days 3–5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of naïve (CD62L<sup>+</sup>CD44<sup>-</sup>), central memory (CD62L<sup>+</sup>CD44<sup>+</sup>) and effector memory (CD62L<sup>-</sup>CD44<sup>+</sup>) CD4<sup>+</sup> T cells in the spleen and MLN were examined by flow cytometry analysis. Representative dot-plots of splenocytes stained with antibodies to CD4, CD44, CD62L are shown. MLN cells were analyzed similarly. Values are presented as mean ± SEM (n=4). \*p < 0.05 compared with PBS, \*\*p < 0.01 compared with PBS, ##p < 0.01 compared with PE+CT, and ###p < 0.001 compared with PE+CT.

T cells, as suggested before (Schulz et al., 2011). The spleen, more distant from the gut, may be less influenced by orally administered sensitizing allergens, but more by direct immunosuppressive effects of TCDD.

It is not clear why thymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells are not affected by TCDD-induced changes of the T cell precursor differentiation program. Many costimulatory signals have been shown to be important in the development and lineage commitment of thymic Foxp3<sup>+</sup> Treg cells, including CD28 ligation by CD80/CD86, cytokine signaling (IL-2 and other common γ-chain-dependent cytokines, TGF-β), IL-2R, thymic stromal-derived lymphopoietin receptor, CD154, glucocorticoid-induced tumor necrosis factor receptor and signal transducer and activator of transcription 5 (Bettini and Vignali, 2010; Poojary et al., 2010; Wirnsberger et al., 2011; Workman et al., 2009). However, until now the decisive

signaling pathway leading to Foxp3<sup>+</sup> Treg cell differentiation has not been elucidated. Therefore, it is difficult to pinpoint a mechanism explaining the lack of susceptibility of thymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells to TCDD. However, IL-2 signaling might play a key role, because TCDD has been shown to increase thymic IL-2 gene expression signaling in vitro and in vivo (Jeon and Esser, 2000) and to increase IL-2R in thymocytes of mice perinatally exposed to TCDD (Camacho et al., 2004). Interestingly, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells cannot secrete IL-2, but constitutively express the IL-2R chain CD25 (Bettini and Vignali, 2010; Poojary et al., 2010; Wirnsberger et al., 2011). Since IL-2 has been shown to be very important in the development and maintenance of these cells, this might explain why TCDD treatment preserves CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells from deletion. Interestingly, the immunosuppressant rapamycin, which exerts its

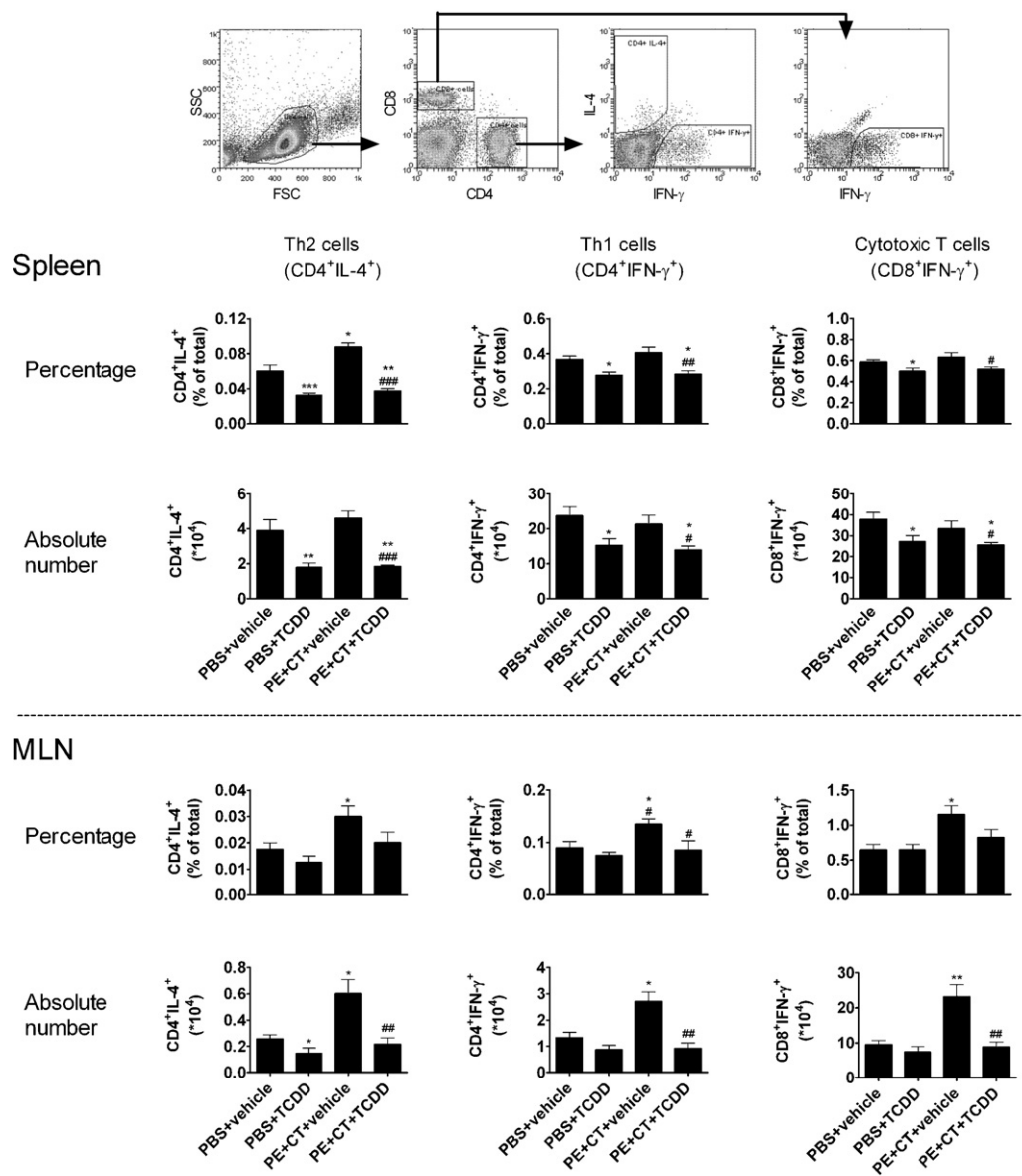


**Fig. 7.** Effect of AhR activation by TCDD on peripheral T helper cells during allergic sensitization. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE + CT) on days 3–5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of Th2 (CD4<sup>+</sup>IL-4<sup>+</sup>), Th1 (CD4<sup>+</sup>IFN-γ<sup>+</sup>) and cytotoxic T cells (CD8<sup>+</sup>IFN-γ<sup>+</sup>) cells were examined by flow cytometry analysis. Representative dot-plots of splenocytes stained with antibodies to CD4, CD8, IL-4 and IFN-γ are shown. MLN cells were analyzed similarly. Values are presented as mean ± SEM (n = 4). \*p < 0.05 compared with PBS, \*\*p < 0.01 compared with PBS, \*\*\*p < 0.001 compared with PBS, #p < 0.05 compared with PE + CT, ##p < 0.01 compared with PE + CT, ###p < 0.001 compared with PE + CT.

immunosuppressive effects by blocking cell growth and proliferation in response to growth factors like IL-2 (Abraham and Wiederrecht, 1996; Bierer et al., 1990; Chung et al., 1992), has been shown to exert similar effects as TCDD on CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, major thymic T cell subsets and effector T cells (Battaglia et al., 2006; Coenen et al., 2007; Tian et al., 2004; Valmori et al., 2006).

In conclusion, TCDD treatment preserves intrathymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, increases the absolute number of splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and decreases peripheral T helper and cytotoxic T cells. We postulate that the intrathymic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg population, which seems preserved from TCDD-mediated depletion, contributes to the increase of the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in the spleen, but not in the MLN. Future research should reveal which effect of TCDD, preservation, expansion or induction of (thymic) Treg cells contributes most to the peripheral presence of Treg cells. Of course, this may depend on the particular situation, e.g. the disease-dependent

circumstances, and hence influence the therapeutic outcome of the suppressive effect of AhR activation.

### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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