The Aryl Hydrocarbon Receptor and Food Allergy

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The Aryl Hydrocarbon Receptor and Food Allergy

De Aryl Hydrocarbon Receptor en Voedselallergie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 7 maart 2013 des ochtends te 10.30 uur

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Chapter 1

Introduction

The immune system is important for protection against pathogens and malignant cells. However, malfunction of the immune system can also result in detrimental auto-immune diseases, inflammatory diseases, cancers and allergies. The aryl hydrocarbon receptor (AhR), present in numerous tissues and cell subsets, including cells of the immune system, plays an important role in the functioning of the immune system. Activation of the AhR is for example associated with various effects on dendritic cells, regulatory T cells and the Th1/Th2 cell balance. These cells play a major role in the development of food allergy, which is an adverse immune response to a food protein. This thesis will address whether the AhR might be a possible target to interfere in food allergic reactions.

1 The aryl hydrocarbon receptor

1.1 The aryl hydrocarbon receptor and toxicity

The AhR, a cytosolic ligand-activated transcription factor, is a member of the eukaryotic Per-ARNT-Sim (PAS) domain protein family that was discovered in the 1970s and is present in numerous tissues and cell subsets throughout the body [1,2]. It mediates the toxic responses of a variety of chemical pollutants, including polyhalogenated aromatic hydrocarbons (PHAHs) and polycyclic aromatic hydrocarbons (PAHs). The persistent PHAH compound 2,3,7,8-tetrachlorodibenzo-[p]-dioxin (TCDD) is one of the most potent AhR ligands known [3]. Exposure to TCDD has been shown to induce a wide variety of toxic effects in various species, as shown in table 1 [3].

Table 1. Toxic effects associated with exposure to TCDD and TCDD-like chemicals in various species.
Immunotoxicity
Hepatotoxicity
Cardiotoxicity
Reproductive toxicity
Dermal toxicity
Teratogenesis
Endocrine disruption
Lethality
Wasting syndrome
Carcinogenesis
Porphyria
Diabetes

1.2 Endogenous functions of the aryl hydrocarbon receptor

Next to mediating the toxicity of xenobiotics, the AhR plays a major role in developmental processes and physiology, including hematopoiesis, circadian rhythm, and in cell processes, such as cell growth and differentiation [4-7]. This is illustrated by AhR knockout mice, which have hypertrophy and hyperplasia of the heart, liver and gastrointestinal tract, vascular lesions in the uterus, small spleens and skin lesions [8,9]. Furthermore, the AhR may be relevant in immunology for the differentiation and balance of T cells subsets in ongoing immune responses and the decision for active immunity or the induction of tolerance [10]. Dietary and endogenous AhR ligands, for example indoles, tetrapyroles, arachidonic acid metabolites, appear to play a role in these different processes [3,4].

1.3 Aryl hydrocarbon receptor dependent gene transcription

The AhR is associated with the chaperone proteins hsp90, hsp23 and the X-associated protein 2. Upon ligation, the AhR complex translocates to the nucleus, where the ligand:AhR dimerizes with AhR nuclear translocator (ARNT) and dissociates from the other subunits of the complex (figure 1). This will transform the ligand:AhR:ARNT complex into a high affinity DNA-binding form, which subsequently binds to a specific

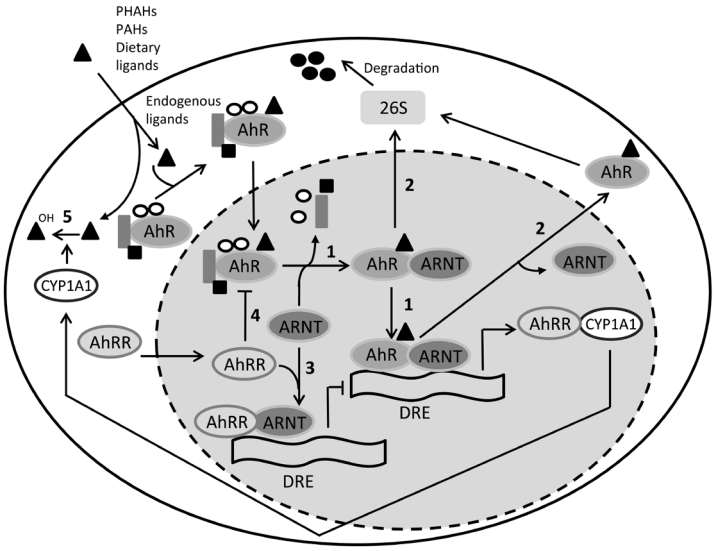

Figure 1. Schematic overview of mechanisms involved in AhR activation. Scheme 1: After ligation of the AhR with an agonist, the ligand:AhR complex translocates to the nucleus where it dimerizes with ARNT. Then, hsp90, hsp23 and the X-associated protein 2 dissociate from the complex and the ligand:AhR:ARNT complex binds to DREs, resulting in the transcription of genes. Scheme 2: Proteolytic degradation of the AhR by the 25S proteosome. Scheme 3 and 4: AhR-mediated induction of the AhRR reduces the formation of the ligand:AhR:ARNT complex. Scheme 5: AhR ligands are metabolically depleted by CYP450 enzymes. Adapted from Mitchell et al 2009 [11].

site on the DNA, the dioxin response element (DRE). There exist multiple DREs and these DREs are present upstream of AhR responsive genes such as CYP1A1 or AhR repressor protein (AhRR) [3,5,11]. Prolonged AhR activation can be suppressed by dissociation of the AhR complex from the DRE, followed by export of the AhR into the cytosol and ubiquitin-mediated AhR proteosomal degradation. Furthermore, the AhR-mediated induction of CYP450 enzymes facilitates a negative feedback loop, resulting in the metabolic degradation of AhR agonists. However, in contrast to for instance PAHs, PHAHs like TCDD are resistant to metabolic degradation because of their halogenation. AhR-dependent induction of AhRR can reduce the formation of the ligand:AhR:ARNT complex (figure 1) [5,11].

Next to this, AhR activation can interact and crosstalk with other nuclear proteins and signaling factors. For example, the AhR has been shown to cross-talk with estrogen receptor signaling pathways [12] and to interact with the NF-κβ subunit RelB [13]. Some of these interactions with other genes and pathways are ligand specific. For

instance, the AhR ligands TCDD and 3,3'-diindolylmethane (DIM) have different ability to activate distinct AhR-controlled pathways, including degree of activation of CYP450 enzymes and activation of the estrogen receptor degradation pathway [14]. In summary, AhR activation results in the transcription of various genes and different pathways essential for numerous physiological functions, thereby exerting very diverse biological effects, including toxicity.

1.4 Ligands for the aryl hydrocarbon receptor

There are a wide variety of AhR ligands, which differ in structure, binding affinity and metabolic stability. Ligands of the AhR can be divided into two major categories, synthetic AhR ligands (including both halogenated and non-halogenated) and dietary/endogenous AhR ligands. Structures of AhR ligands described and used in this thesis are shown in figure 2.

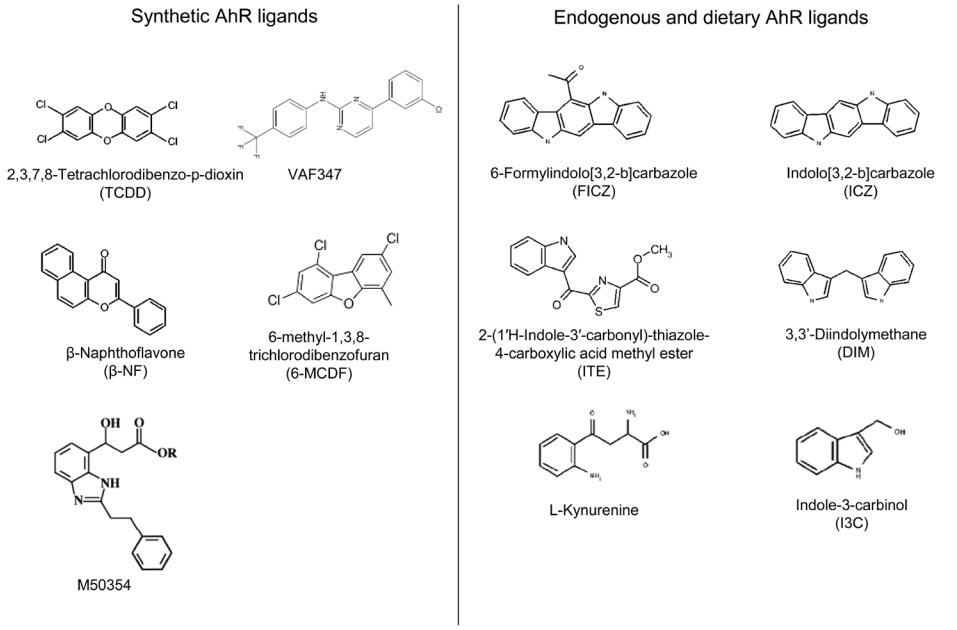

Figure 2. Structures of synthetic, endogenous and dietary AhR ligands described this thesis.

1.4.1 Synthetic aryl hydrocarbon receptor ligands

Synthetic AhR ligands, e.g. PHAHs, PAHs and PAH-like chemicals, are in general associated with toxic effects, mediated through the AhR. Polychlorinated and polybrominated dibenzo-p-dioxins (e.g. TCDD), dibenzofurans and biphenyls (all PHAHs) have the highest binding affinity for the AhR [15]. These compounds are environmental contaminants and their extensive halogenation makes these compounds environmentally and metabolically stable [15,16]. In addition, the presence of halogens at the lateral positions of the coplanar rings is associated with strong AhR binding potency [16]. PAHs, for example β-naphthoflavone, have reduced AhR binding affinity and are less metabolically stable when compared to PHAHs. Furthermore, the electronic and thermodynamic properties of these compounds are important in determining their AhR binding affinity [17]. Three-dimensional quantitative structure-activity relationship studies revealed that planar, aromatic and hydrophobic ligands with a maximal dimension of 14 Å x 12 Å x 5 Å can fit into the ligand binding pocket of the AhR [18].

1.4.2 Dietary and endogenous aryl hydrocarbon receptor ligands

The structure and physiochemical properties of endogenous and dietary AhR ligands differ markedly from synthetic AhR ligands [15]. In general, most endogenous and dietary AhR ligands have low affinity for the AhR and only induce modest AhR-dependent gene transcription when compared to TCDD [3,16]. 2-(1'H-Indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), UV-products of tryptophan (such as 6-formylindolo[3,2-b]carbazole [FICZ]), metabolites from arachondic acid and heme degradation products are examples of endogenous AhR ligands. Furthermore, cruciferous vegetables like broccoli and cabbages contain indole-3-carbinol (I3C), which is condensed into the high affinity AhR ligands diindolylmethane (DIM) and indolocarbazole (ICZ) by stomach acids [3]. The AhR-dependent induction of CYP450 enzymes (e.g. CYP1A1) makes that these endogenous and dietary AhR ligands facilitate their own metabolism [16], thereby greatly affecting their bio-availability.

2. Aryl hydrocarbon receptor and the adaptive immune system

As mentioned previously, the AhR could play an important functional role in the immune system, such as in food allergic responses. The AhR is present in a variety of cells of the immune system, including dendritic cells, T cells and B cells.

2.1 Aryl hydrocarbon receptor and dendritic cells

Dendritic cells (DC) are professional antigen-presenting cells (APC) linking innate immune responses with adaptive immune responses mediated by T and B lymphocytes. Immature DC continuously sample foreign antigens. Encounter of a foreign antigen could induce DC to mature and to migrate to the lymph node. Here, DC present the foreign antigen as peptides on major histocompatibility complex (MHC) molecules to matching antigen-specific T cells. These maturated DC also upregulate co-stimulatory molecules that are required for interaction with and activation of antigen-specific T cells. In the absence of inflammation, factors released by epithelial and mesenchymal cells (prostaglanding E2, transforming growth factor [TGF]-β, IL-10) induce only partial maturation of the DC. This results in the induction of tolerance via induction of regulatory T cells. However, in the presence of pro-inflammatory factors (IL-1, IL-6, IL-8), induced by interaction of pathogens with toll-like receptors present on epithelial cells, mesenchymal cells and macrophages, maturated DC induce active immunity by activating effector T cells [19,20].

In the mouse, five major subsets of DC have been defined so far and each subset may have a specific role in the immune system [21]. In food allergy, the balance between tolerogenic $CD103^+$ DC and inflammatory $CD11b^+$ DC is important, because oral sensitization to peanut is accompanied by an increase of inflammatory $CD11b^+$ DC and a decrease of $CD103^+$ DC in the intestine [22]. Moreover, mucosal $CD103^+$ DC are important in the induction of oral tolerance, since they have been shown to induce $Foxp3^+$ regulatory T (T_{reg}) cells in a TGF-β and retinoic acid dependent manner [20,23,24]. These T_{reg} cells regulate the intensity of immune responses and play an important role in regulating allergic sensitization [25].

Little is known about AhR gene expression in DC subsets. However, DC in the spleen have been shown to express the AhR [26]. Moreover, $CD103^+$ DC in the mesenteric lymph nodes (MLN) have been shown to increase AhRR upon *in vivo* AhR activation by TCDD [27].

In vitro AhR activation affects maturation and activation of bone marrow cultured DC by increasing expression of MHC class II, CD80 and CD86 [28-30]. Moreover, *in vitro* AhR activation of DC decreases the production pro-inflammatory cytokines, for example IL-1β, IL-6, TNF-α and IL-12, increases the gene expression of regulatory factors, including TGF-β and indoleamine 2,3-dioxygenase (IDO), and increases the frequency of $Foxp3^+$ T_{reg} cells [29,30].
Effects of *in vivo* AhR activation on DC resemble the effects of *in vitro* AhR activation on DC. *In vivo* TCDD treatment reduces the amount of splenic DC and induces activation-like changes in splenic DC (increase of MHCII, CD86, CD40, CD24, CD54), which may enhance their ability to provide activation signals to T cells resulting in dysregulation of immune responses [31,32]. Furthermore, DC isolated from the lung draining lymph nodes of TCDD-treated mice reduce T cell activation [33]. Next, *in vivo* AhR activation is associated with the induction of tolerogenic splenic $CD103^+$ DC and the subsequent induction of $CD4^+CD25^+Foxp3^+$ T_{reg} cells. An increase of retinoic acid production by DC and/or an increase of the immunosuppressive IDO gene expression induced by AhR activation are proposed mechanisms for the increase of T_{reg} cells and the suppression of the immune response [34-37].

2.2. Aryl hydrocarbon receptor and T cells

$CD8^+$ and $CD4^+$ T cells develop in the thymus and after migration to periphery they may become activated by APC presenting antigen on MHC class I or MHC class II, respectively, in the presence of co-stimulatory signals. Activated $CD8^+$ T cells can be cytotoxic T cells, which are important for destroying tumor cells and virus infected cells. Activated $CD4^+$ T cells can develop into T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) or T_{reg} cells, which direct and exert different types of immune responses by the secretion of specific cytokines (figure 3). AhR expression is higher in $CD8^+$ T cells than in $CD4^+$ T cells [27]. Within the $CD4^+$ T cell population, Th17 cells have the highest AhR expression [38,39].

2.2.1 Aryl hydrocarbon receptor and thymic T cells

In the thymus, T cells develop from $CD4^-CD8^-$ (double-negative) cells to $CD4^+CD8^+$ (double-positive) cells before differentiation into either $CD4^+$, including $CD4^+CD25^+Foxp3^+$ T_{reg} cells, or $CD8^+$ single-positive cells. For decades, it is known that AhR activation by TCDD results in thymus atrophy [40,41]. AhR activation by TCDD alters thymic selection by affecting thymic precursor T cells, e.g. by increasing

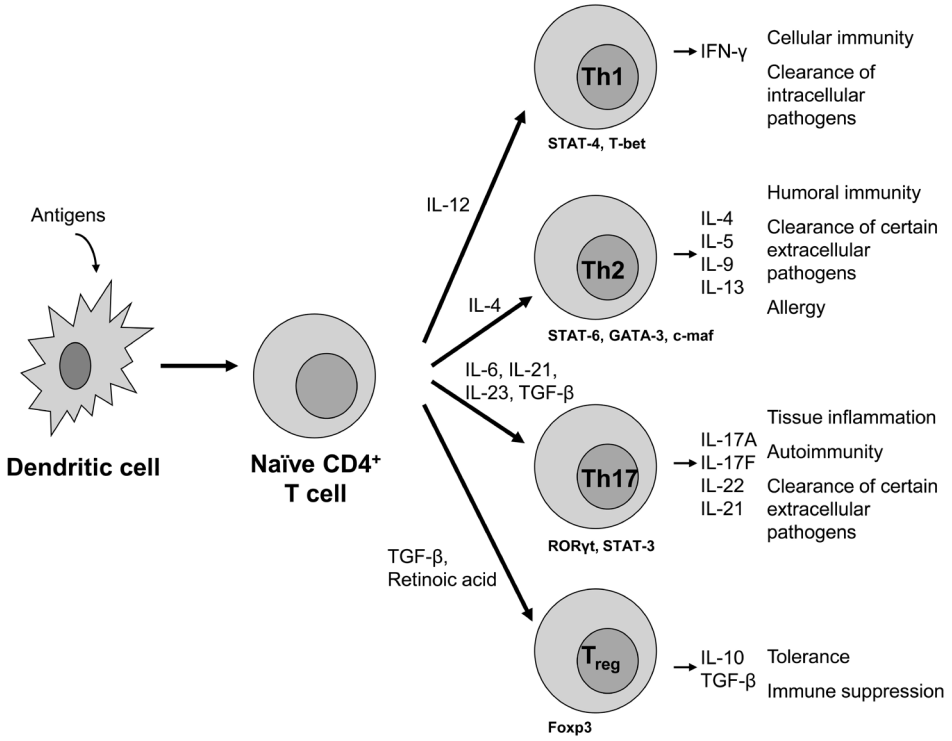

Figure 3. Differentiation of $CD4^+$ cells into different effector T cells; T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) or regulatory T (T_{reg}) cells. Differentiation of these T cell subsets is mediated by the interaction of dendritic cells with naïve $CD4^+$ T cells, the presence of specific cytokines and mediators and cell-type specific transcription factors. By secreting specific cytokines, effector T cells mediate their regulatory or activating properties, as shown on the right. Adapted from Jetten et al., 2009 [52].

percentages of $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$ cells and by decreasing percentages of $CD4^+CD8^+$ cells [42,43]. Apoptosis of precursor T cells via binding of FasL to Fas is proposed as one of the underlying mechanism for TCDD-induced thymic atrophy [40]. AhR activation by TCDD induces upregulation of FasL via NF-κB on stromal cells, resulting in increased apoptosis of thymic T cells [40]. The epithelium of the thymic stroma is involved in negative and positive selection of thymocytes. Conversely, epithelial cells need the presence of thymocytes to maintain their integrity. TCDD exposure disturbs the cortical and the medullary epithelium, thereby contributing to thymic atrophy and disturbing thymocyte differentiation [45]. Next to apoptosis of precursor T cells, reduced thymic seeding could be another explanation for TCDD-

induced thymic atrophy, because TCDD treatment has been shown to affect thymocyte precursors from the bone marrow [46]. Remarkably, effects of AhR activation on thymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells have not been studied yet.

2.2.2 General effects of aryl hydrocarbon receptor activation on peripheral $CD4^+$ T cells

Besides suppressing T cell development in the thymus, AhR activation by TCDD can also affect mature T cells in the periphery resulting in suppression of immune responses. *In vivo* TCDD treatment suppresses survival, activation, differentiation, proliferation and cytokine production of $CD4^+$ T cells [47-51]. Similar to thymic precursor T cells, there is evidence that TCDD suppresses $CD4^+$ T cell mediated immune responses via Fas/FasL. AhR activation induces Fas via activation of the NF-κB pathway and downregulates the cellular FLICE inhibitory protein (c-FLIP), an inhibitor of apoptosis [44,48,49]. Next to these general suppressive effects of AhR activation on T cells, AhR activation can skew T cell responses by affecting the balance between Th1 and Th2 cells, and Th17 and T_{reg} cells.

2.2.3 Aryl hydrocarbon receptor and Th1/Th2 cell balance

Th1 cells secrete IFN-γ and are important for mediating cellular immunity and the clearance of intracellular pathogens. Th2 cells secrete IL-4, IL-5, IL-9 and IL-13 and are involved in humoral immunity, allergy and the clearance of extracellular pathogens (figure 3) [52,53].

At subcellular level, development of Th cells is regulated by transcription factors, for Th1 cells signal transducer and activator of transcription-4 (STAT-4) and T-bet. These transcription factors are usually activated when IL-12 is produced by DC. In contrast, Th2 cell development is regulated by STAT-6, GATA-3 and c-maf and these transcription factors are usually activated in the presence of IL-4.

The transcription factors controlling Th1 or Th2 cell differentiation are antagonistic and conceivably Th1 and Th2 cell development are mutually regulated. Furthermore, the presence of thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 from non-lymphoid sources (e.g. epithelial cells, mast cells, basophils, eosinophils) are important for Th2 cell differentiation [54]. Manipulation of the Th1/Th2 balance can be beneficial for food allergic responses, because an enhanced Th1 response may suppress food allergic responses [55].

AhR activation impairs Th2-mediated immune responses by suppressing Th2-related cytokines and antigen-specific antibody production by B cells [49]. Concomitantly, AhR activation increases antigen-induced IFN-γ production [50,56,57]. Interestingly, M50354, a transient but full AhR agonist, skews the differentiation of naïve T cells towards Th1 cells by inhibiting GATA-3 expression in T cells [57-59]. This indicates that the AhR may play a significant role in suppressing food allergic responses by affecting the balance of Th1 and Th2 cells.

2.2.4 Aryl hydrocarbon receptor and T_{reg}/Th17 cell balance

Next to Th3 regulatory T cells and T regulatory 1 cells, there exist $CD4^+CD25^+Foxp3^+$ T_{reg} cells. There are two types of $CD4^+CD25^+Foxp3^+$ T_{reg} cells, natural and inducible $CD4^+CD25^+Foxp3^+$ T_{reg} cells. Foxp3 expression is essential for T_{reg} cell differentiation in the thymus and periphery and their suppressor function [60]. Natural $CD4^+CD25^+Foxp3^+T_{reg}$ cells develop in the thymus during the course of positive and negative selection like other T cells. Their development is influenced by co-stimulatory molecules, cytokines, T cell receptor (TCR) and antigen affinity, and the location and context of encountering self antigen within the thymus [61-63]. Inducible $CD4^+CD25^+Foxp3^+$ T_{reg} cells develop from naïve $CD4^+$ T cells in the periphery upon strong TCR stimulation in the presence of high amounts of TGF-β, IL-2 and/or retinoic acid [60]. $Foxp3^+$ T_{reg} cells secrete IL-10 and TGF-β and these cells are important for the induction of oral tolerance and the suppression of immune-mediated disorders, including food allergy (figure 3) [60,25].

Th17 cells develop in the periphery in the presence of low levels of TGF-β, IL-23 and IL-21 or IL-6 [65]. Th17 cells are characterized by retinoic acid-related orphan receptor (ROR)γt and STAT-3 and secrete mainly IL-17A, IL-17F, IL-21 and IL-22. Th17 cells are involved in autoimmunity and tissue inflammation by expanding and recruiting innate immune cells such as neutrophils [65,66]. Th17 cells may also play a role in the pathogenesis of various allergic diseases, including allergic dermatitis and asthma by recruiting neutrophils. However, whether IL-17 or Th17 cells play a role in food allergy is not clear yet [67] (figure 3).

Development of $Foxp3^+$ T_{reg} cells and Th17 cells can be reciprocally regulated, e.g. low concentrations of TGF-β together with IL-6 and IL-21 favors Th17 differentiation, whereas high concentrations of TGF-β induces $Foxp3^+$ T_{reg} cell differentation [52,65]. However, development of Th17 cells in the presence of IL-6 and IL-23, but in the absence of TGF-β, has also been reported [68].

Interestingly, the AhR is involved in peripheral differentiation of both Th17 and T_{reg} cells. In various disease models, e.g. colitis [69,70], various autoimmune-mediated diseases [34,39,71,72], graft versus host disease [73] and viral immunopathology [74] AhR activation by TCDD, the water-soluble derivate of VAF347 (VAG539) or ITE suppresses these immune-mediated disorders by increasing the percentage of $Foxp3^+$ T_{reg} cells (table 2). In contrast, AhR activation by FICZ enhances disease by increasing the percentage of Th17 cells in an experimental autoimmune encephalomyelitis (EAE) model [38,39] (table 2). These divergent effects of AhR activation on $Foxp3^+$ T_{reg} and Th17 cell development may result from the presence or absence or IL-6 and TGF-β during AhR activation, or from effects of the AhR ligand itself [39,52,65].

AhR ligand	Disease model	Suppression/ exacerbation	Parameter	Reference
TCDD	Dextran sodium sulphate (DSS) colitis	Suppression	Increase % $Foxp3^+$ T_{reg} cells	Singh et al., 2011 [69]
TCDD	2,4,6-trinitrobenzenesulfonic acid (TNBS) murine model of colitis	Suppression	Increase % $Foxp3^+$ T_{reg} cells	Benson et al., 2010 [70]
TCDD	Experimental Autoimmune Uveoretinitis (EAU)	Suppression	Increase % $Foxp3^+$ T_{reg} cells	Zhang et al., 2010 [71]
TCDD	Type 1 diabetes	Suppression	Increase % $Foxp3^+$ T_{reg} cells	Kerkvliet et al., 2009 [72]
TCDD	Viral immunopathology (herpes keratitis lesions)	Suppression	Preservation of # $Foxp3^+$ T_{reg} cells Decrease # effector T cells	Veiga-Parga et al., 2011 [74]
TCDD	Experimental Autoimmune Encephalomyelitis (EAE)	Suppression	Increase % $Foxp3^+$ T_{reg} cells	Quintana et al., 2008 [39]
FICZ	Experimental Autoimmune Encephalomyelitis (EAE)	Exacerbation	Increase % Th17 cells	Quintana et al., 2008 [39]
FICZ	Experimental Autoimmune Encephalomyelitis (EAE)	Exacerbation	Increase % Th17 cells	Veldhoen et al., 2008 [38]
ITE	Experimental Autoimmune Encephalomyelitis (EAE)	Suppression	Increase % $Foxp3^+$ T_{reg} cells, partly mediated DC secreting retinoic acid	Quintana et al., 2010 [34]
VAG539	Graft versus Host Disease	Suppression	Increase % $Foxp3^+$ T_{reg} cells, partly mediated by DC	Hauben et al., 2008 [73]

Table 2: Effects of AhR activation on Th17 and T_{reg} cells in various disease models as described in text.

Furthermore, it appears that the AhR has an important regulatory function in the commitment of Th17 cells by negatively regulating STAT-1 activation *in vitro*. STAT1 activation, induced by IFN-γ, inhibits Th17 cell development [75]. In addition, induction of human $Foxp3^+$ T_{reg} cells *in vitro* is coordinated by AhR-mediated upregulation of the transcription factors Smad1 and Aiolos, which are important for the induction of $Foxp3^+$ T_{reg} cells [76]. However, so far only relative effects of AhR activation on T_{reg} cells and Th17 cells have been shown. Therefore, it is not clear whether their increased presence is a consequence of a decrease of other cells resulting in an increased percentage of Th17 and T_{reg} cells. The finding by Veiga-Parga

et al. that TCDD does not increase the actual numbers of T_{reg} cells suggest the latter [74] (table 2). In conclusion, AhR activation can suppress or enhance disease in various immune-mediated disease models via T_{reg} and Th17 cells, probably dependent on the AhR ligand and the presence or absence of certain cytokines during AhR activation.

2.3 Aryl hydrocarbon receptor and B cells

Interaction of antigen with a mature naïve B cell, as well as interactions with T cells, induces activation and differentiation of B-cell clones for the specific antigen. This occurs through several stages, each stage representing a change in the genome content at the antibody loci, ultimately resulting in the generation of plasma effector cells, memory B cells and generation of antibodies (humoral immunity).

TCDD suppresses humoral immune responses, characterized by suppression of IgM and IgG secretion and decreased B cell differentiation into Ig-secreting cells [77]. Sulentic et al. recently reviewed the direct effects of TCDD on B cells [78]. Briefly, TCDD affects B cells at multiple stages of maturation and differentiation and impairs B cell proliferation and antibody production. B cell activation induces marked upregulation of AhR and B cells are most susceptible to TCDD during activation and less susceptible as they progress towards terminal differentiation. Furthermore, TCDD probably affects AhR-dependent gene transcription in B cells and induces biochemical changes independent of AhR-mediated gene transcription, thereby affecting regulators important for the B cell differentiation program [78]. Next to these direct suppressive effects on B cells, AhR activation can also indirectly suppress B cell function and activation by suppressing Th cells and Th-related cytokines as mentioned before.

2.4 Aryl hydrocarbon receptor and the mucosal immune system

The intestine is the largest contact surface between the body and the external environment, which is rich in commensals, nutrients and fluids, but also pathogens. Proper functioning of the intestinal immune system requires a coordinated balance of tolerance and immunity and is crucial for protection against infections and intestinal diseases, for instance inflammatory bowel disease and food allergies [79-81].

Recently, it was proposed that AhR activation by dietary ligands plays a major role in the postnatal expansion of RORγt innate lymphoid cells (ILC) and is necessary to maintain intraepithelial lymphocytes (IEL) in the intestine [82,83]. By producing IL-22,

RORγt ILC maintain the epithelial barrier, which is required for protection against intestinal infections and food allergy [83,84]. IEL are important as a first line of defense, epithelial barrier organization, wound repair and food allergic sensitization [82,85]. The importance of the AhR in the intestinal immune system is illustrated by the fact that AhR activation by FICZ or β-NF ameliorates TNBS- and DSS-induced colitis by suppressing inflammatory cytokines [86] and by enhancing production of IL-22 [86,87]. In addition, TCDD treatment of mice before induction of TNBS-induced colitis suppresses colonic inflammation and this was accompanied by an increase of $Foxp3^+$ T_{reg} cells [70].

Furthermore, as mentioned before, mucosal $CD103^+$ DC can also induce $Foxp3^+$ T_{reg} cells in a TGF-β and retinoic acid dependent manner. These inducible $Foxp3^+$ T_{reg} cells are thought to play a major role in the induction of tolerance [23,24,88]. However, it is not clear yet whether there is a role for the AhR in the induction of $Foxp3^+$ T_{reg} cells by $CD103^+$ DC in the intestine.

3 Food allergy

3.1 Prevalence and risk factors

Food allergy is defined as an 'adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food' [89]. Currently, food allergy is a major health problem, with an estimated prevalence of about 5% in young children and 3-4% in adults and the prevalence is increasing [90,91]. The major food allergens are milk, egg, peanut three nuts, shellfish, fish, wheat and soy [90]. Exposure to these food allergens causes only mild allergic reactions in most cases. However, in severe cases anaphylaxis can be induced, mainly in people allergic to peanut and tree nuts [92].

Allergies are the result of a complex interplay between genetic predisposition and environmental factors. The development of food allergy is determined by genetic predisposition to allergies, but the specific genetic loci that may modulate individual risk of food allergy remain to be identified [93]. Furthermore, reported effects of breastfeeding on food allergy are contradicting, as some report no protective effects and others do [94,95]. However, delayed introduction of solid foods in infants is associated with higher risk of food allergic diseases [96]. In addition, early colonization of the intestine by appropriate microbiotica is important for the development of a

healthy immune system, including the development of oral tolerance to food antigens [96]. Other risk factors for the development of food allergy are the dose, the route of exposure (e.g. allergic sensitization might occur via inflamed skin), concomitant exposure to drugs and the biochemical nature of a food allergen [96,97]. Furthermore, the acid content of the stomach plays a role in allergic sensitization, because an increased pH in the stomach is associated with increased allergic sensitization [98,99]. Nevertheless, it is not yet elucidated how all these factors exactly play a role in the development of food allergy [96].

3.2 Allergic sensitization versus oral tolerance

After ingestion, most food proteins are digested by gastric acids and enzymes in the stomach and intestine. The remaining food proteins and peptides are subsequently transferred from the lumen to the mucosa via gut epithelial cells, by specialized M cells present in Peyer's Patches or by direct sampling of mucosal DC. In the mucosa, DC process these proteins and peptides, move to T cell areas and present them on MHCII where they can interact with naïve T cells [19]. The presence of costimulatory molecules is important in determining the subsequent immune response: interaction of CD28 present on T cells with CD80 and CD86 present on DC induces T cell activation, whereas interaction of CD80 and CD86 with CTLA-4 present on T cells down-regulates T cell activation [100,101]. CTLA-4 signaling has been shown to be important for the regulation of the intensity of food allergic responses, because blocking of CTLA-4 during the induction of food allergy increases food allergic responses [64].

In normal individuals, presentation of processed proteins and peptides by DC to naïve T cells will lead to the induction of oral tolerance, a state of active inhibition of immune responses to antigens encountered in the intestine. Two types of oral tolerance are known: high-dose tolerance and low-dose tolerance. A high dose of protein results in tolerance induced by T cell clonal anergy and apoptosis, whereas a low dose of protein results in active suppression of the immune reaction via induction of T_{reg} cells (Th3, Tr1, $CD4^+CD25^+$, $CD4^+CD25^+Foxp3^+$) (figure 4).

However, in certain individuals, caused by factors mentioned before, presentation of a food protein by DC on MHCII may result in the induction and activation of Th2 cells. By secreting cytokines (e.g. IL-4, IL-5, IL-13), these Th2 cells stimulate B cells to produce allergen-specific IgE which is distributed systemically. This allergen-specific IgE binds to high affinity IgE receptors (FcεRI) present primarily on mast cells and

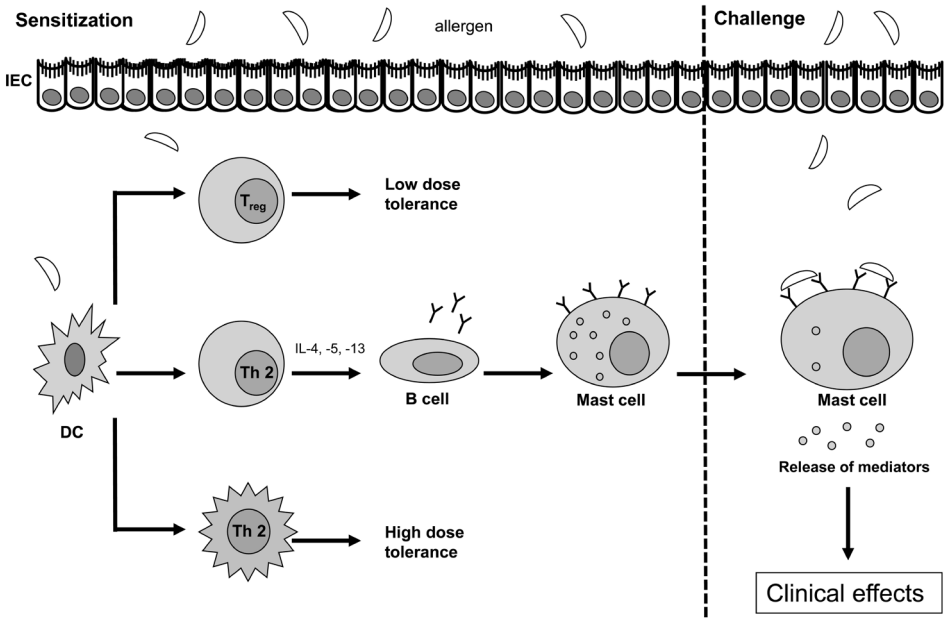

Figure 4. Schematic representation of an IgE-mediated allergic reaction to food allergens. In normal individuals, presentation of processed proteins and peptides by DC to naïve T cells will lead to the induction of high-dose tolerance (induced by T cell clonal anergy and apoptosis) or low-dose tolerance (active suppression of the immune reaction via induction of T_{reg} cells). However, in certain individuals, presentation of a food protein by DC on MHCII may result in the induction and activation of Th2 cells. By secreting cytokines (e.g. IL-4, IL-5, IL-13), these Th2 cells stimulate B cells to produce allergen-specific IgE which is distributed systemically. This allergen-specific IgE binds to high affinity IgE receptors (FcεRI) present primarily on mast cells and basophils. This process is known as allergic sensitization. Re-exposure (challenge) to the food antigen results in crosslinking of the IgE bound on FcεRI, which provokes degranulation of mast cells and basophils and the release of mediators (such as histamines, cytokines, leukotrines, prostaglandins and platelet-activating factor). This leads to a variety of clinical effects.

basophils. This process is known as allergic sensitization. Re-exposure (challenge) to the food antigen results in crosslinking of the IgE bound on FcεRI, which provokes degranulation of mast cells and basophils and the release of mediators (such as histamines, cytokines, leukotrines, prostaglandins and platelet-activating factor). The release of mediators may lead to a variety of symptoms occurring within minutes to hours affecting the skin (eczema, angioedema, urticarial), gastrointestinal tract (nausea, vomiting, diarrhea) and airways (cough, wheeze). In severe cases, anaphylaxis is induced. Anaphylaxis can include any of these symptoms and additionally may include cardiovascular symptoms (figure 4) [89,102-104].

3.3 Foxp3$^+$ regulatory T cells and food allergy

The importance of Foxp3$^+$ T_{reg} cells in food allergic responses is demonstrated in several ways. Individuals that suffer from the immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome do not have functional Foxp3$^+$ T_{reg} cells and some of these people suffer from severe food allergy [105]. Furthermore, Foxp3 and IL-10 gene expression (measured in nucleated cells from peripheral blood) from children with IgE mediated food allergy is lower compared to healthy children and children that acquired tolerance to food express more Foxp3 than children with food allergy [106].

3.4 Therapy

Currently, the primary recommendation for food allergy is strict avoidance of the causal food. Antihistamines and corticosteroids are used to treat acute allergic symptoms and in anaphylaxis epinephrine is used a first line treatment. However, these drugs only ameliorate symptoms and do not stop or cure progression of disease [90,91].

A potentially curative treatment for (food) allergy is allergen-specific immunotherapy (SIT). SIT is already used for more than 100 years as a desensitizing therapy [107] and is presently successfully used to treat allergic rhinitis, asthma and wasp and bee venom hypersensitivity [108]. The aim of SIT is to induce peripheral T cell tolerance and to increase the thresholds for IgE mediated mast cell and basophil activation [109]. The high success rate of SIT (varying from 80-90%, depending on the antigen) and the interruption of allergic sensitization is promising. However, disadvantages are the long duration of the treatment (3-5 years), the amount of allergen injections needed (about 80) and allergic side effects [110]. Key mechanisms of successful SIT are the induction of regulatory T cells ($CD4^+CD25^+Foxp3^+$ and Tr1 cells) and an increase of IgG4 (in humans) that may capture the allergen before reaching effector cell-bound IgE [109].

In food allergy, especially peanut allergy, immunotherapy has not been successfully applied yet, because of the unacceptable high risk for side effects, including anaphylactic reactions [111]. Therefore, immune modulators are used to lower these side effects and to improve safety and efficacy of immunotherapy. For example, *Nigella sativa* seed supplementation may improve immunotherapy by increasing the activity of polymorphonuclear leukocytes [112]. In addition, probiotics, oligomannose-coated liposomes and vitamin D3 have been shown to improve immunotherapy by

increasing Th1 and T_{reg} cells and decreasing Th2 cells [113-116]. Moreover, activation of the innate immune system by toll-like-receptor (TLR) agonists, including TLR-4 agonist MPL and a phosphorothioate oligodeoxyribonucleotide immunostimulatory sequence of DNA containing a CpG motif, appear to improve immunological and clinical responses in allergic patients [117,118]. Furthermore, omalizumab, a humanized, monoclonal anti-IgE antibody that binds to circulating IgE molecules, improves safety and efficacy of immunotherapy by interrupting the allergic cascade downstream the IgE production of B cells [119].

Next to SIT, treatments involving activation of the intestinal innate immune system via TLRs might be effective in suppressing food allergy by skewing immune responses from Th2 towards Th1 [120]. For instance, a specific herbal formula reduces peanut-induced anaphylaxis via IFN-γ producing $CD8^+$ T cells [121]. In addition, dietary intervention by the use of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (GOS/FOS) in dietary products suppresses food allergic responses via $CD25^+$ T_{reg} cells and an increase of Th1 responses [122,123]. In infants, this formula decreases plasma levels of total IgE, IgG1, IgG2 and IgG3, but not IgG4 [124].

Furthermore, retinoic acid might represent a potential new target to treat food allergy, because retinoic acid imprints T and B cells with gut-homing signatures and induces, in the presence of TGF-β, $Foxp3^+$ T_{reg} cells via $CD103^+$ DC [125,126]. In addition, high levels of retinoic acid in the intestine can promote class switching of B cells to IgA [127]. IgA binds and neutralizes food proteins that retained their immunogenic epitopes after digestion [128].

Finally, vitamin D deficiency has been epidemiologically linked to an increase of food allergy [129]. Vitamin D acquired through skin exposure to sunlight or via the diet, plays an important role in the immune system. The vitamin D receptor is expressed by T cells, activated B cells, monocytes, DC and epithelial cells. Activation of this receptor results in regulation of both innate and adaptive immune responses. Therefore, it has been proposed that early correction of vitamin D deficiency might decrease food allergy by enhancing proper functioning of the (intestinal) immune system [129].

However, all these suggested treatments or targets of treatments are still in the experimental or preclinical phase of research.

4 Mouse model for food allergy

The process of allergic sensitization, including cell functioning and associated processes that take place in the intestine and other lymphoid organs, cannot be studied in humans for practical and ethical reasons. Therefore, animal models are widely used to study mechanisms of allergic sensitization and to identify therapeutic strategies.
In this thesis, a mouse model for IgE-mediated hypersensitivity responses to peanut was used in order to investigate the role of the AhR in food allergic sensitization and to study the therapeutic potential of the AhR in food allergic responses. In this model, mice are exposed several times to peanut extract (PE) in the presence of the mucosal adjuvant cholera toxin [130]. This results in the induction of PE-specific IgE and mast cell degranulation upon challenge. The model of peanut allergic sensitization using cholera toxin is functional in both C57BL/6 mice and C3H/HeOuJ mice and mimics the clinical and immunological characteristics of peanut allergy in human patients [131]. The role of the AhR in this food allergy model was studied by using the prototypical AhR ligand TCDD, because it is one of the most potent AhR ligand known, it is hardly metabolized and it is relatively slowly excreted (half life time in mice 10-12 days) [3,132,133].

5 Scope of this thesis

AhR activation may alter immune responses by affecting the functionality of DC, the Th1/Th2 cell balance and the Th17/T_{reg} cell balance. In food allergy, these cell subsets are important in regulating allergic sensitization and oral tolerance. Therefore, the aim of this thesis was to investigate whether and how AhR activation suppresses allergic sensitization and if AhR activation could improve immunotherapy for food allergy.
In **chapter 2** we investigated whether AhR activation by the prototypical AhR ligand TCDD suppresses peanut allergic sensitization. The role of $CD4^+CD25^+Foxp3^+$ T_{reg} cells was studied by depleting these T_{reg} cells using the mouse antibody anti-CD25. In **chapter 3**, we looked whether AhR activation increased the absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells or decreased other T cells and preserved $CD4^+CD25^+Foxp3^+$ T_{reg} cells. Because AhR activation has been shown to suppress or to exacerbate

immune responses, probably depending on the AhR ligand and the cytokine milieu, we studied the potential of the non-dioxin-like AhR ligands FICZ, β-NF and 6-MCDF to suppress allergic sensitization in **chapter 4**. We also studied the possible role of metabolism, affecting the bioavailability of these non-dioxin-like AhR ligands in this chapter. In **chapter 5**, the effect of AhR activation by TCDD on DC subsets and their functionality in peanut allergic sensitization is described. In **chapter 6**, we investigated whether AhR activation can be used to improve immunotherapy for peanut allergy. Finally, the studies described in this thesis are summarized and discussed in **chapter 7**.

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Chapter 2

Activation of the aryl hydrocarbon receptor suppresses sensitization in a mouse peanut allergy model

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Abstract

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_{reg}) cells. In addition, it has been shown that activation of the aryl hydrocarbon receptor (AhR) regulates T cell responses by induction of T_{reg} cells. Therefore, we hypothesized that activation of the AhR-pathway 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. C3H/HeOuJ mice (AhR^{b-2}) 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 BW) on day -3 and 11 orally. The functional role of $CD4^+CD25^+Foxp3^+$ T_{reg} 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, IgG2a and PE-induced IL-5, IL-10, IL-13). The percentage, but not the number, of $CD4^+CD25^+Foxp3^+$ T_{reg} cells dose-dependently increased by AhR activation in both spleen and mesenteric lymph nodes. Depletion of $CD4^+CD25^+Foxp3^+$ T_{reg} 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 $CD4^+$ cell population towards $CD4^+CD25^+Foxp3^+$ T_{reg} 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

Introduction

In Western Countries, food allergy affects about 5% of young children and 3-4% of adults [1]. Most of these people only have mild allergic reactions after exposure to a food allergen, but in severe cases anaphylaxis can be induced [1-3]. The major allergens causing food allergy are peanut, tree nuts, shellfish, fish, wheat, milk, egg and soy [1]. These allergens can enter the body via the gastrointestinal mucosal immune system (GALT). 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 [2,4,5]. 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 (DRE) [6]. 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 [7,8]. Recently, it has been shown that activation of the AhR by TCDD, VAG539 or ITE results in the induction of regulatory (T_{reg}) cells in experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveoretinitis (EAU) and graft versus host disease (GvHD) [9-14]). In contrast, activation of the AhR by the tryptophan photoproduct FICZ has been shown to induce Th17 cells [11,15]. So, the AhR can regulate both T_{reg} and Th17 differentiation, dependent on the ligand.
In food allergy T_{reg} 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 (IPEX) syndrome, which can be accompanied by severe food allergy [16]. 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 [5]. 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 [17]. 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 [18-24]. In addition, one epidemiological study found a negative correlation between serum IgE and dioxin-like compounds in humans [25]. 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) [6,7,26].
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 towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells may underlie this suppression.

Materials and methods

Mice and reagents
Female 4-5 week old C3H/HeOuJ mice (AhR^{b-2}), purchased from Charles River (France), were maintained under controlled conditions (relative humidity of 50-55%, 12h light/dark cycle, temperature of 23±2 ºC) 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, 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% vol/vol anisole). Anisole diluted in corn oil was used as vehicle-control (0.07% vol/vol).
Peanut protein extract (PE) (30 mg/ml) was prepared from peanuts from the Golden Peanut Plant (USA, provided by Intersnack Nederland BV, The Netherlands) as described previously [27]. Peanut extracts were prepared according to standard procedures and checked for protein content by BCA analysis (Pierce, IL). Cholera toxin (CT) was purchased from List Biological Laboratories (Inc, CA).

Experimental design

In setup 1 (figure 1, setup 1), C3H/HeOuJ mice (n=6-8) were sensitized to PE by oral exposure to PE (6 mg PE, 200 µl/mouse) with CT (15 µg/mouse) on three consecutive days (day 0, 1, 2) followed by weekly dosing (day 7, 14, 21, 28). On day -3 and 11 mice were exposed orally to different doses of TCDD (0.6, 1.7, 5 or 15 µg/kg body weight (BW)).

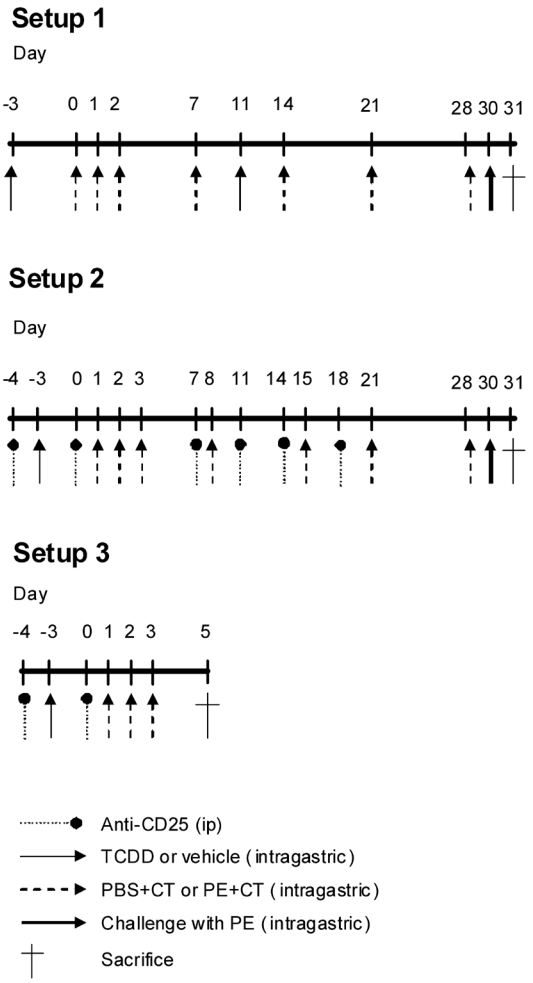


Figure 1. Treatment protocol of setup 1, setup 2 and setup 3 as described in ' Materials and Methods' section.

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 (figure 1, setup 2) [5,28,29]. Anti-CD25 mAb treatment depletes mostly $CD4^+CD25^+Foxp3^+$ cells. C3H/HeOuJ mice (n=6-8 per group) were treated ip with purified rat anti-CD25 (IL-2Rα) mAb (clone PC61, provided by Bioceros B.V., The Netherlands) (200 µg/mouse) on day -4, 0, 7, 11, 14 and 18. In setup 2, TCDD (15 µg/kg BW) was administered orally only on day -3 to prevent interference in the T_{reg} cell depletion by anti-CD25 mAb.
In both setups, mice were challenged intragastrically with PE (12 mg/mouse) on day 30 and blood samples were taken after 30 minutes. 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 $CD4^+CD25^+Foxp3^+$ T_{reg} cells during sensitization to peanut were investigated. C3H/HeOuJ mice (n=4 per group) were treated ip with purified rat anti-CD25 (IL-2Rα) mAb (clone PC61, 200 µg/mouse, provided by Bioceros B.V., The Netherlands) or control antibody (purified rat anti-GL113 mAb, 200 µg/mouse, provided by Bioceros B.V., The Netherlands) on day -4 and 0 and TCDD (15 µg/kg BW) was administrated 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 (50 mM, 1,15% KCL, pH=7.4). The microsomal fraction was obtained from the homogenate by successive centrifugation for 25 minutes at 9000 x g and 85 minutes at 100.000 x g with a Beckman Coulter Optima L-90 K centrifuge. The microsomal fraction was resuspended in a sucrose solution (0.25 M). Protein concentration of the microsomes was determined by the method of Lowry using bovine serum albumin (BSA) as protein standard [30].

EROD activity
EROD activities in liver microsomes were determined in 10 µl sample containing 10-40 µg protein with 90 µl 50 mM Tris buffer (pH 7.4) containing 5 mM $MgCl_2$, 20 µM dicumarol, 2 µM 7-ethoxyresofurin and 1.5 mM NADPH. A standard curve using resofurin was generated to quantify the EROD activity. Fluorescence was measured at 37 ºC at an excitation wavelength of 530 nm and an emission wavelength of 590 nm,

every 80 seconds for 16 minutes in a Fluostar plate reader (BMG Labtechnologies GmbH, Germany). EROD activity was calculated as pmol resofurin/minute/mg 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 [27]. mMCP-1 in plasma from C3H/HeOuJ mice obtained 30 minutes 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 ($2.5*10^6$ cells/ml) were cultured in 200 µl complete RMPI 1640 (10 % FCS) in the presence of medium or PE (100 µg/ml) for 96 hours at 37 ºC, 5% CO_2. Levels of IL-5, IL-10, IL-13, IL-17a and IFN-γ 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.

FACS analysis

Single cell suspensions of spleen (after red blood cell lysis) and MLN ($1*10^7$ cells/ml) were stained with anti-CD4-FITC (clone L3T4, eBioscience, Austria) and anti-CD25-PE (clone PC61, eBioscience, Austria) (setup 1) or anti-CD4-PerCP (clone RM 4-5, BD Biosciences, USA) and anti-CD25-FITC (clone 3C7, BD Biosciences, USA) (setup 3) in FACS-buffer (PBS containing 0.25 % BSA, 0.05% NaN_3, 0.5 mM EDTA) for 30 minutes at 4 ºC. Subsequently, cells were washed with FACS-buffer and stained intracellularly for Foxp3 (Foxp3-APC, clone FJK-16s, eBioscience, Austria) according to the manufacturer's instructions. Analysis was performed on a FACScan with standard FACSflow using CellQuest software (BD Biosciences, USA).

Statistical analysis

Results are presented as the mean ± 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 (figure. 1). Compared with PE-sensitized vehicle control mice, TCDD treatment slightly decreased body weight (BW) at doses of 5 and 15 µg/kg BW (21.31±0.38 vs 19.26±0.37 (p<0.05) and 19.49±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, figure 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 µg/kg BW (figure 2).

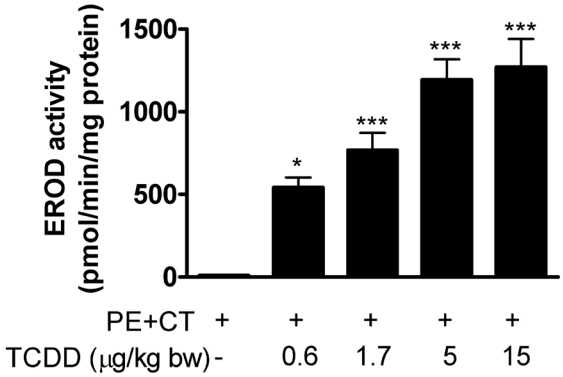


Figure 2. Activation of the AhR by TCDD dose-dependently increased EROD activity in hepatic microsomes. Mice were sensitized for peanut (PE+CT) and treated with 0.6, 1.7, 5 or 15 µg/kg BW TCDD on day -3 and 11 (setup 1). On day 31, livers were isolated and EROD activity was measured in liver microsomes.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

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 µg/kg BW) during sensitization (figure 1, setup 1). PE-sensitized mice had increased levels of PE-specific IgE, IgG1 and IgG2a (figure 3a-3c), mast cell degranulation (measured by mMCP-1) (figure 3d) and PE-induced Th2-type T-cell responses (*ex vivo* release of IL-5, IL-10, and IL-13) (figure 3e-3f) compared with nonsensitized vehicle control mice. Treatment with TCDD during PE-sensitization dose-dependently suppressed PE-specific IgE (5 and 15 µg/kg BW), IgG1 (15 µg/kg BW) and IgG2a (15 µg/kg BW) (figure 3a-c). Mast cell degranulation (mMCP1) was significantly suppressed at 5 and 15 µg/kg BW TCDD (figure 3d). Furthermore, isolated spleen cells that were exposed to TCDD *in vivo* significantly suppressed PE-induced IL-5 (at 1.7, 5 and 15 µg/kg BW), IL-10, IL-13 and IL-17a (all at 15 µg/kg BW) (figure 3e-3h). Particularly at lower doses of TCDD (0.6, 1.7 and 5 µg/kg BW), a significant increase of PE-induced IFN-γ was observed compared with PE-sensitized mice (figure 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.

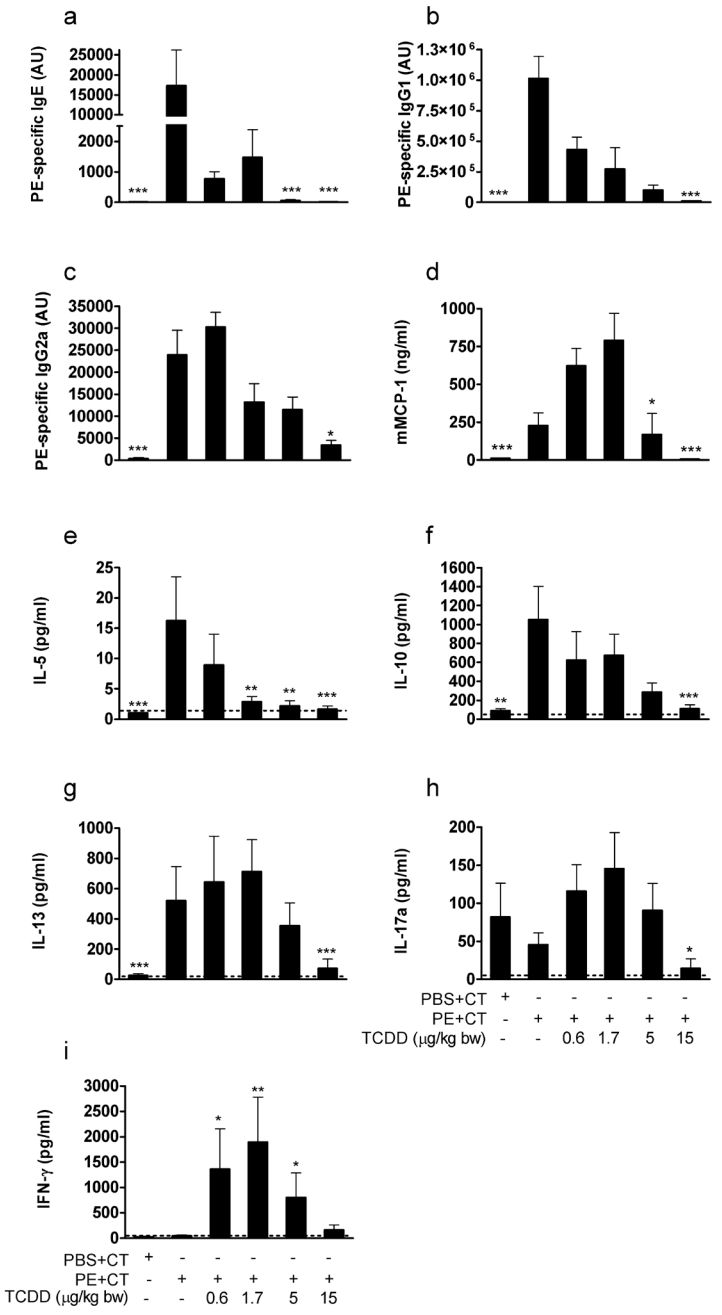


Figure 3. Activation of the AhR suppressed peanut specific antibody responses, mast cell degranulation, Th2-type cytokine responses and increased IFN-γ production (continued).

Figure 3. (continued). 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 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 minutes after challenge. Single cells suspensions of spleen cells (day 31) were cultured for 96 hours in the presence of PE. The supernatant was analyzed for IL-5 (e), IL-10 (f), IL-13 (g), IL-17a (h) and IFN-γ (i) by ELISA. No cytokine production could be detected when splenocytes were cultured in the absence of PE. 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.

Activation of the AhR by TCDD increases the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in MLN and spleen

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

Depletion of $CD4^+CD25^+Foxp3^+$ T_{reg} cells abrogates the suppressive effect of AhR activation by TCDD on the peanut allergic response

Then, we determined whether the increased percentages of $CD4^+CD25^+Foxp3^+$ T_{reg} cells observed after AhR activation were responsible for suppression of the peanut allergic response. For this purpose, $CD4^+CD25^+Foxp3^+$ T_{reg} cells were depleted using T_{reg} cell-depleting anti-CD25 mAb before and during sensitization to peanut and TCDD treatment (15 µg/kg BW).

The early effects of TCDD and/or anti-CD25 treatment during sensitization to PE were investigated on day 5 (figure 1, setup 3). Anti-GL113 mAb was used as a control antibody. TCDD treatment of PE-sensitized mice increased the percentage of

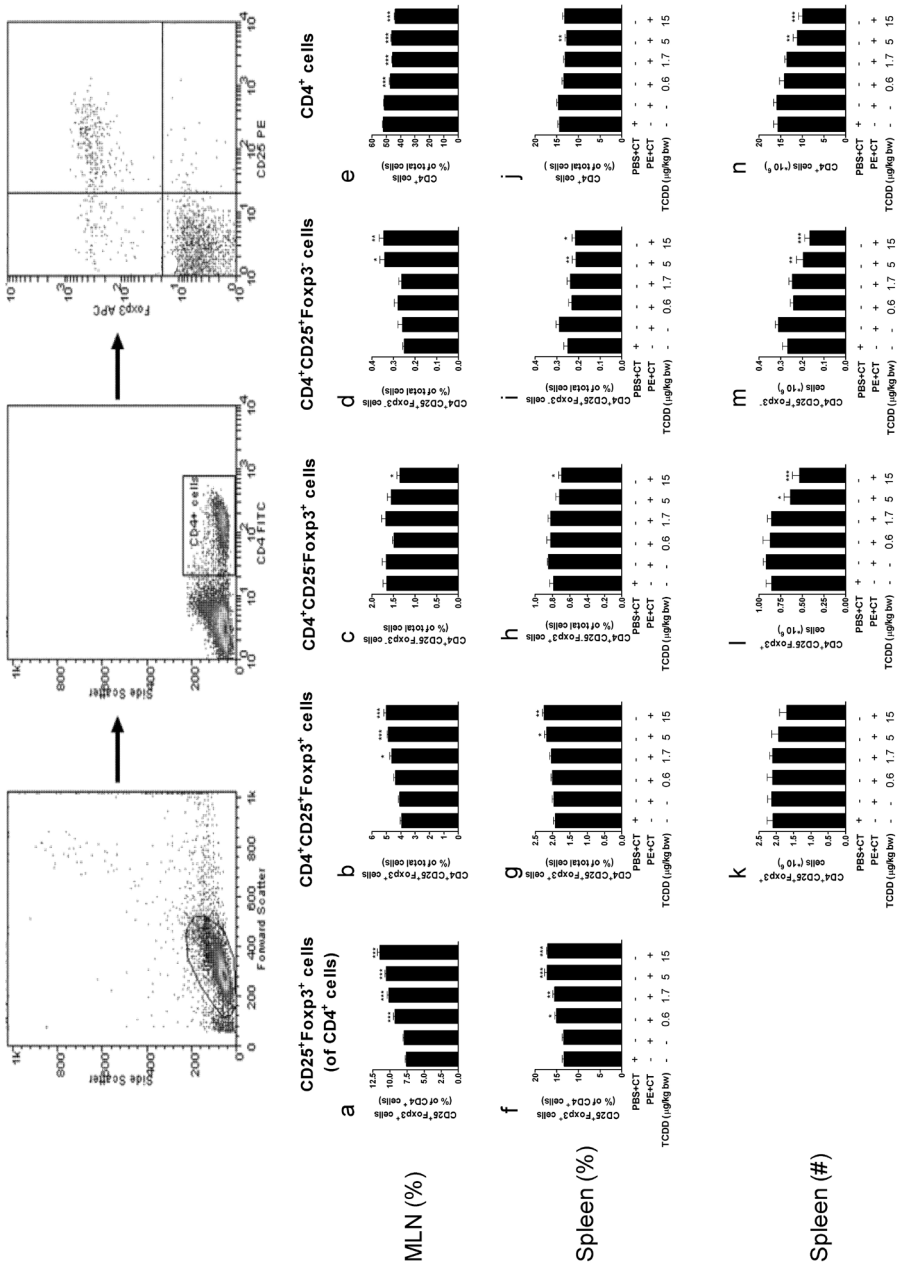


Figure 4. Activation of the AhR by TCDD increased the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in spleen and MLN (continued).

Figure 4 (continued). Mice were sensitized for peanut (PE+CT) and treated with 0.6, 1.7, 5 or 15 µg/kg BW TCDD on day -3 and 11 (setup 1). On day 31, mice were sacrificed and the percentage of $CD25^+Foxp3^+$ cells of $CD4^+$ cells (a, f), $CD4^+CD25^+Foxp3^+$ cells of total isolated cells (b, g), $CD4^+CD25^-Foxp3^+$ cells of total isolated cells (c, h), $CD4^+CD25^+Foxp3^-$ cells of total isolated cells (d, i) and $CD4^+$ cells of total isolated cells (e, j) in MLN and spleen were examined by FACS analysis. The numbers of $CD4^+CD25^+Foxp3^+$ cells (k), $CD4^+CD25^-Foxp3^+$ cells (l), $CD4^+CD25^+Foxp3^-$ cells (m) and $CD4^+$ 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.

$CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen from 9.05% to 11.37% ($p < 0.01$) (figure 5a, 5c), whereas no effect of TCDD was observed on the number of $CD4^+CD25^+Foxp3^+$ T_{reg} (Fig. 6e). Treatment of PE-sensitized mice with anti-CD25 mAb decreased the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells from 9.05% to 2.94% ($p < 0.001$), whereas $CD4^+CD25^+Foxp3^-$ and $CD4^+CD25^-Foxp3^+$ cells were not significantly affected (figure 5b). This shows that anti-CD25 mAb treatment specifically depleted $CD4^+CD25^+Foxp3^+$ T_{reg} cells.

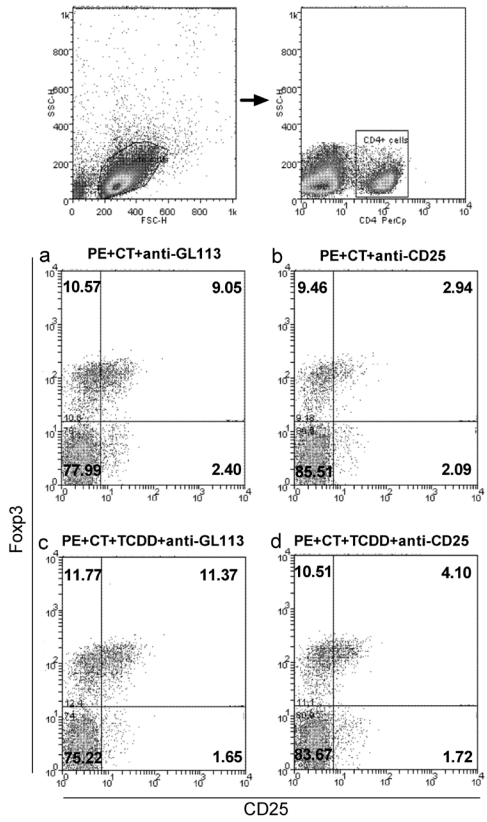


Figure 5. Early effect of anti-CD25 mAb treatment (clone PC61) (day -4 and 0) in mice sensitized for peanut (PE+CT) and treated with 15 µg/kg BW TCDD (day -3) on the percentage of T_{reg} cells. On day 5, mice were sacrificed and the percentage of $CD4^+CD25^+Foxp3^+$ cells of total cells in spleens from mice sensitized for peanut (PE+CT) treated with control mAb (anti-GL113) (a), depleted for $CD25^+$ cells (b) or/and treated on day -3 with 15 µg/kg BW TCDD (c, d) was examined by FACS analysis (setup 3). For detection of CD25 expression, clone 3C7 was used. Numbers within the quadrants indicate mean percentages of total spleen cells (n=4).

In PE-sensitized mice that were treated with TCDD and anti-CD25 mAb (figure 5d), the percentage $CD4^+CD25^+Foxp^+$ T_{reg} cells was 4.10% versus 2.94% in PE-sensitized mice that were treated with anti-CD25 mAb only (figure 5b).In MLN, anti-CD25 mAb treatment decreased the percentage $CD4^+CD25^+Foxp3^+$ T_{reg} cells (from 5.55% to 0.97%, $p < 0.001$), whereas no effect of TCDD treatment on the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells was observed (5.55% versus 5.9%) (data not shown). The percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in 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 $CD4^+$ and $CD8^+$ cells in the spleen and slightly increased the percentage of $CD19^+$ cells on day 5 (figure 6).

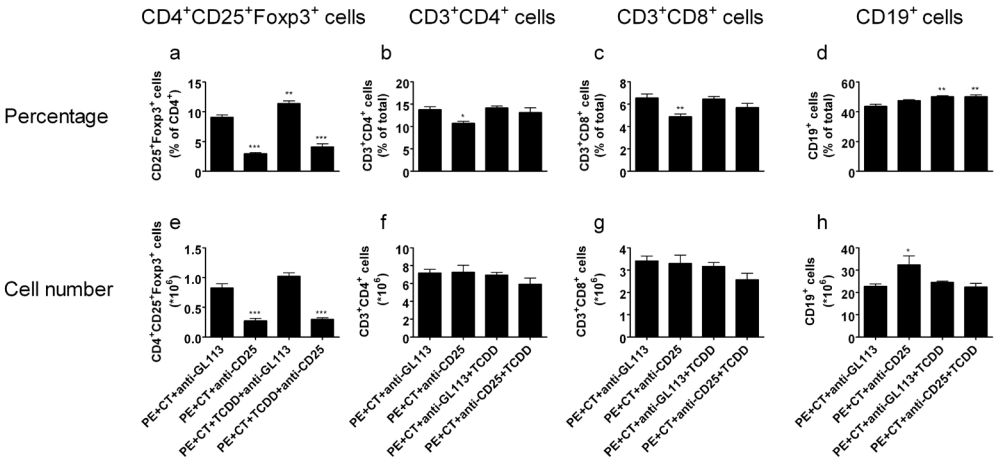


Figure 6. TCDD treatment increased the percentage, but not the number, of $CD4^+CD25^+Foxp3^+$ T_{reg} cells and did not affect the percentage and number of $CD3^+CD4^+$ and $CD3^+CD8^+$ and the number of $CD19^+$ cells in the spleen on day 5. Mice were injected with anti-CD25 (clone PC61) mAb or control 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 $CD4^+CD25^+Foxp3^+$ (a, e), $CD3^+CD4^+$ (b, f) $CD3^+CD8^+$ (c, g) and $CD19^+$ (d, 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.

The effects of TCDD and/or anti-CD25 treatment on the peanut allergic response were investigated on day 31 (figure 1, setup 2). PE-specific serum levels of IgE, IgG1 (figure 7a-7b) and PE-induced T-cell responses (*ex vivo* release of cytokines IL-5, IL-10, IL-13, IL-17a and IFN-γ, figure 7e-7i) were markedly increased by depletion of

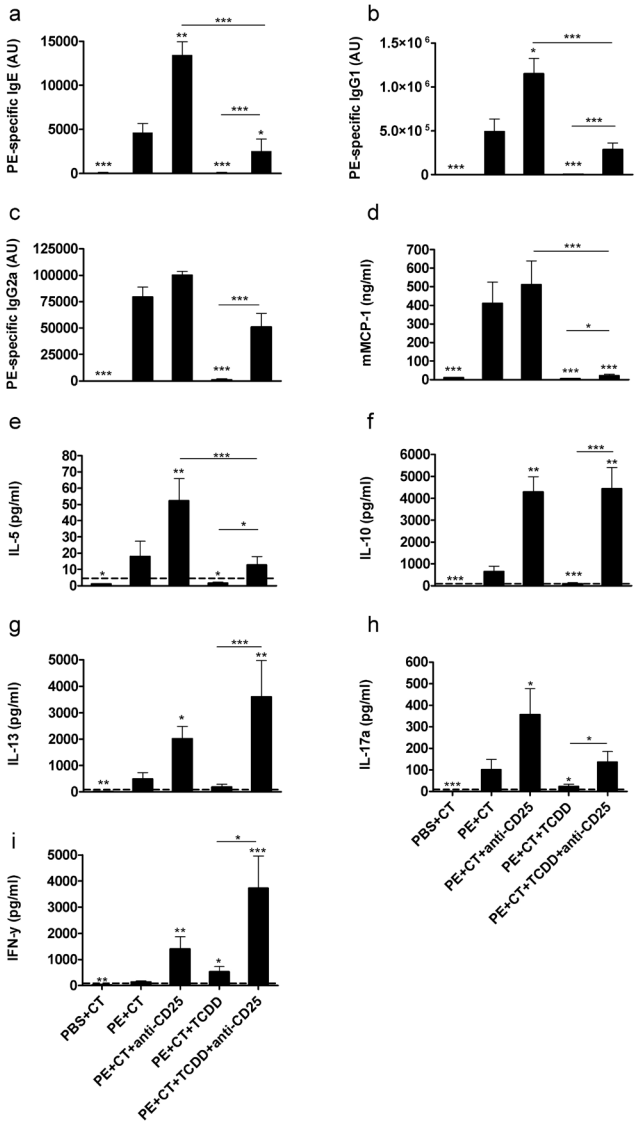
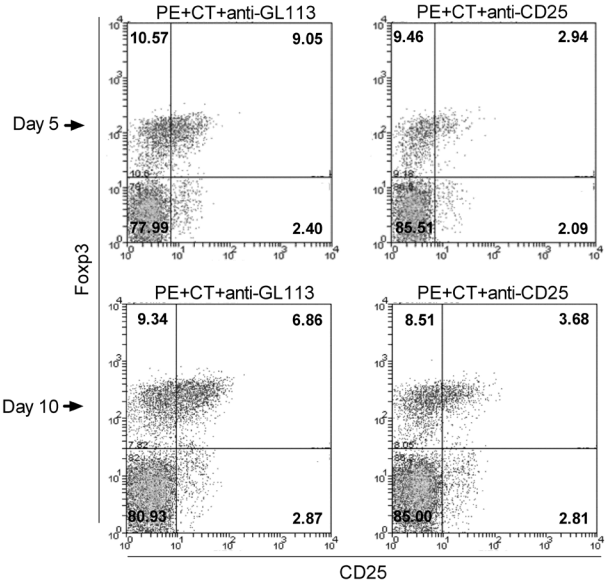


Figure 7. Depletion of $CD4^+CD25^+Foxp3^+T_{reg}$ cells abrogated the suppressive effect of AhR activation by TCDD on the peanut allergic response. Mice were injected with anti-CD25 (clone PC61) mAb or control antibody (mAb anti-GL113) prior and during sensitization to peanut and treated with 15 µg/kg 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 minutes after challenge. Single cells suspensions of spleen cells (day 31) were cultured for 96 hours in the presence of PE. The supernatant was analyzed for IL-5 (e), IL-10 (f), IL-13 (g), IL-17a (h) and IFN-γ (i) by ELISA. No cytokine production could be detected when splenocytes were cultured in the absence of PE. 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.

$CD4^+CD25^+Foxp3^+$ T_{reg} cells. This confirms earlier findings that T_{reg} cells are involved in the sensitization of allergic sensitization [5]. 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 (figure 7a, 7b) and IL-5 (figure 7e) was partly reversed after depletion of $CD4^+CD25^+Foxp3^+$ T_{reg} 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 (figure 7c, 7f-7i). It must be noted that anti-CD25 mAb treatment depletes T_{reg} cells for about 7-10 days after the last injection (day 18), which allowed $CD4^+CD25^+Foxp3^+$ T_{reg} cells to have partly recovered on day 31 [5,28].

However, supplementary data show that 11 days after the last anti-CD25 mAb treatment the effect of CD25 depletion is still apparent (Supplementary data 1). Together, these findings clearly show that TCDD increases the percentage of $CD4^+CD25^+Foxp3^+$ T_{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.



Supplementary data 1. Effect of anti-CD25 mAb treatment on the percentage of $CD4^+CD25^+Foxp3^+T_{reg}$ cells in the spleen. Mice were injected with anti-CD25 (clone PC61) mAb or control antibody (mAb anti-GL113) prior (day -4 and 0) and during sensitization to peanut (day 0, 1, 2). On day 5 and day 10 mice were sacrificed and the effect of anti-CD25 mAb treatment on the percentage of $CD4^+CD25^+Foxp3^+T_{reg}$ cells in the spleen was investigated. For detection of CD25 expression, clone 3C7 was used. Numbers within the quadrants indicate mean percentages of total spleen cells (n=4).

Discussion

T_{reg} cells have been shown to play an important role in allergic sensitization and the intensity of food allergic responses [5,16,17]. Recently, it has been shown that activation of the AhR results in the induction of T_{reg} cells [9-13]. Therefore, we examined whether activation of the AhR by TCDD suppresses Th2-mediated food allergic responses through the induction of T_{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 $CD4^+CD25^+Foxp3^+$ T_{reg} cells dose-dependently increased due to TCDD treatment. Importantly, depletion of $CD4^+CD25^+Foxp3^+$ T_{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 $CD4^+$ cell population towards $CD25^+Foxp3^+$ T_{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 [18-24]. However, no role for T_{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 µg/kg BW vs 15 µg/kw BW in our experiments) suppresses GvHD and Th1/Th17-mediated EAE and EAU through a relatively modest induction of $CD4^+Foxp3^+$ T_{reg} cells [10-12,14]. However, in these studies, the effect of AhR activation on $CD4^+Foxp3^+$ T_{reg} cells was only shown in percentages, whereas no data about absolute numbers of $CD4^+Foxp3^+$ T_{reg} cells were provided. Therefore, it is not clear whether AhR activation also increased the number of $CD4^+Foxp3^+$ T_{reg} cells or only induced a shift within the $CD4^+$ population towards $CD4^+Foxp3^+$ cells, as seen in our experiments.

Genes linked to immunoregulation such as Foxp3, transforming growth factor (TGF)-β and IL-10 contain dioxin-responsive elements (DREs) in their promotor regions [7,8]. Therefore, it is likely that immunoregulatory cells such as $Foxp3^+$ T_{reg} cells can be induced directly by AhR activation. In support of the importance of AhR in the actual generation of $Foxp3^+$ T_{reg} cells, naïve T cells from AhR KO mice showed impaired T_{reg} cell development *in vitro* [31]. Also, mutant AhR $CD4^+Foxp3^-$ cells with reduced affinity for AhR ligands appeared less capable of differentiating into $CD4^+Foxp3^+$ both *in vivo* and *in vitro* [14]. In addition, it has recently been shown that AhR activation in naïve

human T cells induces $Foxp3^+$ T_{reg} cells in the presence of TCDD and TGF-β1 [32]. Despite the strong indications that $CD4^+CD25^+Foxp3^+$ T_{reg} 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. $CD4^+CD25^+Foxp3^-$ cells [9], which were also increased in our study by TCDD treatment in MLN, and Tr1 cells producing regulatory IL-10 [32,33]. 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 [34]. 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 $CD4^+$ T cells after TCDD treatment may result from an effect of TCDD on thymocyte differentiation [35-37] or from a direct suppressive effect of TCDD on peripheral T cells on day 4-5 [38-40]. Importantly, in our experiments, TCDD did not affect the percentage and number of splenic $CD4^+$ and $CD8^+$ T cells on day 5. Interestingly, the percentage, but not the number, of $CD4^+CD25^+Foxp3^+$ T_{reg} cells was already increased on this day, i.e during initiation of sensitization. However, our data cannot exclude that TCDD 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 [18,23,24]. Interestingly, a role for the AhR in skewing the Th1/Th2 cytokine balance towards Th1 has been described by others [22,23]. 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 [41]. Therefore, shifting the Th1/Th2 cytokine balance towards 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 [42].

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 $CD4^+$ 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 centres [43] and plasma cell differentiation [44]. However, 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 (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 DCs producing retinoic or indoleamine 2,3-dioxygenase (IDO), both associated with the development of T_{reg} cells [14,45,46]. In other studies TCDD has been shown to activate DCs in the presence and absence of an antigen [47], to selectively decrease $CD11c^{high}CD8\alpha^-33D1^+$ splenic DCs specialized at activating $CD4^+$ T cells [48] and also to reduce the amount of splenic DCs [48,49]. 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 [50]. 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 [6]. The AhR is strongly expressed along the small intestine [51] 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, provided that 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 [11]. Moreover, the existing cytokine milieu (especially the presence of IL-6 and/or TGF-β) appears to be very important in deciding whether AhR activation results in an inflammatory or a regulatory immune response [32,33,52]. Furthermore, it has been reported that TCDD impairs maintenance of oral tolerance against OVA in a high-dose oral tolerance

model [51]. 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 towards $CD4^+CD25^+Foxp3^+$ 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.

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Chapter 3

Activation of the aryl hydrocarbon receptor reduces the number of precursor and effector T cells, but preserves thymic $CD4^+CD25^+Foxp3^+$ regulatory T cells

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Abstract

Aryl hydrocarbon receptor (AhR) activation suppresses immune responses, including allergic sensitization, by increasing the percentage of regulatory (T_{reg}) cells. Furthermore, AhR activation is known to affect thymic precursor T cells. However, the effect of AhR activation on intrathymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells is unknown. Therefore, we investigated the effect of AhR activation on the percentage and number of $CD4^+CD25^+Foxp3^+$ T_{reg} 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 $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ precursor T cells, but not the number of thymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells. TCDD treatment increased the number of splenic $CD4^+CD25^+Foxp3^+$ T_{reg} 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 $CD4^+CD25^+Foxp3^+$ T_{reg} cells, Th1, Th2 and cytotoxic T cells induced by peanut sensitization.

Together, TCDD treatment preserves thymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells and decreases peripheral T helper and cytotoxic T cells. This effect of TCDD may contribute to the increased influence of $CD4^+CD25^+Foxp3^+$ T_{reg} cells on immune mediated responses and to the understanding of how AhR activation modulates immune mediated diseases, including food allergy.

Key words:

Aryl hydrocarbon receptor, TCDD, regulatory T cells, thymus, spleen, mesenteric lymph nodes.

Introduction

The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix protein and upon ligation the AhR acts as a transcription factor by 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) [1]. 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 $CD4^+Foxp3^+$ regulatory (T_{reg}) cells [2-5]. However, a numerical increase of $CD4^+Foxp3^+$ T_{reg} cells after AhR activation has not been demonstrated in these studies. Possibly, the increase of the percentage of T_{reg} cells observed after AhR activation results from a decrease of other cell subsets [6]. Nevertheless, *in vitro* treatment of naïve $CD4^+$ T cells with TCDD or the endogenous AhR ligand 6-formylindolo[3,2-b]carbazole (FICZ) induces $CD4^+Foxp3^+$ T_{reg} cells from naïve T cells in the presence, but not in the absence of TGF-β [7]. Currently, the question whether AhR activation induces, expands or preserves T_{reg} cells, in particular $CD4^+CD25^+Foxp3^+$ T_{reg} 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 [8-15]. A single dose of 0.1 µg/kg BW TCDD is already effective in decreasing thymocyte numbers in mice [16]. 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 [17]. Notably, the effect of AhR activation on $Foxp3^+$ T_{reg} cells in the thymus has not been described in any of the previously mentioned *in vivo* studies. Quintana et al. only demonstrated that the frequency of $Foxp3^+$ T_{reg} cells was unchanged in mice with reduced AhR ligand binding capacity [4]. The lack of studies into the effects of AhR ligands on thymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells is remarkable given the known alterations induced by AhR activation on T cell precursors in the thymus and the substantial contribution of thymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells to the peripheral $CD4^+CD25^+Foxp3^+$ T_{reg} population [18,19].

Activation of the AhR has been shown to suppress Th2-type immune responses by decreasing antigen-specific antibody levels and Th2-related cytokines [20-26]. In this Th2-mediated immune response, including allergic sensitization, T_{reg} cells play an important role [27,28]. Recently, we have shown AhR activation suppresses allergic sensitization at least in part by increasing the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells [29].

In light of the prominent thymocyte depleting effect of TCDD, we here investigated the effect of TCDD on the intrathymic $CD4^+CD25^+Foxp3^+$ T_{reg} 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 [28].

Materials and methods

Mice and reagents

4-5 week old female C3H/HeOuJ mice (AhR^{b-2}), purchased from Charles River (France), were maintained under controlled conditions (relative humidity of 50-55%, 12h light/dark cycle, temperature of 23±2 ºC) 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).

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 (day 3, 4, 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 [29,30]. 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 wellfare committee. Experiments were performed in duplicate, with similar results.

Flow cytometry analysis

Single cell suspensions of thymus, spleen (after red blood cell lysis) and MLN ($2.5*10^6$ cells for extracellular stainings, $1*10^7$ cells/ml for intracellular stainings) were incubated for 15 minutes with anti-mouse CD16/32 (clone 93, eBioscience, Austria) in fluorescence activated cell sorting (FACS) buffer (PBS containing 0.25 % BSA, 0.05% NaN_3, 0.5 mM EDTA) and subsequently stained extracellularly in FACS-buffer for 30 minutes at 4 ºC 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]) $1*10^7$ cells/ml were stimulated for 4 h at 37 ºC 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 manufactur's instructions. Analysis of all stainings was performed on a BD FACSCanto II using BD FACS Diva software (BD Biosciences, USA).

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.

Results

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 (figure 1). In the MLN, TCDD treatment decreased cell numbers independent of PE-sensitization (figure 1). Together, these results show that, also during PE-sensitization, TCDD treatment affects the cellularity in the thymus and the MLN, but not in the spleen.

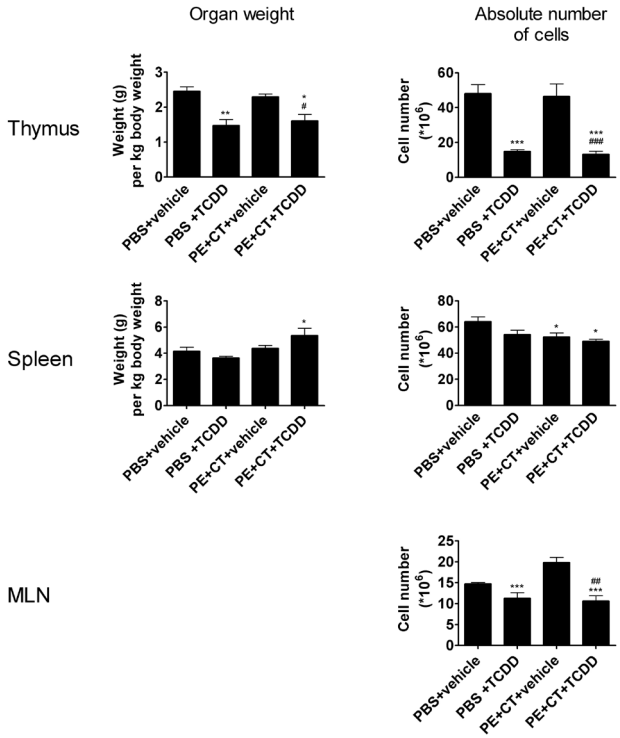

Figure 1: Effect of AhR activation by TCDD on thymus and spleen weight and cell number in spleen, thymus and MLN. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE+CT) on day 3, 4, 5 or treated with PBS. On day 8, mice were sacrificed and thymus, spleen and MLNs were isolated. Relative weights of thymus and spleen and cellularity of thymus, spleen and MLN were determined. Values are presented as mean ± 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.

Effect of AhR activation by TCDD on thymic T cell precursors and $CD4^+CD25^+Foxp3^+$ T_{reg} 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 $CD4^+CD25^+Foxp3^+$ T_{reg} cells. In both non-sensitized and PE-sensitized mice, TCDD treatment decreased the absolute number of $CD4^+CD8^+$ cells, followed by $CD4^+CD8^-$, $CD4^-CD8^-$ and $CD4^-CD8^+$ cells, respectively (figure 2).

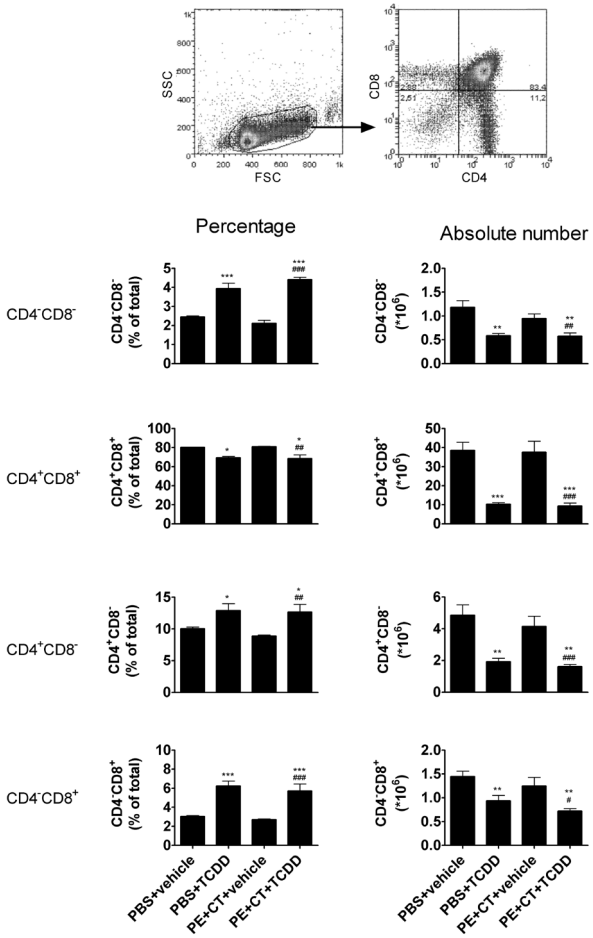

Figure 2: Effect of AhR activation by TCDD on thymic T cell precursors. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE+CT) on day 3, 4, 5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ 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, ### $p < 0.001$ compared with PE+CT.

As a consequence of the strong decrease of the number of $CD4^+CD8^+$ cells, the frequencies of the other cell populations, in particular $CD4^-CD8^-$ and $CD4^-CD8^+$ cells were increased. TCDD treatment did not affect the absolute number of thymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells compared to their appropriate controls, but increased the percentage of these cells independent of PE-sensitization (figure 3). Combined, these data show that TCDD treatment decreased the absolute number of major thymocyte subsets but did not affect the absolute number of $CD4^+CD25^+Foxp3^+$ cells. This appeared to be independent of allergic sensitization.

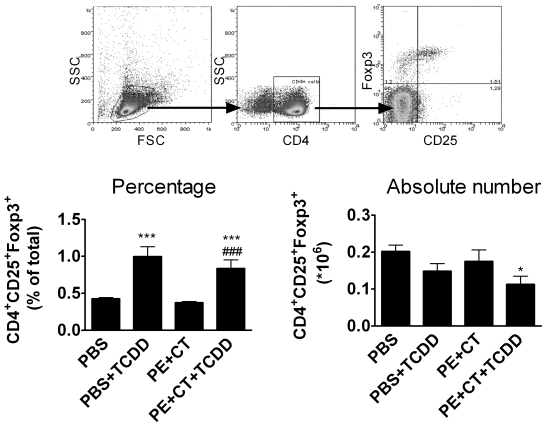

Figure 3: Effect of AhR activation by TCDD on $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the thymus during allergic sensitization. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE+CT) on day 3, 4, 5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the thymus was examined by flow cytometry analysis. Representative dot-plots of thymus cells stained with antibodies to CD4, CD25 and Foxp3 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, ### $p < 0.001$ compared with PE+CT.

Effect of AhR activation by TCDD during allergic sensitization on B cells, $CD8^+$ and $CD4^+$ T cells and $CD4^+CD25^+Foxp3^+$ T_{reg} 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 $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen and MLN after 8 days. PE-sensitization decreased the absolute number of B cells and $CD8^+$ T cells in the spleen and increased the absolute number of $CD8^+$ T cells in the MLN. The absolute number of $CD4^+$ T cells in the spleen and MLN was not affected by PE-sensitization (figure 4). In PE-sensitized mice, TCDD treatment increased the percentage of $CD19^+$ B cells in the spleen, but not in the MLN (figure 4).

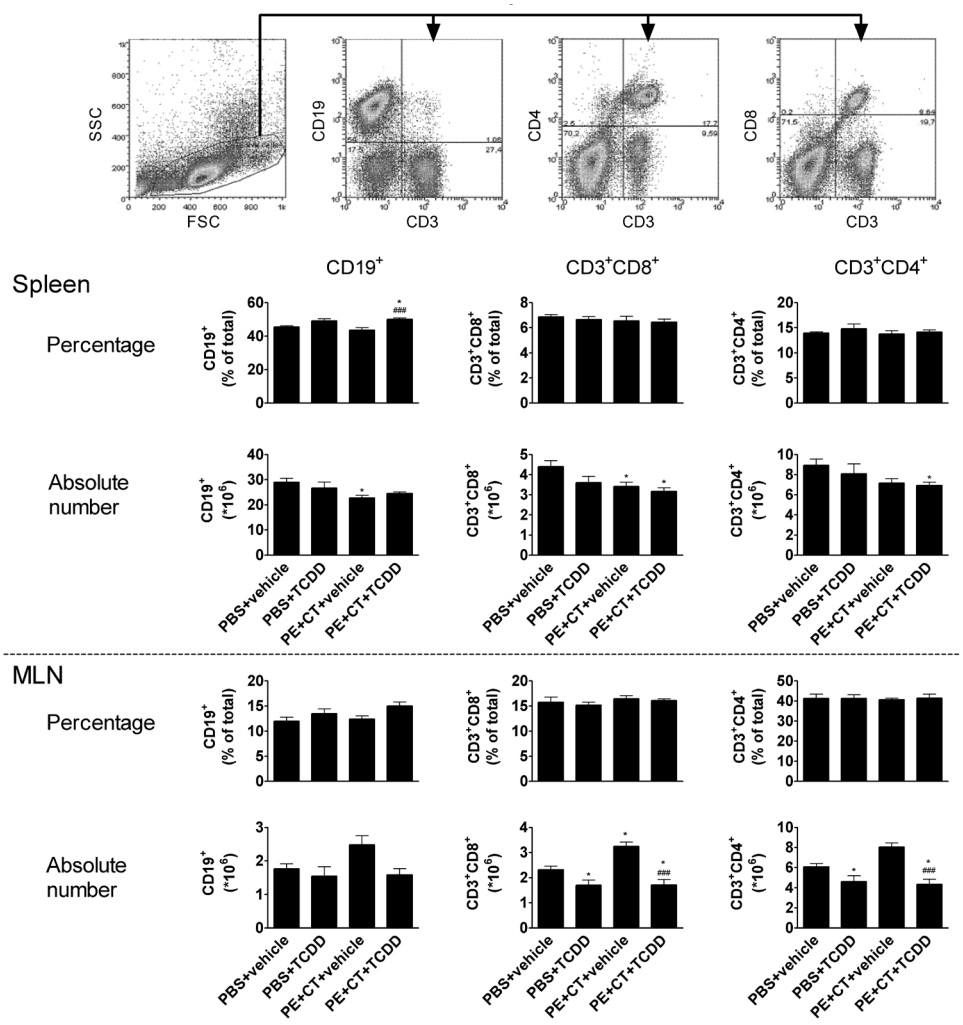

Figure 4: Effect of AhR activation by TCDD during allergic sensitization on B cells, $CD8^+$ and $CD4^+$ T cells in the spleen and MLN. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE+CT) on day 3, 4, 5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of $CD19^+$ B cells and $CD8^+$ and $CD4^+$ 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, ### $p < 0.001$ compared with PE+CT.

No effect of TCDD treatment was observed on the absolute number of $CD19^+$ B cells in the spleen and MLN (figure 4). Furthermore, TCDD treatment did not affect the percentage of $CD8^+$ and $CD4^+$ 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 (figure 4). The percentage and absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen and MLN were not affected by PE-sensitization. In the spleen, TCDD treatment increased both the percentage and absolute number of $CD4^+CD25^+Foxp3^+$ cells, independent of PE-sensitization (figure 5). In the MLN, no effect of TCDD was observed on the percentage of $CD4^+CD25^+Foxp3^+$ cells, whereas the absolute numbers were decreased by TCDD treatment in PE-sensitized mice (figure 5). Together, these data show that on day 8 TCDD treatment increased the absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen independent of PE-sensitization and decreased the absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells, $CD4^+$ and $CD8^+$ T cells compared to PBS or PE+CT controls in the MLN.

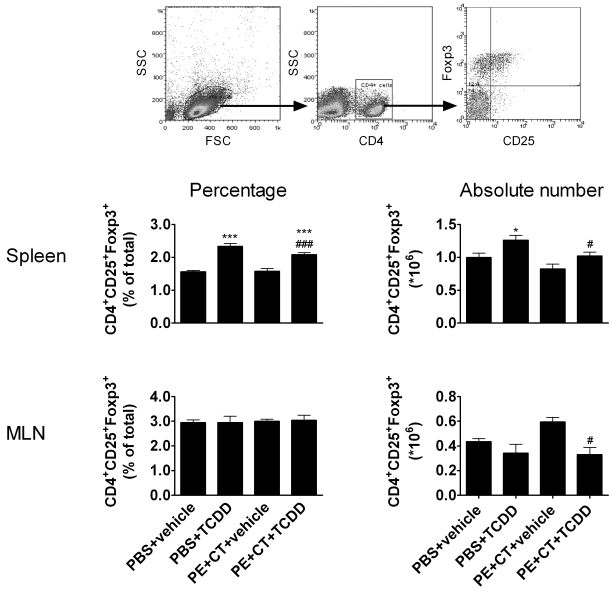

Figure 5: Effect of AhR activation by TCDD on $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen and MLN during allergic sensitization. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE+CT) on day 3, 4, 5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} 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 Foxp3 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, ### $p < 0.001$ compared with PE+CT.

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 ($CD4^+CD62L^+CD44^-$), central memory ($CD4^+CD62L^+CD44^+$) and effector memory ($CD4^+CD62L^-CD44^+$) T cells in the spleen and MLN was studied (according to Lanzavecchia et al., and Sallusto et al., [31,32]).

In the spleen, PE-sensitization decreased the absolute number of naïve $CD4^+$ T cells but did not affect central memory and effector memory $CD4^+$ T cells, whereas in the MLN PE-sensitization increased the absolute number of naïve and central memory $CD4^+$ T cells (figure 6).

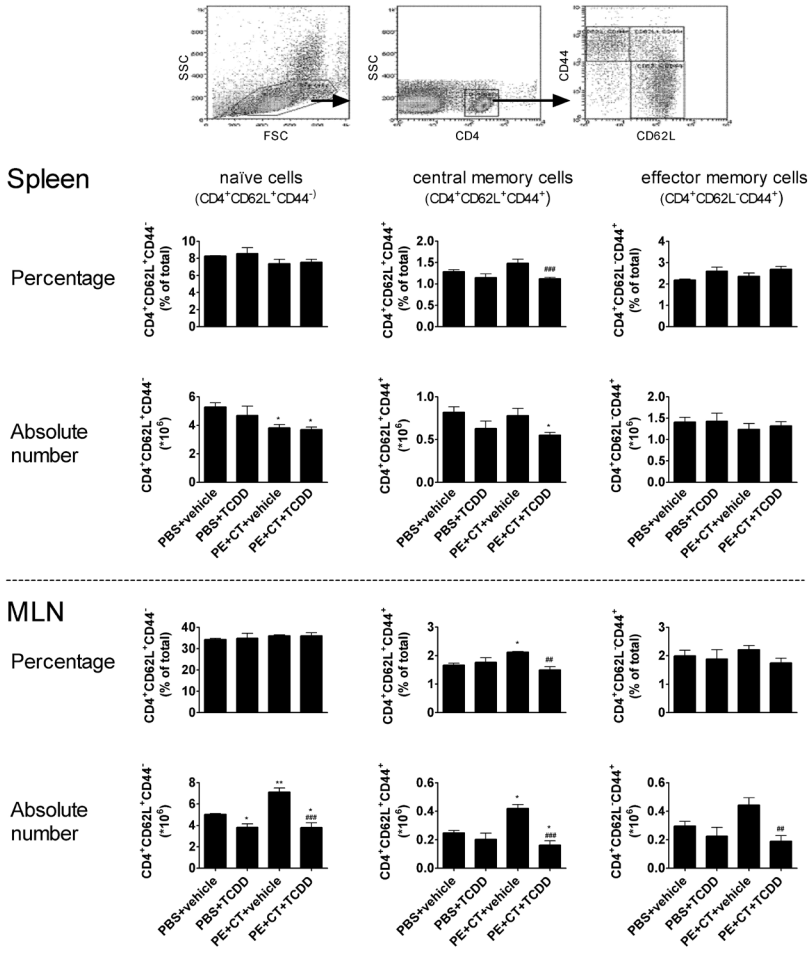

Figure 6: Effect of AhR activation by TCDD on peripheral naïve, central memory, effector memory, Th1, Th2 and cytotoxic T cells during allergic sensitization (continued).

Figure 6. (continued). Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE+CT) on day 3, 4, 5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of naïve ($CD62L^+CD44^-$), central memory ($CD62L^+CD44^+$) and effector memory ($CD62L^-CD44^+$) $CD4^+$ T cells in the spleen an 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, ### $p < 0.001$ compared with PE+CT.

Compared to PE-sensitized mice, TCDD treatment did not affect the absolute number of naïve, central memory and effector memory $CD4^+$ T cells in the spleen (figure 6). In the MLN, the increase of naïve and central memory $CD4^+$ T cells induced by PE-sensitization was decreased by TCDD treatment (figure 6). Finally, the effect of TCDD treatment on Th1 ($CD4^+IFN\text{-}\gamma^+$), Th2 ($CD4^+IL\text{-}4^+$) and cytotoxic T cells ($CD8^+IFN\text{-}\gamma^+$) 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 (figure 7). In the MLN, TCDD treatment prevented the increase of PE-sensitization induced Th2, Th1 and cytotoxic T cells (figure 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.

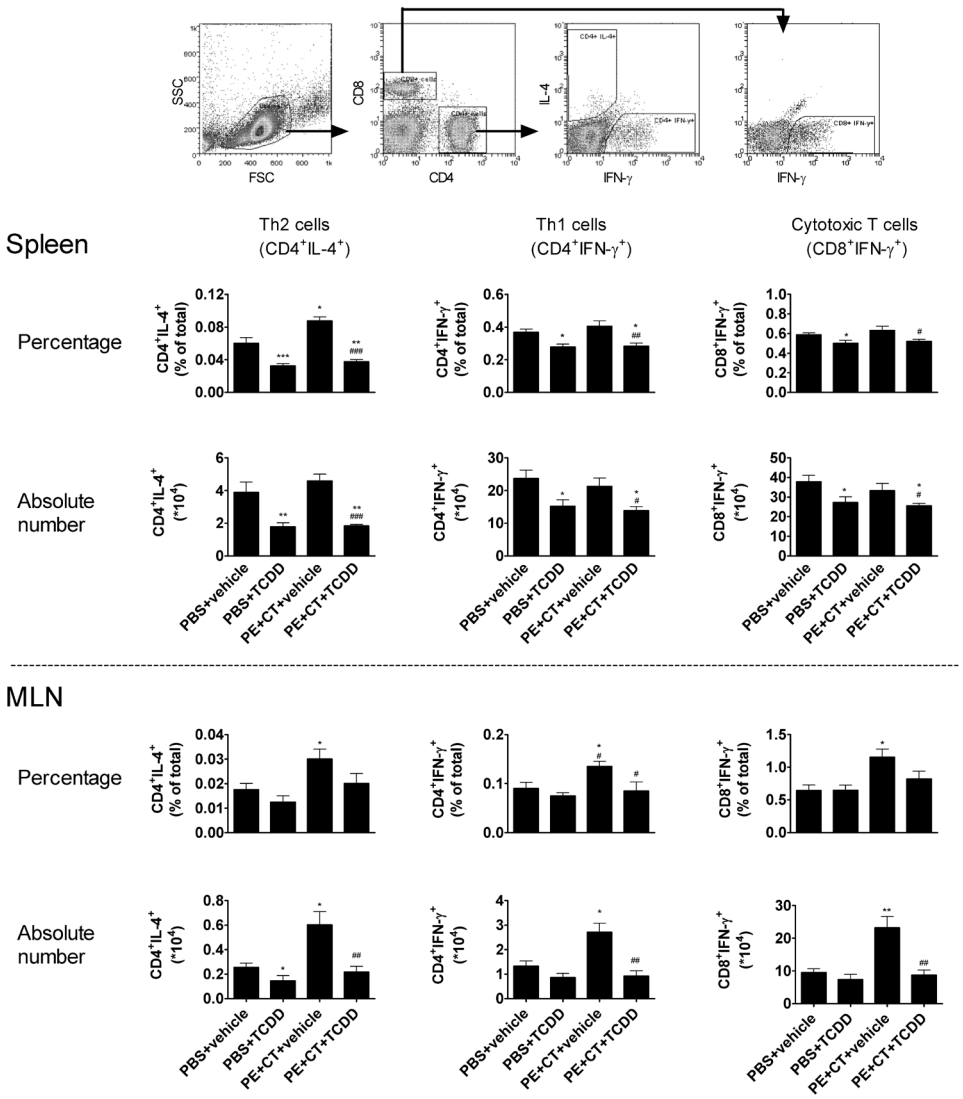

Figure 7: Effect of AhR activation by TCDD on peripheral T helper cells during allergic sensitization. Mice were treated with vehicle or TCDD (15 µg/kg BW) on day 0 and sensitized to PE (PE+CT) on day 3, 4, 5 or treated with PBS. On day 8, mice were sacrificed and the percentage and absolute number of Th2 ($CD4^+IL\text{-}4^+$), Th1 ($CD4^+IFN\text{-}\gamma^+$) and cytotoxic T cells ($CD8^+IFN\text{-}\gamma^+$) 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.

Discussion

AhR activation suppresses a variety of immune responses accompanied by an increase in the percentage of $Foxp3^+$ T_{reg} cells [2-5] and regulates the generation of type 1 regulatory T cells together with c-MAF [33]. Foxp3, transforming growth factor (TGF)-β and IL-10, all important for T_{reg} induction, contain DREs in their promoter regions [34,35] and AhR activation of naïve T cells has been shown to increase the percentage of $Foxp3^+$ T_{reg} cells *in vitro* [4,7,36]. Furthermore, tolerogenic dendritic cells producing retinoic acid or indoleamine 2,3-dioxygenase after AhR activation have been shown to increase the percentage of splenic $Foxp3^+$ T_{reg} cells *in vivo* and $Foxp3^+$ gene expression in the spleen, respectively [4,37].

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 $CD4^+CD25^+Foxp3^+$ T_{reg} cells in spleen and MLN [29]. In line with this, others also suggested that the increased percentage of $Foxp3^+$ T_{reg} cells observed after AhR activation results from a decrease of other cell subsets [6]. 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 [17]. In addition, the thymus contributes substantially to the peripheral $CD4^+CD25^+Foxp3^+$ T_{reg} population [18,19]. However, the effect of AhR activation on the intrathymic $CD4^+CD25^+Foxp3^+$ T_{reg} 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 $CD4^-CD8^-$, $CD4^+CD8^-$ and $CD4^-CD8^+$ cells and the decreased percentage of $CD4^+CD8^+$ cells in the thymus after TCDD treatment are in line with findings of others [38,39]. However, the present study shows for the first time that the absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the thymus is not changed shortly after TCDD treatment. Moreover, we show that the percentage of 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 $Foxp3^+$ T_{reg} 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 $CD4^+CD25^+Foxp3^+$ T_{reg} 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 and colleagues [40], using a viral infection model, have shown that a single intraperitoneal TCDD treatment increases the percentage of splenic $CD4^+Foxp3^+$ T_{reg} cells, without affecting the number of splenic $CD4^+Foxp3^+$ T_{reg} 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 $CD4^+CD25^+Foxp3^+$ T_{reg} cells .

Temchura and colleagues have shown that TCDD treatment decreases the absolute number of $CD4^+$ recent thymic emigrants (RTE) in the spleen and MLN about two-fold, but they did not analyze $CD4^+CD25^+Foxp3^+$ T_{reg} cells within these RTE [39]. Our present data suggest that during allergic sensitization and AhR activation by TCDD, the increase in the absolute number of splenic $CD4^+CD25^+Foxp3^+$ T_{reg} cells may indeed result from the enhanced presence of $CD4^+CD25^+Foxp3^+$ T_{reg} cells within the diminished population of RTE. This would imply that increased occurrence of the naturally occuring $CD4^+CD25^+Foxp3^+$ T_{reg} 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 $CD4^+CD25^+$ T_{reg} cells by TCDD [30], for instance by increased production of TGF-ß [3,7]). Intriguingly, not all $CD4^+CD25^+$ T_{reg} cells identified by Funatake and colleagues were $Foxp3^+$, suggesting that TCDD stimulates other regulatory T cells as well. However, the absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} 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 $Foxp3^+$ T_{reg} cell emigrants in the spleen is approximately three times higher in the spleen than in the MLN [41].

Previously, others have described the suppressive effects of AhR activation on $CD4^+$ and $CD8^+$ T cells in the periphery [42-45]. 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 $CD4^+$ and $CD8^+$ 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 [46]. 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 T_{reg} cells, or by direct immunosuppressive effects on effector T cells, as suggested before [29]. 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 $CD4^+CD25^+Foxp3^+$ T_{reg} 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 $Foxp3^+$ T_{reg} 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 [47-50]. However, until now the decisive signaling pathway leading to $Foxp3^+$ T_{reg} cell differentiation has not been elucidated. Therefore, it is difficult to pinpoint a mechanism explaining the lack of susceptibility of thymic $CD4^+CD25^+Foxp3^+$ T_{reg} 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* [51] and to increase IL-2R in thymocytes of mice perinatally exposed to TCDD [52]. Interestingly, $CD4^+CD25^+Foxp3^+$ T_{reg} cells cannot secrete IL-2, but constitutively express the IL-2R chain CD25 [47-49]. 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 $CD4^+CD25^+Foxp3^+$ T_{reg} cells from deletion. Interestingly, the immunosuppressant rapamycin, which exerts its immunosuppressive effects by blocking cell growth and proliferation in response to growth factors like IL-2 [53-55], has been shown to exert similar effects as TCDD on $CD4^+CD25^+Foxp3^+$ T_{reg} cells, major thymic T cell subsets and effector T cells [56-59].

In conclusion, TCDD treatment preserves intrathymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells, increases the absolute number of splenic $CD4^+CD25^+Foxp3^+$ T_{reg} cells and decreases peripheral T helper and cytotoxic T cells. We postulate that the intrathymic $CD4^+CD25^+Foxp3^+$ T_{reg} population, which seems preserved from TCDD-mediated depletion, contributes to the increase of the number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen, but not in the MLN. Future research should reveal which effect of TCDD, preservation, expansion or induction of (thymic) T_{reg} cells contributes most to the peripheral presence of T_{reg} 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.

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Chapter 4

Non-dioxin-like AhR ligands in a mouse peanut allergy model

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Abstract

Recently, we have shown that AhR activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) suppresses sensitization to peanut at least in part by inducing a functional shift towards $CD4^+CD25^+Foxp3^+$ T cells. Next to TCDD, numerous other AhR ligands have been described. In this study, we investigated the effect of three structurally different non-dioxin-like AhR ligands, e.g. 6-formylindolo[3,2-b]carbazole (FICZ), β-naphthoflavone (β-NF) and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), on peanut sensitization.

Female C57BL/6 mice were sensitized by administering peanut extract (PE) by gavage in the presence of cholera toxin. Before and during peanut sensitization, mice were treated with FICZ, β-NF or 6-MCDF. AhR gene transcription in duodenum and liver was investigated on day 5, even as the effect of these AhR ligands on $CD4^+CD25^+Foxp3^+$ T_{reg} cells in spleen and mesenteric lymph nodes (MLN). Mice treated with TCDD were included as a positive control. Furthermore, the murine reporter cell line H1G1.1c3 (CAFLUX) was used to investigate the possible role of metabolism of TCDD, FICZ, β-NF and 6-MCDF on AhR activation *in vitro*.

TCDD, but not FICZ, β-NF and 6-MCDF, suppressed sensitization to peanut (measured by PE-specific IgE, IgG1, IgG2a and PE-induced IL-5, IL-10, IL-13, IL-17a, IL-22, IFN-γ). In addition, FICZ, β-NF and 6-MCDF treatment less effectively induced AhR gene transcription (measured by gene expression of AhR, AhRR, CYP1A1, CYP1A2, CYP1B1) compared with TCDD-treated mice. Furthermore, FICZ, β-NF and 6-MCDF did not increase the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in spleen and MLN compared with PE-sensitized mice, in contrast to TCDD. Inhibition of metabolism *in vitro* increased AhR activation.

Together, these data shows that TCDD, but not FICZ, β-NF and 6-MCDF suppresses sensitization to peanut. Differences in metabolism, AhR binding and subsequent gene transcription might explain these findings and warrant further studies to investigate the role of the AhR in food allergic responses.

Key words

Aryl hydrocarbon receptor, peanut sensitization, food allergy, FICZ, β-NF, 6-MCDF, TCDD, $CD4^+CD25^+Foxp3^+$ T_{reg} cells

Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor and is best known for its role in mediating the toxicity of xenobiotics such as halogenated aromatic hydrocarbons (HAH) and polycyclic aromatic hydrocarbons (PAH). Exposure to the HAH 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent AhR ligand known, results in the induction of a range of species- and tissue-specific toxic and biological effects, the majority of which are AhR-dependent [1]. One of these effects is the suppression of immune responses, including T cell-dependent immune responses (e.g. food allergy) [2].

Food allergy affects about 5% of young children and 3-4% of adults and the prevalence is increasing. Most of these people have only mild reactions after exposure to a food allergen, but in severe cases anaphylaxis can be developed [3-5]. Peanut allergy is responsible for the majority of fatal food-induced allergic reactions [5]. Recently, we have shown that activation of the AhR by TCDD suppresses sensitization to peanut and that this is partly mediated by the induction of a functional shift within the $CD4^+$ population towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells [6]. Similar effects of this persistent AhR ligand on T_{reg} cells have been described previously in a mouse model for experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveoretinitis (EAU) and graft versus host disease (GvHD) [7-10]. Like TCDD, an increase of the percentage of T_{reg} cells has been observed with the AhR ligands VAG539, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) and indirubin in a GvHD model, EAE model and in healthy mice, respectively [11-13]. In addition, it has been shown that the AhR ligands 6-formylindolo[3,2-b]carbazole (FICZ) (in the presence of transforming growth factor-β) and kynurenine induce $Foxp3^+$ T_{reg} cells *in* vitro [14,15]. Furthermore, FICZ (administrated ip) has been shown to ameliorate TNBS-induced colitis and DSS-induced colitis by suppressing inflammatory cytokines and increasing IL-22 [16]. The AhR ligand β-naphthoflavone (β-NF) has also been demonstrated to attenuate DSS-induced colitis [17]. In contrast, activation of the AhR by FICZ has also been described to induce Th17 cells *in vitro* and to enhance disease in an EAE model by inducing Th17 cells [7,14,18]. Thus, depending on the AhR ligand and the disease, activation of the AhR appears to result in attenuation or initiation of T-cell dependent immune responses. Therefore, we investigated whether the non-dioxin-like AhR ligands FICZ,

β-NF and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) suppress peanut sensitization similar to the classical AhR ligand TCDD.

Materials and methods

Mice and reagents

Female C57BL/6 mice (4-5 week old) purchased from Charles River (France) were maintained under controlled conditions (relative humidity of 50%-55%, 12h light/dark cycle, temperature of 23±2 ºC) 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, 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% vol/vol anisole). Anisole diluted in corn oil was used as vehicle control (0.07% vol/vol). FICZ (Biomol International, USA) was dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml and diluted in corn oil to the final exposure concentration (1% v/v DMSO). For oral treatment, FICZ was dissolved in DMSO at 2 mg/ml and diluted in corn oil to the final exposure concentration (2.5% vol/vol DMSO). β-NF (Sigma Aldrich, The Netherlands) was dissolved in DMSO at 20, 60 or 200 mg/ml and diluted with corn oil to the final exposure concentration (2,5% vol/vol DMSO). 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) was dissolved directly in corn oil to the final exposure concentration. Final exposure concentrations of FICZ, β-NF and 6-MCDF were based on previously published *in vivo* studies [7,18-21].

Peanut protein extract (PE) (30 mg/ml) was prepared from peanuts from the Golden Peanut Plant (USA, provided by Intersnack Nederland BV, The Netherlands) as described previously [22]. 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. (Cambell, CA).

Experimental design

According to our standard PE sensitization protocol, mice were sensitized to PE by intragastric exposure to PE (6 mg PE, 200 µl/mouse) with CT (15 µg/mouse) on three consecutive days (day 0, 1 and 2) followed by weekly dosing (days 7, 14, 21 and 28). One day before termination, mice were challenged intragastrically with PE (12 mg/mouse). Mice were killed on day 31 or day 36 by cervical dislocation and blood and spleen were isolated. From experience we know that the day of termination (31 or 36) has no influence on the results obtained with this peanut allergy model. Before and during sensitization to peanut, mice (n=6-8 per group) were treated with TCDD (15 µg/kg body weight [BW]) by gavage, on days -1 and 13), FICZ (50 µg/kg BW, ip, on days -1 and 13 or on days -1, 2, 6, 9, 13, 16, 20, 23, 27, 30 and 33) (figure 1, setup 1), β-NF (5, 15 or 50 mg/kg BW, by gavage, on days -3, 0, 4, 8 and 11) (figure 1, setup 2) or 6-MCDF (12.5 or 50 mg/kg BW, by gavage, on days -3 and 11) (figure 1, setup 3). To detect the effect of FICZ, β-NF and 6-MCDF on $CD4^{+}CD25^{+}Foxp3^{+}$ T_{reg} cells, early cytokine production and AhR-related gene expression, mice were exposed before and during initiation of sensitization to peanut to FICZ (500 µg/kg BW), β-NF (5 mg/kg BW) or 6-MCDF (150 mg/kg BW) by gavage on days -3, 0 and 4. TCDD (15 µg/kg BW) was given by gavage only on day -3. On day 5, mice were sacrificed by cervical dislocation and blood, spleen, duodenum and liver were isolated (figure 1, setup 4).

Preparation of liver microsomes

Excised livers were homogenized in Tris/HCL (50 mM, 1,15% KCL, pH=7.4). The microsomal fraction was obtained from the homogenate by successive centrifugation for 25 minutes at 9000 x g and 85 minutes at 100.000 x g with a Beckman Coulter Optima L-90 K centrifuge. The microsomal fraction was resuspended in a sucrose solution (0.25 M). Protein concentration of the microsomes was determined by the method of Lowry using BSA as protein standard [23].

Ethoxyresofurin-O-deethylase activity in liver microsomes

Transcriptional activation of the AhR *in vivo* was determined by measuring ethoxyresorufin-O-deethylase (EROD) activity in liver microsomes in 10 µl sample containing 10-40 µg protein with 90 µl 50 mM Tris buffer (pH 7.4) containing 5 mM $MgCl_2$, 20 µM dicoumarol, 2 µM 7-ethoxyresofurin and 1.5 mM NADPH. A standard curve using resofurin was generated to quantify the EROD activity. Fluorescence was measured at 37 ºC at an excitation wavelength of 530 nm and an emission wavelength

of 590 nm, every 80 seconds for 16 minutes in a Fluostar plate reader (BMG Labtechnologies GmbH, Germany). EROD activity was calculated as picomoles resofurin per minute per milligram protein.

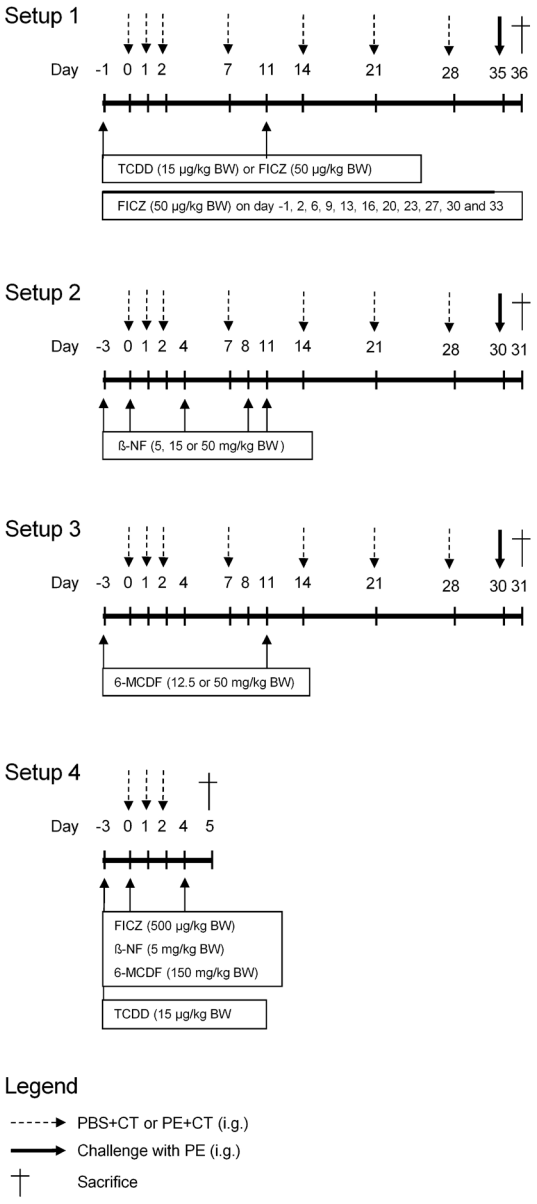


Figure 1. Treatment protocols as described in 'Materials and Methods' section.

Measurement of PE-specific IgE, IgG1 and IgG2a antibody levels in serum
PE-specific IgE, IgG1 and IgG2a antibodies in serum were detected as previously described [24].

Splenic cell culture and analysis of cytokine production
After red blood cell lysis, single-cell spleen suspensions ($2.5*10^6$ cells/ml) were cultured in 200 µl complete RPMI 1640 (10% Fetal Calf Serum) in the presence of medium or PE (100 µg/ml) for 96 h at 37 ºC and 5% CO_2. Levels of IL-5, IL-10, IL-13, IL-17a, IL-22 and IFN-γ 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.

Flow cytometry analysis
Single cell suspensions of spleen (after red blood cell lysis) and mesenteric lymph nodes (MLN) ($1*10^7$ cells/ml) were stained with anti-CD4-FITC (clone L3T4, eBioscience) and anti-CD25-PE (clone PC61, eBioscience) in FACS buffer (PBS containing 0.25 % BSA, 0.05% NaN_3, 0.5 mM EDTA) for 30 min at 4 ºC. 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 BD FACSCanto II using BD FACS Diva software (BD Biosciences, USA).

RNA isolation
Isolated duodenum and liver were homogenized two times for one minute at 20 Hertz in 500 µl RNA Insta-Pure (Eurogentec, USA) using a Mixermill (Retsch, Germany). The homogenized tissue was centrifuged for 10 minutes at 12.000 g. The supernatant, containing RNA in RNA Insta-Pure was transferred to a new vial and RNA was isolated using phenol-chloroform extraction. The amount of RNA was determined using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA).

Real Time-PCR
Complementary DNA (cDNA) was synthesized from 1 µg RNA using the iScript™cDNA Synthesis Kit from Biorad (Bio-Rad Laboratories, CA). Amplification reactions were set up with 9 µl mastermix (7,5 µl iQ™SYBR Green Supermix from Biorad, 0.3 µl d H_2O, 0.6 µl (10 µM) forward primer (FW), 0.6 µl (10 µM) reverse primer (RV)) and 6 µl first strand cDNA (10x diluted). Primer sequences: AhR: FW-CGGCTTCTTGCAAAACACAGT

and RV-GTAAATGCTCTCGTCCTTCTTCATC; AhRR: FW-GTTGGATCCTGTAGGGAGCA and RV-AGTCCAGAGGCTCACGCTTA CYP1A1: FW-GGTTAACCATGACCGGGAACT and RV-TGCCCAAACCAAAGAGAGTGA; CYP1A2: FW-ACATTCCCAAGGAGCGCTGTATCT and RV-GTCGATGGCCGAGTTGTTATTGGT; CYP1B1: FW-GTGGCTGCTCATCCTCTTTACC and RV-CCCACAACCTGGTCCAACTC; β-actin: FW-ATGCTCCCCGGGCTGTAT and RV-CATAGGAGTCCTTCTGACCCATTC. Reactions were performed in Hard-shell PCR 96-well plates (Biorad, USA) capped with optical cap and amplified for 40 cycles with the standard PCR parameters (thermal profile: 95 ˚C for 3 minutes [1x per sample], 95 ˚C for 10 seconds [40 x per sample], 60 ˚C for 45 seconds [40 x per sample]). Following amplification, the melting curves of PCR products were determined from 60 ˚C to 95 ˚C to detect possible contaminations in the samples and to determine the specificity of the amplification. The data generated from reactions was analyzed by plotting ΔRn (normalized) fluorescence signal versus cycle number. An arbitrary threshold was set at the midpoint of the log ΔRn versus cycle number plot. The Ct values (thresholds) calculated from this plot were used to determine relative quantitation of gene expression by applying comparative Ct method (ΔΔCt). ΔCt was calculated by substracting the Ct of the reference gene from the Ct of the target gene. As a reference gene was β-actin was used. Next, the average ΔCt of the control group was substracted from treated-groups (ΔΔCt) and fold changes in gene expression relative to the control group were calculated according to the 2-ΔΔCt method.

CAFLUX cell line

The murine reporter cell line H1G1.1c3 was created by stable transfection of mouse hepatoma cells (Hepa1c1c7) with the AhR-eGFP (enhanced green fluorescent protein) reporter plasmid pGreen and were kindly provided by Dr. M.S. Denison (UC Davis, CA, USA). Cells were cultured in 75 cm^2 Tissue Culture Flasks (Greiner Bio-One, The Netherlands) in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, The Netherlands) supplemented with 10% Fetal Calf Serum, 1% Pen/Strep (Pen 10.000 U/ml, Strep 10.000 µg/ml, Invitrogen, The Netherlands) in an incubator (37 ºC, 5% CO_2).

Exposure of the CAFLUX cell line to AhR ligands and CYP1A2 inhibitor

For exposure, $1*10^5$ cells/ml were seeded in flatbottom 96-well plates and the next day exposed to concentrations series of various AhR ligands for 24 h. TCDD (Cambridge Isotope Lab, USA), FICZ (Biomol International, USA) and 6-MCDF (kindly

provided by S. Safe) were dissolved in DMSO. β-NF (Sigma Aldrich, The Netherlands) was dissolved in acetone. The final exposure concentration of each compound was 0.5% vol/vol. Every sample concentration was assayed in triplicate. After 24 h, fluorescence was measured in PBS with a spectrophotometer (Polarstar Galaxy, BMG labtechnologies, Germany) using excitation at 485 nm and emission at 510 nm. Optical density values were obtained using corresponding software (FLUOstar Galaxy, version 4.30-0) and corrected for background levels. The role of metabolism of TCDD, FICZ, β-NF and 6-MCDF on AhR activation was investigated by exposing cells to the EC_{50} value of these compounds in the presence of the CYP1A2 inhibitor furafylline (3-(2-furanylmethyl)-3,7-dihydro-1,8,-dimethyl-1H-purine-2,6-dione) (Calbiochem, Germany) (dissolved in DMSO, 0.5 % vol/vol). EROD activity was measured by adding EROD medium (DMEM, containing 0.1% 10 mM dicumarol [dissolved in DMSO] and 0.5% 1 M $MgCl_2$, all supplied by Sigma-Aldrich, The Netherlands) to the cells. Resofurin concentrations were measured eight times during 609 seconds, with excitation at 530 nm and emission at 590 nm at 37 ºC. OD-values were corrected for background levels. A standard curve using resofurin was generated to quantify the EROD activity. EROD activity was calculated as picomoles resofurin per minute per milligram protein.

Statistical analysis

Results are presented as the mean ± the standard error of the mean (SEM) of six to eight mice per group. In vitro data are presented as the mean ± the standard error. All data were logarithmically transformed to achieve normal distribution. Data 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

Immunomodulation of peanut sensitization by β-NF and 6-MCDF, but not FICZ

We first investigated the effect of the AhR ligands FICZ, β-NF and 6-MCDF on sensitization to peanut in an established mouse model of food allergy. As a positive control, the potent AhR ligand TCDD was included. PE sensitization of the vehicle-control group resulted in increased serum levels of PE-specific IgE, IgG1 and IgG2a

(figure 2) and increased cytokine (IL-5, IL-10, IL-13, IL-17a, IL-22 and IFN-γ) production by spleen cultures incubated with PE (figure 3) compared with the non-sensitized vehicle control group.

Treatment with TCDD during sensitization to PE suppressed all these parameters, except IL-22 and IFN-γ (figure 2 and 3). PE-specific IgE, IgG1 and IgG2a levels were not affected in mice treated with FICZ and β-NF during sensitization to peanut, whereas treatment with 6-MCDF decreased PE-specific IgG1 levels (figure 2). Interestingly, treatment of mice with β-NF increased PE-induced IL-5, IL-13 (at 1000 µg β-NF) and IL-17a production (at 300 µg and 1000 µg β-NF) (figure 3). FICZ and 6-MCDF treatment of mice during PE-sensitization showed no effect on PE-induced cytokine production (figure 3). In addition, repeated oral treatment of mice with FICZ (500 µg/kg BW, on

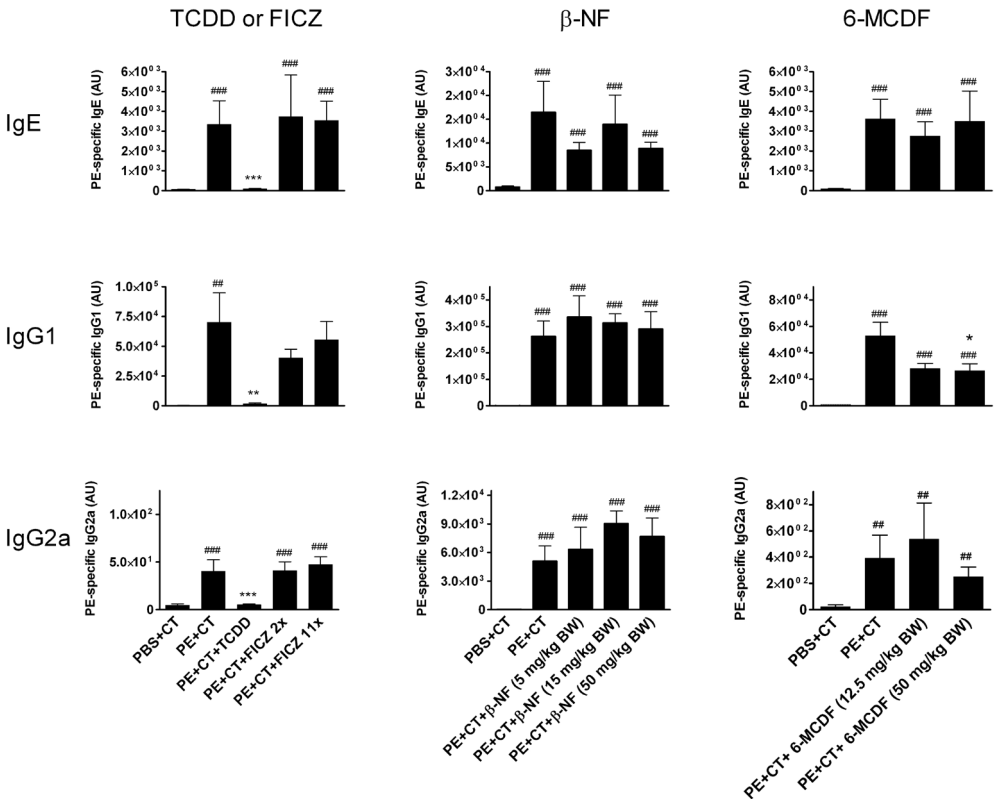


Figure 2. Effect of FICZ, β-NF and 6-MCDF treatment before and during peanut sensitization on peanut-specific IgE, IgG1 and IgG2a levels. Before and during sensitization to peanut (PE+CT) mice were treated with TCDD (15 µg/kg BW, setup 1), FICZ (50 µg/kg BW, setup 1), β-NF (5, 15 or 50 mg/kg BW, setup 2) or 6-MCDF (12.5 or 50 mg/kg BW, setup 3). Peanut specific IgE, IgG1 and IgG2a were determined in the serum collected on the day of sacrificing by ELISA. Values are presented as mean ± SEM (n=6-8 per group). ## $p < 0.01$ compared with PBS+CT, ### $p < 0.001$ compared with PBS+CT, * $p < 0.05$ compared with PE+CT, ** $p < 0.01$ compared with PE+CT, *** $p < 0.001$ compared with PE+CT.

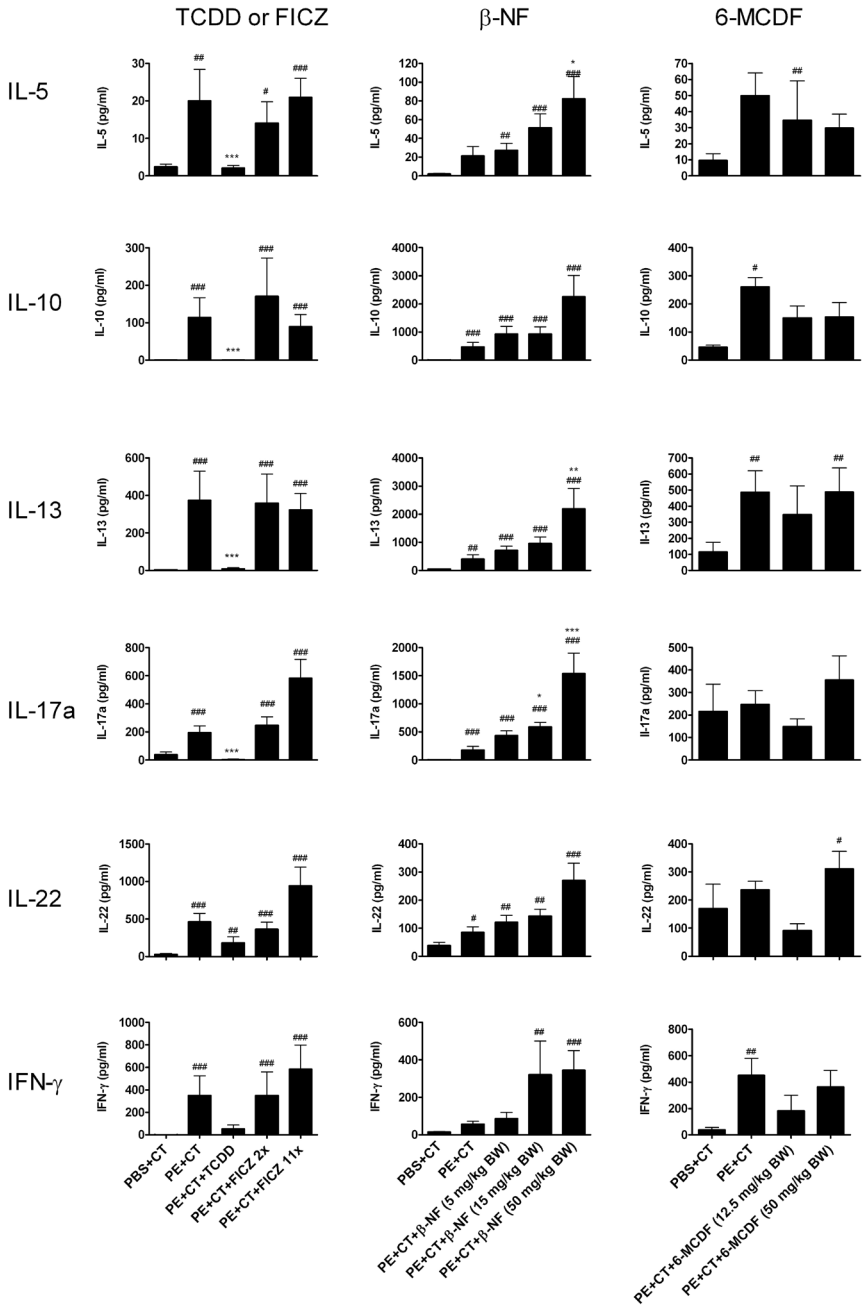


Figure 3. Effect of FICZ, β-NF and 6-MCDF treatment before and during peanut sensitization on PE-induced IL-5, IL-10, IL-13, IL-17a, IL-22 and IFN-γ cytokine responses (continued).

Figure 3 (continued). Mice were sensitized for peanut (PE+CT) and treated with TCDD (15 µg/kg BW, setup 1), FICZ (50 µg/kg BW, setup 1), β-NF (5, 15 or 50 mg/kg BW, setup 2) or 6-MCDF (12.5 or 50 mg/kg BW, setup 3). Single cell suspensions of spleen cells isolated on the day of sacrificing were cultured for 96 h in the presence of PE. The supernatant was analyzed for IL-5, IL-10, IL-13, IL-17a, IL-22 and IFN-γ. No cytokine production could be detected when splenocytes were cultured in the absence of PE. Values are presented as mean ± SEM (n=6-8 per group). # $p < 0.05$ compared with PBS+CT, ## $p < 0.01$ compared with PBS+CT, ### $p < 0.001$ compared with PBS+CT, * $p < 0.05$ compared with PE+CT, *** $p < 0.001$ compared with PE+CT.

days -3, 0, 4, 8, 11), before and during PE sensitization, did also not affect the peanut allergic response (data not shown). Together, these results show that β-NF and 6-MCDF, but not FICZ, affect parameters of peanut sensitization to limited extent at the dose and frequency of administration used in these experiments. However, these ligands are not as effective as TCDD in lowering peanut sensitization.

FICZ, β-NF and 6-MCDF do not increase the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in spleen and MLN in PE-sensitized mice

Next, we investigated the effects of FICZ, β-NF and 6-MCDF treatment on the induction of $CD4^+CD25^+Foxp3^+$ T_{reg} cells during peanut sensitization in the spleen and MLN on day 5. In addition, the effect of FICZ, β-NF and 6-MCDF treatment on PE-induced cytokine production of splenic cells on day 5 was also investigated. Again, TCDD was included as a positive control. PE-sensitization did not increase the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen and MLN on day 5 (figure 4). As expected, TCDD treatment increased the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen and MLN compared with PE-sensitized mice (figure 4). FICZ, β-NF and 6-MCDF did not increase the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen and MLN compared with PE-sensitized mice. However, FICZ and 6-MCDF treatment before and during PE-sensitization increased the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in spleen and MLN compared with non-sensitized vehicle control mice, whereas β-NF treatment only increased the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in spleen (figure 4). In addition, we investigated the early effects of FICZ, β-NF and 6-MCDF treatment on initiation of peanut sensitization by measuring PE-induced cytokine production on day 5. PE sensitization of the vehicle-control group resulted in increased cytokine (IL-5, IL-10, IL-13, IL-17a and IFN-γ) production by spleen cultures incubated with PE (supplementary data 1) compared with the non-sensitized vehicle control group. Again, treatment with TCDD prior to PE-sensitization suppressed this increase (supplementary data 1). FICZ, β-NF and 6-MCDF, however, did not affect PE-

induced IL-5, IL-10, IL-13, IL-17a and IFN-γ on day 5 (supplementary data 1). Together, these data show that in contrast to TCDD, treatment of mice with FICZ, β-NF and 6-MCDF does not increase the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in PE-sensitized mice and does not affect PE-induced cytokine production during the initiation of sensitization.

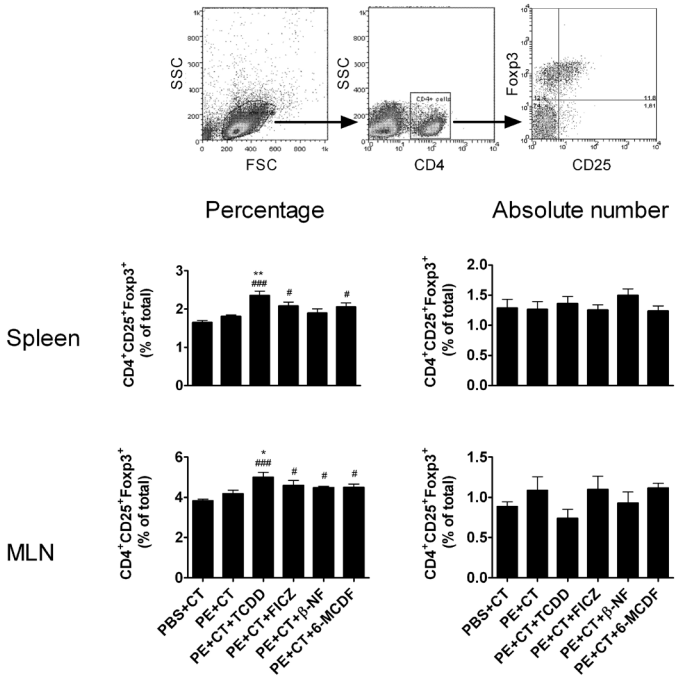


Figure 4. Effect of FICZ, β-NF and 6-MCDF treatment before and during peanut sensitization on $CD4^+CD25^+Foxp3^+$ T_{reg} cells. Before and during sensitization to peanut (PE+CT) mice were treated with TCDD (15 µg/kg BW), FICZ (500 µg/kg BW), β-NF (5 mg/kg BW) or 6-MCDF (150 mg/kg BW) (setup 4). On day 5, mice were sacrificed and the percentage and absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen and MLN were examined by FACS analysis. Values are presented as mean ± SEM (n=4 per group). # $p < 0.05$ compared with PBS+CT, ### $p < 0.001$ compared with PBS+CT, * $p < 0.05$ compared with PE+CT, ** $p < 0.01$ compared with PE+CT.

In vivo treatment of mice with FICZ, β-NF and 6-MCDF less effectively induce AhRR and cytochrome P450 gene expression compared with mice treated with TCDD

Since FICZ, β-NF and 6-MCDF were not as effective as TCDD in lowering peanut sensitization, we investigated whether or not these AhR ligands were able to activate the AhR in the duodenum and liver on day 5, i.e. 24 h after the last administration of a non-dioxin-like AhR ligand. Mice treated with TCDD were included as a positive control. TCDD treatment strongly increased AhRR, CYP1A1, CYP1A2 and CYP1B1 gene

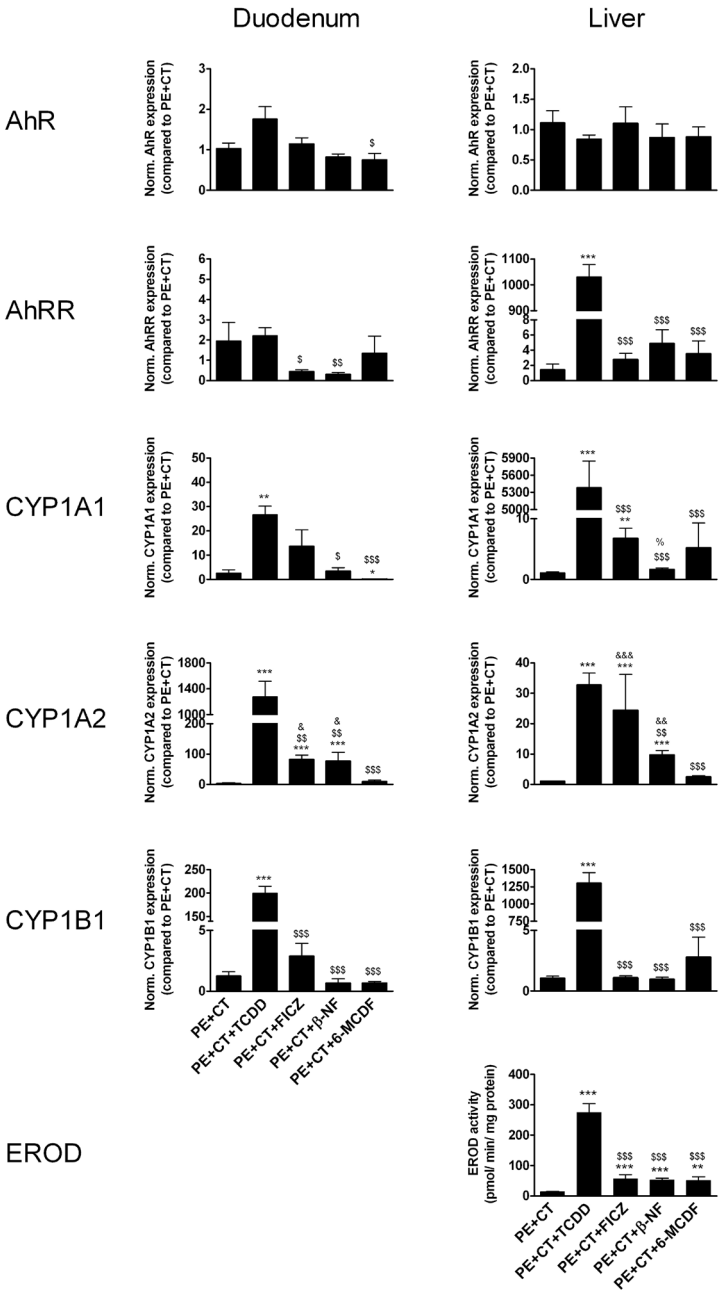


Figure 5. Effect of FICZ, β-NF and 6-MCDF treatment on AhR-related gene expression in liver and duodenum. (continued).

Figure 5 (continued). Before and during sensitization to peanut (PE+CT) mice were treated with TCDD (15 µg/kg BW), FICZ (500 µg/kg BW), β-NF (5 mg/kg BW) or 6-MCDF (150 mg/kg BW) (setup 4). On day 5, mice were sacrificed and duodenum and liver were isolated. Total RNA was extracted and the expression of AhR, AhRR, CYP1A1, CYP1A2 and CYP1B1 compared with vehicle-treated mice (PE+CT) was examined by real-time PCR. Also EROD activity was measured in liver microsomes. Values are presented as mean ± SEM (n=6 per group). * $p < 0.05$ compared with PE+CT, ** $p < 0.01$ compared with PE+CT, *** $p < 0.001$ compared with PE+CT, $ $p < 0.05$ compared with PE+CT+TCDD, $$ $p < 0.01$ compared with PE+CT+TCDD, $$$ $p < 0.001$ compared with PE+CT+TCDD, & $p < 0.05$ compared with PE+CT+6-MCDF, && $p < 0.01$ compared with PE+CT+6-MCDF, &&& $p < 0.001$ compared with PE+CT+6-MCDF, % $p < 0.05$ compared with

expression in both the duodenum and the liver compared with vehicle-treated mice (figure 5). Also, EROD activity measured in liver microsomes was strongly increased after TCDD treatment (figure 5). Treatment of mice with FICZ, unlike treatment with β-NF, increased CYP1A1 expression in the liver and both FICZ and β-NF treatment increased CYP1A2 expression in the duodenum and liver (figure 5). In contrast, 6-MCDF treatment decreased CYP1A1 expression in the duodenum, whereas 6-MCDF did not affect CYP1A2 expression in either the duodenum or the liver. FICZ, β-NF and 6-MCDF treatment enhanced EROD activity measured in liver microsomes. Together, these results show that treatment of mice with FICZ, β-NF and 6-MCDF results in limited AhR activation compared with mice treated with TCDD.

Inhibition of metabolism in vitro increases AhR activation

Because sensitization to peanut could not be suppressed by repeated administration of FICZ, β-NF and 6-MCDF, we investigated the possible role of metabolism of FICZ, β-NF and 6-MCDF on AhR activation *in vitro*, as many non-halogenated ligands for the AhR facilitate their own metabolism by inducing CYP450 enzymes. Treatment of the mouse H1G1.1c3 cells with TCDD, FICZ, β-NF or 6-MCDF resulted in concentration-dependent activation of the AhR after 24 h exposure, as indicated by the production of eGFP controlled by AhR transactivation (figure 6a). The estimated EC_{50} value to activate the AhR was lowest for TCDD (19.9×10^{-12} M), followed by FICZ (9.65×10^{-10} M), β-NF (4.69×10^{-10} M) and 6-MCDF (4.66×10^{-7} M). The concentration-response curve for TCDD had a maximal height compared with the other AhR ligands, indicating that the efficacy of TCDD to activate the AhR was highest. The role of metabolism on AhR activation by TCDD, FICZ, β-NF and 6-MCDF was investigated by using the CYP1A2 inhibitor furafylline. Cells were incubated for 24 h with the EC_{50} values of TCDD, FICZ, β-NF and 6-MCDF and with an increasing concentration of furafylline. As expected, no effect on AhR activation (measured by eGFP) by TCDD was observed when CYP1A2 was inhibited, because it is generally known that TCDD is very slowly metabolized

(figure 6b, c). Inhibition of CYP1A2 activity increased AhR activation by FICZ, β-NF and 6-MCDF (figure 6b, c). In all test-conditions, cell viability was not affected (data not shown). Together, these data show that inhibition of CYP1A2 metabolism results in increased *in vitro* AhR activation by FICZ, β-NF and 6-MCDF, but not by TCDD.

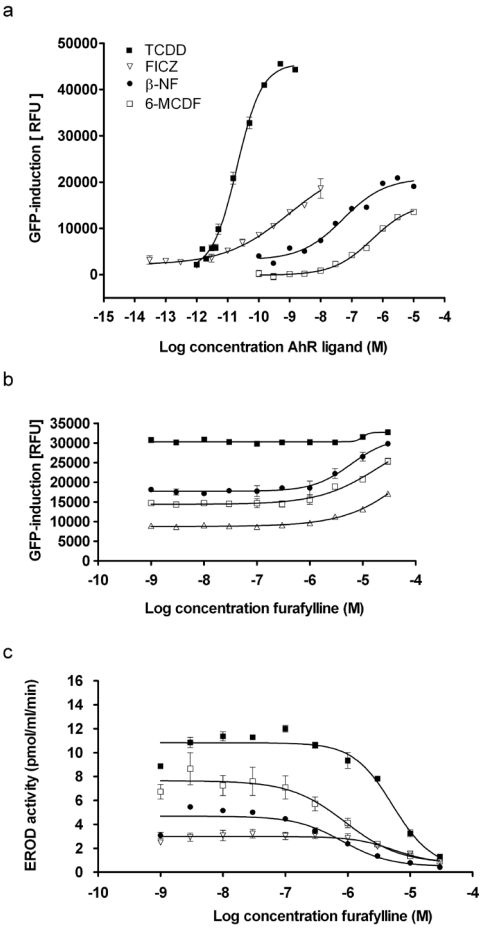
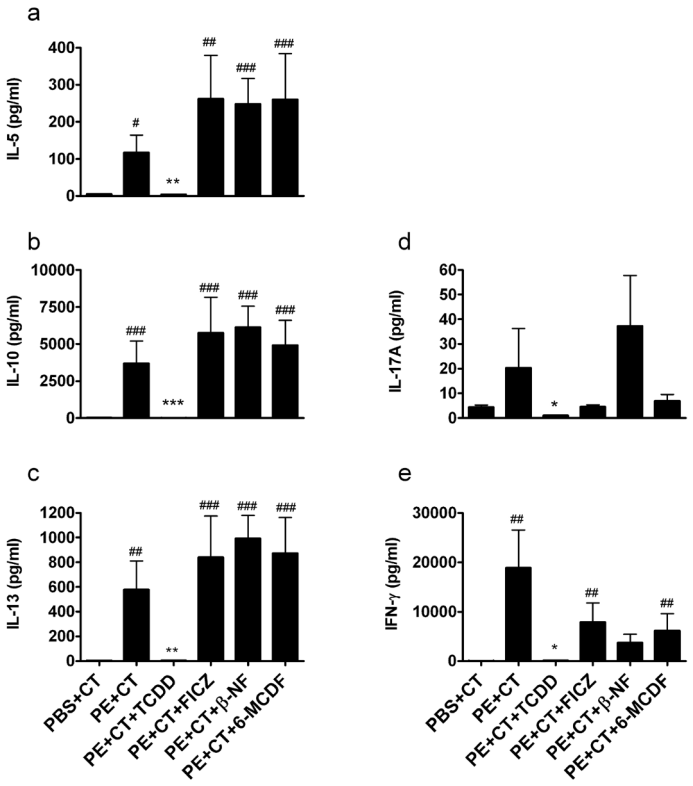


Figure 6. Effect of FICZ, β-NF and 6-MCDF on AhR activation and the role of CYP1A2 metabolism on AhR activation. H1G1.1c3 cells containing the AhR-eGFP reporter plasmid pGreen were seeded in a 96-well plate. The next day, cells were exposed to concentration series of TCDD, FICZ, β-NF and 6-MCDF (0.5 % v/v) and after 24 h exposure AhR activation was examined by measuring eGFP (a). The role of CYP1A2 metabolism on AhR activation was investigated by exposing cells for 24 h to the EC_{50} values of TCDD (15 pM), FICZ (1 nM), β-NF (200 nM) and 6-MCDF (2 µM) in the presence of an increasing dose of furafylline. After 24 h of exposure AhR activation was examined by measuring eGFP (b) and CYP1A2 metabolism was examined by measuring EROD activity (c). Values are presented as mean ± SE (n=3 per concentration).



Supplementary data 1. Effect of FICZ, β-NF and 6-MCDF treatment before and during peanut sensitization on PE-induced IL-5, IL-10, IL-13, IL-17a and IFN-γ cytokine responses on day 5. Mice were sensitized for peanut (PE+CT) and treated with TCDD (15 µg/kg BW), FICZ (500 µg/kg BW), β-NF (5 mg/kg BW) or 6-MCDF (150 mg/kg BW) (setup 4). On day 5, spleens were isolated and single cell suspensions of spleen cells were cultured for 96 h in the presence of PE. The supernatant was analyzed for IL-5 (a), IL-10 (b), IL-13 (c), IL-17a (d) and IFN-γ (e). No cytokine production could be detected when splenocytes were cultured in the absence of PE. Values are presented as mean ± SEM (n=6 per group). # $p < 0.05$ compared with PBS+CT, ## $p < 0.01$ compared with PBS+CT, ### $p < 0.001$ compared with PBS+CT, * $p < 0.05$ compared with PE+CT, ** $p < 0.01$ compared with PE+CT, *** $p < 0.001$ compared with PE+CT.

Discussion

Previously, we have shown that activation of the AhR by TCDD suppresses sensitization to peanut and that this is partly mediated by a TCDD-induced increase of the percentage of $CD4^+CD25^+Foxp3^+$ cells [6]. Besides TCDD, numerous other exogenous and endogenous AhR ligands are described. Here, we examined the effect of a number of non-dioxin-like AhR ligands that are structurally distinctly different

from TCDD, i.e. FICZ, β-NF and 6-MCDF, on peanut sensitization. The present study shows that in contrast to TCDD, these AhR ligands do not suppress sensitization to peanut, do not increase the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in PE-sensitized mice and less effectively induce AhR-related gene expression *in vivo* and *in vitro*.
Compared with TCDD, the AhR ligands FICZ and β-NF only marginally increased AhR-related gene expression and EROD activity *in vivo*. The selective AhR modulator (SAhRM) 6-MCDF, that retains the antiestrogenic effects but lacks the transcriptional effects of TCDD associated with toxicity, had no effect on AhR-related expression in the liver and down-regulated CYP1A1 expression in the duodenum, although EROD activity in the liver was increased. The finding that inhibition of CYP1A2 metabolism *in vitro* increased AhR activation by FICZ, β-NF and 6-MCDF, but not by TCDD, indicates a clear link between metabolism and AhR activation by FICZ, β-NF and 6-MCDF. This might suggest that these AhR ligands are metabolized too fast *in vivo* to have a comparable effect as TCDD on peanut sensitization and $CD4^+CD25^+Foxp3^+$ T_{reg} cells. In relation to this, it could be argued that the frequency of administration and the dose of FICZ, β-NF and 6-MCDF used was not high enough to exert similar effects as TCDD on peanut sensitization and $CD4^+CD25^+Foxp3^+$ T_{reg} cells. However, in other disease models, effects of FICZ, β-NF and 6-MCDF on the immune system have been described at comparable doses and frequency of administration. For example, in our model FICZ was administrated multiple times ip (50 μg/kg BW), whereas Monteleone et al. have shown that a single administration of approximately 50 μg/kg BW FICZ ip already ameliorates TNBS-induced colitis and relapses DSS-induced [16]. In addition, it has been shown that the presence of 600 ng FICZ in the antigen emulsion accelerates and increases pathology of EAE [18]. Furthermore, in the peanut allergy model administration of 50 mg/kg BW β-NF every 2-3 days affected some allergic parameters, as β-NF treatment increased PE-induced IL-5, IL-13, IL-17a production of splenocytes. However, no effect was observed on antibody levels, suggesting that the effect of β-NF on cytokine level is not sufficient to affect the food allergic response. Interestingly, in a DSS-induced colitis model, a similar dose of β-NF (oral administration, every day) has been shown to suppress IL-6, TNF-α and IL-1β expression in colon tissue resulting in attenuated disease [17]. Further, while 6-MCDF is mainly known for its anti-(breast) cancer effects [20,25], it has also been reported that a single administration of approximately 10 mg/kg BW 6-MCDF caused 26% inhibition of the splenic plaque-forming cell response to sheep erythrocytes [19]. This suggests that 6-MCDF might induce modest immunosuppressive effects. However, in

our studies 6-MCDF suppressed PE-specific IgG1 at a dose of 50 mg/kg BW, but did not affect PE-specific IgE, PE-specific IgG2a and *ex vivo* PE-cytokine production, indicating that 6-MCDF does not suppress allergic sensitization to peanut. Together, these findings indicate that next to metabolism, the type of the disease might play a role in determining the effect of FICZ, β-NF and 6-MCDF on the immune response.

Another possibility for the finding that TCDD, but not FICZ, β-NF and 6-MCDF, suppresses sensitization to peanut is that these three ligands interact differently with the AhR compared with TCDD, because it has been reported that high- and low-affinity ligands interact with different residues of the AhR ligand-binding pocket [26,27]. As a result, it is possible that TCDD induces different genes or distinct pathways than FICZ, β-NF and 6-MCDF, which has been reported for TCDD compared with FICZ, 3,3'-diindolylmethane (DIM) and PCB126 [28-30]. Furthermore, proinflammatory cytokines have been shown to affect the expression of most AhR-dependent xenobiotic metabolizing enzymes, thereby affecting metabolism of xenobiotics and thus binding of these xenobiotics to the AhR and the subsequent effect on the immune system [31]. In addition, cytokines can influence the level of AhR expression itself which could also impact effects of AhR ligands on the immune system [7,15,32,33]. For the rest, it should be taken into account that metabolism of AhR ligands such as FICZ and β-NF results in the generation of metabolites, which could subsequently interact with the AhR or other receptors and pathways, thereby possibly exerting beneficial or harmful effects. This, together with the different expression of AhR in species, tissues and various cell types and the importance of transcriptional crosstalk in shaping cell-specific AhR responses, makes the effect of AhR ligands on the immune system still very unpredictable [34-38]. All together, the diversity of the AhR-linked pathways may result in different effects on immune responses, depending on the AhR ligand, the cytokine milieu, the type of disease, the route of administration and probably the timing of administration.

In summary, the present study shows that TCDD, but not FICZ, β-NF and 6-MCDF, suppresses sensitization to peanut. Differences in metabolism, AhR binding and subsequent gene transcription might explain the difference between TCDD and the AhR ligands FICZ, β-NF and 6-MCDF on the peanut allergic response. This warrants further studies to investigate the role of the AhR in food allergic responses.

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Chapter 5

Aryl hydrocarbon receptor activation affects the dendritic cell phenotype and function during allergic sensitization

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Submitted

Abstract

Aryl hydrocarbon receptor (AhR) activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) suppresses peanut sensitization by affecting T cell subsets. However, effects of AhR activation on dendritic cells (DC) in an allergic setting were not investigated yet. Therefore, we analyzed the effects of AhR activation on DC phenotype *in vivo*, as well as their *ex vivo* potency to stimulate allergen-specific splenic T cells and to induce $CD4^+CD25^+Foxp3^+$ regulatory (T_{reg}) cells.
C3H/HeOuJ mice were treated with TCDD by gavage and subsequently sensitized to peanut extract (PE). After eight days, mice were sacrificed and DC in spleen and mesenteric lymph nodes (MLN) were characterized or cocultured with PE-specific $CD4^+$ T cells.
AhR activation almost doubled the absolute number of $CD103^+$ DC, while not affecting $CD11b^+$ DC, the absolute number of DC and the expression of the activation makers MHCII, CD86, CD80, CD40, CD54 and CD8α on $CD11c^+$ DC in the spleen. In the MLN, TCDD decreased the absolute number of DC and $CD103^+$ DC, while not affecting $CD11b^+$ DC and the expression of activation markers on DC. PE-pulsed splenic DC from TCDD-treated mice suppressed IL-5, IL-13 and IFN-γ production by PE-specific T cells, but did not induce $CD4^+CD25^+Foxp3^+$ T_{reg} cells.
Combined, these results indicate that AhR activation suppresses the initiation of food allergic responses by affecting DC and their interaction with effector T cells.

Key words
Aryl hydrocarbon receptor, CD103, dendritic cells, mesenteric lymph nodes, regulatory T cells, spleen.

Introduction

Normally, exposure to food antigens results in the induction of tolerance. However, in some individuals exposure will result in allergic sensitization [1-3]. With a prevalence of about 5% among children and 3-4% among adults food allergy is a significant public health problem [4,5]. Dendritic cells (DC) play an important role in the induction of tolerance or allergic sensitization to food allergens. These cells sample antigen from the gut lumen or underlying tissue, migrate to lymphoid organs and present there antigen to naïve T cells. This will result either in tolerance by induction of regulatory T (T_{reg}) cells expressing Foxp3 or in allergic sensitization by induction of T helper 2 (Th2) cells [6].
Different subsets of DC have been described and each subset has a specific role in the immune system [7]. The balance between tolerogenic $CD103^+$ DC and inflammatory $CD11b^+$ DC is important in food allergic sensitization, because oral sensitization to peanut is accompanied by an increase of inflammatory $CD11b^+$ DC and a decrease of $CD103^+$ DC in the intestine [8]. Mucosal $CD103^+$ DC are important for the induction of oral tolerance, since they have been shown to induce $Foxp3^+$ T_{reg} cells in a TGF-β and retinoic acid dependent manner [9,10].
The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor which is expressed by cells of the innate and adaptive immune system and is best known for its role in mediating toxicity of xenobiotics, in particular of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Nowadays, it is evident that the AhR to plays an important role in the immune system [11-13]. Previously, we have shown that activation of the AhR by TCDD suppresses sensitization to peanut by decreasing precursor and effector T cells and by inducing a functional shift towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells [14,15]. Interestingly, activation of the AhR has been associated with the induction of tolerogenic DC and $CD4^+CD25^+Foxp3^+$ T_{reg} cells. For example, in a mouse model for graft versus host disease (GvHD) transfer of splenic $CD11c^+$ cells from mice treated with the AhR ligand VAG539 increased the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells and promoted graft acceptance [16]. Moreover, splenic DC isolated from mice treated with the AhR ligand 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) expressed more CD103. In addition, these DC increased the percentage of $Foxp3^+$ T_{reg} cells *in vitro* dependent on retinoic acid [17]. However, it was also reported that TCDD treatment reduces the number of

splenic DC, increases the expression of CD86, CD40 and CD54 on splenic DC and enhances their ability to provide activation signals to T cells, resulting in dysregulation of immune responses [18,19]. Altogether, these findings show that the role of DC in AhR-mediated suppression of immune responses is yet not clear and may depend on many factors, including the disease model and the AhR ligand used.

Because DC play an important role in allergic sensitization and AhR activation has been shown to affect functions of DC, we investigated the effect of AhR activation on DC in a mouse model for peanut allergy. For this purpose, we studied the effect of TCDD on DC phenotype and populations *in vivo* during sensitization, their interaction with peanut-specific $CD4^+$ T cells *ex vivo* and their capacity to induce $CD4^+CD25^+Foxp3^+$ T_{reg} cells *ex vivo*.

Materials and methods

Mice and reagents

Female C3H/HeOuJ mice (4-5 week old) purchased from Charles River (France) were maintained under controlled conditions (relative humidity of 50%-55%, 12h light/dark cycle, temperature of 23±2 ºC) 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 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 was used as vehicle control (0.07% v/v).

Peanut extract (PE) (30 mg/ml) was prepared from peanuts (Intersnack Nederland BV, The Netherlands) as described previously [20] and checked for protein content by BCA analysis (Pierce, IL). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc. (CA).

Experimental design

For phenotypic analysis of DC after TCDD treatment, mice (n=3 per group) were treated with vehicle or TCDD (15 µg/kg BW) on day 0 by gavage. The dose of

TCDD used was based on previous research [14,21]. On day 8 mice were sacrificed by cervical dislocation and spleens and mesenteric lymph nodes (MLN) were isolated.

To detect possible effects of TCDD treatment on DC subsets in the spleen and MLN, mice (n=4 per group) were treated with phosphate buffered saline (PBS) or sensitized to PE (6 mg PE, 200 µl/mouse) mixed with CT (15 µg/mouse) on three consecutive days (day 3, 4, 5) by gavage. TCDD (15 µg/kg BW) was administrated on day 0 by gavage. On day 8, mice were sacrificed by cervical dislocation and spleens and MLNs were isolated.

Flow cytometry analysis

Single cell suspensions of spleen (after red blood cell lysis) and MLN ($2.5*10^6$ cells/ml for extracellular stainings, $1*10^7$ cells/ml for intracellular stainings) were incubated for 15 minutes with anti-mouse CD16/32 (clone 93, eBioscience, Austria) in fluorescence activated cell sorting (FACS) buffer (PBS containing 0.25 % BSA, 0.05% NaN_3, 0.5 mM EDTA) and subsequently stained extracellularly in FACS-buffer for 30 minutes at 4 ºC using the following FACS-antibodies: CD11c-APC (clone N418, eBioscience, Austria), CD103-PE (clone 2E7, eBioscience, Austria), MHCII-FITC (clone M5/114.15.2, eBioscience, Austria), CD86-PerCP (clone GL-1, Biolegend, USA), CD80-PE (clone 16-10A1, BD Pharmingen, USA), CD40-FITC (clone 3/23 BD Pharmingen, USA), CD54-FITC (clone 3E2, BD Pharmingen, USA), CD11b-PE (clone M1/70, BD Pharmingen, USA), CD8α-PerCP (clone 53-6.7, BD Pharmingen, USA), CD4-FITC (clone L3T4, eBioscience, Austria), CD25-PE (clone PC61.5, eBioscience, Austria), CD3e-FITC (clone 145-2C11, eBioscience, Austria), CD8α-PE (clone 53-6.7, eBioscience, Austria), CD103-APC (clone 2E7, eBioscience, Austria). Next, cells were washed with FACS-buffer and fixed with 1% formaline in FACS buffer or subsequently stained intracellularly for Foxp3-APC (clone FJK-16s, eBioscience, Austria) according to the manufacturer's instructions. Appropriate isotype controls were used in conjunction with the primary antibody staining. Cells were analyzed on a BD FACSCanto II using BD FACS Diva software (BD Biosciences, USA).

Coculture of splenic DC with PE-specific $CD4^+$ T cells

Spleens were isolated 8 days after treatment of mice with vehicle or TCDD (15 µg/kg BW). Pooled splenic single cell suspensions were put on a density

gradient and $CD11c^+$ DC were subsequently isolated by negative selection using a Dynabeads Mouse DC Enrichment Kit according to manufacturer's instructions (Invitrogen, The Netherlands). PE-specific $CD4^+$ T cells were enriched by immunizing mice with PE/alum (100 µg PE, 4 mg aluminium hydroxide and 4 mg magnesium hydroxide per mouse [Thermoscientific, The Netherlands]). On day 28-38, mice were sacrificed and spleens were isolated. $CD4^+$ T cells were isolated from the spleen by negative selection using a Dynal Mouse CD4 Negative Isolation Kit according to manufacturer's instructions (Invitrogen, The Netherlands). Isolated splenic $CD11c^+$ DC ($6.67*10^5$ cells/ml) were plated in a 12-well plate and pulsed with PE (50 µg/ml) for 24 h. Next, DC were scraped, washed with PBS and plated in a 48-well plate ($8*10^5$ cells/ml) together with freshly isolated $CD4^+$ T cells ($8*10^6$ cells/ml) (1:10) as described before [22]. After 3 days, the supernatant was collected and the frequency of $CD4^+CD25^+Foxp3^+$ T_{reg} cells was determined by flow cytometry.

Analysis of cytokine production

Levels of IL-5, IL-10, IL-13 and IFN-γ in collected supernatants were determined by commercially available sandwich ELISA (eBioscience, Austria) according to the manufacturer's instructions.

Statistical analysis

Results are presented as the mean ± standard error of the mean (SEM) of 3-4 mice per group. All data were logarithmically transformed to achieve normal distribution and were analyzed by a t-test or a 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 AhR activation on surface marker expression on DC in the spleen and MLN.

To investigate effects of AhR activation on DC activation, antigen-presentation and co-stimulation, we studied the expression of the molecules MHCII, CD86, CD80, CD40, CD54 and CD8α on $CD11c^+$ DC 8 days after TCDD treatment.

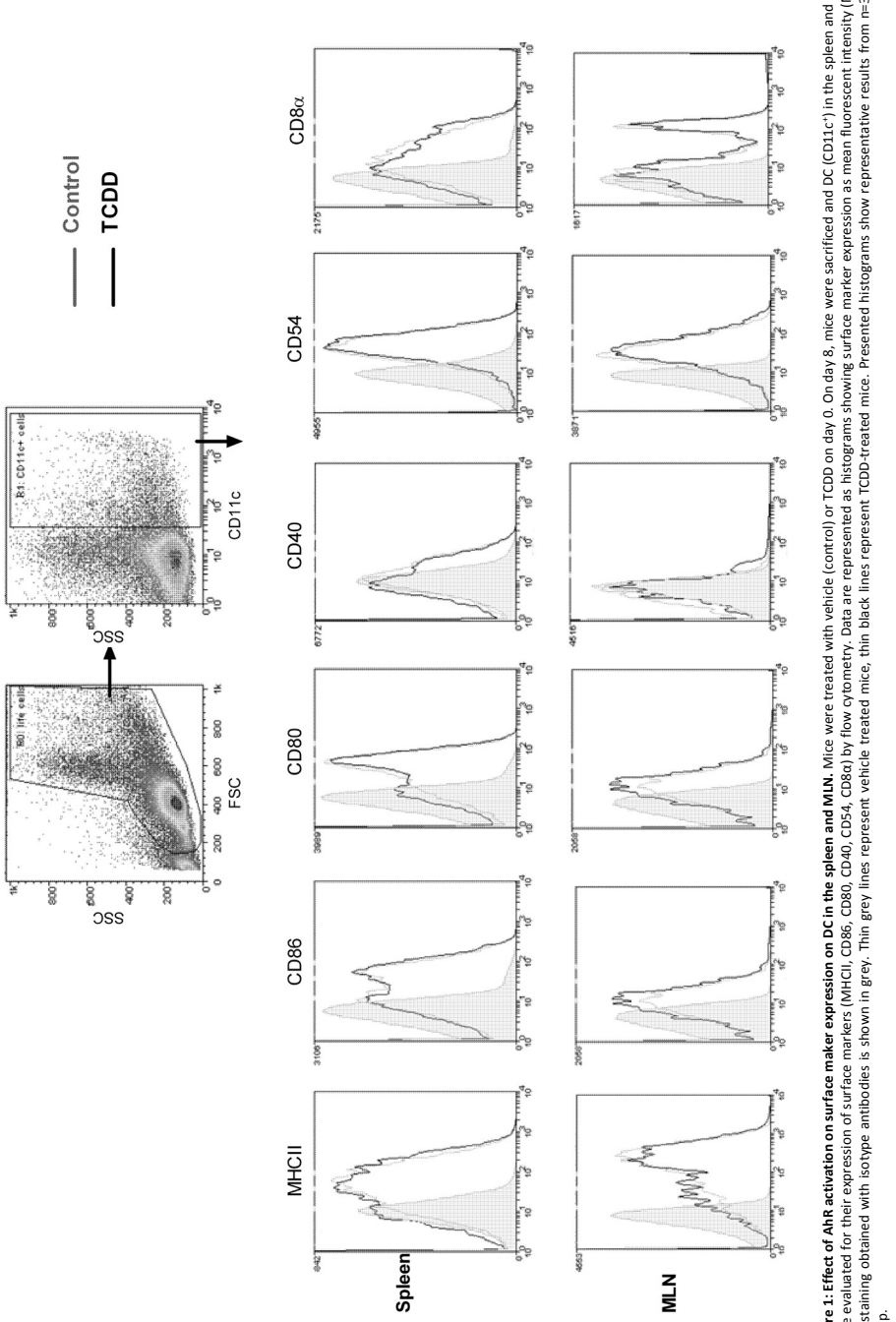

Figure 1: Effect of AhR activation on surface maker expression on DC in the spleen and MLN. Mice were treated with vehicle (control) or TCDD on day 0. On day 8, mice were sacrificed and DC ($CD11c^+$) in the spleen and MLN were evaluated for their expression of surface markers (MHCII, CD86, CD80, CD40, CD54, CD8α) by flow cytometry. Data are represented as histograms showing surface marker expression as mean fluorescent intensity (MFI). The staining obtained with isotype antibodies is shown in grey. Thin grey lines represent vehicle treated mice, thin black lines represent TCDD-treated mice. Presented histograms show representative results from n=3 per group.

Compared to control mice, TCDD treatment did not significantly affect the expression of MHCII, CD86, CD80, CD40, CD54 and CD8α on DC in the spleen and MLN (figure 1).

Effect of AhR activation on CD103$^+$ and CD11b$^+$ DC in the spleen and MLN.
Because AhR activation could increase CD103$^+$ DC in the spleen and CD103$^+$ and CD11b$^+$ DC play an important role in allergic sensitization [8,10,17,23], the effect of AhR activation on these DC subsets was studied in the spleen and MLN during peanut sensitization 8 days after TCDD treatment. Peanut sensitization did not affect the percentage and absolute number of CD11c$^+$ DC and no effects were observed on CD103$^+$ and CD11b$^+$ DC subsets in the spleen and MLN. TCDD treatment did also not affect CD11c$^+$ DC in the spleen, but decreased the percentage and absolute number of CD11c$^+$ DC in the MLN (figure 2). CD11b$^+$ DC were not affected by TCDD treatment in the spleen and MLN (figure 2). Interestingly, AhR activation by TCDD almost doubled the percentage and absolute number of CD103$^+$ DC in the spleen compared to control mice, independent of PE-sensitization (figure 2). However, the percentage of CD103$^+$ DC did not change after TCDD treatment in the MLN and the absolute number of these cells even decreased in PE-sensitized mice treated with TCDD.

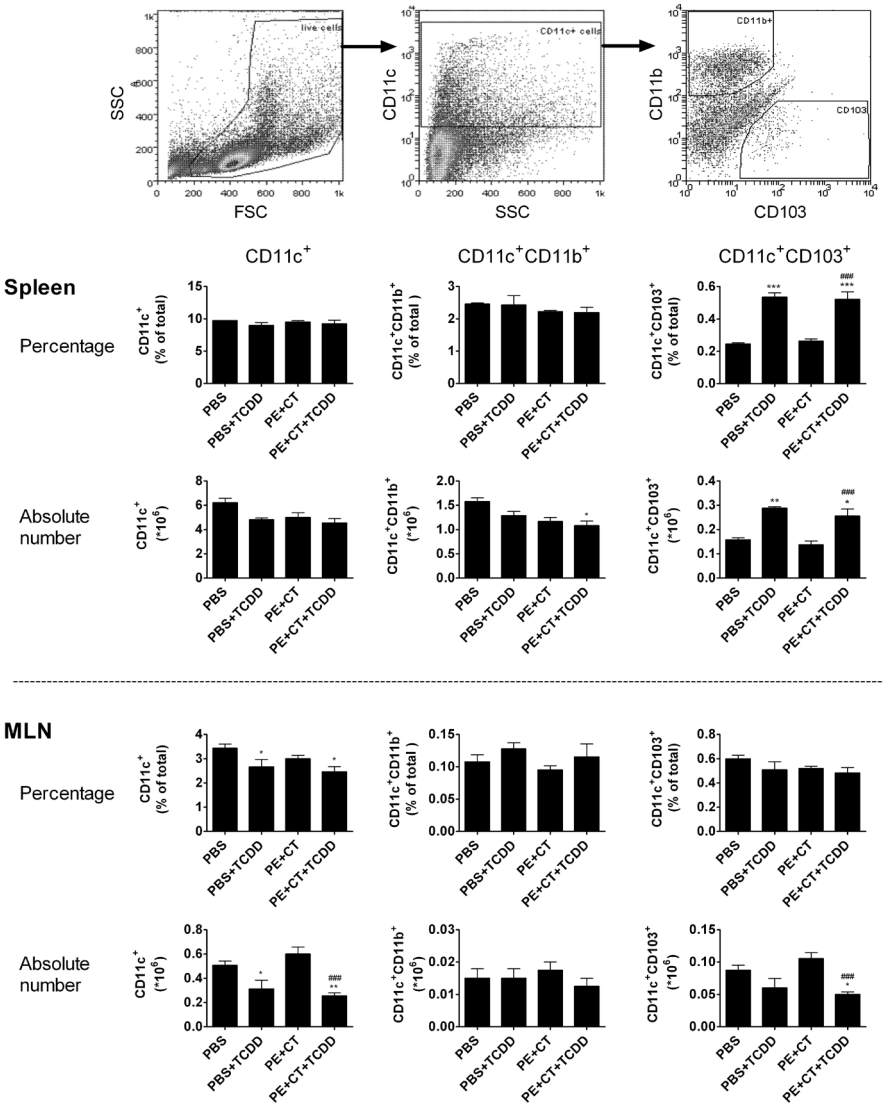

Figure 2: Effect of AhR activation by TCDD on CD11b$^+$ and CD103$^+$ DC in the spleen and MLN. Mice were treated with vehicle or TCDD (15 µg/kg BW) and subsequently sensitized to peanut (PE+CT). On day 8, mice were sacrificed and the percentage and absolute number of DC (CD11c$^+$), CD11b$^+$ DC and CD103$^+$ DC in the spleen and MLN were examined by flow cytometry. Values are presented as mean ± SEM (n=4 per group). * $p < 0.05$ compared to PBS, ** $p < 0.01$ compared to PBS, *** $p < 0.001$ compared to PBS, ### $p < 0.001$ compared to PE+CT.

Effect of in vivo TCDD treatment on splenic DC and their interaction with PE-specific CD4$^+$ T cells.

Next, the functionality of splenic DC from TCDD-treated mice was investigated by culturing *ex vivo* PE-pulsed splenic DC in the presence of PE-specific splenic CD4$^+$ T cells. PE-specific CD4$^+$ T cells cultured with PE-pulsed control DC produced IL-5, IL-10, IL-13 and IFN-γ compared to PE-specific CD4$^+$ T cells cultured with non-pulsed control DC (figure 3). PE-specific CD4$^+$ T cells produced significantly less IL-5, IL-13 and IFN-γ, but similar amounts of IL-10, after incubation with PE-pulsed DC from TCDD-treated mice (figure 3).

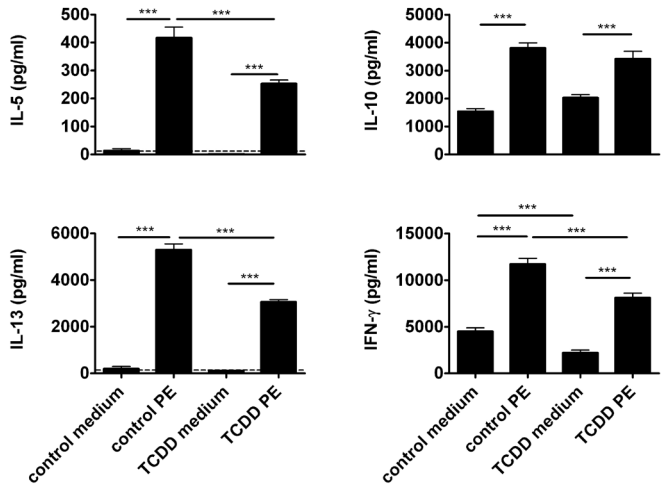

Figure 3: Effect of *in vivo* TCDD treatment on splenic DC and their interaction with PE-specific CD4$^+$ T cells. Splenic DC were isolated from control or TCDD-treated mice and pulsed with PE for 24 h. Next, splenic CD4$^+$ T cells were isolated from PE-immunized mice and cocultured for 3 days with PE-pulsed control or TCDD DC. The supernatant was analysed for IL-5, IL-10, IL-13 and IFN-γ by ELISA. Non-pulsed control and TCDD DC were included as a negative control. Data are representative of three separate experiments. Values are presented as mean ± SEM (n=3-4 per medium pulsed group, n=7 per PE-pulsed group). *** $p < 0.001$

Furthermore, compared to PE-pulsed control DC, incubation of PE-specific CD4$^+$ T cells with PE-pulsed DC from TCDD-treated mice did not affect the frequency of CD4$^+$CD25$^+$Foxp3$^+$ T_{reg} cells *in vitro* (6.57% ±0.44 vs 5.96% ±0.29) (figure 4). Combined, these results show that *in vivo* TCDD treatment affects the capacity of DC to stimulate cytokine production by allergen-specific CD4$^+$ T cells, but does not affect the induction of CD4$^+$CD25$^+$Foxp3$^+$ T_{reg} cells by DC.

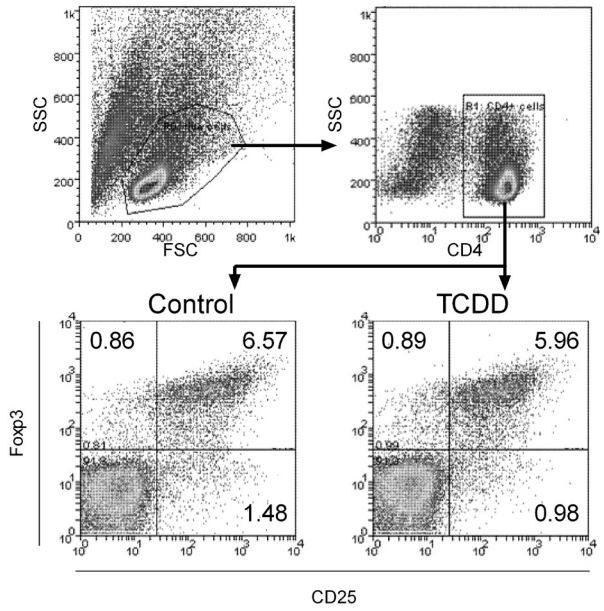

Figure 4: Effect of *in vivo* TCDD treatment on splenic DC and their effect on $CD4^+CD25^+Foxp3^+$ T_{reg} cells in an in vitro coculture with PE-specific $CD4^+$ T cells. Splenic DC were isolated from control or TCDD-treated mice and pulsed with PE for 24 h. Next, splenic $CD4^+$ T cells were isolated from PE-immunized mice and cocultured for 3 days with PE-pulsed control or TCDD DC. Then, the frequency of $CD4^+CD25^+Foxp3^+$ T_{reg} cells was determined by flow cytometry. Data are representative of three separate experiments. Values are presented as mean (n=5 per group).

Discussion

Activation of the AhR by TCDD suppresses peanut sensitization by inducing a functional shift towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells by preserving $CD4^+CD25^+Foxp3^+$ T_{reg} cells and by suppressing precursor and effector T cells [14,15]. However, possible effects of AhR activation on DC during allergic sensitization were not investigated yet, despite their importance during this process [8,24]. Previously, it has been shown that splenic DC express AhR and that *in vivo* AhR activation can modulate splenic DC and their function, resulting in either attenuation or exacerbation of immune responses, dependent on the disease-model and AhR ligand used [16-19,25-27]. Therefore, we investigated the effect of AhR activation on DC during allergic

sensitization in a mouse model for peanut allergy and studied their interaction with allergen-specific T cells *ex vivo*.

The present study shows that *in vivo* TCDD treatment almost doubles the absolute number of $CD103^+$ DC, but does not affect the absolute number of total DC and $CD11b^+$ DC and the expression of MHCII, CD86, CD80, CD40, CD54 and CD8α on $CD11c^+$ DC in the spleen. Comparable effects of AhR activation by ITE on the percentage of $CD103^+$ DC and MHCII, CD86, CD80 and CD40 expression on DC in the spleen have been reported by Quintana et al. [17]. Because coculturing of splenic allergen-specific T cells with DC isolated from TCDD-treated mice suppressed IL-5, IL-13 and IFN-γ but did not induce $CD4^+CD25^+Foxp3^+$ T_{reg} cells, it can be concluded that *in vivo* TCDD treatment affects the T-cell stimulating capacity of DC. Our results suggest that a change in proportion of $CD103^+$ DC versus $CD11b^+$ DC in the spleen induced by AhR activation might be responsible for this.

AhR activation reduces the trafficking of DC from the lung to the mediastinal lymph nodes [25]. Therefore, the reduced number of $CD11c^+$ DC and $CD103^+$ DC in the MLN after AhR activation by TCDD observed in our experiments might be a consequence of reduced trafficking of DC from the intestine to the MLN. On the other hand, Chmill et al. showed that treatment of mice with TCDD does not affect the percentage of total DC in the MLN 10 and 14 days after TCDD treatment, and increased the percentage of $CD103^+MHCII^+$ DC in the MLN [28]. Benson et al. also reported an increased frequency of $CD103^+MHCII^+$ DC in the MLN from TCDD-treated mice [29]. Differences in mouse models and time of analysis might explain these and our findings.

$CD103^+$ DC in the spleen and MLN appear to have different functions. In the intestine and MLN $CD103^+$ DC play an important role in the induction of oral tolerance via induction of $Foxp3^+$ T_{reg} cells [10,23]. Factors released from the epithelium such as TGF-β and retinoic acid induce the development of these tolerogenic DC [30]. In the spleen 50-70% of $CD8\alpha^+$ DC in the T cell zone express $CD103^+$ and these DC are specialized in cross-presenting exogenous antigen to $CD8^+$ T cells, resulting in a cytotoxic T lymphocyte response or in T cell deletion/anergy [31]. In addition, $CD8\alpha^+CD103^+$ DC have been shown to stimulate allogenic $CD4^+$ T cells [32]. The observed increase of $CD103^+$ DC in the spleen after AhR activation might result from interfering effects of AhR activation on systemic retinoid homeostasis[17,33-35]. Quintana et al. showed that splenic DC isolated from ITE treated mice induced $Foxp3^+$ T_{reg} cells in an

retinoic acid dependent manner *ex vivo* in the presence of IL-2, antigen and TGF-β1 after 5-6 days [17]. However, it seems that many stimuli, including TGF-β, IL-2, IL-10, retinoic acid and indoleamine 2,3-dioxygenase play a role in the peripheral induction of T_{reg} cells [27,36,37]. This makes it complicated to study the induction of T_{reg} cells by DC *in vitro*. In our setting, splenic DC isolated from TCDD-treated mice did not induce $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the absence of additional artificial stimuli. Recently, we showed that AhR activation by TCDD suppresses allergic sensitization by reducing precursor and effector T cells and not by inducing $CD4^+CD25^+Foxp3^+$ T_{reg} cells [14,15]. Findings of the present study support this, as AhR activation affects functioning of effector T cells by decreasing the capacity of DC to activate T cells rather than by inducing $CD4^+CD25^+Foxp3^+$ T_{reg} cells. Together, this shows that AhR activation suppresses allergic sensitization by affecting various cells of the immune system.

In summary, AhR activation almost doubles the number of $CD103^+$ DC in the spleen and suppresses the initiation of food allergic responses by affecting the T-cell stimulatory capacity of DC. Furthermore, AhR activation decreases the absolute number of DC in the MLN. This study identified DC as an important target of AhR activation during allergic sensitization and future research should emphasize how AhR activation by TCDD increases splenic $CD103^+$ DC and affects the capacity of DC to stimulate cytokine production by $CD4^+$ T cells.

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Chapter 6

Effects of aryl hydrocarbon receptor activation on immunotherapy for food allergy

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Submitted

Abstract

Food allergy is an increasing health problem and currently there are no approved treatments available. Immunotherapy might be promising but is not successful applied in food allergy yet because of high rate of anaphylactic reactions. In immunotherapy suppression of T helper 1/ T helper 2 cells and induction of regulatory T (T_{reg}) cells are important mechanisms. Recently, we have shown that aryl hydrocarbon receptor (AhR) activation by increases $CD4^+CD25^+Foxp3^+$ T_{reg} cells and decreases T helper 2 cells, resulting in suppression of allergic sensitization. Therefore, we investigated whether AhR activation can be used to improve immunotherapy.

C3H/HeOuJ mice were sensitized to peanut extract (PE) and were treated with TCDD and /or immunotherapy by subcutaneous PE injections. PE-specific antibody levels in the serum and mast cell degranulation (mMCP-1) were analyzed after immunotherapy and anaphylaxis was measured after a systemic challenge with PE.

AhR activation did not affect the increase of PE-specific IgG1 induced by immunotherapy, but reversed the increase of PE-specific IgE and IgG2a and the decrease of mMCP-1 (after intragastric challenge) induced by immunotherapy. Interestingly, the amelioration of systemic anaphylaxis by immunotherapy was not reversed by AhR activation. Treatment of allergic mice with only TCDD did not affect PE-specific antibody levels, mMCP-1 and systemic anaphylaxis.

These findings suggest AhR activation as a possibility to improve immunotherapy for food allergy.

Key words

Anaphylaxis, aryl hydrocarbon receptor, food allergy, immunotherapy, mast cell degranulation, TCDD

Introduction

Food allergy is an increasing health problem in the world with an estimated prevalence of about 5% in young children and 3-4% in adults. Most food allergies are outgrown in children, however, peanut allergy is persistent to adulthood [1,2]. Although food allergy diminishes quality of life and life-threatening anaphylaxis can occur in severe cases, there is no approved treatment available for food allergy [3]. Currently, the primary recommendation is strict avoidance of the causal food and the use of epinephrine as a first line treatment in anaphylaxis [1,2].

It is known for about 100 years that allergen-specific immunotherapy can be used as a desensitizing therapy in allergy [4]. In allergen-specific immunotherapy an appropriate dose of an allergen, administrated subcutaneously, sublingually or epicutaneously, induces a state of clinical and immune tolerance to that allergen [5-7]. The aim of allergen-specific immunotherapy is to induce peripheral T cell tolerance and to increase the thresholds of mast cell and basophil activation [8]. An increase of IgG4 (in humans), regulatory B cells and regulatory T (T_{reg}) cells ($CD4^+CD25^+$, $CD4^+CD25^+Foxp3^+$ and Tr1) have been shown to be key mechanisms in this process [8]. Supposedly, IgG4 captures the allergen before it reaches IgE bound on effector cells such as mast cells and basophils. Regulatory B cells may regulate the development, proliferation and maintenance of $CD4^+$ effector, memory and T_{reg} cells via the secretion of IL-10. $CD4^+CD25^+$ T_{reg}, $CD4^+CD25^+Foxp3^+$ T_{reg} and IL-10 producing Tr1 cells inhibit the development of T helper 1 (Th1) and T helper 2 (Th2) cell responses, suppress IgE production by B cells and directly or indirectly control the activity of effector cells during allergic inflammation, e.g. eosinophils, basophils and mast cells [8,9].

Immunotherapy is successful for allergic rhinitis, asthma, and venom hypersensitivity [10]. Recently, oral immunotherapy for peanut allergy has proven to be effective, although accompanied by relatively high occurrence of adverse side effects such as anaphylaxis [11,12]. For this reason, immunotherapy has not been successfully applied yet in food allergy [13]. Therefore, immune modulators are used to improve safety and efficacy of immunotherapy. For example probiotics, oligomannose-coated liposomes and vitamin D3 have been shown to improve immunotherapy by increasing Th1 and T_{reg} cells and by decreasing Th2 cells [14-17]. In addition, activation of the innate immune system by toll-like-receptor (TLR) agonists during immunotherapy, including TLR-4 agonist monophosphoryl lipid A and a phosphorothioate oligodeoxyribonucleotide immunostimulatory sequence of DNA containing a CpG

motif, appears to improve immunological and clinical responses in allergic patients [18,19]. Furthermore, omalizumab, a humanized, monoclonal anti-IgE antibody that binds to circulating IgE molecules, improves safety and efficacy of immunotherapy by interrupting the allergic cascade downstream the IgE production of B cells [20].
Recently, we have shown that activation of the aryl hydrocarbon receptor (AhR) by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) suppresses peanut sensitization by increasing the relative number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells and by decreasing effector T cells [21,22]. Because the increase of $CD4^+CD25^+Foxp3^+$ T_{reg} cells and the suppression of Th1/Th2 cell responses and IgE production may play a key role in immunotherapy, we hypothesized that AhR activation can be used to improve immunotherapy in a mouse model for peanut allergy. TCDD, the prototypical AhR ligand, was used to study the role of the AhR, because it is the most potent AhR ligand known, it is hardly metabolized and relatively slowly excreted (half life time in mice 10-12 days) [23-25].

Materials and Methods

Mice and reagents
4-5 week old female C3H/HeOuJ mice (AhR^{b-2}), purchased from Charles River (France), were maintained under controlled conditions (relative humidity of 50-55%, 12h light/dark cycle, temperature of 23±2 ºC) 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. 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) and diluted in corn oil (Sigma Aldrich, The Netherlands) to the final exposure concentration. The final anisole exposure concentration was 0.07% v/v. Anisole diluted in corn oil (0.07% v/v) was used as vehicle-control. Peanut extract (PE) was prepared from peanuts (Intersnack Nederland BV, The Netherlands) as described previously [26]. 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).

Experimental design

C3H/HeOuJ mice (n=6-8 per group) were sensitized to PE (6 mg PE, 200 µl/mouse) mixed with CT (15 µg/mouse) by gavage on three consecutive days (day 0, 1, 2) followed by weekly dosing (day 7, 14, 21, 28). Vehicle or TCDD (15 µg/kg BW) [21,27] was administrated on day 39 intragastrically (i.g.) or subcutaneously (s.c.). From day 42-60, mice were treated with immunotherapy (0.09 mg PE, s.c.) or PBS three times a week. On day 70, mice were challenged i.g. with PE (12 mg/mouse) and on day 77 mice were challenged systemically (0.1 mg/mouse, i.p.). Blood was taken on day 32, 65 and 70 (figure 1).

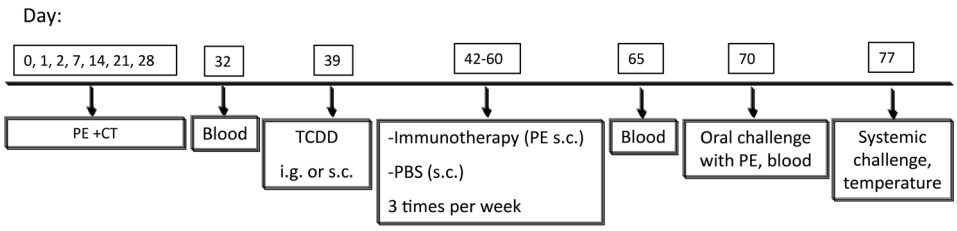

Figure 1. Treatment protocol as described in 'Materials and Methods' section.

Measurement of PE-specific antibody and mMCP-1 levels in serum

PE-specific IgE, IgG1 and IgG2a antibodies in the serum were detected as previously described [28]. mMCP-1 levels in the serum, obtained 30 minutes after i.g. challenge with PE, were determined by using an ELISA kit (eBioscience, Austria). The ELISA was performed according to the manufacturer's instructions.

Measurement of systemic anaphylaxis

Mice were challenged with 0.1 mg PE intraperitoneally (i.p.) on day 77. As an objective parameter of anaphylactic shock, body temperature was measured by means of rectal thermometry every 10–20 minutes for 60 minutes after challenge as described before [28].

Statistical analysis

Results are presented as the mean ± standard error of the mean (SEM) of 6-8 mice per group. Antibody and mMCP-1 levels were logarithmically transformed and statistically

analyzed by a one-way ANOVA and Bonferroni as a post-hoc test. Temperature curves were statistically analyzed using a repeated measures ANOVA followed by Bonferroni as a 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 AhR activation on PE-specific antibody levels in mice after immunotherapy.
To examine the effect of AhR activation on subcutaneous immunotherapy, PE-specific IgE, IgG1 and IgG2a levels were measured before and after TCDD and subcutaneous PE treatment. In line with previous research TCDD was administrated intragastrically [21,29], but to compare possible therapeutic routes of exposure TCDD was also administrated subcutaneously. Before TCDD treatment and the start of subcutanous PE administrations all PE-sensitized mice showed similar levels of PE-specific IgE, IgG1 and IgG2a (figure 2) and only TCDD treatment did not affect these antibody levels on days 65 and 70 (figure 2). Immunotherapy caused a clear increase of PE-specific IgE, IgG1 and IgG2a levels on days 65 and 70 compared to day 32, whereas TCDD treatment of mice receiving immunotherapy decreased PE-specific IgE (on days 65 and 70) and IgG2a (day 65). This was independent of the route of TCDD exposure. Notably, PE-specific IgG1 in mice receiving immunotherapy was not suppressed by TCDD treatment on day 70. Together, these data show that TCDD treatment suppresses the increase of especially PE-specific IgE antibodies induced by immunotherapy, but does not affect the increase of PE-specific IgG1.

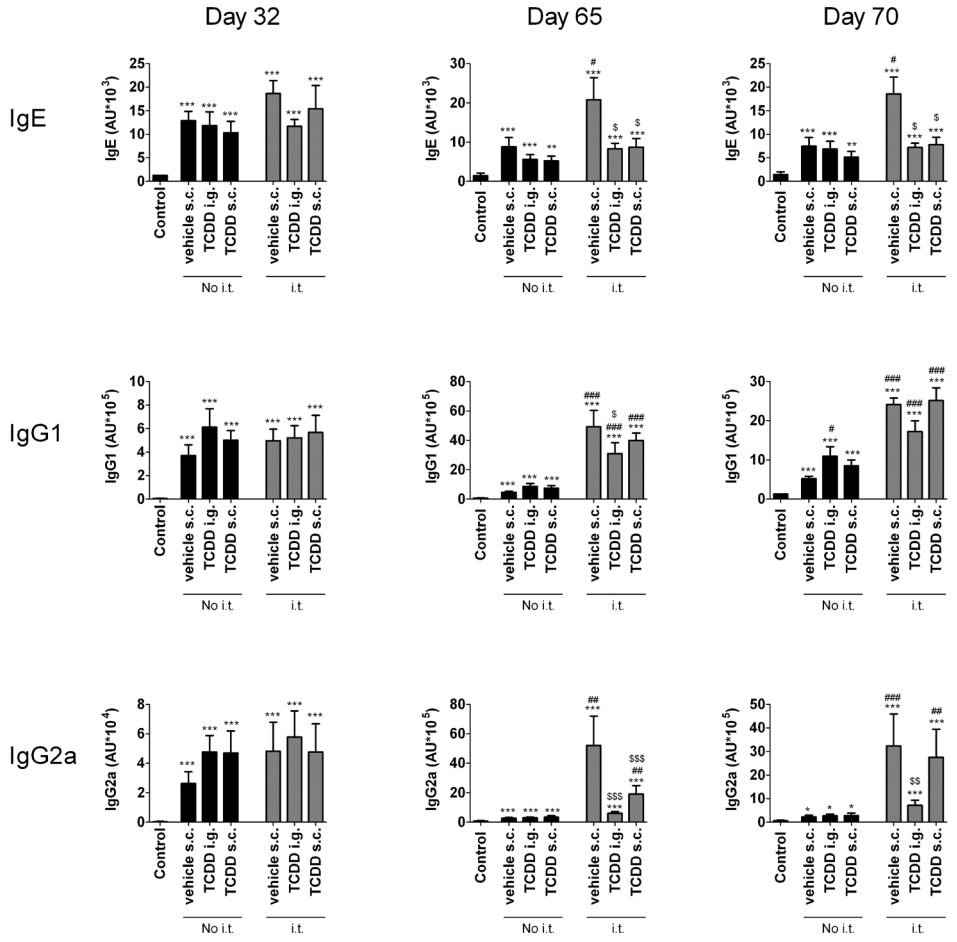

Figure 2. Effect of AhR activation on PE-specific antibody levels after immunotherapy. Mice were sensitized for peanut (PE+CT) and subsequently treated with vehicle or TCDD (15 µg/kg BW) administrated i.g. or s.c.. Then, mice received immunotherapy (i.t., grey bars) or only PBS injections (no i.t., black bars). Control mice (PBS+CT, vehicle+PBS) were not sensitized for peanut and were treated with vehicle and PBS injections. Peanut specific IgE, IgG1 and IgG2a were determined by ELISA in the serum collected on day 32, 65 and 70. Values are presented as mean ± SEM (n=6-8 per group). * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control, # $p < 0.05$ compared to vehicle s.c. no i.t., ## $p < 0.01$ compared to vehicle s.c. no i.t., ### $p < 0.001$ compared to vehicle s.c. no i.t., $ $p < 0.05$ compared to vehicle s.c. i.t., $$ $p < 0.01$ compared to vehicle s.c. i.t., $$$ $p < 0.001$ compared to vehicle s.c. i.t..

Effect of AhR activation on mast cell degranulation in mice after immunotherapy.
To analyze the effect of AhR activation on mast cell degranulation after subcutaneous immunotherapy, mMCP-1 levels were determined in the serum after intragastric challenge with PE. PE-sensitized mice challenged with PE had increased mMCP-1 levels in the serum compared to control mice, which was not affected by only TCDD treatment (figure 3). Immunotherapy suppressed mMCP-1 levels in PE-sensitized mice after PE challenge. However, TCDD treatment reversed the suppressive effect of immunotherapy on mMCP-1 levels after challenge (figure 3).

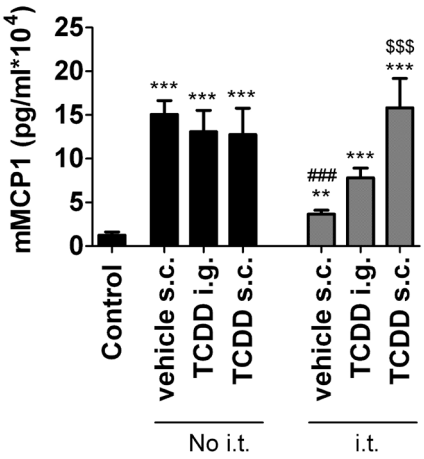

Figure 3. Effect of AhR activation on mast cell degranulation after immunotherapy. Mice were sensitized for peanut (PE+CT) and subsequently treated with vehicle or TCDD (15 µg/kg BW) administrated i.g. or s.c.. Then, mice received immunotherapy (i.t., grey bars) or only PBS injections (no i.t., black bars). Control mice (PBS+CT, vehicle+PBS) were not sensitized for peanut and were treated with vehicle and PBS injections. On day 70, mice were challenged i.g. with PE and mMCP-1 levels were determined in the serum collected 30 minutes after challenge. Values are presented as mean ± SEM (n=6-8 per group). ** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control, ### $p < 0.001$ compared to vehicle s.c. no i.t., $$$ $p < 0.001$ compared to vehicle s.c. i.t..

Effect of AhR activation on systemic anaphylaxis in mice after immunotherapy.
To assess whether AhR activation influences the protective effect of immunotherapy on systemic anaphylaxis, mice were challenged systemically with PE and body temperature was monitored at regular intervals. PE-sensitized mice showed a severe drop in temperature after challenge compared to control mice and only TCDD treatment did not reverse this (figure 4, left). As expected, PE-sensitized mice receiving immunotherapy were protected from anaphylactic reactions (figure 4, right).

Remarkably, TCDD treatment of mice receiving immunotherapy did not reverse the protective effect of immunotherapy on systemic anaphylaxis.

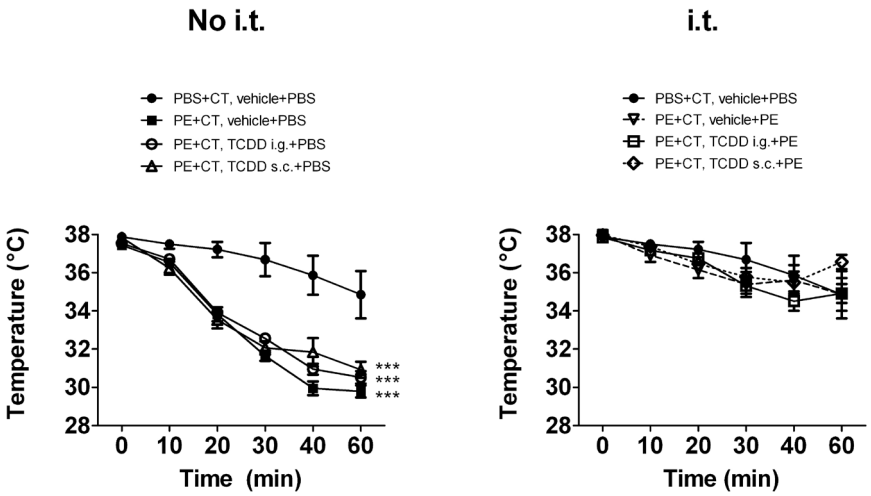

Figure 4. Effect of AhR activation on systemic anaphylaxis after immunotherapy. Mice were sensitized for peanut (PE+CT) and subsequently treated with vehicle or TCDD (15 µg/kg BW) administrated i.g. or s.c.. Then, mice received immunotherapy (i.t., grey bars) or only PBS injections (no i.t., black bars). Control mice (PBS+CT, vehicle+PBS) were not sensitized for peanut and were treated with vehicle and PBS injections. On day 77, mice were challenged i.p. with PE and body temperature was measured by means of rectal thermometry every 10–20 minutes for 60 minutes after challenge. Values are presented as mean ± SEM (n=6-8 per group). *** $p < 0.001$ compared to control.

Discussion

Immunotherapy is successful for several allergic disorders and is accompanied by an increase of IgG4 (in humans), regulatory B cells and regulatory T (T_{reg}) cells ($CD4^+CD25^+Foxp3^+$ and Tr1), and a decrease in Th1/Th2 cell responses and IgE production by B cells [8-10,13]. However, immunotherapy is not yet applied for food allergy due to the high risk for severe side effects [10,13]. Immune modulators are used to reduce side effects and to improve safety and efficacy of immunotherapy. Because AhR activation suppresses Th2 responses by decreasing effector T cells, by suppressing allergen-specific antibody responses and by inducing a functional shift within the $CD4^+$ population towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells [21,22,30-33], we hypothesized that AhR activation may improve immunotherapy.

AhR activation suppresses a variety of Th2- and Th1/Th17-immune-mediated diseases when the AhR is activated before, simultaneously or during the progression of disease-induction [21,27,31,32,34,35]. However, to our knowledge, the effect of AhR activation on an established immune response such as food allergy was not studied before. The present data indicate that AhR activation does not suppress established food allergy, because TCDD treatment did not suppress PE-specific IgE, IgG1 and IgG2a, mast cell degranulation and anaphylaxis.

However, when combined with immunotherapy TCDD treatment affected various serological allergic parameters. AhR activation suppressed the increase of especially PE-specific IgE antibodies induced by immunotherapy, but did not affect the increase of PE-specific IgG1 by immunotherapy. Moreover, AhR activation reversed the suppressive effect of immunotherapy on mast cell degranulation after intragastric challenge. This finding on mast cell degranulation suggests that AhR activation prevents the curative effect of immunotherapy on peanut allergy. Strikingly however, mice receiving immunotherapy that were treated with TCDD were protected from systemic anaphylaxis, showing that AhR activation does not suppress the clinical effect of immunotherapy.

This suggests that AhR activation interferes with the pathophysiology of mast cell degranulation. In anaphylaxis a variety of mediators released by mast cells are involved, for instance cytokines, histamine, tryptase, chymase, heparin, carboxipeptidase A3, platelet activating factor, prostaglandin and leukotriene. Importantly, anaphylaxis depends on cellular responses to these mediators [36]. Because the AhR is present in a variety of cells in the body [37], it can be hypothesized that AhR activation affects cellular responses to mediators released by mast cells. Furthermore, a recent study shows that mast cells express AhR and that *in vivo* AhR activation by 6-formylindolo[3,2-b]carbazole (FICZ) exacerbates mast cell degranulation measured by histamine release, whereas repeated FICZ administration attenuates histamine release upon challenge [38]. Together, this indicates that AhR activation interferes with the release of mediators by mast cells and might suppresses the response of cells involved in anaphylaxis to these mediators. Whether a decrease in effector T cells and an increase in the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells as a result of AhR activation are involved in the different effects of AhR activation on serological parameters and clinical parameters remains to be investigated.

The present study addressed the AhR as a possible immune modulator to improve immunotherapy for food allergic responses. Notably, AhR ligands have a highly ligand-

specific effect on gene expression, degranulation and calcium influxes of mast cells [39], which makes their effect on mast cell degranulation unpredictable. Importantly, the greatest source of exposure to AhR ligands comes from our diet [23]. Daily products, like potatoes, cruciferous vegetables, bread, hamburgers and grape fruit induce AhR activation [40]. Although most natural AhR ligands are weak agonists and are metabolized relatively fast after ingestion [23], it can be hypothesized that the safety and efficacy of immunotherapy can be influenced by AhR ligands present in our diet. Therefore, the contribution of the diet during immunotherapy should not be underestimated. In addition, the route of exposure to an AhR ligand might influence immunotherapy, because our results show that the effect of immunotherapy on IgG2a and mMCP-1 is influenced by the route of TCDD exposure. Furthermore, FICZ is produced by skin cells from tryptophan on exposure to UV and visible lights [41], suggesting that exposure to sunlight might influence immunotherapy via AhR ligands.
In conclusion, our data demonstrate for the first time AhR activation suppresses the increase of allergen-specific antibodies (especially IgE) induced by immunotherapy and increases mast cell degranulation after challenge, but does not affect the protective effect of immunotherapy on the clinical response. Future research will focus on the AhR as a possible immune modulator to improve immunotherapy for food allergy.

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Chapter 7

General discussion

Summary

For decades, aryl hydrocarbon receptor (AhR) activation is known to suppress a variety of immune responses [1,2]. Today, together with an increase in immunological knowledge, it is clear that the AhR affects the functioning of dendritic cells (DC) and the development of and balance between Th1, Th2, Th17 cells and regulatory T (T_{reg}) cells [3-6]. Evidently, DC, T_{reg} cells and the Th1/Th2 cell balance are important in allergy, because of their involvement in the induction of either tolerance or sensitization [6-9]. Despite an increase in the knowledge behind allergic sensitization, food allergy has become a major public health problem in the world for which no cure or approved treatment is available [10,10,11].
Therefore, the aim of this thesis was to investigate whether and how AhR activation suppresses allergic sensitization and if AhR activation could improve immunotherapy for food allergy. The prototypical AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was used to study the role of the AhR [12-14].
In **chapter 2** it was shown that AhR activation by TCDD dose-dependently suppressed peanut allergic sensitization and that this was accompanied by a dose-dependent increase of the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells. Depletion of these T_{reg} cells reversed the suppressive effect of TCDD on peanut sensitization, showing that AhR activation suppressed allergic sensitization by inducing a functional shift within the $CD4^+$ population towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells. However, AhR activation did not increase the absolute number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells. Moreover, the suppression of some peanut allergic parameters by AhR activation was not completely abrogated in the absence of $CD4^+CD25^+Foxp3^+$ T_{reg} cells. This could mean that AhR activation also suppressed peanut sensitization independent of $CD4^+CD25^+Foxp3^+$ T_{reg} cells. The effect of AhR activation by TCDD on precursor T cells, effector T cells and T_{reg} cells was therefore investigated in more detail in **chapter 3**. It is known for a long time that AhR activation by TCDD suppresses thymic T cell precursors. However, the effect of TCDD on thymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells, which substantially contribute to the peripheral T_{reg} population, has not been studied before. Strikingly, data presented in this thesis showed that thymic $CD4^+CD25^+Foxp3^+$ T_{reg} cells were preserved from a TCDD-induced decrease of the number of precursor T cells and effector T cells. This resulted in an increased percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the thymus and spleen, but not in the MLN. The effects of AhR activation on T cell subsets in the thymus and the spleen were independent of allergic sensitization. In contrast, the

decrease of the absolute number of effector T cells after AhR activation in the MLN was dependent of allergic sensitization.

AhR activation has been shown to suppress but also to exacerbate immune responses and this may depend on the AhR ligand and the disease model used. Therefore, the effect of the AhR ligands 6-formylindolo[3,2-b]carbazole (FICZ), β-naphthoflavone (β-NF) and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), which do not induce dioxin-like-toxicity, on peanut sensitization was investigated in **chapter 4**. In contrast to TCDD, FICZ, β-NF and 6-MCDF did not suppress allergic sensitization and did not increase the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells. In addition, these AhR ligands only marginally increased AhR-dependent gene transcription *in vivo,* measured by CYP1A1, CYP1A2 and CYP1B, and were metabolically unstable *in vitro*, when compared to TCDD. Vulnerability of these non-dioxin-like AhR ligands to AhR-induced metabolism, differences in AhR binding affinity and subsequent gene transcription might explain why TCDD, but not FICZ, β-NF and 6-MCDF, suppressed allergic sensitization.

In **chapter 5**, the effect of AhR activation by TCDD on DC was investigated. Interestingly, AhR activation did not affect the total number of DC in the spleen, but drastically increased the proportion of splenic $CD103^+$ DC. Moreover, AhR activation of splenic DC affected their interaction with effector T cells, which suppressed the initiation of food allergic responses. In contrast, AhR activation decreased the number of DC in the MLN.

Finally, it was investigated whether AhR activation improved allergen-specific immunotherapy in **chapter 6**. AhR activation suppressed the increase of allergen-specific antibody levels, especially IgE, induced by immunotherapy, but reversed the suppressive effect of immunotherapy on mast cell degranulation after oral challenge. Strikingly, the effect of immunotherapy on systemic anaphylaxis was not reversed by AhR activation. In addition, AhR activation without immunotherapy did not lower food allergic responses.

Together, the data described in this thesis show that AhR activation by dioxin-like compounds suppresses allergic sensitization by suppressing the absolute number of precursor and effector T cells, by preserving $CD4^+CD25^+Foxp3^+$ T_{reg} cells and by affecting DC and their interaction with effector T cells. Furthermore, data in this thesis show that AhR activation affects immunotherapy for food allergy.

The AhR as a therapeutic target for food allergy: consequences and risks

Data presented in this thesis shows that AhR activation can be used to suppress peanut allergic sensitization and suggests that activation of the AhR modifies immunotherapy. However, it should be taken into account that not all AhR ligands suppress allergic sensitization and induce AhR-dependent gene expression comparable to TCDD as shown in chapter 3. Moreover, AhR activation may also exacerbate disease in some cases. For instance, FICZ exacerbates experimental autoimmune encephalomyelitis (EAE) by increasing Th17 cells [3,5]. Especially the chemical structure of the AhR ligand and the cytokine environment during AhR activation appear to determine the ultimate effects of AhR activation on the immune system.

Obviously, the chemical structure of an AhR ligand determines the stability and binding affinity to the AhR, but it also determines subsequent gene transcription, the interaction of the AhR with other genes and pathways and possible toxic effects [12,15]. Moreover, AhR ligands can bind different in AhR binding pockets, dependent on their chemical structure. This differential binding can result in ligand-dependent differences in the overall functionality of the AhR, including differences in co-activator recruitment and transcriptional activity [16]. Ligand-dependent differences have been shown for example for the AhR ligands TCDD and 3,3'-diindolylmethane (DIM). These AhR ligands have a different ability to activate distinct AhR-controlled pathways, including activation of CYP450 enzymes and activation of the estrogen receptor degradation pathway [15]. Because estrogens are associated with an increased risk for allergies [17], the influence of certain AhR ligands on this pathway should be taken into account. Besides, initially low affinity AhR ligands can be converted into high affinity AhR ligands. For example, the AhR ligands indolo-(3,2,-b)-carbazole (ICZ) and 3,3'-diindolylmethane (DIM) are acidic condensation products formed from the weak AhR ligand indole-3-carbinol (I3C) which is present in cruciferous vegetables [12]. This may result in unpredictable effects of dietary AhR ligands on food allergic responses. Furthermore, non-halogenated AhR ligands induce their own metabolism by activating CYP450 enzymes, thereby reducing their bio-availability, in contrast to the metabolically stable halogenated AhR ligands [18-22].

Another important factor determining the effect of AhR activation on the immune response is the cytokine environment. Pro-inflammatory cytokines can affect the expression of most AhR-dependent metabolizing enzymes, thereby affecting

metabolism of AhR ligands and thus ligand binding to the AhR and subsequent effects on the immune system [23]. In addition, cytokines can directly influence the level of AhR expression. For instance TNF-α increases AhR expression fibroblast-like synoviocytes [24]. This cytokine dependent expression of the AhR suggests that the cytokine milieu as associated with certain diseases plays a major role in determining the effect of AhR activation on the immune system. Whether the Th2 cytokines, that initiate food allergy, affect AhR expression is not known yet.
Altogether, cautiousness is needed when considering the AhR as a potential therapeutic target to treat food allergy: chemical structure of the AhR ligand, the cytokine milieu, the time (i.e. the presence or absence of certain cytokines) and route of administration (i.e. the vulnerability for metabolism) could affect the possible therapeutic effects of AhR activation on food allergy. However, data presented in this thesis indicate the AhR as a potential therapeutic target to prevent or treat food allergy.

The intestinal immune system, dietary AhR ligands and food allergy

The gut is the first exposure site after food ingestion. Importantly, the greatest source of exposure to AhR ligands comes from our diet [12]. Daily products, like potatoes, cruciferous vegetables, bread, hamburgers and grape fruit induce AhR activation [25]. Because most natural AhR ligands are metabolized relatively fast after ingestion and have various degrees of AhR binding affinity, it is difficult to estimate the exposure of individuals to AhR ligands. Nevertheless, these dietary AhR ligands might influence the onset of intestinal diseases such as food allergy.
The default immune pathway of the intestinal immune system is the induction of oral tolerance to food antigens and harmless commensals. In this process, intestinal epithelial cells condition the development of tolerogenic $CD103^+$ DC via TGF-β and retinoic acid (RA) [26]. These $CD103^+$ DC promote the differentiation of T_{reg} cells, resulting in oral tolerance [27-30]. The balance between $CD103^+$ DC and other DC subsets, including $CD11b^+$ DC, may be important in food allergic sensitization. This is illustrated by the fact that oral sensitization to peanut is accompanied by an increase of inflammatory $CD11b^+$ DC and a decrease of $CD103^+$ DC in the intestine [31]. In the spleen, AhR activation increases tolerogenic $CD103^+$ DC which induce $Foxp3^+$ T_{reg} cells [4]. In addition, AhR activation decreases the functionality of the total splenic DC

population to activate antigen-specific T cells (Chapter 5). In the intestine, AhR activation also increases the frequency of $CD103^+$ DC [32]. However, whether AhR activation induces tolerogenic $CD103^+$ DC in the intestine and stimulates T_{reg} cells in our models, thereby playing a role in food allergic responses, is not clear yet.

Recently, it became clear that the AhR plays a crucial role in intestinal innate immunity and that dietary AhR ligands derived from vegetables interact with the intestinal immune system by regulating the postnatal expansion of innate lymphoid cells (ILC) secreting IL-22 [33-36]. ILC, including nonconventional natural killer cells and lineage marker-negative lymphoid tissue inducer cells, produce IL-22 upon activation by IL-23 secreted by DC. One of the effects of IL-22 on the intestinal immune system is maintaining the epithelial barrier by increasing epithelial cell growth and survival [34,37]. Maintenance and condition of the epithelial barrier is important for the prevention of food allergy, because defects in the intestinal epithelial barrier contribute to the development of food allergy [38]. Therefore, it can be hypothesized that AhR activation, especially by dietary AhR ligands, may suppress allergic sensitization by enhancing the intestinal epithelial barrier via ILC secreting IL-22.

Furthermore, the AhR and dietary AhR ligands are important for the maintenance of another innate cell in the intestine: intraepithelial lymphocytes (IEL) [36]. Interestingly, IEL (e.g. γδ T cells) are involved in food allergic sensitization [39]. In addition, unpublished data from our lab (by L. Spronck et al.) show that AhR ligands directly interact with intestinal epithelial cells, because AhR ligands suppress the secretion of pro-inflammatory IL-6 by MODE-K cells (a small intestinal epithelial cell line) dose-dependently upon TLR4 ligation by LPS (figure 1). Intestinal epithelial cells can shape immune responses towards regulatory or inflammatory immune responses [26]. By decreasing the secretion of the pro-inflammatory cytokine IL-6 by intestinal epithelial cells, AhR activation might skew immune responses towards a less inflammatory and/or more regulatory type thereby contributing to the suppression of food allergic responses (figure 2).

Together, given the importance of the AhR in the intestinal immune system, it could be speculated that the presence of natural AhR ligands in our diet influences the development of food allergy by affecting the intestinal immune system via epithelial cells, ILC, IEL, DC and T_{reg} cells.

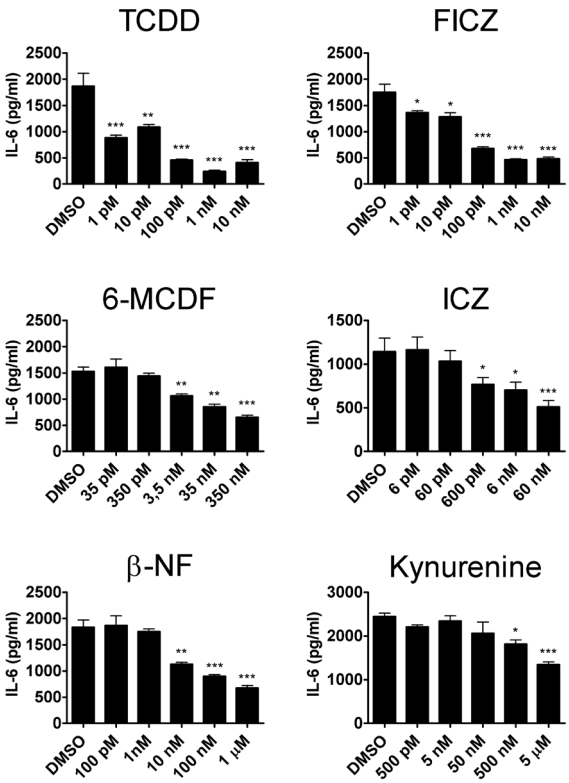

Figure 1. Effect of various AhR ligands on LPS induced IL-6 production by MODE-K cells (a mouse small intestinal epithelial cell line). MODE-K cells were exposed to 10 ng/ml LPS in the presence of increasing concentrations TCDD, FICZ, 6-MCDF, ICZ, β-NF and kynurenine. After 24 hours the supernatant was taken and the amount of IL-6 was determined by ELISA. No IL-6 could be detected in the absence of LPS stimulation. Values are expressed as mean ± standard error. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to DMSO control.

Concluding remarks

A hypothetical overview of the mechanisms responsible for the interference of the AhR with the development of food allergic responses is shown in figure 2, as described in this thesis and in the literature. Upon gastrointestinal exposure to an AhR ligand, the intestinal barrier is maintained and conserved by ILC and IEL [33,36]. This might contribute to a lower exposure of allergen to the subsequent immune system. Furthermore, decreased production of inflammatory cytokines by intestinal epithelial

cells as a result of AhR activation during the establishment of allergic disease (using the adjuvant CT) results in a less pro-inflammatory cytokine milieu. In addition, AhR activation induced an increase in tolerogenic $CD103^+$ DC accompanied by a relative reduction in the number inflammatory DC (Chapter 5). This is followed by the observed AhR-induced suppression of the capacity of DC to activate T cells, and Th2 cells in particular (Chapter 5) [27,31,40]. Others have shown that AhR activation increases tolerogenic $CD103^+$ DC, which in turn induce $Foxp3^+$ T_{reg} cells in an retinoic acid (RA) dependent manner [4]. We observed that dependent on the AhR ligand used, AhR activation *in vivo* induces a shift within the $CD4^+$ T cell population towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells by preserving these T_{reg} cells and suppressing the number of T cells and Th2 cells in particular (Chapter 2, 3, 4). This shift within the $CD4^+$ population towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells is accompanied by a decrease in the production of IL-5 and IL-13 by Th2 cells (Chapter 2). As a result of reduced Th2 cytokine production, or by direct AhR stimulation, B cell activation and subsequent IgE production are decreased (Chapter 2) [2]. The consequence of reduced IgE levels is reduced IgE-loading of mast cells and decreased mast cell degranulation upon allergen challenge (Chapter 2). Moreover, $CD4^+CD25^+Foxp3^+$ T_{reg} cells can suppress mast cell degranulation via OX40 ligand [41,42]. This suggests that the shift within the $CD4^+$ population towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells induced by AhR activation actively contributes to suppression of mast cell degranulation.

Recent studies show that AhR activation can directly increase or decrease mast cell degranulation and that this is ligand-specific and dependent on the frequency of exposure [43,44]. Interestingly, when combined with immunotherapy, AhR activation reverses diminished mast cell degranulation without affecting the protective effect of immunotherapy on the clinical anaphylactic response (Chapter 6). In anaphylaxis, a variety of mediators released by mast cells are involved, including histamine, proteases, platelet activating factor, prostaglandins and leukotrienes. Subsequent anaphylaxis depends on cellular responses to these mediators [45]. Therefore, it can be speculated that although AhR activation reverses the therapy-induced suppression of mMCP-1 release, it at the same time interferes with the release of other mediators and effector cells involved in anaphylaxis. Interestingly, it must be noted that mucosal mast cell degranulation as measured by mMCP-1 is not always correlated with systemic anaphylaxis, which indicates the involvement of other factors than mucosal mast cell degranulation in anaphylaxis [46].

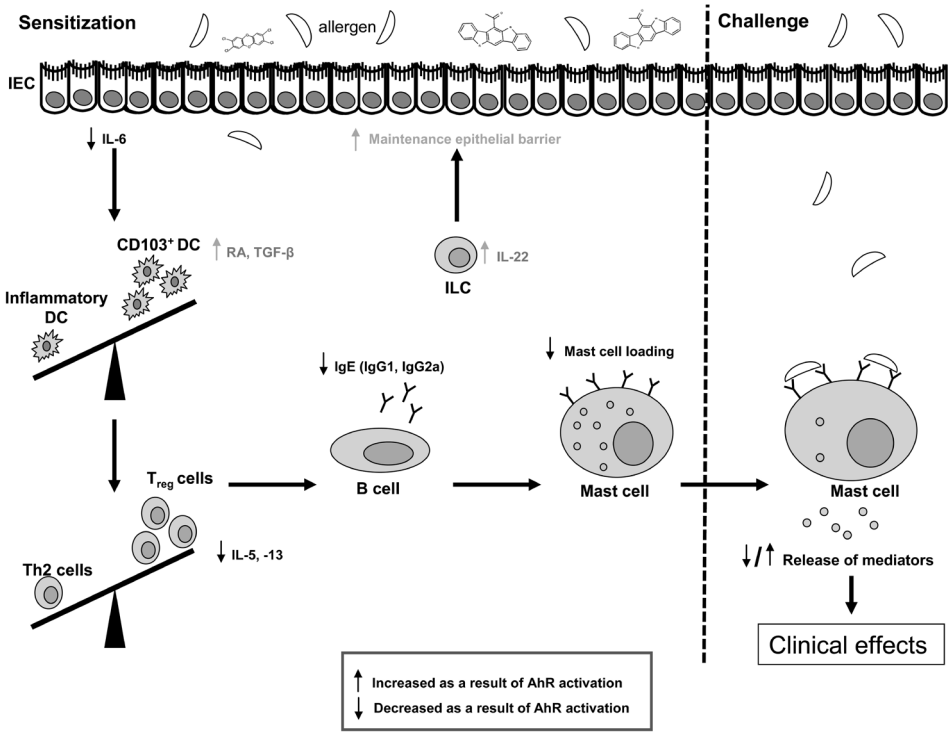

Figure 2. Hypothetical overview of mechanisms responsible for the interference of the AhR with the development of food allergic responses. Upon gastrointestinal exposure to an AhR ligand, the intestinal barrier is conserved by increased production of IL-22 by ILC and enhanced presence of IEL. This might contribute to a lower exposure of allergen to the subsequent immune system. Furthermore, AhR activation suppresses IL-6 production by intestinal epithelial cells (IEC), resulting in a less pro-inflammatory cytokine milieu. AhR activation induces an increase of $CD103^+$ DC which is followed by decreased capacity to activate T cells, in particular Th2 cells. In addition, others have shown that AhR activation increases tolerogenic $CD103^+$ DC, which in turn induce $Foxp3^+$ T_{reg} cells in an retinoic acid (RA) and TGF-β dependent manner. Furthermore, AhR activation induces a shift within the $CD4^+$ T cell population towards $CD4^+CD25^+Foxp3^+$ T_{reg} cells, which is accompanied by a decrease in the production of IL-5 and IL-13 by Th2 cells. As a result of reduced Th2 cytokine production, or by direct AhR stimulation, B cell activation and subsequent IgE production are decreased. The consequence of reduced IgE levels is reduced IgE-loading of mast cells and decreased mast cell degranulation upon allergen challenge. Interestingly, when combined with immunotherapy, AhR activation reverses diminished mast cell degranulation without affecting the protective effect of immunotherapy on the clinical anaphylactic response. Moreover, others have shown that AhR activation can directly increase or decrease mast cell degranulation and this is ligand-specific and dependent on the frequency of exposure (black: results from this thesis, grey: findings from literature).

In conclusion, AhR activation by persistent high affinity ligands, such as dioxin-like compounds, suppresses allergic sensitization via complex interactions with various cells of the immune system and may improve allergen specific immunotherapy for food allergy. As mentioned before, critical factors determining the effect of AhR on food allergic reactions are the AhR binding site of an AhR ligand, binding affinity of AhR ligands to the AhR, subsequent gene transcription, metabolic stability, dose, timing and route of administration and cytokine milieu.

Questions and future directions

- *What is the effect of AhR ligands present in our diet on allergic sensitization?*

An optimal epithelial barrier function is essential for the prevention of food allergic sensitization. As mentioned before, the AhR may play a major role in the intestinal immune system by maintaining the intestinal epithelial barrier [33,36]. Because our diet is rich in AhR ligands [12,25] it can be hypothesized that our diet greatly influences the induction of either tolerance or active immunity via the AhR, thereby playing a major role in food allergic responses. Interestingly, preliminary data show that various AhR ligands, including FICZ, β-NF and 6-MCDF, suppress TRL4 induced IL-6 production by small intestinal cells *in vitro* (figure 1), suggesting that AhR activation can affect immune responses of the intestinal epithelial barrier. However, results of this thesis also showed that oral administration of FICZ, β-NF and 6-MCDF did not affect food allergic sensitization *in vivo* (Chapter 4), probably as a result of less sustained AhR activation and decreased metabolic stability compared to TCDD. Nevertheless, via our diet we are constantly exposed to AhR ligands and this could influence the functioning of the intestinal immune system. Therefore future research should focus on the effect of AhR activation on intestinal epithelial cells ILC and IEL, and how this affects the functioning of ($CD103^+$) DC, Th cells and $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the intestinal immune system during the induction of oral tolerance or allergic sensitization. Obviously, the choice of AhR ligands or specific diets supplemented with AhR ligands is of importance.

- *Can AhR activation be used to improve immunotherapy for food allergy?*

Findings in this thesis show that AhR activation suppresses the increase of allergen-specific IgE but reverses the diminished mast cell degranulation induced by

immunotherapy, without affecting the protective effect of immunotherapy on the clinical response (Chapter 6). Future research using different doses of various AhR ligands, suboptimal doses of immunotherapy, and/or doses of immunotherapy that cause serious side effects should reveal whether AhR activation can be used to improve immunotherapy. In addition, direct effects of AhR activation on mast cell degranulation should be studied, because it is important to know when AhR activation suppresses or increases mast cell degranulation [43,44]. Furthermore, it has to be investigated if the relative increase of $CD4^+CD25^+Foxp3^+$ T_{reg} cells and the decrease of effector T cells after AhR activation contribute to the beneficial effects of AhR activation on immunotherapy. In addition, it could be investigated whether exposure to sunlight affects immunotherapy, because the endogenous high affinity AhR ligand FICZ is produced by skin cell exposure to UV and visible lights [47].

- *What is the ideal AhR ligand to prevent/treat food allergy?*

The chemical structure of an AhR ligand determines stability, binding to the AhR and subsequent gene transcription, as mentioned previously [12,15,18]. This can greatly influence the effect of AhR activation on the immune system either in a harmful or a beneficial way. To be able to suppress allergic sensitization, the ideal AhR ligand should be relatively stable, activate the AhR persistently and induce the relevant down-stream-pathways as TCDD. Obviously, this AhR ligand should not cause the toxic effects associated with dioxin. Metabolic stability of potential AhR ligands can be pre-screened by measuring AhR activation after exposing a hepatic cell line to the AhR ligand in the presence of CYP450 inhibitors (Chapter 4). Effects of AhR ligands on relevant down-stream-pathways of the AhR in cells of the immune system, for example pathways important for DC and T cell differentiation and cytokine expression, can be compared by detailed mechanistic studies including microarrays.

- *To what extent contributes the cytokine environment to the effect of AhR activation on the immune system, including food allergy?*

As mentioned before, the cytokine milieu as associated with certain diseases can play a major role in determining the effect of AhR activation on the immune system by influencing AhR expression and metabolism of AhR ligands [23,24]. This may also determine whether AhR activation results for instance in the induction of $Foxp3^+$ T_{reg} cells (TGF-β) or Th17 cells (TGF-β and IL-6) [3,48]. Therefore, it is crucial to elucidate how the cytokine milieu shapes the effect of AhR activation on immune responses.

Currently, it is not clear if, how and when pro- and anti-inflammatory cytokines play a role in food allergy, and whether this determines the suppressive effect of AhR activation on food allergic responses.

- *Can AhR activation affect food allergic responses in humans?*

The AhR differs in structure and function between species and even within species [49]. For instance, C56BL/6 mice express an AhR with high binding affinity for ligands, whereas DBA mice express a low-affinity AhR. The dissociation constant of the AhR for TCDD is approximately 6-10 higher in DBA mice [50,51]. Notably, the human AhR resembles the structure of the DBA AhR more than the C57BL/6 AhR and its dissociation constant for TCDD is almost similar to the DBA AhR [51,52], suggesting that humans are not very susceptible for AhR-mediated effects. However, TCDD induces CYP1A1 expression more potently in human lymphocytes than in lymphocytes from DBA mice and rats *in vitro* [53]. It is possible that AhR ligands interfere differently with coactivators, transcription factors and chaperone proteins in different species [54,55]. Therefore, cautiousness is needed when extrapolating effects of AhR activation in rodents to effects of AhR activation in humans. Furthermore, in the human population there is a wide range of variation in responses regulated by the AhR [56]. However, until now only one pair of polymorphisms has been shown to affect AhR-mediated responses. Unidentified polymorphisms, especially in the transactivation domain, might affect the expression of genes and responses via the AhR. In addition, genetically based variations in the AhR structure might contribute to differences in human responsiveness [56]. This makes it complicated to assess the effect of AhR activation in humans.

Strikingly, the AhR plays an important role in human T cell differentiation *in vitro*, because AhR activation promotes the differentiation of $CD4^+Foxp3^+$ T_{reg} cells in the presence of TGF-β and the differentiation of $CD4^+Foxp3^-$ T cells producing IL-10 which control effector T cells [57]. Moreover, human epidemiological studies indicate that early life exposure to AhR ligands, including dioxins and polychlorinated biphenyls, alters immune function in children measured by decreased thymic size, increased susceptibility to infections, reduced antibody responses upon vaccination and increased cancer susceptibility [58]. Moreover, a negative correlation between serum IgE and dioxin-like compounds in humans has been reported [59]. Interestingly, it has been hypothesized that the increased susceptibility to infections as a result of AhR activation prevents the development of allergic diseases [60]. Together, this suggests

that AhR activation can influence the functioning of the human immune system, including allergic responses.

To conclude

Activation of the AhR does not unambiguously result in the suppression of immune responses. The chemical structure of the AhR ligand, the time and route of administration, and the cytokine milieu appear to play major roles in determining beneficial or harmful effects of AhR activation on the immune system. The results in this thesis have contributed to knowledge about the role of the AhR in the immune system, especially in the context of food allergic responses. Future research should elucidate whether and how AhR activation can be used to interfere in food allergic responses in humans. This may lead to new prevention strategies and therapeutic possibilities for food allergy.

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Nederlandse Samenvatting

In het onderzoek beschreven in dit proefschrift is onderzocht of en hoe activatie van een bepaald eiwit, de aryl hydrocarbon receptor (AhR), voedselallergie mogelijk kan voorkomen en genezen. Dit is onderzocht met behulp van een proefdiermodel, waarbij muizen allergisch worden gemaakt voor pinda's. Het gebruik van proefdieren hierbij is noodzakelijk omdat onderzoek naar voedselallergieën bij mensen maar zeer beperkt mogelijk is en wegens de betrokkenheid van veel verschillende typen cellen onmogelijk met celkweken kan worden nagebootst.

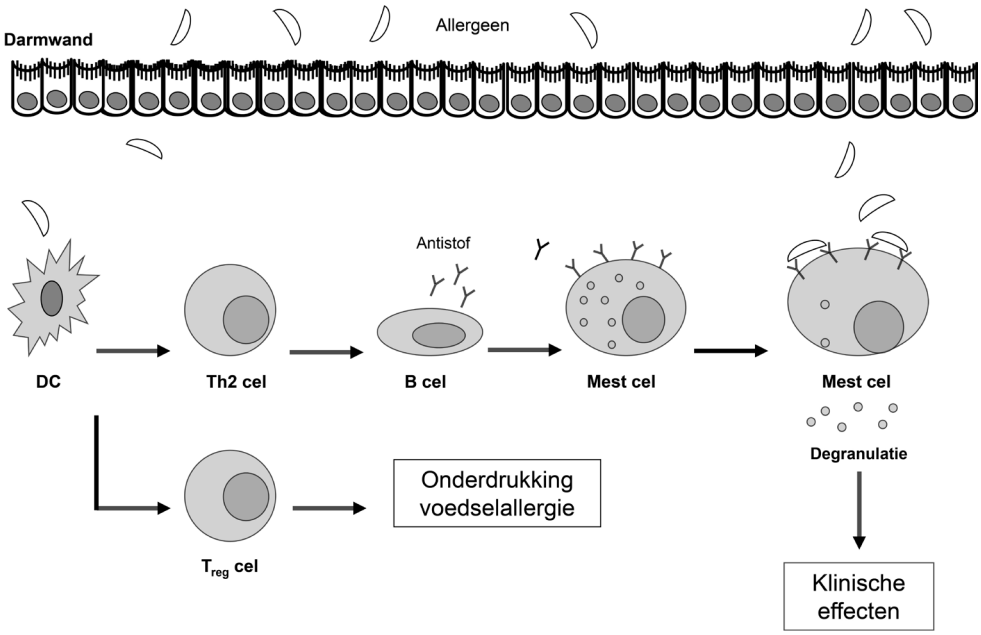
Aryl hydrocarbon receptor en immuunsysteem

De AhR is in veel verschillende cellen en weefsels aanwezig. De AhR staat ook wel bekend als de dioxine-receptor, omdat deze de sterk giftige milieuverontreinigende dioxines (o.a. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, afgekort TCDD) bindt en de activatie van dit eiwit een scala aan gezondheidsschadelijke effecten veroorzaakt. Naast dioxines zijn er nog veel meer stoffen die aan de AhR kunnen binden (ook wel liganden genoemd). Hieronder vallen zowel andere milieuverontreinigende stoffen, bijvoorbeeld gechloreerde dibenzofuranen en PCBs, maar ook stoffen die van nature voorkomen in ons lichaam en onze dagelijkse voeding. Sinds kort is duidelijk dat de AhR een zeer belangrijke rol speelt in de werking en regulatie van het immuunsysteem. Zo kan activatie van de AhR auto-immuunziekten die veroorzaakt worden door een niet goed functionerend immuunsysteem (bijvoorbeeld reuma) onderdrukken.

Voedselallergie

Bij mensen met een voedselallergie functioneert het immuunsysteem ook niet goed. Het classificeert namelijk bepaalde ongevaarlijke eiwitten (allergenen) in ons voedsel als gevaarlijk. Als reactie hier op produceert het immuunsysteem antistoffen tegen deze allergenen. Deze antistoffen binden aan mest cellen en bij volgende blootstelling aan het desbetreffende allergeen degranuleren deze mest cellen. Het gevolg is een ongewenste allergische reactie, die kan variëren van jeuk en uitslag tot een anafylactische shock met mogelijk een dodelijke afloop (figuur 1). Op dit moment heeft ongeveer 5% van de kinderen last van een voedselallergie, bij volwassenen is dit 3-4%. Vermijding van het allergeen en symptoombestrijding zijn momenteel de enige

oplossingen die een bescherming geven. Wel wordt er op dit moment onderzoek gedaan naar de mogelijkheid om immunotherapie toe te passen bij mensen met een voedselallergie. Bij deze immunotherapie wordt de patiënt minder allergisch gemaakt voor het desbetreffende allergeen door middel van herhaalde toediening van een lage dosis van dit allergeen. Momenteel wordt immunotherapie al wel veel toegepast bij mensen met een pollen-, wespen- of bijengifallergie. Bij voedselallergieën is immunotherapie vanwege het hoge risico op een anafylactische shock echter nog niet toepasbaar.



Figuur 1. Schematisch overzicht van cellen die betrokken zijn bij een voedselallergische reactie. In de darmwand nemen dendritische cellen (DC) tal van voedselcomponenten op, waardoor ze herkenbaar voor T helper cellen. Bij mensen met een voedselallergie ontstaan er T helper 2 (Th2) cellen, die op hun beurt weer B cellen aanzetten om antistoffen te maken specifiek tegen dit allergeen. De antistoffen binden aan mest cellen en bij een volgende blootstelling aan het allergeen, bindt het allergeen aan de antilichamen die op de mest cel zitten. Dit resulteert in het degranuleren van mest cellen waarbij allerlei stoffen, onder andere histamine, vrijkomen. Deze stoffen zorgen voor een ongewenste allergische reactie, variërend van jeuk en uitslag tot een anafylactische shock. Daarnaast kunnen onder invloed van bepaalde stoffen die door cellen van het immuunsysteem worden uitgescheiden de zogenaamde regulatoire T (T_{reg}) cellen ontstaan. Deze T_{reg} cellen kunnen een te agressieve werking van het immuunsysteem onderdrukken waardoor een voedselallergische reactie zou kunnen worden verminderd of uit zou kunnen blijven.

Aryl hydrocarbon receptor en voedselallergie

Bij het ontstaan van een voedselallergie zijn verschillende cellen van het immuunsysteem betrokken. Activatie van de AhR kan de werking van een deel van deze immuuncellen beïnvloeden. Zo heeft AhR activatie bijvoorbeeld invloed op de functie van dendritische cellen (DC). In de darmwand nemen DC tal van voedselcomponenten op en verwerken deze zodanig, dat ze herkenbaar worden voor de zogenaamde T helper cellen. Er zijn verschillende soorten T helper cellen (T helper 1, T helper 2, T helper 17), die ieder een bepaalde functie hebben in het immuunsysteem. Bij mensen met een voedselallergie ontstaan er zogenaamde T helper 2 (Th2) cellen, die op hun beurt weer andere cellen (B cellen) aanzetten om antistoffen te maken (figuur 1). Onderzoek heeft uitgewezen dat activatie van de AhR de Th2 cellen kan onderdrukken.

Verder kunnen onder invloed van bepaalde stoffen die door cellen van het immuunsysteem worden uitgescheiden de zogenaamde regulatoire T (T_{reg}) cellen ontstaan. Deze T_{reg} cellen kunnen een te agressieve werking van het immuunsysteem tegen gaan waardoor een allergische reactie zou kunnen worden verminderd of uit zou kunnen blijven. Eén van de hypotheses over het ontstaan van voedselallergieën is dat bij mensen met voedselallergie de balans tussen Th2 en T_{reg} cellen verstoord is. Interessant is dat eerder onderzoek heeft aangetoond dat activatie van de AhR deze T_{reg} cellen kan induceren.

Dit proefschrift

Omdat er nog geen therapie voor handen is om voedselallergie te voorkomen of te genezen en activatie van de AhR relevante cellen van het immuunsysteem die een rol spelen in voedselallergie (DC, Th2 en T_{reg}) kan beïnvloeden, is in dit proefschrift onderzocht of (een al bestaande) voedselallergie door middel van AhR activatie kan worden onderdrukt.

Hoofdstuk 2 laat zien dat AhR activatie door TCDD het ontstaan van een pinda-allergie dosis-afhankelijk onderdrukt. Dit gaat gepaard met een procentuele toename van T_{reg} cellen in de milt en in de lymfeklieren bij de darm. Als deze T_{reg} cellen uit de muis worden verwijderd, zijn de onderdrukkende effecten van de AhR activatie op pinda-allergie deels opgeheven. Dit toont aan dat activatie van de AhR pinda-allergie voor een deel kan remmen door voor een procentuele toename van T_{reg} cellen te zorgen.

In **hoofdstuk 3** wordt aangetoond dat de procentuele toename van T_{reg} cellen na AhR activatie door TCDD een gevolg is van een afname van andere T cellen naast het behoud van T_{reg} cellen in de thymus. De thymus is het orgaan waar T cellen ontwikkelen.
Zoals eerder genoemd zijn er heel veel verschillende AhR liganden. Uit nader onderzoek is echter gebleken dat niet elk AhR ligand een zelfde effect op het immuunsysteem heeft. Daarom is in **hoofdstuk 4** het effect van drie andere AhR liganden, 6-formylindolo[3,2-b]carbazole (FICZ), β-naphthoflavone (β-NF) en 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), op het ontstaan van een pinda-allergie onderzocht. Deze drie niet-dioxine-achtige AhR liganden zijn gekozen omdat eerder onderzoek heeft uitgewezen dat deze liganden de werking van het immuunsysteem kunnen beïnvloeden. Theoretisch zouden deze stoffen dus ook het ontstaan van pinda-allergie kunnen remmen of verhinderen. Uit het onderzoek blijkt dat in tegenstelling tot TCDD, deze AhR liganden het ontstaan van een pinda-allergie niet onderdrukken. Ook hebben FICZ, β-NF en 6-MCDF geen effect op het percentage T_{reg} cellen en zorgen deze AhR liganden voor een lagere expressie van genen, welke worden aangezet na AhR activatie, ten opzichte van TCDD. Het ontbreken van remmende effecten van FICZ, β-NF en 6-MCDF op een pinda-allergie ten opzichte van TCDD zou verklaard kunnen worden door bijvoorbeeld een snellere afbraak van deze niet dioxine AhR liganden door het lichaam, een minder sterke binding aan de AhR of een verschil in genexpressie.
Uit **hoofdstuk 5** blijkt dat de AhR activatie door TCDD het aantal DC in de lymfeklieren bij de darm van de muis vermindert en de aanwezigheid van het eiwit CD103 op de buitenkant van DC in de milt verhoogt. Ook beïnvloedt deze AhR activatie de functionaliteit van DC en hun interactie met Th2 cellen. Dit mechanisme zou mogelijk een reden kunnen zijn voor de onderdrukking van het ontstaan van pinda-allergie. Of dit direct het gevolg is van de verhoging van het eiwit CD103 op DC is nog niet duidelijk.
In **hoofdstuk 6** is onderzocht of AhR activatie immunotherapie voor voedselallergie kan verbeteren. Dit is gedaan door muizen allergisch te maken voor pinda en deze vervolgens te behandelen met TCDD in combinatie met immunotherapie. Uit deze studie blijkt dat AhR activatie de verhoging van antistoffen tegen pinda als gevolg van immunotherapie tegengaat, zonder het gunstige genezende effect van immunotherapie (tegengaan van een anafylactische shock) op de pinda-allergie te beïnvloeden.

Samenvattend kan worden geconstateerd dat het in dit proefschrift beschreven onderzoek heeft aangetoond dat activatie van de AhR het ontstaan van pinda-allergie op verschillende manieren kan onderdrukken. Enerzijds doordat de balans van T_{reg} en Th2 cellen verschuift in de richting van T_{reg} cellen, maar anderzijds ook door remming van de interactie van DC met Th2 cellen. Daarnaast suggereert het beschreven onderzoek dat AhR activatie immunotherapie voor voedselallergie zou kunnen verbeteren door mogelijk het risico op ongewenste neven effecten van immunotherapie te beperken. Hierbij zou echter wel het risico op ongewenste "dioxine toxiciteit" tegelijkertijd vermeden moeten worden. Vooralsnog biedt het in dit proefschrift beschreven onderzoek geen duidelijke mogelijkheden hiervoor. Wel kunnen de resultaten op het gebied van werkingsmechanisme in de toekomst hopelijk een bijdrage leveren aan de ontwikkeling van preventiemethoden en therapieën ter voorkoming van voedselallergieën.

Curriculum Vitae

Veronica Schulz was born in Hagen, Germany, on the 6th of December in 1984. In 2003 she graduated from the Lambert Franckens College located in Elburg, after which she started the study Biomedical Sciences at Utrecht University. In 2006 she received her Bachelor's degree and she continued with the Master Toxicology and Environmental Health in Biomedical Sciences. During this Master she did an internship at the Institute for Risk Assessment Sciences in Utrecht and the Institute for Food Safety in Wageningen where she investigated the effect of estrogen intake on the development of cancer. She did this under supervision of Dr. Majorie van Duursen and Dr. Marjoke Heneweer. At Pepscan Therapeutics in Lelystad she did her second internship. Here, she studied fibril growth induced by prion peptides under the supervision of Dr. Ronald Boshuizen and Dr. Hans Langedijk. After receiving her Master's degree in 2008, she directly continued with her PhD at the Immunotoxicology group at the Institute for Risk Assessment Sciences under the supervision of Dr. Raymond Pieters and Dr. Joost Smit. She investigated whether and how AhR activation suppresses allergic sensitization and if AhR activation could improve (immuno)therapy for food allergy. During her PhD she took courses of the Postgraduate Education in Toxicology in order to obtain the certification as Toxicologist at graduation. She also participated in the Utrecht Center for Food allergy and was trained in immunology at the Eijkman Graduate School.

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