

Dioxin and immune regulation

Emerging role of aryl hydrocarbon receptor in the generation of regulatory T cells

Nikki B. Marshall¹ and Nancy I. Kerkvliet^{1,2}

Author Information

1. Department of Microbiology
2. Department of Environmental and Molecular Toxicology and Environmental Health Sciences Center, Oregon State University, Corvallis, Oregon, USA

*Address for correspondence: Nancy Kerkvliet, Oregon State University, 1007 ALS Bldg, Corvallis, OR 97331. Voice: +1541-737-4387; fax: +1541-737-0497.
Nancy.Kerkvliet@oregonstate.edu

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Abstract

The immune toxicity of the ubiquitous environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), commonly referred to as dioxin, has been studied for over 35 years but only recently has the profound immune suppression induced by TCDD exposure been linked to induction of regulatory T cells (Tregs). The effects of TCDD are mediated through its binding to the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor. The subsequent AHR-dependent effects on immune responses are determined by the cell types involved, their activation status, and the type of antigenic stimulus. Collectively, studies indicate that TCDD inhibits CD4⁺ T cell differentiation into T helper (Th)1, Th2, and Th17 effector cells, while inducing Foxp3-negative and/or preserving Foxp3⁺ Tregs. Although it is not yet clear how activation of AHR by TCDD induces Tregs, there is a potential therapeutic role for alternative AHR ligands in the treatment of immune-mediated disorders.

Introduction to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and the aryl hydrocarbon receptor

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), commonly referred to as dioxin, is generally recognized as a toxic, persistent, and ubiquitous environmental contaminant. It is an unintentional byproduct of various industrial, combustion, and natural processes and can be detected in air, water, soil, and sediment worldwide. Municipal, medical, and hazardous waste incineration is the main source of dioxin contamination today. TCDD is probably best known as a contaminant of Agent Orange, the herbicide that was widely sprayed during the Vietnam War. More recently, TCDD was used in an

attempted assassination of then Ukrainian presidential candidate Viktor Yushchenko. Fortunately he survived, although he suffered a severe disfigurement after developing a skin condition called chloracne, a hallmark symptom of dioxin poisoning in humans.

Humans are exposed to small amounts of dioxin daily, the majority through consumption of food.¹ TCDD is highly lipid soluble (K_{ow} 7.0) and therefore concentrates in the fat found in meat, dairy, fish, and shellfish. The World Health Organization has established a tolerable TCDD daily intake of 1–4 pg/kg body weight (ppq). The average lipid-adjusted body burden of TCDD in people living in North America and Europe is 2 ppt.² The half-life of TCDD in the human body ranges from 7–10 years and is affected by dose, age, exposure duration, health status, and diet.³ Levels of dioxin in the United States and Europe continue to decrease in both the population and the environment, reflecting regulatory decisions that have reduced the production and use of dioxin-contaminated substances.

The effects observed in animals following exposure to TCDD have intrigued toxicologists for over 50 years.⁴ TCDD produces a broad spectrum of effects at very low concentrations, leading to TCDD's moniker as an “environmental hormone.” Lethal doses of TCDD cause a slow death as a result of a wasting syndrome that is characterized by thymic atrophy, lipolysis, and altered intermediary metabolism. At nonlethal doses, reproductive and developmental effects, hepatocarcinogenesis, tumor promotion, and immune suppression are observed. This spectrum of toxicities associated with TCDD exposure are now known to be mediated through the ligation and activation of the aryl hydrocarbon receptor (AHR), first identified by Poland and colleagues in 1976.⁵ The interesting odyssey that led to the discovery of the AHR was recently recounted in a review by Okey.⁶ Activation of AHR induces a variety of drug-metabolizing enzymes, termed the *AHR battery*.⁷ Unlike most AHR ligands that induce their own metabolism, TCDD is resistant to these enzymes and its persistent occupancy of AHR is postulated to contribute to its potent toxicity.

AHR belongs to the basic helix-loop-helix-PER-ARNT-SIM family of proteins and functions as a ligand-activated transcription factor*derived from three functional domains.⁹ The DNA-binding domain is made up of the basic helix-loop-helix motif found in a variety of transcription factors.¹⁰ The PAS-A and PAS-B domains, homologous to *Drosophila* proteins Per and Sim, make up the ligand-binding domain.^{11,12} A third glutamine-rich region contains the transactivation domain involved in co-activator recruitment.¹³

Located in the cytoplasm of most cells, nonligand-bound AHR forms a receptor complex with several proteins, including a 90-kDa heat shock protein dimer (HSP90), hepatitis B virus X-associated protein 2 (XAP2; also known as AHR-interacting protein [AIP]), and phosphoprotein p23 (reviewed by Beischlag *et al.*¹⁴). Once bound by ligand, the ligand–receptor complex undergoes a conformational change and translocates to the nucleus where HSP90 is exchanged for the AHR nuclear translocator protein (ARNT) to form a heterodimer. This heterodimer binds cis elements of DNA with the core sequence 5'-GCGTG-3' known as xenobiotic- or dioxin-responsive elements (DREs), which can be found in the promoter or enhancer regions of responsive genes.¹⁵ The AHR/ARNT transcriptional complex recruits other proteins (e.g., SRC-1, CBP, NCoA2) that modulate

transcriptional activity and chromatin structure.¹⁴ The result is enhanced or repressed expression of AHR/ARNT-responsive genes. The most commonly used biomarker for AHR activation is induction of cytochrome p450 member Cyp1a1 and, more recently, AHR repressor (AHRR).¹⁶ The absence of TCDD toxicity in mice carrying a mutation in the DNA-binding domain of the AHR¹⁷ supports the hypothesis that inappropriate transcriptional enhancement or repression of AHR-responsive genes mediates the toxic effects of TCDD.

However, some studies indicate that AHR-mediated changes in gene expression are not limited to AHR/ARNT-dependent transcriptional activity. AHR has also been shown to interact directly with proteins in other signaling pathways, including nuclear factor (NF)- κ B,^{18,19} retinoblastoma protein,²⁰ and estrogen receptor.^{21,22} AHR has also been reported to act as part of a ligand-dependent E3 ubiquitin ligase complex that regulates protein degradation.²³ Clearly, we are only beginning to understand the diversity in AHR activity and function that coalesce to create complex mechanisms of altered gene expression.

Aryl hydrocarbon receptor-mediated effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the immune system

The immune toxicity of TCDD has been studied for more than 35 years as this small molecule is one of the most potently immunosuppressive chemicals known. Some of the reported effects of TCDD include thymic involution, decreased host resistance to pathogens and tumors, suppressed fetal lymphocyte development and maturation, and suppressed adaptive immune responses, including antibody production, cytotoxic T lymphocyte (CTL) activity, and delayed hypersensitivity responses.^{24–26} AHR is not required for the development of a functional immune system but its absence precludes the immunosuppressive effects of TCDD.²⁷ Thymic involution, a hallmark immunotoxic effect of TCDD in all species examined,²⁶ is dependent upon AHR expression in hematopoietic cells.^{28,29} AHR is expressed by all major cell types of the immune system, including B cells, T cells, dendritic cells (DCs), macrophages, granulocytes, and natural killer cells,²⁵ and many genes involved in immune regulation contain multiple DREs in their promoter region.³⁰ However, these regions of DNA are not necessarily accessible for AHR/ARNT binding. Because TCDD primarily affects immune cells responding to stimulation, the windows of promoter and enhancer availability created by other signaling events likely dictate when the presence of TCDD produces an AHR-mediated effect. Ultimately, the specific effects of AHR activation by TCDD on an immune response are context dependent, determined by what cell types are involved, the activation status of the cells, and the type of antigenic stimulation.

Dendritic cells

The innate immune system consists of cells and mechanisms that protect a host from infection by a broad spectrum of pathogens. Cells, including macrophages, natural killer cells, neutrophils, and DCs, are part of this first line of host defense that can be affected by TCDD exposure.^{25,26,31} DCs are an important link between the innate and adaptive

immune systems. DCs migrate to lymph nodes and present antigen to T cells on major histocompatibility complex (MHC) class II molecules, while providing additional costimulation to allow full CD4⁺ helper T cell activation. In turn, activated CD4⁺ T cells help B cells develop into antibody-producing cells and can license DCs to activate CD8⁺ T cells to develop into CTLs.

Several studies have shown that TCDD alters the function of DCs. Splenic DCs isolated from mice exposed to TCDD expressed increased levels of MHC class II, adhesion molecules intercellular adhesion molecule type 1 (ICAM-1) and CD24, and costimulatory molecule CD40.³² DCs exposed to TCDD also produced increased levels of IL-12 and enhanced T-cell proliferation in a mixed lymphocyte reaction. Antigen processing appeared unaffected as phagocytosis of latex beads and antigen presentation were not altered by TCDD.³³ Bone marrow-derived DCs exposed to TCDD were also shown to express increased MHC II, CD86, CD40, and CD54 (ICAM-1) with increased T-cell-stimulating ability.^{34,35} Taken together, these results suggest that TCDD enhances the activation and T-cell stimulatory capacity of DCs. However, the number of DCs in the spleen of TCDD-treated mice was significantly reduced 4–7 days after treatment,³³ and bone marrow-derived DCs treated with TCDD were shown to undergo increased Fas-mediated apoptosis.³⁵ This premature loss of DCs would likely reduce the strength and duration of a T-cell-mediated response.

Some of the AHR-mediated effects of TCDD on DCs, and likely other cell types, appear to involve altered signaling of the NF- κ B pathway.^{34,36} NF- κ B activity is induced by canonical and noncanonical pathways of which both have been shown to interact with AHR.^{18,19,37,38} The canonical NF- κ B pathway involves nuclear translocation of Rel-A/p50 heterodimers that activate transcription of genes containing NF- κ B-responsive elements. Interestingly, AHR was shown to associate with Rel-A and to prevent its nuclear translocation, while preserving DNA binding of p50 homodimers in the DC2.4 cell line in response to tumor necrosis factor (TNF)- α or anti-CD40.³⁶ The p50 homodimers act as transcriptional repressors and are associated with immune tolerance to endotoxin and suppression of inflammatory responses.^{39–42} The noncanonical NF- κ B pathway involves nuclear translocation of Rel-B/p52 heterodimers. AHR was shown to interact with Rel-B in a complex that bound Rel-B/p52-response elements and induced DRE-mediated transcriptional activity in the presence of TCDD.⁴³ The absence of AHR appears to promote premature degradation of Rel-B, resulting in enhanced inflammatory responses,^{14,44} suggesting an endogenous role for AHR in the NF- κ B pathway.

The noncanonical NF- κ B pathway is also associated with the induction of indoleamine 2,3-dioxygenase (IDO) expression by DCs.⁴⁵ IDO is the first and rate-limiting step of tryptophan catabolism and is associated with suppression of T-cell responses.³⁸ This suppression is associated with the generation of tolerogenic DCs that induce regulatory T cells (Tregs).^{46–48} Furthermore, tolerogenic IDO⁺ DCs are induced as a consequence of engaging CTLA-4 or glucocorticoid-induced TNF receptor (GITR) expressed on the surface of Tregs.^{49–51} Naturally occurring Tregs are a subpopulation of suppressive CD4⁺CD25⁺ T cells whose phenotype and function are governed by the forkhead family transcription factor Foxp3.^{52,53} Recently, Vogel and colleagues showed that activation of

AHR by TCDD for 10 days induced IDO1 and IDO-like protein IDO2 in lung and spleen of C57Bl/6 (B6) mice, which correlated with a 2.5-fold increase in expression of Foxp3 transcript in the spleen.⁵⁴ The increase in Foxp3 expression was prevented when IDO activity was inhibited. Both the *IDO1* and *IDO2* genes contain putative DREs;^{30,54} thus, AHR may play a direct role in the induction of IDO expression by DCs.

Tolerogenic DCs appear to be induced by the low-molecular weight compound VAF347 and its water-soluble homologue VAG539, which have been shown to activate AHR.⁵⁵ VAG539 was shown to suppress allergic lung inflammation in AHR^{+/+} but not AHR-deficient (AHR^{-/-}) mice. VAG539 also promoted allograft acceptance in mice that correlated with an increase in Foxp3⁺ Treg frequency.⁵⁶ This tolerance could be transferred with CD11c⁺ DCs or CD4⁺ CD25⁺ T cells from VAG539-treated mice but not with CD4⁺ T cells or CD19⁺ B cells. Furthermore, the transfer of DCs from VAG539-treated mice increased the frequency of Foxp3⁺ T cells in nontreated mice. These findings implicate AHR activation in the induction of tolerogenic DCs that may play a role in expansion or preservation of Foxp3⁺ Tregs.

T cells

Adaptive immunity consists of activation, effector differentiation, and clonal expansion of antigen-specific populations of lymphocytes, including CD4⁺ T cells, CD8⁺ T cells, and B cells. As the cells encounter their specific antigen and are exposed to costimulatory signals and cytokines, they differentiate into effector cells capable of carrying out functions best suited to clear the antigenic stimulus. B cells were identified in the 1980s as direct cellular targets of TCDD because the effects of TCDD on B-cell differentiation could be easily observed in culture.^{57–59} T cells on the other hand were thought to be indirect targets until *in vivo* studies showed that suppression of effector T-cell functions in an acute graft-versus-host response (GVHR) required the presence of AHR in the donor T cells themselves.⁶⁰

The mechanisms for suppression of effector T-cell differentiation by TCDD are still not well understood. Upon antigenic challenge, both CD4⁺ and CD8⁺ T cells proliferate normally in TCDD-treated mice; however, a significant decline in their numbers occurs on day 4–5 of the immune response that appears to reflect a cessation of proliferation rather than apoptosis.^{61–63} Furthermore, activation of CD8⁺ CTL precursors is suppressed as early as day 5 in a CD4⁺ T cell-dependent tumor allograft response⁶⁴ that is not explained by insufficient IL-2 or deletion of CD8⁺ T cells.^{65,66} Suppressed CTL development was also observed in a CD4⁺ T cell-independent CD8⁺ T cell response to influenza^{67,68} that was also not explained by increased apoptosis.⁶⁸ Thus, TCDD causes a premature cessation of T-cell proliferation and inhibition of CTL activation, which does not appear to be linked to increased T cell death.

Extensive chromatin remodeling occurs during T-cell activation that may explain why activated T cells are particularly sensitive to the effects of AHR activation by TCDD compared to resting T cells.^{61,62,69–71} As T cells differentiate into effectors during the early stages of an immune response, it is likely that direct AHR–DRE-mediated effects occur throughout this time period rather than only in the first few hours following T-cell receptor ligation. A recent review highlights some of the genes in CD4⁺ T cells that show

altered expression following TCDD exposure both *in vivo* and *in vitro*. These genes encode lineage-specific transcription factors, cytokines, cytokine receptors, and signaling kinase families, many of which contain multiple DREs in their promoters.⁷² This complex network of genetic and epigenetic changes that occurs during T-cell differentiation in the presence of TCDD ultimately determines T-cell fate.

Effects of AHR activation on CD4⁺ T cell effector differentiation and disease

The immunosuppressive effects of TCDD are undesirable in terms of host resistance where increased susceptibility to bacterial and viral infections as well as increased tumor growth have been observed in some animal models. During inappropriate immune responses, however, the effects of AHR activation by TCDD are beneficial for preventing development of disease. TCDD has been shown to suppress allograft responses,^{60,65} allergic responses,^{73,74} and autoimmune responses in animal models of multiple sclerosis (experimental autoimmune encephalomyelitis [EAE])⁷⁵ and type I diabetes.⁷⁶ These disease conditions are associated with different types of CD4⁺ T cells, suggesting that TCDD suppresses Th1, Th2, and Th17 T-cell-mediated responses *in vivo*. On the other hand, Treg development appears to be enhanced in the presence of TCDD.^{75–77} Given the therapeutic potential for Treg induction to suppress undesirable immune responses, there is considerable interest in furthering our understanding of how TCDD acts through AHR to suppress CD4⁺ T cell differentiation but to enhance the development of Tregs.

Suppression of Th2-mediated responses

Type 2 CD4⁺ T cells (Th2) predominate in antibody-mediated immune responses, including responses to extracellular bacteria and viruses, parasitic infections, as well as allergens that cause immediate hypersensitivity. TCDD has been shown to suppress Th2-mediated immune responses, including allergic response to dust mite antigen,⁷³ development of atopic dermatitis,⁷⁴ and antibody responses to ovalbumin in alum adjuvant.^{78,79} Suppressed production of Th2 cytokines, including IL-4 and IL-5, has been shown in TCDD-treated mice^{74,78} at doses as low as 0.3 µg/kg.⁷⁹ IgE production was suppressed in TCDD-treated NC/Nga mice prone to develop atopic dermatitis⁷⁴ and in TCDD-treated rats sensitized to dust mite antigens.⁷³ Interestingly, the anti-allergic drug M50354 and its derivative M50367 have been shown to act as AHR agonists that suppress Th2 development.^{80,81} Although Treg-mediated suppression of Th2 responses has been described,⁸² no link has yet been established between suppressed Th2 responses and induction of Tregs in TCDD-treated mice.

Suppression of Th1-mediated allograft responses

Much work has been carried out in our laboratory studying the effects of TCDD on allograft immunity. The type 1 CD4⁺ T-cell (Th1)-dependent CTL- and alloantibody-mediated responses to P815 mastocytoma (H-2^d haplotype) are suppressed in B6 mice (H-2^b) treated with TCDD.⁶⁵ To observe suppression of the CTL response, TCDD must be given within the first 3 days of the allograft being introduced and the animals must express AHR.³² The primary target for the early-stage suppression by TCDD appears to

be the development of Th1 cells that are required during the first 3 days of the allograft response to activate the CTL precursors.⁶⁵ These findings suggest that once CTL precursors have become activated, TCDD does not inhibit their clonal expansion or cytolytic activity.

A second allograft model we have used in our lab is an acute GVHR model in which donor T cells from B6 mice (H-2^b) are injected intravenously into B6D2F1 (F1) mice of mixed haplotype (H-2^{b/d}). Alloreactive donor T cells respond to H-2^d alloantigens expressed by host tissues inducing an anti-H-2^d Th1-dependent CD8⁺ CTL response. When host mice were treated with TCDD within 24 h before the adoptive transfer of donor T cells, the allospecific CTL response was suppressed.⁶⁰ If however, the donor T cells were AHR^{-/-}, the CTL response was unaffected by TCDD, demonstrating that AHR in the donor T cells is the direct target of TCDD for suppression of the CTL response. When the donor CD4⁺ T cells from B6 mice were adoptively transferred with CD8⁺ T cells from B6 AHR^{-/-} mice (or vice versa), the CTL response was partially impaired, indicating that TCDD acts directly on both alloreactive CD4⁺ T cells to impair their ability to support CTL development and alloreactive CD8⁺ T cells to suppress their development into CTL.

Effects of AHR on the generation of adaptive Treg

The direct effects of TCDD on the response of T cells to alloantigen stimulation was examined using flow cytometric and functional analysis of CD4⁺ and CD8⁺ donor T cells following their injection into F1 hosts.^{77,83,84} Phenotypic analysis of proliferating alloreactive donor T cells revealed significant increases in the frequency of CD25⁺ T cells (both CD4⁺ and CD8⁺) and in the level of CD25 expressed per cell that peaked 48 h after adoptive transfer into TCDD-treated host mice. When pre-existing CD25⁺ cells were depleted from the donor inoculum prior to adoptive transfer, there was no effect on the generation of the CD25^{hi} population, suggesting *de novo* induction of CD25 expression rather than expansion of a pre-existing CD25⁺ population. The CD25^{hi} cells also expressed increased levels of CTLA-4, GITR, and downregulated CD62-L expression compared to cells from vehicle-treated mice.⁷⁷ These phenotypic changes were not seen with AHR^{-/-} donor T cells, suggesting that AHR activation in the T cells by TCDD was inducing the development of adaptive Tregs.

The donor T cells in TCDD-treated host mice did not express the Treg transcription factor Foxp3 yet showed significant suppressive activity when isolated and tested *in vitro*.⁷⁷ Both CD4⁺ and CD8⁺ donor T cells suppressed the proliferation of naive CD4⁺ T-cell responders stimulated with anti-CD3 even more potently than a population of natural CD25⁺ CD4⁺ regulatory T cells.^{77,84} Furthermore, the donor CD4⁺ T cells significantly suppressed proliferation of naive CD4⁺ and CD8⁺ responder T cells stimulated with semi-allogeneic F1-DCs.⁸³ The donor CD4⁺ T cells did not express IL-2 at the mRNA or protein level at 48 h but produced significantly more IL-10 in response to allostimulation at both the transcript and protein levels. Interestingly, these characteristics are similar to Foxp3-negative, IL-10-producing Tregs (Tr-1) that have been previously described in mice.⁸⁵ Thus, alloreactive donor CD4⁺ and CD8⁺ T cells exposed to TCDD during acute GVHR are both phenotypically and functionally consistent with Tregs.

These GVHR studies were the first to link AHR activation by TCDD with the induction of CD4⁺ and CD8⁺ Tregs. Because these Treg-like cells do not express Foxp3,⁸³ it suggests that AHR may act as an alternative transcription factor to induce Treg phenotype and function. Interestingly, the phenotypic changes that occurred in both CD4⁺ and CD8⁺ donor T cells exposed to TCDD were primarily dependent on AHR expression in the donor CD4⁺ T cells.⁸⁴ Thus, it is possible CD8⁺ T cells were converted to Treg-like cells through direct interactions with the CD4⁺ T cells or indirectly through interactions with CD4⁺ T-cell-licensed DCs. However, the AHR status of the host did not influence the ability of TCDD to suppress the GVHR,⁸⁶ indicating that TCDD did not act on host antigen-presenting cells to mediate the induction of the Tregs. Thus, direct AHR-mediated effects of TCDD on donor CD4⁺ T cells are necessary for the induction of Tregs in the acute GVHR model.

An important feature of Tregs is their lack of IL-2 production despite their high expression of CD25; Tregs must instead rely on the IL-2 produced by other T cells, which is essential for Treg development and expansion.^{87,88} Given that TCDD induces IL-2 expression through AHR interactions with dioxin-responsive elements of the IL-2 gene,⁸⁹ an early increase in IL-2 production could promote the induction of Tregs. In fact, an early increase in IL-2 production by donor CD4⁺ T cells is seen in TCDD-treated mice at 20 h post-adoptive transfer (Funatake and Kerkvliet, unpublished observations); however, the effect is short lived as the donor T cells no longer express IL-2 by 48 h when they acquire Treg phenotype and function.⁸³ Furthermore, excess IL-2 given in the first 3 days of the GVHR does not recapitulate the effects of TCDD on donor T-cell phenotype or suppression of GVHR (Funatake and Kerkvliet, unpublished observations). Thus, any role played by IL-2 to enhance AHR-mediated Treg development or expansion remains to be determined.

Effects of AHR on Foxp3⁺ Tregs

CD25^{hi} Foxp3⁺ CD4⁺ Tregs constitute 5–10% of peripheral CD4⁺ T cells and play an important role in self-tolerance. Foxp3⁺ Tregs suppress cell- and antibody-mediated immune responses and protect a host against autoimmunity. Although Foxp3⁺ Tregs are derived from the thymus, Foxp3 expression can be induced in peripheral T cells by stimulation in the presence of transforming growth factor (TGF)- β ⁹⁰ and IL-2.⁹¹ AHR is expressed in Foxp3⁺ Tregs^{92,93} and DRE sequences in the Foxp3 promoter are capable of binding AHR,⁷⁵ suggesting AHR can directly influence Foxp3 gene expression. TCDD alone at 100 nM was reported to induce a small increase in the frequency of Foxp3⁺ T cells *in vitro*,⁷⁵ while another laboratory found that co-treatment with TGF- β was needed for TCDD (160 nM) to increase Foxp3⁺ T-cell frequency.⁹⁴ In contrast, preliminary data from our laboratory show the percentage of CD4⁺ T cells that express Foxp3 is significantly reduced when splenocytes from B6 mice are cultured with 20 nM TCDD in the presence of TGF- β and IL-2 for 3 days and is reduced even further when IL-6 is added to the cultures. Interestingly, the frequency of Foxp3⁺ CD8⁺ T cells of the same cultures was only suppressed in the presence of IL-6, suggesting that the conditions that influence AHR regulation of Foxp3 expression differ in CD4⁺ and CD8⁺ T cells.

The majority of studies on TCDD have been performed with B6 mice that are homozygous for a high-affinity AHR allele (AHR^b). However, AHR is polymorphic both

in mice and humans, and a low-affinity allele (AHR^d), which is approximately 10-fold less responsive to TCDD, is expressed in some commonly used mouse strains (e.g., DBA/2, SJL). Interestingly, Foxp3⁺ T-cell frequency was reported to be increased in AHR^b B6 mice compared to congenic AHR^d B6 mice.⁷⁵ However, the frequency of Foxp3⁺ Tregs in B6 AHR^{-/-} mice was not altered in comparison to AHR^{+/+} B6 mice,⁹⁵ suggesting AHR does not play a necessary role in maintenance of Foxp3⁺ Treg populations. Quintana and colleagues also reported that administration of 1 µg of TCDD (approximately 50 µg/kg) increased Foxp3⁺ Treg frequency and inhibited development of EAE in B6 mice.⁷⁵ However, no increase was seen at the 0.1-µg dose of TCDD, which is an immunosuppressive dose for AHR^b B6 mice. Thus, the relationship between Foxp3 expression and mechanisms of TCDD-induced immune suppression requires further study.

An increase in Foxp3⁺ Treg frequency was also found in the pancreatic lymph nodes of nonobese diabetic (NOD) mice chronically treated with TCDD, which correlated with suppression of the development of type 1 diabetes.⁷⁶ The body burden of TCDD in the NOD mice (AHR^d) was maintained at approximately 15 µg/kg over the course of 30 weeks as blood glucose was monitored. Mice that were taken off TCDD treatment at 21 weeks developed diabetes over the next 8 weeks as the body burden of TCDD dropped below an estimated 4 µg/kg (0.06 µg/25g mouse). At 30 weeks, the induction of Cyp1A1 was no longer evident in these mice, indicating that AHR was no longer activated. These data indicate that TCDD must be present at a sufficient concentration to sustain AHR activation, which in turn maintains the elevated frequency of Foxp3⁺ Tregs. The elevated Treg frequency likely counters the continued emergence of differentiating effector T cells in the periphery. It is not yet clear whether these results reflect a preservation or induction of Foxp3⁺ Tregs. Because TCDD has little effect on fully differentiated T cells, natural Foxp3⁺ Tregs may be relatively resistant to AHR-mediated effects of TCDD. This could explain their increased frequency *in vivo* in TCDD-treated mice during autoimmune responses.

Effects of AHR on Th17 development

IL-17-secreting T cells (Th17) are a recently identified lineage of effector T cells. Th17 cells are generally found in the skin and GI tract and are involved with inflammatory and autoimmune conditions, such as inflammatory bowel disease, multiple sclerosis and rheumatoid arthritis.⁹⁶ Th17 cells can be generated *in vitro* upon co-treatment with TGF-β and IL-6 and/or IL-21.^{97–99} Although activation of T cells in general increases their expression of AHR,⁸⁰ AHR was shown to be highly upregulated in Th17-polarized T-cell cultures.^{75,94} The implications of this increased AHR expression during Th17 differentiation is not known but it could confer enhanced sensitivity to TCDD on the Th17 effector pathway compared to other T-cell effector subsets. However, Kimura and colleagues showed only a small effect of TCDD on the induction of IL-17-producing cells *in vitro*.⁹⁴ Furthermore, it is important to bear in mind that treatment of mice with TCDD does not induce Th17-like effector activity but rather appears to suppress Th17 differentiation.⁷⁵

Another high-affinity ligand of AHR, 6-formylindolo[3,2-b]carbazole (FICZ), is an endogenous photoproduct of tryptophan, which, unlike TCDD, was shown to exacerbate

the onset and severity of EAE.^{75,95} The effects of FICZ were AHR dependent and correlated with an increased frequency of Th17 cells. FICZ has also been shown to enhance Th17 cell generation in T-cell cultures treated with TGF- β and IL-6.^{75,94,95} Kimura and colleagues showed that FICZ enhanced TGF- β /IL-6-induced Th17 development to approximately the same small degree as TCDD.⁹⁴ These effects were not seen when the cells were AHR^{-/-}. FICZ also inhibited TGF- β -induced Treg development *in vitro*.⁷⁵ The differential effects of TCDD and FICZ on Th17 and Treg development are not yet understood; however, the rapid metabolism of FICZ by AHR-induced enzymes¹⁰⁰ is one plausible explanation for the discrepancies between the two ligands.⁷² The finding that TCDD enhanced Th17 generation *in vitro* but inhibited Th17 development during EAE is contradictory; however, it is likely that TCDD affects other cell types in the animal to influence Th17 generation. For example, IL-6 production is affected by TCDD exposure in different cell types^{101,102} in contrast to the direct addition of IL-6 to the *in vitro* cultures.

Alternative natural AHR ligands

A known high-affinity endogenous ligand of AHR has not been identified, thus AHR is still considered to be an orphan receptor. The ligand-binding site of AHR is promiscuous; structurally diverse, synthetic, and naturally occurring AHR ligands have been identified. TCDD, as the most potent ligand of AHR, is a good prototype for studying the effects of AHR activation as there is reduced chance for high-dose off-target effects by a lower affinity ligand or confounding effects as a result of ligand metabolism. Given the profound immunotoxicity of TCDD, however, there is interest in studying the effects of less suppressive alternative AHR ligands on the immune system to not only identify putative natural endogenous ligands of AHR but also explore the potential for alternative AHR ligands to alter disease outcome.

In addition to other halogenated aromatic hydrocarbon ligands of AHR, like TCDD, there are numerous naturally occurring AHR ligands that we are exposed to both through endogenous biological processes and in our diet. Some of these compounds are converted in the gut to high-affinity AHR ligands. Indole-3-carbinol, a metabolite of glucobrassicin found in cruciferous vegetables, is a weak AHR ligand that is converted to its acid condensation product indole[3,2-b]carbazole that binds and activates AHR with high affinity.¹⁰³ The flavonoids are a large group of dietary AHR ligands that includes flavones, flavanols, flavanones, and isoflavones, which are agonists and antagonists of AHR.^{104,105} Resveratrol, a known antagonist of AHR in the flavonoid family, was found to inhibit both TGF- β - and TGF- β /IL-6-mediated induction of Treg and Th17 cells in culture, respectively.⁷⁵

AHR ligands are also produced during different endogenous biological processes. The essential amino acid tryptophan (Trp) is metabolized and photo-oxidized into multiple AHR ligands. One such photoproduct is FICZ, which was found to promote Th17 differentiation.^{75,94,95} Trp photoproducts generated in cell culture media, like FICZ, have been shown to affect AHR activity.¹⁰⁶ The enzyme IDO catalyzes degradation of Trp into products such as kynurenine, which have been implicated in immune suppression and tolerance induction.^{48,107–109} Interestingly, kynurenine has been shown to activate AHR¹¹⁰ and induce a Treg phenotype.¹¹¹ The anti-allergic drug Tranilast® is a derivative of the

Trp metabolite 3-hydroxyanthranilic acid, which binds and activates AHR⁷² and has been shown to suppress EAE in a mechanism linked to Tregs.¹¹² These studies support the plausibility of the AHR as a target for treatment of immune-mediated diseases.

Emerging story for AHR activation and Treg development

The mechanism(s) underlying the enhanced Treg induction in TCDD-treated mice is not yet understood. Activation of AHR by TCDD has been shown to induce Foxp3-negative adaptive Tregs and is associated with increased numbers of Foxp3⁺ Tregs in different mouse models. Increased IL-2 expression in T cells as well as induction of IDO expression in DCs are some of the effects of TCDD that may contribute to Treg induction. AHR-mediated alterations in gene expression patterns during effector T-cell differentiation interfere with effector T-cell development and may result in the generation of adaptive Tregs by default. Whether pre-existing Foxp3⁺ Tregs are resistant to AHR-mediated effects of TCDD and thus are functionally preserved during an immune response to explain increased frequency is also not yet known. Ultimately, furthering our understanding of how AHR acts to suppress immune responses and specifically preserves and/or induces Tregs may open up new approaches for drug development for treatment of conditions such as autoimmunity, allergic reactions, and transplant rejection.

Conflict of interest

The authors declare no conflicts of interest.

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