

# Activation of the Aryl Hydrocarbon Receptor Suppresses Sensitization in a Mouse Peanut Allergy Model

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Food allergy is an increasing health problem in Western countries. Previously, it has been shown that the intensity of food allergic reactions can be regulated by regulatory T (T<sub>reg</sub>) cells. In addition, it has been shown that activation of the aryl hydrocarbon receptor (AhR) regulates T-cell responses by induction of T<sub>reg</sub> cells. Therefore, we hypothesized that activation of the AhR pathway can suppress development of food allergic responses through the induction of T<sub>reg</sub> cells. This was investigated by using a mouse model for peanut allergy. C3H/HeOuJ mice (AhR<sup>b-2</sup>) were sensitized to peanut by administering peanut extract (PE) by gavage in the presence of cholera toxin and were treated with the prototypical AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (0.6, 1.7, 5, and 15 µg/kg body weight) on days 3 and 11 orally. The functional role of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells was investigated by depleting these cells with anti-CD25 mAb during sensitization to PE. TCDD treatment dose dependently suppressed sensitization to peanut (PE-specific IgE, IgG1, and IgG2a and PE-induced IL-5, IL-10, and IL-13, respectively). The percentage, but not the number, of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells dose dependently increased by AhR activation in both spleen and mesenteric lymph nodes. Depletion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells markedly reversed the suppressive effect of TCDD on PE-specific antibody levels and PE-induced IL-5, IL-10, and IL-13 cytokine production. Present data demonstrate for the first time that activation of the AhR by TCDD suppressed the development of Th2-mediated food allergic responses. A functional shift within the CD4<sup>+</sup> cell population toward CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells appeared to underlie this effect. This suggests that the AhR pathway might provide potential therapeutic targets to treat food allergic diseases.

**Key Words:** aryl hydrocarbon receptor; peanut allergy; regulatory T cells; TCDD.

In Western countries, food allergy affects about 5% of young children and 3–4% of adults (Sicherer and Sampson, 2010). Most of these people only have mild allergic reactions after exposure to a food allergen, but in severe cases, anaphylaxis can be induced (Lee and Burks, 2009; Shaker and

Woodmansee, 2009; Sicherer and Sampson, 2010). The major allergens causing food allergy are peanut, tree nuts, shellfish, fish, wheat, milk, egg, and soy (Sicherer and Sampson, 2010). These allergens can enter the body via the gastrointestinal mucosal immune system. At this location, immune cells must distinguish harmless food antigens and commensal bacteria from pathogens. Disruption of this delicate balance can result in the breakdown of oral tolerance, resulting in the development of food allergy (Shaker and Woodmansee, 2009; Sicherer and Sampson, 2009; Van Wijk *et al.*, 2007).

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor regulating the expression of a wide range of genes via the transcription of dioxin-responsive elements (DREs) (Denison and Nagy, 2003). Cells participating in innate and adaptive immune responses express the AhR and many genes for cytokines, transcription factors, and mediators contain DRE sequences in their promoter regions (Kerkvliet, 2009; Sun *et al.*, 2004). Recently, it has been shown that activation of the AhR by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), VAG539, or 2-(1'-H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) results in the induction of regulatory T (T<sub>reg</sub>) cells in experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveoretinitis (EAU), and graft versus host disease (GvHD) (Funatake *et al.*, 2005; Hauben *et al.*, 2008; Marshall *et al.*, 2008; Quintana *et al.*, 2008, 2010; Zhang *et al.*, 2010). In contrast, activation of the AhR by the tryptophan photoproduct 6-formylindolo[3,2-*b*]carbazole has been shown to induce Th17 cells (Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). So, the AhR can regulate both T<sub>reg</sub> and Th17 differentiation, dependent on the ligand.

In food allergy, T<sub>reg</sub> cells have been shown to regulate allergic sensitization and the intensity of the food allergic response. Individuals with mutations in the *FOXP3* gene suffer from the immune dysregulation polyendocrinopathy enteropathy X-linked syndrome, which can be accompanied by severe food allergy (Torgerson and Ochs, 2007). Furthermore,

depletion of  $CD4^+CD25^+$   $T_{reg}$  cells during sensitization to peanut increases allergen-specific antibody levels and mast cell degranulation after oral challenge with peanut in mice (Van Wijk *et al.*, 2007). In addition, it has been shown that whey-specific  $CD25^+$   $T_{reg}$  cells induced by dietary intervention with prebiotics are involved in the suppression of cow milk allergy in mice (Schouten *et al.*, 2010). Previously, it has been shown that activation of the AhR impairs Th2-type immune responses by suppressing 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 addition, one epidemiological study found a negative correlation between serum IgE and dioxin-like compounds in humans (Van Den Heuvel *et al.*, 2002). However, no role for  $T_{reg}$  cells was described or suggested in any of these studies. Because  $T_{reg}$  cells play an important role in food allergic diseases and activation of the AhR is associated with the induction of  $T_{reg}$  cells, we hypothesized that activation of the AhR can suppress development of food allergic responses through the induction of  $T_{reg}$  cells. This was investigated by using a mouse model for peanut allergy. The prototypical AhR ligand TCDD was used to study the role of the AhR because it is the most potent AhR ligand known (thereby reducing high-dose off-target effects), it is hardly metabolized (the effects observed are not confounded by ligand metabolism) and relatively slowly excreted (half life time in mice 10–12 days) (Denison and Nagy, 2003; Kerkvliet, 2009; Van Den Berg *et al.*, 1994).

First, the effect of different doses TCDD on peanut allergic sensitization was investigated. Next, the role of  $CD4^+CD25^+Foxp3^+$   $T_{reg}$  cells in TCDD-mediated suppression of the peanut allergic response was studied by depleting these cells with anti-CD25 mAb. Collectively, our data demonstrate that activation of the AhR by TCDD suppresses the development of food allergic responses and that a functional shift within the  $CD4^+$  cell population toward  $CD4^+CD25^+Foxp3^+$   $T_{reg}$  cells may underlie this suppression.

## MATERIALS AND METHODS

**Mice and reagents.** C3H/HeOJ mice (4- to 5-week-old;  $AhR^{b-2}$ ), purchased from Charles River (France), were maintained under controlled conditions (relative humidity of 50–55%, 12 h light/dark cycle, and temperature of  $23 \pm 2^\circ\text{C}$ ) in filter-topped macrolon cages with wood chip bedding. Food pellets and drinking water were available *ad libitum*. Prior to the start of the experiments, mice were acclimatized. All experiments were approved by the animal experiments committee of the Faculty of Veterinary Medicine, Utrecht University.

2,3,7,8-TCDD (Cambridge Isotope Lab) was dissolved in anisole (Sigma-Aldrich, The Netherlands) at  $20.5 \mu\text{g/ml}$  and diluted in corn oil (Sigma-Aldrich) to the final exposure concentration ( $0.07\%$  vol/vol anisole). Anisole diluted in corn oil was used as vehicle control ( $0.07\%$  vol/vol).

Peanut extract (PE) (30 mg/ml) was prepared from peanuts from the Golden Peanut Plant (provided by Intersnack Nederland BV, The Netherlands) as described previously (Van Wijk *et al.*, 2005). Peanut extracts were prepared

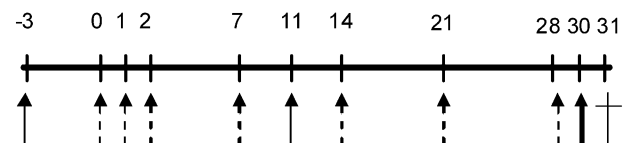
according to standard procedures and checked for protein content by BCA analysis (Pierce, Rockford, IL). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc. (Campbell, CA).

**Experimental design.** In setup 1 (Fig. 1, setup 1), C3H/HeOJ mice ( $n = 6$ –8 per group) were sensitized to PE by oral exposure to PE (6 mg PE,  $200 \mu\text{l}$  per mouse) with CT ( $15 \mu\text{g}$  per mouse) on three consecutive days (days 0, 1, and 2) followed by weekly dosing (days 7, 14, 21, and 28). On days 3 and 11, mice were exposed orally to different doses of TCDD ( $0.6$ ,  $1.7$ ,  $5$ , or  $15 \mu\text{g/kg}$  body weight [BW]).

The role of  $CD4^+CD25^+Foxp3^+$   $T_{reg}$  cells was investigated by treating mice with anti-CD25 mAb to deplete  $T_{reg}$  cells during sensitization with PE (Fig. 1, setup 2) (Setiady *et al.*, 2010; Tenorio *et al.*, 2010; Van Wijk *et al.*, 2007). Anti-CD25 mAb treatment depletes mostly  $CD4^+CD25^+Foxp3^+$  cells. C3H/HeOJ mice ( $n = 6$ –8 per group) were treated ip with purified rat anti-CD25 (IL-2R $\alpha$ ) mAb (clone PC61, provided by Bioceros B.V.) ( $200 \mu\text{g}$  per mouse) on days 4, 0, 7, 11, 14, and 18. In setup 2, TCDD ( $15 \mu\text{g/kg}$  BW) was

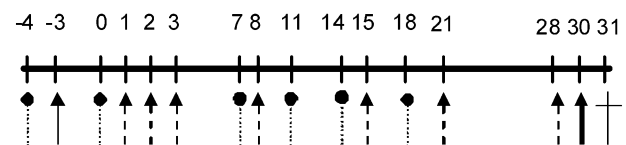
### Setup 1

Day



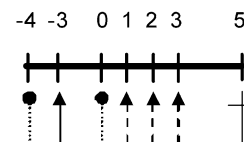
### Setup 2

Day



### Setup 3

Day



- .....● Anti-CD25 (ip)
- TCDD or vehicle (intra-gastric)
- ▶ PBS+CT or PE+CT (intra-gastric)
- Challenge with PE (intra-gastric)
- ⊥ Sacrifice

**FIG. 1.** Treatment protocol of setups 1, 2, and 3 as described in “Materials and Methods” section.

administered orally only on day 3 to prevent interference in the T<sub>reg</sub> cell depletion by anti-CD25 mAb.

In both setups, mice were challenged intragastrically with PE (12 mg per mouse) on day 30, and blood samples were taken after 30 min. On day 31, mice were sacrificed by cervical dislocation and blood, thymus (only in setup 2), spleen, mesenteric lymph nodes (MLN), and liver were isolated.

In setup 3, the early effects of TCDD and anti-CD25 mAb treatment on CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells during sensitization to peanut were investigated. C3H/HeOJ mice ( $n = 4$  per group) were treated ip with purified rat anti-CD25 (IL-2R $\alpha$ ) mAb (clone PC61, 200  $\mu$ g per mouse, provided by Bioceros B.V.) or control antibody (purified rat anti-GL113 mAb, 200  $\mu$ g per mouse, provided by Bioceros B.V.) on days 4 and 0, and TCDD (15  $\mu$ g/kg BW) was administered orally on day 3. On day 5, mice were sacrificed by cervical dislocation and spleen and MLNs were isolated.

**Preparation of liver microsomes.** Excised livers were homogenized in Tris/HCL (50mM, 1.15% KCL, pH = 7.4). The microsomal fraction was obtained from the homogenate by successive centrifugation for 25 min at 9000  $\times$  g and 85 min at 100,000  $\times$  g with a Beckman Coulter Optima L-90 K centrifuge. The microsomal fraction was resuspended in a sucrose solution (0.25M). Protein concentration of the microsomes was determined by the method of Lowry using bovine serum albumin (BSA) as protein standard (Lowry *et al.*, 1951).

**Ethoxyresorufin-O-deethylase activity.** Ethoxyresorufin-O-deethylase (EROD) activities in liver microsomes were determined in 10  $\mu$ l sample containing 10–40  $\mu$ g protein with 90  $\mu$ l 50mM Tris buffer (pH 7.4) containing 5mM MgCl<sub>2</sub>, 20 $\mu$ M dicumarol, 2 $\mu$ M 7-ethoxyresorufin, and 1.5mM nicotinamide adenine dinucleotide phosphate (reduced form). A standard curve using resorufin was generated to quantify the EROD activity. Fluorescence was measured at 37°C at an excitation wavelength of 530 nm and an emission wavelength of 590 nm, every 80 s for 16 min in a Fluostar plate reader (BMG Labtechnologies GmbH, Germany). EROD activity was calculated as picomoles resorufin per minute per milligram protein.

**Measurement of PE-specific IgE, IgG1 and IgG2a antibody levels in serum and mMCP-1 in plasma.** PE-specific IgE, IgG1, and IgG2a antibody levels in serum were detected as previously described and are depicted in arbitrary units (AU) (Van Wijk *et al.*, 2005). mMCP-1 in plasma from C3H/HeOJ mice obtained 30 min after challenge with PE was determined by using an ELISA kit (Moredun Scientific Ltd., Great Britain). The ELISA was performed according to the manufacturer's instructions.

**Splenic cell culture and analysis of cytokine production.** After red blood cell lysis, single-cell spleen suspensions ( $2.5 \times 10^6$  cells/ml) were cultured in 200  $\mu$ l complete RPMI 1640 (10% Fetal Calf Serum) in the presence of medium or PE (100  $\mu$ g/ml) for 96 h at 37°C and 5% CO<sub>2</sub>. Levels of IL-5, IL-10, IL-13, IL-17a, and IFN- $\gamma$  in collected supernatant were determined by commercially available sandwich ELISA (eBioscience, Austria) according to the manufacturer's instructions. Levels of IL-4 were below detection limit.

**Fluorescence-activated cell sorting analysis.** Single-cell suspensions of spleen (after red blood cell lysis) and MLN ( $1 \times 10^7$  cells/ml) were stained with anti-CD4-FITC (clone L3T4, eBioscience) and anti-CD25-PE (clone PC61, eBioscience) (setup 1) or anti-CD4-PerCP (clone RM 4-5, BD Biosciences) and anti-CD25-FITC (clone 3C7, BD Biosciences) (setup 3) in Fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.25% BSA, 0.05% NaN<sub>3</sub>, 0.5mM EDTA) for 30 min at 4°C. Subsequently, cells were washed with FACS buffer and stained intracellularly for Foxp3 (Foxp3-APC, clone FJK-16s, eBioscience) according to the manufacturer's instructions. Analysis was performed on a FACScan with standard FACSflow using CellQuest software (BD Biosciences).

**Statistical analysis.** Results are presented as the mean  $\pm$  standard error (SE) of four to eight mice per group. All data were logarithmically transformed (except percentages of FACS data) to achieve normal distribution and were analyzed by one-way ANOVA followed by a Bonferroni *post hoc* test. A value

of  $p < 0.05$  was considered as statistically significant. All statistical analyses were performed using Graphpad Prism software.

## RESULTS

### Effect of TCDD Treatment on Body, Spleen, and Thymus Weight and EROD Activity in Liver Microsomes

To evaluate possible overt toxic effects of TCDD, body and spleen weights were examined in experimental setup 1 on day 31 (Fig. 1). Compared with PE-sensitized vehicle control mice, TCDD treatment slightly decreased BW at doses of 5 and 15  $\mu$ g/kg BW ( $21.31 \pm 0.38$  vs.  $19.26 \pm 0.37$  [ $p < 0.05$ ] and  $19.49 \pm 0.38$  [ $p < 0.05$ ], respectively). Relative weights of spleen or thymus (only measured in setup 2) were not affected by TCDD on day 31 (data not shown). The effect of TCDD on transcriptional activation of the AhR *in vivo* was investigated by measuring hepatic EROD activity (day 31, Fig. 1, setup 1). No EROD activity was observed in liver microsomes from PE-sensitized mice. EROD activity increased dose dependently in TCDD-treated mice reaching maximal activity at 5 and 15  $\mu$ g/kg BW (Fig. 2).

### Activation of the AhR Affects Peanut-Specific Antibody Responses, Mast Cell Degranulation, and Cytokine Responses

We first investigated whether activation of the AhR inhibited the development of peanut allergy by administering increasing doses of TCDD (0.6, 1.7, 5, or 15  $\mu$ g/kg BW) during sensitization (Fig. 1, setup 1). PE-sensitized mice had increased levels of PE-specific IgE, IgG1, and IgG2a (Figs. 3a–c), mast cell degranulation (measured by mMCP-1) (Fig. 3d), and PE-induced Th2-type T-cell responses (*ex vivo* release of IL-5, IL-10, and IL-13) (Figs. 3e–f) compared with nonsensitized vehicle control mice. Treatment with TCDD during PE-sensitization dose dependently suppressed PE-specific IgE (5 and 15  $\mu$ g/kg BW), IgG1 (15  $\mu$ g/kg BW), and IgG2a (15  $\mu$ g/kg BW) (Figs. 3a–c). Mast cell degranulation (mMCP-1) was significantly suppressed

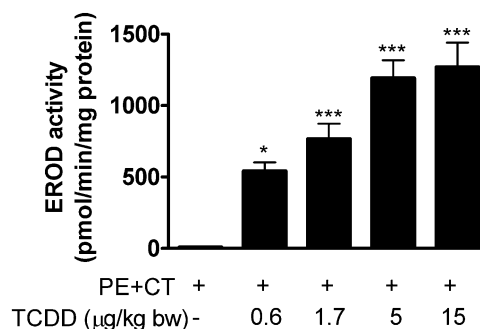


**FIG. 2.** Activation of the AhR by TCDD dose dependently increased EROD activity in hepatic microsomes. Mice were sensitized for peanut (PE + CT) and treated with 0.6, 1.7, 5, or 15  $\mu$ g/kg BW TCDD on days 3 and 11 (setup 1). On day 31, livers were isolated and EROD activity was measured in liver microsomes. Values are presented as mean  $\pm$  SE ( $n = 4$ –5). \* $p < 0.05$  compared with PE + CT, \*\*\* $p < 0.001$  compared with PE + CT.

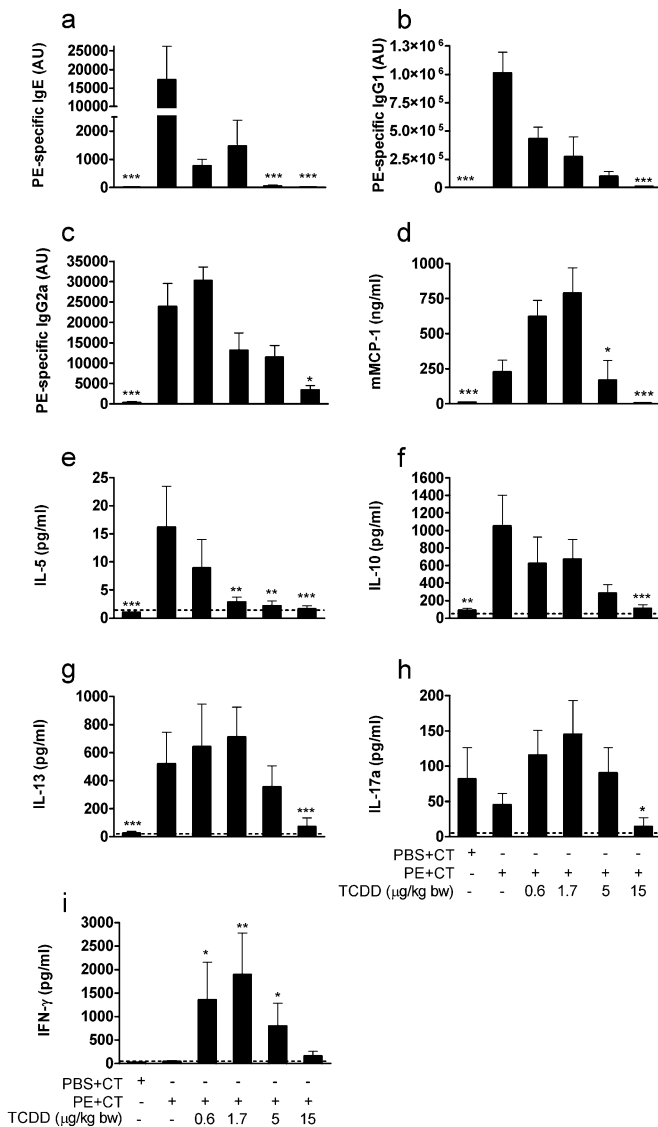


**FIG. 3.** Activation of the AhR suppressed peanut-specific antibody responses, mast cell degranulation, and Th2-type cytokine responses and increased IFN- $\gamma$  production. Mice were sensitized for peanut (PE + CT) and treated with 0.6, 1.7, 5, or 15  $\mu$ g/kg BW TCDD on days 3 and 11 (setup 1). On day 30, mice were challenged with PE. Peanut-specific IgE (a), IgG1 (b), and IgG2a (c) were examined in serum from day 31 by ELISA. mMCP-1 (d) was determined by ELISA in plasma taken 30 min after challenge. Single-cell suspensions of spleen cells (day 31) were cultured for 96 h in the presence of PE. The supernatant was analyzed for IL-5 (e), IL-10 (f), IL-13 (g), IL-17a (h), and IFN- $\gamma$  (i) by ELISA. No cytokine production could be detected when splenocytes were cultured in the absence of PE. Values are presented as mean  $\pm$  SE ( $n = 6-8$ ). \* $p < 0.05$  compared with PE + CT, \*\* $p < 0.01$  compared with PE + CT, \*\*\* $p < 0.001$  compared with PE + CT.

at 5 and 15  $\mu$ g/kg BW TCDD (Fig. 3d). Furthermore, isolated spleen cells that were exposed to TCDD *in vivo* significantly suppressed PE-induced IL-5 (at 1.7, 5, and 15  $\mu$ g/kg BW), IL-10, IL-13, and IL-17a (all at 15  $\mu$ g/kg BW) (Figs. 3e-h). Particularly, at lower doses of TCDD (0.6, 1.7, and 5  $\mu$ g/kg BW), a significant increase of PE-induced IFN- $\gamma$  was observed compared with PE-

sensitized mice (Fig. 3i). Together, these data show that AhR activation by TCDD suppresses PE-specific antibody levels, mast cell degranulation, and PE-induced cytokine responses differently, dependent on the dose of TCDD.

#### *Activation of the AhR by TCDD Increases the Percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> Cells in MLN and Spleen*

Next, we investigated whether AhR activation affected CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells on day 31 (Fig. 1, setup 1). Treatment with TCDD during sensitization to PE increased the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells in MLN starting at a dose of 1.7  $\mu$ g/kg BW and in spleen starting at a dose of 5  $\mu$ g/kg BW (Figs. 4b and g). Within the CD4<sup>+</sup> population, the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells was increased in both organs at all doses of TCDD (Figs. 4a and f). In the spleen, no effect on the absolute number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells was observed (Fig. 4k). TCDD treatment decreased the percentage and number of CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> (Figs. 4h and l) and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> (Figs. 4i and m) in the spleen at 5 and 15  $\mu$ g/kg BW. In the MLN, the percentage of CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> cells was decreased at 15  $\mu$ g/kg BW (Fig. 4c), whereas the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> cells was increased at 5 and 15  $\mu$ g/kg BW TCDD (Fig. 4d). The percentage of CD4<sup>+</sup> cells in the MLN and the absolute number of these cells in the spleen decreased dose dependently by TCDD treatment (Figs. 4e and n). Together, these data show that TCDD treatment during sensitization to peanut results in a shift toward CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells within the CD4<sup>+</sup> T-cell population.

#### *Depletion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> Cells Abrogates the Suppressive Effect of AhR Activation by TCDD on the Peanut Allergic Response*

Then, we determined whether the increased percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells observed after AhR activation were responsible for suppression of the peanut allergic response. For this purpose, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells were depleted using T<sub>reg</sub> cell-depleting anti-CD25 mAb before and during sensitization to peanut and TCDD treatment (15  $\mu$ g/kg BW).

The early effects of TCDD and/or anti-CD25 treatment during sensitization to PE were investigated on day 5 (Fig. 1, setup 3). Anti-GL113 mAb was used as a control antibody. TCDD treatment of PE-sensitized mice increased the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells within the CD4<sup>+</sup> population in the spleen from 9.05 to 11.37% ( $p < 0.01$ ) (Figs. 5a and c), whereas no effect of TCDD was observed on the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> (Fig. 6e). Treatment of PE-sensitized mice with anti-CD25 mAb decreased the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells within the CD4<sup>+</sup> population from 9.05 to 2.94% ( $p < 0.001$ ), whereas CD25<sup>+</sup>Foxp3<sup>-</sup> and CD25<sup>-</sup>Foxp3<sup>+</sup> cells were not significantly affected (Fig. 5b). This shows that anti-CD25 mAb treatment specifically depleted CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells. In PE-sensitized mice that

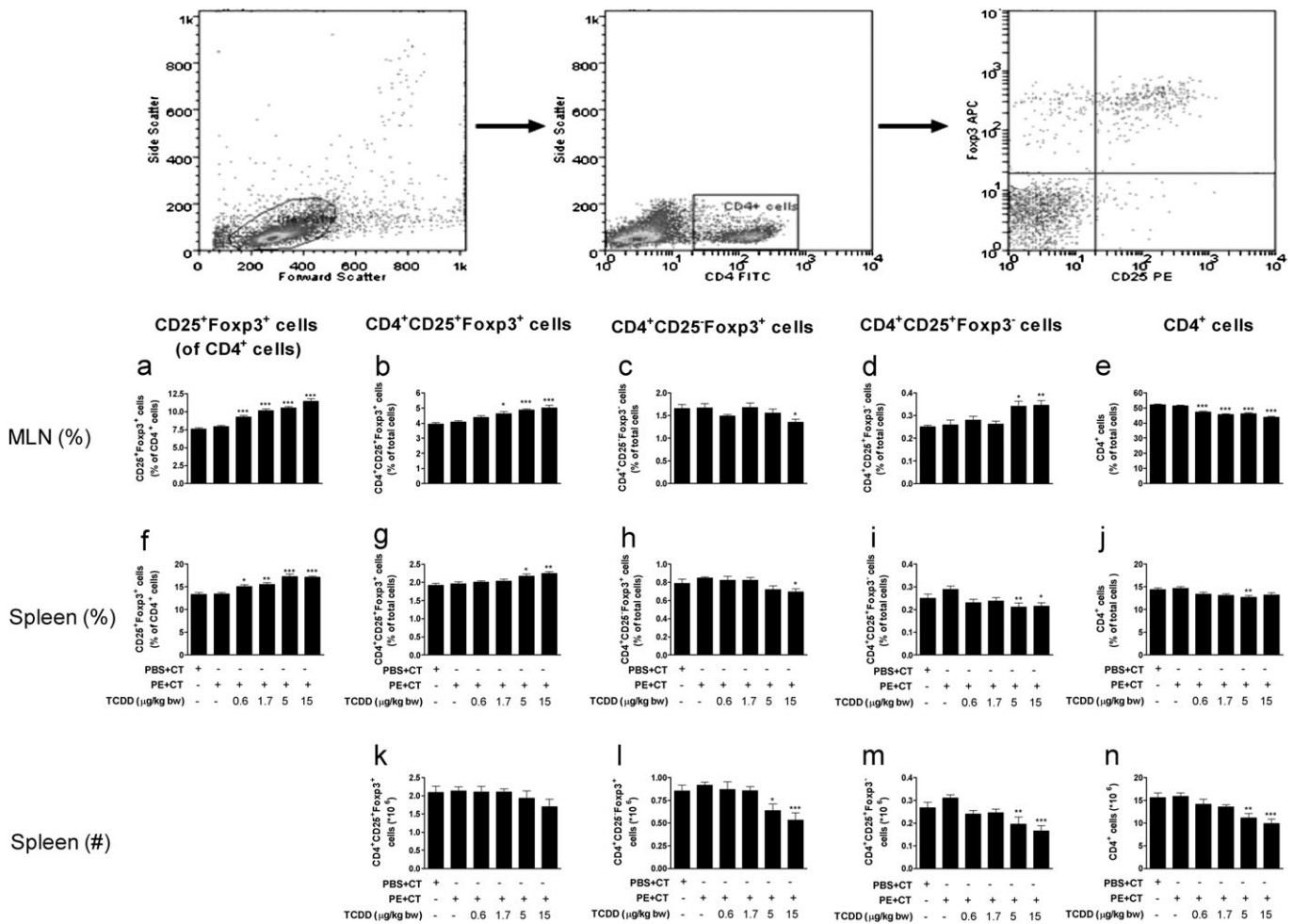


**FIG. 4.** Activation of the AhR by TCDD increased the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells in spleen and MLN. Mice were sensitized for peanut (PE + CT) and treated with 0.6, 1.7, 5, or 15 μg/kg BW TCDD on days 3 and 11 (setup 1). On day 31, mice were sacrificed and the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> cells of CD4<sup>+</sup> cells (a and f), CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells of total isolated cells (b and g), CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells of total isolated cells (c and h), CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells of total isolated cells (d and i), and CD4<sup>+</sup> cells of total isolated cells (e and j) in MLN and spleen were examined by FACS analysis. The numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells (k), CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells (l), CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells (m), and CD4<sup>+</sup> cells (n) of total isolated spleen cells were also determined. For detection of CD25 expression, clone PC61 was used. Values are presented as mean ± SE (n = 6–8). \*p < 0.05 compared with PE + CT, \*\*p < 0.01 compared with PE + CT, \*\*\*p < 0.001 compared with PE + CT.

were treated with TCDD and anti-CD25 mAb (Fig. 5d), the percentage CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells within the CD4<sup>+</sup> population was 4.10 versus 2.94% in PE-sensitized mice that were treated with anti-CD25 mAb only (Fig. 5b). Within the CD4<sup>+</sup> population in the MLN, anti-CD25 mAb treatment decreased the percentage CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells (from 5.55 to 0.97%, *p* < 0.001), whereas no effect of TCDD treatment on the percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells was observed (5.55 vs. 5.9%) (data not shown). The percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells within the CD4<sup>+</sup> population MLN from mice treated with TCDD and anti-CD25 mAb was 0.81% (data not shown). Furthermore, TCDD treatment did not affect the percentage and number of CD4<sup>+</sup> and CD8<sup>+</sup> cells in the spleen and slightly increased the percentage of CD19<sup>+</sup> cells on day 5 (Fig. 6).

The effects of TCDD and/or anti-CD25 treatment on the peanut allergic response were investigated on day 31 (Fig. 1,

setup 2). PE-specific serum levels of IgE, IgG1 (Figs. 7a–b), and PE-induced T-cell responses (*ex vivo* release of cytokines IL-5, IL-10, IL-13, IL-17a, and IFN-γ, Figs. 7e–i) were markedly increased by depletion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells. This confirms earlier findings that T<sub>reg</sub> cells are important in regulating allergic sensitization (Van Wijk *et al.*, 2007). This also shows that anti-CD25 treatment did probably not extensively deplete effector T or Th cells. Remarkably, the suppressive effect of TCDD on PE-specific IgE and IgG1 (Figs. 7a and b) and IL-5 (Fig. 7e) was partly reversed after depletion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells, whereas PE-specific IgG2a and PE-induced T-cell cytokine responses (IL-10, IL-13, IL-17a, and IFN-γ) were comparably high as in anti-CD25-treated PE-sensitized mice (Figs. 7c and f–i). It must be noted that anti-CD25 mAb treatment depletes T<sub>reg</sub> cells for about 7–10 days after the last injection (day 18),

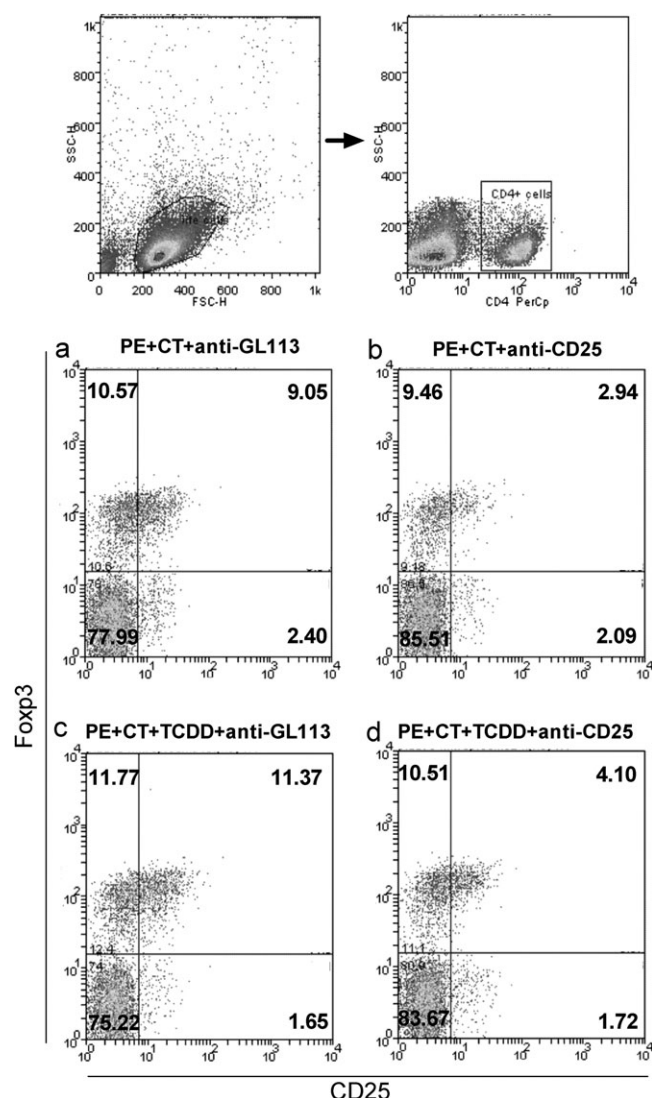


**FIG. 5.** Early effect of anti-CD25 mAb treatment (clone PC61) (days 4 and 0) in mice sensitized for peanut (PE + CT) and treated with 15  $\mu\text{g/kg}$  BW TCDD (day 3) on the percentage of  $T_{\text{reg}}$  cells. On day 5, mice were sacrificed and the percentage of  $\text{CD}25^{+}\text{Foxp}3^{+}$  cells within the  $\text{CD}4^{+}$  population in spleens from mice sensitized for peanut (PE + CT) treated with control mAb (anti-GL113) (a), depleted for  $\text{CD}25^{+}$  cells (b), or/and treated on day 3 with 15  $\mu\text{g/kg}$  BW TCDD (c and d) was examined by FACS analysis (setup 3). For detection of  $\text{CD}25$  expression, clone 3C7 was used. Numbers within the quadrants indicate mean percentages of cells within the splenic  $\text{CD}4^{+}$  population ( $n = 4$ ).

which allowed  $\text{CD}4^{+}\text{CD}25^{+}\text{Foxp}3^{+}$   $T_{\text{reg}}$  cells to have partly recovered on day 31 (Tenorio *et al.*, 2010; Van Wijk *et al.*, 2007). However, supplementary data show that 11 days after the last anti-CD25 mAb treatment the effect of  $\text{CD}25$  depletion is still apparent (Supplementary data 1). Together, these findings clearly show that TCDD increases the percentage of  $\text{CD}4^{+}\text{CD}25^{+}\text{Foxp}3^{+}$   $T_{\text{reg}}$  cells already during the initiation of the immune response and that these cells are involved in the suppression of peanut allergic sensitization after AhR activation by TCDD.

## DISCUSSION

$T_{\text{reg}}$  cells have been shown to play an important role in allergic sensitization and the intensity of food allergic responses (Schouten *et al.*, 2010; Torgerson and Ochs, 2007; Van Wijk *et al.*, 2007). Recently, it has been shown that activation of the AhR results in the induction of  $T_{\text{reg}}$  cells (Funatake *et al.*, 2005; Hauben *et al.*, 2008; Marshall *et al.*, 2008; Quintana *et al.*, 2008; Zhang *et al.*, 2010). Therefore, we examined whether activation of the AhR by TCDD suppresses Th2-mediated food allergic responses through the induction of  $T_{\text{reg}}$  cells. Data of the present study demonstrate for the first time that activation of the AhR by TCDD dose dependently suppressed various parameters of food allergic sensitization. In addition, the proportion, but not the absolute number, of  $\text{CD}4^{+}\text{CD}25^{+}\text{Foxp}3^{+}$   $T_{\text{reg}}$  cells dose dependently increased due to TCDD treatment. Importantly, depletion of  $\text{CD}4^{+}\text{CD}25^{+}\text{Foxp}3^{+}$   $T_{\text{reg}}$  cells markedly reversed the suppressive effect of TCDD on PE-specific antibody levels and Th2-related cytokines. This suggests that activation of the AhR by TCDD induces a moderate but functional shift in the  $\text{CD}4^{+}$  cell population toward  $\text{CD}25^{+}\text{Foxp}3^{+}$   $T_{\text{reg}}$  cells, resulting in suppression of peanut allergic sensitization.

Previously, it has been shown that activation of the AhR (by TCDD or M50354) impairs Th2-type immune responses (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). However, no role for  $T_{\text{reg}}$  cells was described or suggested in these studies. Recently, it has been shown that activation of the AhR by ITE, VAG539, or TCDD (50  $\mu\text{g/kg}$  BW vs. 15  $\mu\text{g/kg}$  BW in our experiments) suppresses GvHD and Th1/Th17-mediated EAE and EAU through a relatively modest induction of  $\text{CD}4^{+}\text{Foxp}3^{+}$   $T_{\text{reg}}$  cells (Hauben *et al.*, 2008; Quintana *et al.*, 2008, 2010; Zhang *et al.*, 2010). However, in these studies, the effect of AhR activation on  $\text{CD}4^{+}\text{Foxp}3^{+}$   $T_{\text{reg}}$  cells was only shown in percentages, whereas no data about absolute numbers of  $\text{CD}4^{+}\text{Foxp}3^{+}$   $T_{\text{reg}}$  cells were provided. Therefore, it is not clear whether AhR activation also increased the number of  $\text{CD}4^{+}\text{Foxp}3^{+}$   $T_{\text{reg}}$  cells or only induced a shift within the  $\text{CD}4^{+}$  population toward  $\text{CD}4^{+}\text{Foxp}3^{+}$  cells, as seen in our experiments.

Genes linked to immunoregulation such as Foxp3, transforming growth factor (TGF)- $\beta$ , and IL-10 contain DREs in their promotor regions (Kerkvliet, 2009; Sun *et al.*, 2004). Therefore, it is likely that immunoregulatory cells such as Foxp3<sup>+</sup>  $T_{\text{reg}}$  cells can be induced directly by AhR activation. In support of the importance of AhR in the actual generation of Foxp3<sup>+</sup>  $T_{\text{reg}}$  cells, naïve T cells from AhR knockout mice showed impaired  $T_{\text{reg}}$ -cell development *in vitro* (Kimura *et al.*, 2008). Also, mutant AhR  $\text{CD}4^{+}\text{Foxp}3^{-}$  cells with reduced affinity for AhR ligands appeared less capable of differentiating into  $\text{CD}4^{+}\text{Foxp}3^{+}$  both *in vivo* and *in vitro* (Quintana *et al.*, 2010). In addition, it has recently been shown that AhR

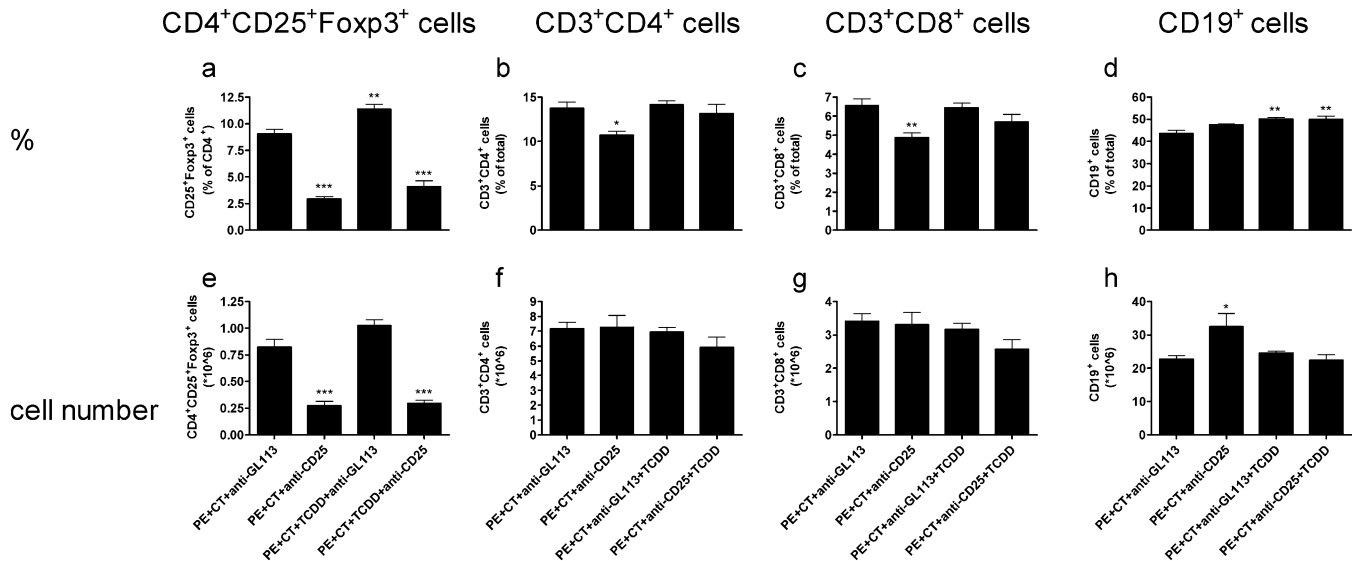


**FIG. 6.** TCDD treatment increased the percentage, but not the number, of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells and did not affect the percentage and number of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> and the number of CD19<sup>+</sup> cells in the spleen on day 5. Mice were injected with anti-CD25 (clone PC61) mAb or control antibody (mAb anti-GL113) prior and during sensitization to peanut and treated with 15 µg/kg BW TCDD on day 3 (setup 3). On day 5, mice were sacrificed and the percentage and number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> (a and e), CD3<sup>+</sup>CD4<sup>+</sup> (b and f) CD3<sup>+</sup>CD8<sup>+</sup> (c and g), and CD19<sup>+</sup> (d and h) cells in the spleen were examined by FACS analysis. For detection of CD25 expression, clone 3C7 was used. Values are presented as mean ± SE (*n* = 4). \**p* < 0.05 compared with PE + CT + anti-GL113.

activation by TCDD in naïve human T cells induces Foxp3<sup>+</sup> T<sub>reg</sub> cells in the presence of TGF-β1 (Gandhi *et al.*, 2010). Despite the strong indications that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells are instrumental in TCDD-induced suppression of sensitization to peanut, it cannot be excluded that TCDD interferes with other cells containing AhR and relevant DRE-containing genes. Among these are other regulatory T cells, e.g., CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−</sup> cells (Funatake *et al.*, 2005), which were also increased in our study by TCDD treatment in MLN and Tr1 cells-producing regulatory IL-10 (Apetoh *et al.*, 2010; Gandhi *et al.*, 2010). However, IL-10 can also stimulate functions of innate immunity and Th2-related immunity (differentiation of B cells), depending on the cell type producing it (Moore *et al.*, 2001). In our experiments, *in vivo* TCDD treatment suppressed *ex vivo* PE-induced IL-10 production dose dependently along with PE-specific antibody levels. This suggests that PE-induced IL-10 is rather a Th2-related cytokine than a regulatory cytokine in the present peanut allergy model.

The observed decrease in numbers of CD4<sup>+</sup> T cells after TCDD treatment may result from an effect of TCDD on thymocyte differentiation (Camacho *et al.*, 2005; Kamath *et al.*, 1998; Temchura *et al.*, 2005) or from a direct suppressive effect of TCDD on peripheral T cells on days 4–5 (Dearstyn and Kerkvliet, 2002; Funatake *et al.*, 2004; Shepherd *et al.*, 2000). Importantly, in our experiments, TCDD treatment did not affect the percentage and number of splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells on day 5. Interestingly, the percentage, but not the number, of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells was already increased on this day, i.e., during initiation of sensitization. However, our data cannot exclude that TCDD treatment

directly deletes or suppresses the function of peripheral (naïve) T cells and/or other cells of the immune system, resulting in suppression of the peanut allergic reaction.

Our data show an increase in IFN-γ production at lower doses (0.6, 1.7, and 5 µg/kg BW) of TCDD in *ex vivo* PE-restimulated splenocyte cultures. Furthermore, IL-5 was already decreased at these doses of TCDD. This agrees with previous findings that *in vivo* TCDD treatment increased antigen-induced IFN-γ production and suppressed antigen-induced IL-5 production by splenocytes (Fujimaki *et al.*, 2002; Negishi *et al.*, 2005; Nohara *et al.*, 2002). Interestingly, a role for the AhR in skewing the Th1/Th2 cytokine balance toward Th1 has been described by others (Kato *et al.*, 2003; Negishi *et al.*, 2005). Unpublished results from our laboratory and published results from others have shown that IFN-γ is involved in regulating the intensity of (peanut) allergic reactions (Yoshida *et al.*, 2002). Therefore, shifting the Th1/Th2 cytokine balance toward Th1 might be an additional mechanism through which TCDD could suppress sensitization to peanut in this study. However, TCDD-induced IFN-γ production by other cells (e.g., phagocytic cells) cannot be excluded (Neff-LaFord *et al.*, 2007).

Because AhR and DREs are present in many different immune cells, it is difficult to pinpoint one of the regulatory players as the crucial one, but importantly, the net result appears to be a proportional shift to a more regulatory phenotype of the CD4<sup>+</sup> T-cell compartment as a whole. Considering the importance of B cells in allergic responses, the situation is further complicated by fact that TCDD can also suppress formation of germinal centers (Inouye *et al.*, 2003) and plasma cell differentiation (North *et al.*, 2009). However,

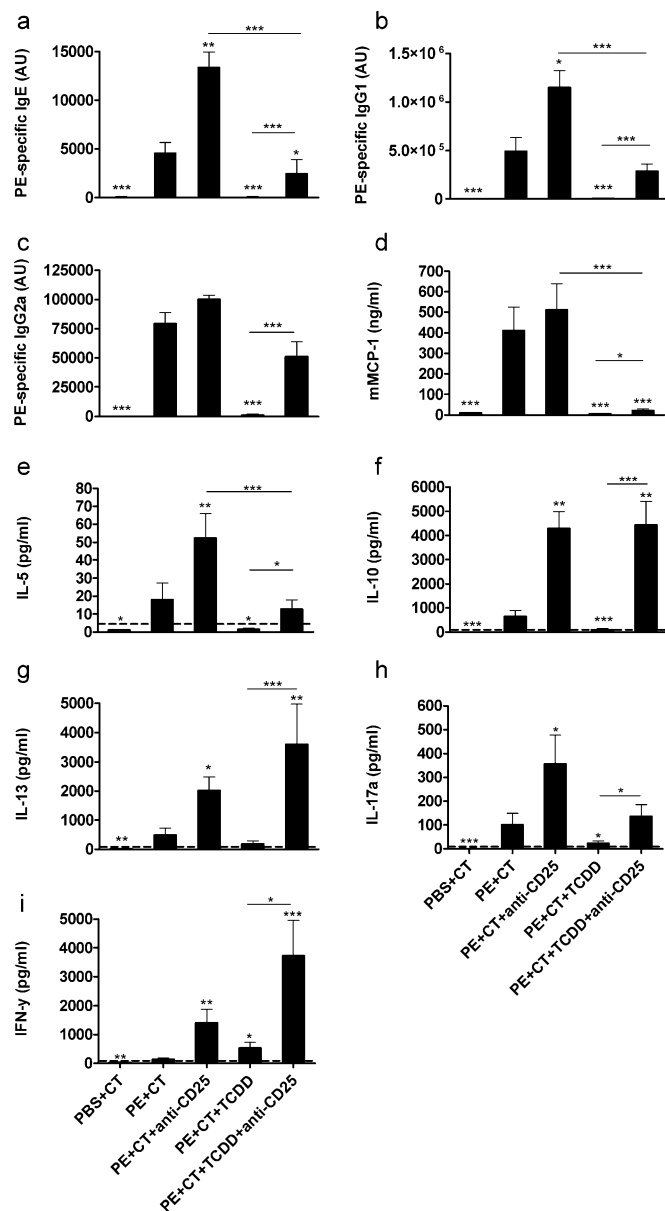


**FIG. 7.** Depletion of  $CD4^+CD25^+Foxp3^+$   $T_{reg}$  cells abrogated the suppressive effect of AhR activation by TCDD on the peanut allergic response. Mice were injected with anti-CD25 (clone PC61) mAb or control antibody (mAb anti-GL113) prior and during sensitization to peanut and treated with 15  $\mu$ g/kg BW TCDD on day 3 (setup 2). On day 30, mice were challenged with PE. Peanut-specific IgE (a), IgG1 (b), and IgG2a (c) were examined in serum from day 31 by ELISA. mMCP-1 (d) was determined by ELISA in plasma taken 30 min after challenge. Single-cell suspensions of spleen cells (day 31) were cultured for 96 h in the presence of PE. The supernatant was analyzed for IL-5 (e), IL-10 (f), IL-13 (g), IL-17a (h), and IFN- $\gamma$  (i) by ELISA. No cytokine production could be detected when splenocytes were cultured in the absence of PE. Values are presented as mean  $\pm$  SE ( $n = 6-8$ ). \* $p < 0.05$  compared with PE + CT, \*\* $p < 0.01$  compared with PE + CT, \*\*\* $p < 0.001$  compared with PE + CT.

TCDD exposure does not decrease B-cell activation and maturation during early peanut sensitization, suggesting that possible effects of TCDD on B cell are secondary to T-cell effects (Schulz, unpublished data).

The finding that TCDD appears to induce an immunoregulatory T-cell compartment, involving  $CD4^+CD25^+Foxp3^+$   $T_{reg}$  cells as important contributors, suggests that activation of the AhR in cells upstream from T cells might play an important role herein. Importantly, activation of the AhR has been shown to induce tolerogenic dendritic cells (DCs) producing retinoic acid or indoleamine 2,3-dioxygenase, both associated with the development of  $T_{reg}$  cells (Nguyen *et al.*, 2010; Quintana *et al.*, 2010; Vogel *et al.*, 2008). In other studies, TCDD has been shown to activate DCs in the presence and absence of an antigen (Vorderstrasse *et al.*, 2003), to selectively decrease  $CD11c^{high}CD8\alpha^{-}33D1^+$  splenic DCs specialized at activating  $CD4^+$  T cells (Bankoti *et al.*, 2010) and to reduce the amount of splenic DCs (Bankoti *et al.*, 2010; Vorderstrasse and Kerkvliet, 2001). The role of DCs in TCDD-induced immunoregulation is clearly not deciphered yet because TCDD-affected DCs may either stimulate  $T_{reg}$  cells or limit activation of effector T cells. Recently, we have demonstrated that DCs are crucial in the allergic response to peanut, by showing that inflammatory  $CD11b^+$  DCs stimulate and regulatory  $CD103^+$  and plasmacytoid DCs inhibit food allergic responses (Smit *et al.*, 2011). This knowledge will be used to study the influence of various DC subsets in TCDD-mediated effects in food allergy further.

Besides TCDD, there are numerous other endogenous and exogenous ligands for the AhR described. Yet, most of these ligands are easily metabolized and therefore probably not sufficiently bioavailable to activate the AhR persistently (Denison and Nagy, 2003). The AhR is strongly expressed along the small intestine (Chmill *et al.*, 2010) and based on present findings, it can be hypothesized that prolonged exposure to AhR ligands via the diet could influence the outcome of food allergic responses. Such dietary AhR ligands may potentially help to alleviate or even treat allergic responses, if they are relatively stable without causing the toxic side effects associated with dioxins. In addition, these AhR ligands should induce  $T_{reg}$  cells and not Th17 cells (Quintana *et al.*, 2008, Veldhoen *et al.*, 2008). Moreover, the existing cytokine milieu (especially the presence of IL-6 and/or TGF- $\beta$ ) appears to be very important in deciding whether AhR activation results in an inflammatory or a regulatory immune response (Apetoh *et al.*, 2010; Gandhi *et al.*, 2010; Veldhoen, 2010). Furthermore, it has been reported that TCDD impairs maintenance of oral tolerance against ovalbumin in a high-dose oral tolerance model (Chmill *et al.*, 2010). Together, this shows that activation of the AhR pathway can affect immune responses differently, depending on the AhR ligand, the cytokine milieu, and the disease model used.

In summary, the present study showed that activation of the AhR pathway suppressed the development of Th2-mediated food allergic responses by inducing a functional shift within the  $CD4^+$  cell population toward  $CD4^+CD25^+Foxp3^+$  cells. This emphasizes the important role of the AhR in shaping the T-cell repertoire and the delicate balance between tolerance and

immunity. Importantly, findings of this study suggest that the AhR pathway might be a unique target for therapeutic manipulation of food allergic diseases. In addition, our data warrant further studies to investigate the therapeutic potential of natural or endogenous AhR ligands in suppressing food allergic responses.

# SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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## Supplementary data 1

