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AHR-mediated immunomodulation: The role of altered gene transcription

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ABSTRACT

The immune system is a sensitive target for aryl hydrocarbon receptor (AHR)-mediated transcriptional regulation. Most of the cells that participate in immune responses express AHR protein, and many genes involved in their responses contain multiple DRE sequences in their promoters. However, the potential involvement of many of these candidate genes in AHR-mediated immunomodulation has never been investigated. Many obstacles to understanding the transcriptional effects of AHR activation exist, owing to the complexities of pathogen-driven inflammatory and adaptive immune responses, and to the fact that activation of AHR often influences the expression of genes that are already being regulated by other transcriptional events in responding cells. Studies with TCDD as the most potent, non-metabolized AHR ligand indicate that AHR activation alters many inflammatory signals that shape the adaptive immune response, contributing to altered differentiation of antigen-specific $CD4^+$ T helper (TH) cells and altered adaptive immune responses. With TCDD, most adaptive immune responses are highly suppressed, which has been recently linked to the AHR-dependent induction of $CD4^+CD25^+$ regulatory T cells. However activation of AHR by certain non-TCDD ligands may result in other immune outcomes, as a result of metabolism of the ligand to active metabolites or to unknown ligand-specific effects on AHR-mediated gene transcription. Based on studies using $AHR^{-/-}$ mice, evidence for a role of endogenous AHR ligands in regulation of the immune response is growing, with bilirubin and lipoxinA4 representing two promising candidates.

1. Introduction

Activation of AHR, a member of a heterodimeric ligand-activated transcription factor, has been recognized for many years as the common underlying mechanism by which numerous environmental contaminants that are structurally similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) suppress the adaptive immune response. Early studies in the 1970s and 1980s showed that various chlorinated congeners of dibenzo-*p*-dioxins, dibenzofurans and biphenyls produced similar immunosuppressive effects on the immune system of mice with a potency directly related to the affinity of the congener for binding to the AHR (reviewed in [1]). Today, TCDD is the AHR ligand of choice for studies aimed at understanding the mechanisms by which the AHR activation influences immune function. TCDD is the most potent AHR ligand, reducing the likelihood of high-dose off-target effects, and its effects are not confounded by ligand metabolism. Many other AHR ligands, such as the polycyclic aromatic hydrocarbons, are rapidly metabolized by AHR-inducible enzymes to active metabolites that produce a different spectrum of effects on the immune system [2–4]. These metabolite-mediated effects

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confound interpretation of the direct role of the AHR during an already-complicated immune response. Thus, the majority of this article is focused on a review of the immunomodulatory roles of the AHR based on studies using TCDD in mouse models. The immunological effects of non-TCDD ligands, including potential endogenous ligands, are considered in more depth later in this paper.

Most, if not all, cells of the immune system express AHR protein, including lymphocytes (T cells and B cells) and myeloid-derived cells [macrophages, dendritic cells (DCs), granulocytes, and natural killer (NK) cells] [5]. These multiple potential target cells may explain why TCDD exerts such potent immunosuppressive effects. For example, the antibody response to prototypic T-dependent antigen, SRBC, which involves collaborative interaction of DC/macrophages as antigen presenting cells (APC), $CD4^+$ T helper cells, and antibody-producing B cells, is highly suppressed following a single dose of TCDD, with an ID50 of 0.7 μg TCDD/kg bw [1]. Similarly, the robust $CD8^+$ cytotoxic T cell (CTL) responses generated to alloantigens or to virus infection, that also depend on DC and/or $CD4^+$ T helper cells, are highly suppressed at single doses of 2–10 μg TCDD/kg bw [6,7]. These immune responses are unaffected by TCDD if the animals do not express a functional AHR demonstrating the necessary role of AHR in mediating the immunosuppressive effects of TCDD [8,9].

Because many different types of cells are involved in immune responses and because most express AHR, understanding the immunological mechanisms that underlie the immune dysfunction induced by AHR activation exposure has been very challenging. The effects of TCDD on different immune endpoints that are measured in isolation can be misleading because effects of AHR activation are context-dependent. For example, interferon gamma (IFNγ) production in response to influenza virus infection is suppressed in the lymph nodes but augmented 10-fold in the lungs of TCDD-treated mice [7,10]. Similarly, TNFα production is not altered in anti-CD3 treated mice [11], whereas it is decreased in P815 tumor-injected mice [12] and significantly increased in LPS-treated mice [13]. These differential effects of TCDD on a single cytokine reflect the different types of cells that are capable of making the cytokine, the state of activation of the cells, and the nature of the activation stimulus. Thus, in order to move beyond descriptive immunological effects, a focused effort is needed to understand what genes, under what conditions of cell activation, are directly regulated by AHR activation in different types of immune cells. A surprisingly large number of immunologically relevant genes contain multiple binding sites for the AHR-ARNT heterodimer (aka, dioxin-response elements (DREs)) upstream of their start site [14]. On the other hand, a surprisingly small number of these candidate genes have ever been examined for direct AHR regulation in immune cells. It is likely that many of these genes can be transcriptionally regulated by AHR activation and that the regulatory effects will differ depending on the type of cell and the activation state of the cell. Since TCDD clearly affects cells that are responding to immunogenic stimuli and has little effect on resting cells, it is critically important to understand the signaling pathways that are directly influenced by AHR activation in the context of defined cellular activation signals if we are to begin to understand the role of AHR in immune function. Furthermore, the common assumption that transcriptional changes occurring several hours or days after exposure to TCDD are not direct AHR-mediated effects must be reconsidered in the context of cells of the immune system that are undergoing differentiation in response to stimulation. As regulatory regions on genes involved in the differentiation of immune cells become more accessible to their normal regulatory factors, these regions may also become newly accessible for AHR/ARNT binding as well. Given the prolonged half-life of TCDD, or continuous exposure to other AHR ligands, it is possible that direct AHR-dependent transcriptional modifications could occur several days after the initial exposure to the AHR ligand. Even though there likely exists alternative non-DRE-dependent pathways by which the ligand-activated AHR influences gene expression, high priority should be given to understanding the direct transcriptional effects via AHR/ARNT binding to DREs, as recent studies suggest that complex toxic effects of TCDD such as thymic involution in vivo are clearly dependent on the ability of AHR to bind DREs [15].

2. Effects of TCDD on innate immune responses

The innate immune response represents the first line of defense against infections by microbial pathogens and is mediated primarily by the myeloid lineage of cells of the immune system, including granulocytes, macrophages, DCs and NK cells. These cells are activated by microbial pathogens through evolutionarily conserved toll-like receptors (TLRs) [16]. TLRs are a family of pattern-recognition receptors that bind structural components shared by many bacteria, viruses, fungi and parasites. Activation of different TLRs initiates various signaling pathways leading to production of proinflammatory cytokines (e.g., TNFα, IL-1, IL-6, IFNγ, IL-8), complement activation, and increased expression of costimulatory molecules on APC that are important for activation of T lymphocytes. Many of the proinflammatory cytokines that are produced following TLR signaling activate NFκB, a ubiquitous transcription factor that expands the inflammatory response and is critical for controlling the infection and eliminating the pathogen. A large number of genes involved in this innate response contain multiple DRE sequences, including complement genes, all 9 TLRS, IL-1Rs and IL-1R associated proteins (Table 1) [14]. However, apart from NFkB, there is no published data on the direct transcriptional effects of activated AHR on expression of these genes. AHR regulation of NFκB signaling is discussed in detail in other papers in this journal and so will not be further discussed here.

The influence of TCDD (and presumptively, AHR activation) on inflammatory responses has been studied for many years. The earliest studies reported that exposure to TCDD greatly enhanced endotoxin (LPS) toxicity [17]. The enhanced lethality of TCDD-treated mice following LPS injection was linked to enhanced TNFα production [13]. Although the mechanism for enhanced TNFα production was not addressed in these studies, direct transcriptional activation of the TNFα gene by AHR is unlikely given only one putative DRE in its promoter

Table 1 – DRE sequences present in genes for inflammatory factors that are involved in activation of the innate immune response.

Innate response genes	DREs[a]	Receptor gene	DREs	Associated molecules	DREs
Tlr1	5				
Tlr2	2				
Tlr3	3				
Tlr4	5				
Tlr5	9				
Tlr6	3				
Tlr7	4				
Tlr8	2				
Tlr9	3				
Il1		Il1r1	5	Irak1	5
		Il1r2	7	Irak1bp	7
				Irak4	4
				Il1rap	12
Il6	3				
Il18	10	Il18r1	3	Il18bp	1
				Il18rap	1
Tnfα	1				

[a] DRE expression from Table S1b, mouse DREs Sun et al. [14].

and no DREs in TNFα receptors. On the other hand, Tang et al. [18] identified an NFkB-independent transcription factor, LPS-Induced TNF-alpha Factor (LITAF), which mediates the expression of TNFα and other inflammatory cytokines induced by LPS. Interestingly, the mouse LITAF gene has 7 DRE sequences in its promoter [14], suggesting the unexplored possibility that increased expression of LITAF is a factor in TCDD-induced endotoxin hypersensitivity. In addition, TRAF6, a transducer of IL-1 and TLR signaling pathways, has 5 DRE sequences in its promoter and may be directly regulated by activated AHR [14]. Other TRAFS except TRAF2 also express multiple DRE sequences and, if regulated by AHR, may influence TNFR2 signaling.

2.1. *Neutrophils*

In addition to heightened LPS sensitivity, TCDD-treated mice exhibit a dose-related increase in neutrophilia following treatment with TCDD [19]. Increased numbers of neutrophils are also found in the peritoneal cavity of TCDD-treated mice following injection of casein [20] or SRBC [21], in the spleen during the allograft response to P815 tumor cells [22] and in the lung following infection with influenza virus [23]. In all of the models, the increase in the number of neutrophils in TCDD-treated mice requires AHR but the mechanisms driving the increase are unknown. An increase in the extramedullary production of neutrophils was observed in the spleen of TCDD-treated mice during the allograft rejection response [22]. This was considered to be a compensatory response to the suppressed adaptive immune response but the mechanism was not determined. The excessive influx of neutrophils into the lungs of influenza-infected TCDD-treated mice did not result from increased local production of several soluble neutrophil chemoattractants, including keratinocyte chemoattractant (KC), macrophage inflammatory protein (MIP)-la, MIP-2, lipopolysaccharide-induced CXC chemokine (LIX), IL-6, or C5a or from altered expression of several adhesion molecules that increase recruitment of neutrophils to the lung in response to the virus (CD1la, CD11b, CD49d, CD31, and CD38) [23]. Changes in the survival of neutrophils through altered apoptosis was also not observed in TCDD-treated mice [23]. Surprisingly, in studies using bone-marrow chimeras of $AHR^{+/+}$ and $AHR^{-/-}$ mice, the recruitment of excess neutrophils to the lung in TCDD-treated mice was not dependent on AHR expression in the bone-marrow derived cells, suggesting that other AHR-expressing cells, presumably in the lung, directed the neutrophilic response [24]. This conclusion was supported by a lack of effect of TCDD on neutrophil production from the bone marrow and the absence of systemic neutrophilia [24]. No DREs have been identified in the granulocyte/macrophage-colony stimulating factor (GM-CSF) or G-CSF genes that specifically promote the production of neutrophils [14]. On the other hand, Vogel et al. [25] found that treatment of mice with TCDD increased macrophage chemotactic protein (MCP)-1 mRNA levels in spleen, lung, kidney, and liver, on day 1 after exposure, which were further increased at day 7. The increase of KC and MCP-1 on day 7 in liver, thymus, kidney, adipose, and heart was associated with elevated levels of the macrophage marker F4/80, suggesting the infiltration of macrophages in these organs. Increased expression of KC in the liver and spleen of mice was shown to be an AHR-dependent process. However the absence of DREs in the KC gene suggests that the increase in KC may be an indirect effect of AHR activation.

Conflicting effects of TCDD on neutrophil oxidative burst and cytolytic activity have been reported that range from enhancement to suppression to no effect [5]. These differing effects likely depend on the activation status of the neutrophils at the time of testing, and further research is required to understand the molecular basis for these differences. It will also be important to elucidate the direct consequences of AHR activation in the neutrophil. For example, genes for neutrophil cytosolic factors (Ncf) 2 and 4 (also known as p67 phox and p40 phox, respectively) have 3 and 5 DRE sequences upstream, respectively. The products of these genes are subunits of the multiprotein NADPH oxidase complex that is responsible for generating superoxide in myeloid cells. Neutrophils from mice with a mutated Ncf4 gene have severe defects in oxidant-

dependent bactericidal activity [26] and the Ncf2 gene is a susceptibility gene for *S. typhimurium* infection in wild mice [27]. If the AHR is found to directly regulate the expression level of these genes, it could potentially explain some of the effects of TCDD on neutrophil functions under different conditions of exposure.

2.2. Macrophages

Like neutrophils, the effects of TCDD on macrophages have been studied for many years with much conflicting data in the literature. Early studies reported that macrophages from TCDD-treated animals showed normal phagocytosis, oxidative burst, and tumor cytolytic activities [5]. In vivo studies of antigen-challenged mice found the exposure to TCDD increased IL-1 and TNF production by macrophages [13,28]. However, more recently, in the context of respiratory viral infection, exposure to TCDD had no effect on IL-1, TNF-α or type I IFN levels in the lung [29].

Recent studies have also examined the influence of AHR activation on gene expression and cell signaling in macrophages treated with TCDD in vitro. Cheon et al. [30] reported that TCDD induced TNFα production in human macrophage cell line by an AHR-EGFR-ERK pathway. Montiero et al. [31] investigated the link between early Ca^{++} elevation and AHR activation by TCDD. They found that mRNA levels for Cyp1b1, Ill1b IL8, Ccl1, b7-integrin, and Ahrr were all increased in primary human macrophages treated with TCDD, and that the increase in gene expression could be blocked by inhibiting Ca^{++}/calmodulin (CaM)-dependent protein kinase. The data suggest that the CaM kinase pathway is required for AHR-mediated transcriptional responses of these genes. These results are intriguing given that the genes Camk, Camk1g and Camkk1 contain 5, 6 and 4 DRE sequences in their promoters, respectively [14]. Changes in the expression of these genes by AHR activation could influence calcium signaling beyond the initial calcium flux associated with TCDD exposure. In other studies using a human macrophage cell line, Vogel et al. [32] reported that TCDD induced the expression of B cell activating factor (Baff), a TNF family member that promotes the survival and differentiation of B and T cells, B cell chemoattractant (Blc), chemokine (C–C motif) ligand 1(Ccl1), and IFNγ response factor (Ifr) 3. The increase in gene expression was associated with an AHR-RelB complex at a novel NFkB binding site in the promoter of these genes that did not involve ARNT [33]. The lack of AHR/ARNT regulation of Baff and Ifr3 is surprising given that there are 4 and 12 DRE sequences in the promoter of these genes, respectively [14]. Taken together, the results of in vitro studies suggest that the transcriptional effects of TCDD do not always involve transcriptional effects via AHR/ARNT binding to DRE, although direct non-DRE mediated effects of TCDD remain to be demonstrated in the intact animal. The DNA-binding domain (dbd) AHR mutants created in the Bradfield laboratory [15] should be useful in investigating the in vivo relevance of alternative pathways of AHR signaling.

2.3. NK cells

The cytolytic activity of NK cells has been reported to be increased, decreased and/or unaffected following TCDD exposure [5]. Several genes for NK receptor subunits (Klra, Klrb1, Klrc, Klrg, and Klrk) have DRE sequences in their promoters that could be directly regulated by AHR [14]. This possibility awaits further research.

2.4. Dendritic cells (DCs)

DCs, often identified by their membrane expression of CD11c, represent a unique niche in the immune system. While having much in common with their macrophage cousins as phagocytic and cytokine-producing cells in response to TLR activation, DCs play a major role as antigen-presenting cells (APC) to link the innate immune response to the adaptive immune response mediated by T and B lymphocytes. As a consequence of TLR stimulation, DCs undergo a series of changes that include decreased phagocytic activity, migration to regional lymph nodes, and processing of antigenic peptides for loading onto major histocompatibility complex (MHC) Class I and Class II molecules for their export to the cell surface. DCs also are induced to up-regulate the expression of a number of molecules that facilitate adhesion and provide co-stimulation to antigen-specific T cells that they encounter in the lymph nodes. The specific cytokines that DCs make, along with co-stimulatory molecules that they express, are determined by the specific TLRs that induce their activation. In this way, the antigen-specific T cells become activated along a pathway that is most effective in killing the pathogen (Fig. 1).

Several studies indicate that exposure to TCDD alters the function of DCs that may result in altered T cell activation and subsequent immune suppression. Vorderstrasse and Kerkvliet [34] characterized the temporal and dose-related effects of TCDD on the expression of costimulatory molecules on dendritic cells (DC), and directly examined the ability of DC from TCDD-treated mice to activate T cells in vitro. Unexpectedly, TCDD increased rather than decreased the expression of several accessory molecules on DC, including MHC Class II, CD54, CD40, and CD24. DC from TCDD-treated mice also produced more IL-12 and stimulated a higher T cell proliferative response in a mixed lymphocyte reaction. These changes in the DC did not translate into an effect on antigen processing as TCDD did not affect the ability of DC to phagocytize latex beads or to present KLH to KLH-specific T cells [35]. The processing and presentation of OVA to OVA peptide-specific transgenic T was also not suppressed by TCDD either in vivo or in vitro [35]. The only presumptively negative consequence of TCDD exposure on DCs was a significant reduction in the number of DCs recovered from spleens of TCDD-treated mice within 1 week after TCDD treatment. It was postulated that this decline in DC number reflected enhanced apoptosis of DCs following their inappropriate activation by TCDD [34]. Since the persistence of activated DCs has been shown to influence the strength and duration of $CD4^+$ T cell responses, a premature loss of DCs in TCDD-treated mice could result in insufficient contact time with T cells to sustain their full activation and differentiation.

Ruby et al. [36] investigated the direct effects of TCDD on the maturation and survival of bone marrow-derived DCs in vitro. The results of these studies showed that TCDD enhanced TNF-α-induced DC maturation and also enhanced Fas-mediated apoptosis. Based on microarray analysis of

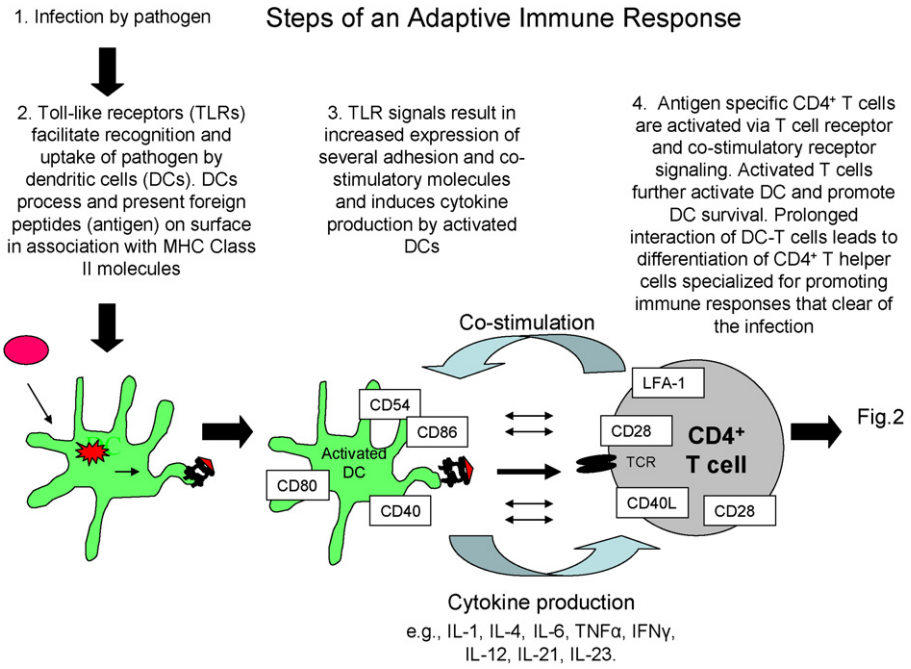

Fig. 1 – Cellular interactions between pathogen-activated dendritic cells (DCs) and CD4⁺ T cells, along with cytokines produced by cells of the innate immune response, determine the outcome of T cell differentiation and the type of adaptive immune response that develops. AHR is expressed in both DCs and in CD4⁺ T cells, and changes in both types of cells have been described following exposure to TCDD (refer to text for details).

genes associated with apoptosis, TCDD increased the expression of several genes in purified DCs, particularly Fadd, Dff40, Ox40l and Caspase9. Of these genes, only Fadd has DRE sequences [4] in its promoter [14]. TCDD also suppressed NF-κB signaling in DCs in response to TNF-α or anti-CD40 stimulation [37], suggesting that altered NF-κB signaling pathways may be an important mechanism for alteration of DC function or survival following TCDD exposure.

3. Effects of TCDD on adaptive immune responses

Adaptive immune responses are dependent on the activation, differentiation and clonal expansion of antigen-specific T and B lymphocytes that occurs over a period of several days following infection. The cornerstone of the adaptive response is the population of CD4⁺ T cells that differentiate along several different pathways depending on the signals received from the antigen-presenting DCs. These alternative pathways lead to the generation of TH1, TH2, TH17 or T regulatory (Treg) cells whose primary roles are to influence the ability of other immune cells to respond to antigen (Fig. 2). TH1 cells assist CD8⁺ T cells to differentiate into CTL effector cells with cytolytic activity directed toward cells that express the foreign antigen (e.g., virus-infected cells) and help B cells make the switch from IgM to IgG2a antibodies. TH2 cells help antigen-specific B cells to produce high affinity antibodies of several different classes appropriate for the pathogen and site of infection as well as IgE antibodies that mediate allergic responses. Recently discovered TH17 cells are proinflammatory cells that are especially prevalent in skin and intestinal tract. Although their natural role is yet to be defined, they appear to play a significant pathogenic role in a number of autoimmune diseases [38]. Finally, Treg cells function to prevent or down-regulate the immune response, by targeting the activation and/or survival of DCs, TH cells, and/or effector T cells [39].

Specific transcription factors have been identified that play critical roles in specifying the differentiation pathway that a given TH cell will follow. These include Tbet for TH1 cells, GATA-3 for TH2 cells, RORc(γt) for TH17 cells, and Foxp3 for Treg cells. These TFs are selectively induced by the signals received by the naïve TH cells at the time of antigen presentation (Fig. 2). GATA-3 has 10 DREs in its promoter, Foxp3 has 5, and Rorc(γt) has 4, indicating their potential to be directly influenced by AHR activation. In fact, Quintana et al. [40] recently reported that the Foxp3 gene is regulated by AHR in the absence of an exogenous ligand. There are no DRE sequences in the Tbet promoter but there are several DREs in the promoters of cytokine and cytokine receptor genes that influence TH differentiation, as well as in Jak, Stat and Socs genes that propagate the cytokine signals (Table 2). Interestingly, expression of the AHR gene itself is significantly up-regulated in CD4⁺ T cells cultured under TH0 [71] or TH17 conditions [40,41]. The signals underlying this up-regulation of AHR expression are not known as the promoter region of the AHR gene has not been thoroughly characterized. Increased AHR expression was also found in Treg cells compared to T effector cells in the pancreatic islets of non-obese diabetic (NOD) mice [42] and in tumor-infiltrating T cells [43].

Several comprehensive reviews of the immunotoxicity of TCDD have been published, most recently in 2007 [5]. These studies will not be reviewed here. Suffice it to say that TCDD is

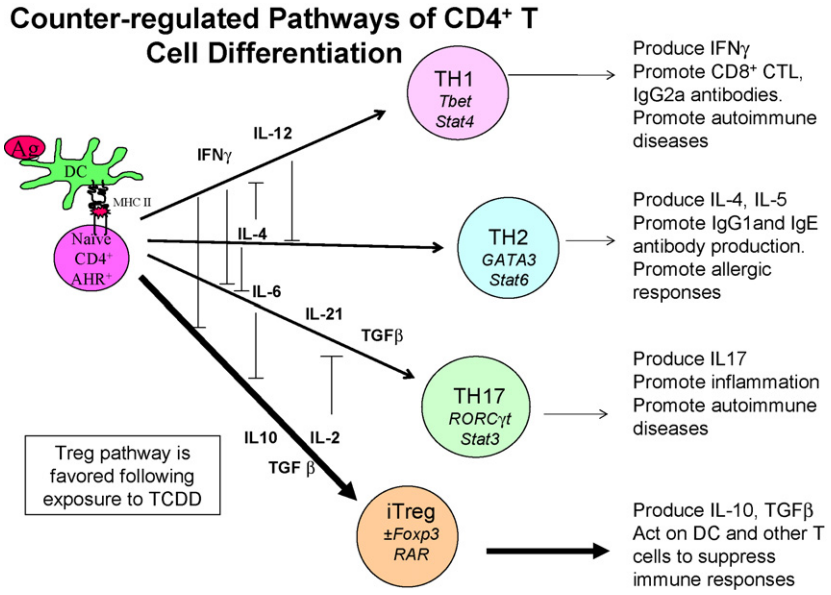

Fig. 2 – T cell differentiation proceeds along distinct pathways following antigen presentation by DCs. Depending on the cytokines that are produced by the DC during naïve T cell activation, expression of specific transcription factors are up-regulated that control T cell differentiation. Other pathways of T cell differentiation are counter-regulated by the presence of cytokines that drive a specific pathway. This assures the development of immune responses that are optimized for clearing the infectious agent that initiated the response. Inducible regulatory T cells (iTreg) develop under conditions where the cytokines that drive other pathways are not produced or co-stimulation by DCs is deficient. The iTreg pathway is enhanced by exposure of CD4+ T cells to TCDD [49,50].

a highly potent immunosuppressive chemical that suppresses essentially all types of adaptive immune responses. While the suppression of adaptive immunity to infectious diseases or to cancer is an undesirable outcome of AHR activation by TCDD, recent studies have shown that TCDD also suppresses allergic responses [44] and is a potent suppressor of autoimmune diseases, including experimental allergic encephalitis (EAE), a model for multiple sclerosis [40] and Type 1 diabetes (Kerkvliet, unpublished data). Thus, understanding the signaling pathways that are influenced by AHR activation to suppress immune responses has taken on new meaning based on the potential applications of novel AHR ligands to the treatment of immune-mediated diseases. The following discussion will therefore center on a series of studies that shed light on this topic.

3.1. *Mechanisms of suppression allograft immunity by TCDD*

The $CD4^+$ T cell-dependent $CD8^+$ CTL-mediated tumor allograft response to P815 mastocytoma was used as a model in the author's laboratory for many years to investigate the mechanisms of TCDD-induced immune suppression. TCDD is a potent suppressor of allospecific CTL responses through a mechanism that is completely dependent on AHR [8]. Furthermore, treatment with TCDD must occur during the first 3 days of the allograft response in order for the $CD8^+$ CTL response to be suppressed on day 10 [12]. Treatment with TCDD on days 4–5 of an ongoing alloresponse has no effect on CTL activity on day 10, even though the terminal differentiation and expansion of $CD8^+$ CTL precursors occurs after day 5. These results were important in demonstrating that, once CTL precursors were fully activated by the $CD4^+$ TH1 cells, TCDD did not affect their clonal expansion or development of cytolytic activity. Taken together, the data suggested that TCDD was suppressing the $CD8^+$CTL response by suppressing the response of $CD4^+$ T helper cells, either through direct effects on $CD4^+$ T cells or indirectly via the APC. This possibility was supported by the finding that TCDD failed to suppress the $CD4^+$ T cell-independent $CD8^+$CTL response to CD86-transfected P815 tumor cells [45]. On the other hand, treatment of mice with exogenous IL-2 as a means to enhance TH1 activation, failed to affect the suppressive activity of TCDD on the CTL response, and unexpectedly suppressed the response of vehicle-treated controls [46]. At the time, the suppressive effects of IL-2 on immune responses and IL-2-dependent induction of Treg cells were not widely appreciated. However, when the IL-2 gene was reported to be directly regulated by TCDD-activated AHR in T cells [47], the possibility that TCDD was enhancing Treg cell induction through enhanced IL-2 production became a subject of interest.

Specific effects of TCDD on alloresponsive $CD4^+$ T cells were investigated in vivo using an acute GVH model in which donor T cells from C57Bl/6 (B6) $H\text{-}2^b$ mice are injected iv into an F1 cross between B6 and DBA/2 (D2) mice (B6D2F1). The F1 host does not recognize the donor B6 T cells as foreign, whereas the donor B6 T cells respond to the Class 1 and Class II alloantigens expressed on the cells of the B6D2 F1 host to generate a donor $CD4^+$ T cell-dependent anti-host allospecific donor $CD8^+$ CTL response. This allospecific CTL response generated by the donor T cells in the F1 host was suppressed by treatment of the

Table 2 – Potential for direct AHR regulation of genes that play major roles in polarization of TH differentiation based on the presence of DRE sequences in their promoters.

Genes involved in polarization of TH differentiation	DREs[a]	Receptor gene	DREs	Associated molecules	DREs
Ifng		Ifngr	11	Ifi47[b]	6
		Ifngr2	5	Igtp[c]	7
Tgfb1	10			Tgfbi[d] Tgfb1i1[e]	12
Tgfb2	15	Tgfbr2	12	Tieg1[f]	10
Tgfb3	5			Tbrg4[g]	13
				Tgif[h]	10
					13
Il2	3	Il2rb	7		
		Il2rg	5		
Il4	2	Il4ra	9		
Il6	6			Il6st[i]	
Il10	3	Il10ra	4	Iltifb[j]	4
Il12a	3	IL12rb1	4	IL12a	3
Il12b	3	IL12rb2	6	IL12b	3
Il17	3	1l17r	6		
Il17b	3	Il17rb	3		
Il17d	8	1l17c	6		
		1l17rd	7		
		Il17re	6		
Il21	4	IL21r	5		
Il23a	5				
		IL27ra	10		
Gata3	10				
Foxp3	5				
Jak1	5				
Jak2	9				
Jak3	20				
Stat1	9				
Stat2	5				
Stat3	5				
Stat4	4				
Stat5a	9				
Stat5b	7				
Stat6	12				
Socs1[k]	18				
Socs2	8				
Socs3	11				

[a] DRE expression from Table S1b, mouse DREs Sun et al. [14].
[b] Interferon gamma inducible protein.
[c] Interferon gamma induced GTPase.
[d] Transforming growth factor beta 1 induced.
[e] Transforming growth factor, beta induced transcript 1.
[f] TGFB inducible early growth response 1.
[g] Transforming growth factor beta regulated gene 4.
[h] TG interacting factor (TGFB-induced factor homeobox 1).
[i] Interleukin 6 signal transducer.
[j] Interleukin 10-related T cell-derived inducible factor beta.
[k] Suppressor of cytokine signaling (Socs).

host with TCDD, similar to the suppression of the CTL response to allogeneic P815 tumor cells described previously [48]. Importantly, however, if the donor T cells were obtained from $AHR^{-/-}$ mice, TCDD had no effect on the CTL response [48]. These data provided the first unequivocal evidence for a direct effect of TCDD on T cells that was mediated by the AHR in the T cell itself.

3.2. *In vivo effects of AHR activation in $CD4^+$ T cells*

Studies to examine the effects of intrinsic AHR activation on the response of donor $CD4^+$ T cells revealed no effect of TCDD on proliferation of the cells but significant changes in markers of T cell differentiation [49]. The most noteworthy changes included a large increase in CD25 expression (reflected in both % positive cells and in fluorescence intensity) and a decrease in CD62L expression that occurred incrementally during cell divisions 2–5. Since high level expression of CD25 is a marker of Treg cells, it suggested that AHR activation by TCDD might be promoting the Treg differentiation pathway. Further analysis showed that the $CD4^+CD25^+$ T cells from TCDD-treated mice shared other attributes of Tregs, co-expressing elevated levels of GITR and CTLA-4, and potently suppressing the proliferation of naïve T cells in vitro [49]. Depletion of

existing natural $CD4^+CD25^+$ Treg cells from the donor population prior to injection into F1 hosts did not influence the subsequent generation of the AHR-dependent $CD4^+$ $CD25^+$ cells, suggesting that TCDD was not simply expanding the natural $CD4^+CD25^+$ $Foxp3^+$ Treg population. Furthermore, the AHR-dependent $CD4^+$ $CD25^+$ cells did not express Foxp3 [50], suggesting that the AHR represents a novel transcription factor for adaptive Treg cell differentiation.

3.3. *In vivo effects of AHR activation in $CD8^+$ T cells*

The response of $CD8^+$ donor T cells was also examined in the studies described above. Interestingly, the effects of TCDD on the donor $CD8^+$ T cells were very similar to the effects on the donor $CD4^+$ T cells, including increased CD25 expression and potent suppressive activity in vitro [51]. These results suggest that AHR activation also induces $CD8^+$ Treg cells. However, despite the similarities in phenotype and function induced by TCDD in $CD4^+$ and $CD8^+$ T cells, intrinsic activation of AHR within each T cell subpopulation was found to produce different outcomes. Based on studies using various combinations of donor $CD4^+$ and $CD8^+$ T cells from $AHR^{+/+}$ and $AHR^{-/-}$ mice, AHR expression in the $CD4^+$ cells was shown to be primarily responsible for the phenotypic and functional changes induced by TCDD in both the $CD4^+$ and $CD8^+$ cells [51]. In contrast, if AHR expression was limited to the $CD8^+$ cells, TCDD effects were modest and restricted to the $CD8^+$ T cells themselves. The ability of AHR activation in $CD4^+$ T cells to induce Treg-like $CD8^+$ T cells instead of CTL effectors is a novel mechanism for suppressing the development of CTL activity.

$CD8^+$ CTL activity in response to influenza virus infection is also highly sensitive to suppression by TCDD, with a time-course of suppression that is very similar to that seen in the allograft models described above [7,9]. However, the primary CTL response to influenza does not depend on $CD4^+$ T cells, and TCDD suppresses the $CD8^+$ CTL response to influenza in the absence of $CD4^+$ T cells (P. Lawrence, personal communication). These findings suggest that AHR-induced $CD4^+$ Tregs are not involved in the suppression of the $CD8^+$ CTL response to influenza virus. Furthermore, AHR expression in the $CD8^+$ T cells is not the primary determinant of the suppression of CTL activity by TCDD in influenza-infected mice [9], suggesting that TCDD does not act directly on the differentiation of $CD8^+$ CTL precursors. One possible explanation for the apparent conflict between the influenza and allograft models is that DCs are the ultimate cells that confer suppression on the $CD8^+$ T cells in both models of the CTL response. In the influenza model, infection of DCs by the virus activates them to become fully functional APC without input from $CD4^+$ T cells. Since DCs express AHR, it is possible that TCDD directly alters the activation of DCs to induce tolerogenic properties that do not support $CD8^+$ CTL differentiation but may instead induce $CD8^+$ Treg-like cells. In contrast, in $CD4^+$ T-dependent $CD8^+$ CTL responses like the allograft response, there is essential cross-stimulation that must occur between DCs and activated $CD4^+$ T cells that licenses DCs to activate the $CD8^+$ T cells. Thus, in the allograft model, it is possible that changes induced by AHR activation in the $CD4^+$ T cells confer a tolerogenic phenotype on DCs, likely in conjunction with additional influences of the AHR in the DC, resulting in failure to induce $CD8^+$ CTL differentiation and instead induction of $CD8^+$ T regs. The specific role that the AHR in DCs might play in either of these models remains to be determined.

3.4. *Changes in gene expression in activated $CD4^+$T Cells following AHR activation in vivo*

Changes in gene expression in donor T cells isolated from TCDD- and vehicle-treated F1 hosts 2 days after adoptive transfer, at the time of peak Treg development in TCDD-treated mice, were analyzed using a TH1-2-3 pathway-specific gene array [50]. Several genes were significantly up-regulated in the AHR-dependent Tregs including Tgfb3, Ccr4, Ccr5, Cd30, Bcl3, Ctla4, Il10, Gata3, Icos, and Cd28 along with several genes associated with the IL-12 signaling pathway (Il12rb2, Stat4, Socs3, jak2) (Table 3). The JAK2/STAT4 pathway is important in the promotion of IFN-γ production and Tbet expression for TH1 differentiation. However, TH1 effectors are suppressed in TCDD-treated mice, and the production of IFNγ by donor T cells from TCDD-treated mice is significantly reduced. These results suggest disruption of the signals downstream of Jak/STAT phosphorylation, or additional constraints imposed on transcription of the IFN-γ gene by AHR-induced signals. For example, Gata-3 transcript, which was up-regulated almost 2-fold in TCDD-exposed cells, induces IL-10 expression, and IL-10 inhibits IFN-γ production mediated by STAT4. Increased expression of SOCS-3 in AHR-Tregs might also interfere with the Jak/STAT signaling. In other assays, expression of the genes for Blimp-1, Granzyme B and AHRR were found to be

Table 3 – Changes in gene expression in donor T cells from TCDD-treated host mice compared to donor T cells from vehicle-treated host mice on day 2 of the GVH response.

Gene	TCDD/Veh[a]	DREs[b]	Gene	TCDD/Veh	DREs
Tgfb3	13.1	5	Ox40l	−2.9	
Il12rb2	9.8	6	I13ral	−2.3	
Gzmb	5.5	3	Cd86	−2.2	
Ccr4	4.7	8	Bcl6	−1.5	2
Ccr9	4.7		Ccl5	−1.4	
Stat4	3.3	4	Il5	−1.4	2
Prdm1	3.0	8	Nfkb1	−1.4	
Ccr5	3.0				
Socs3	2.7	11			
Tnfrsf8 (CD30)	2.6	4			
Bcl3	2.4	4			
Ctla4	1.9				
IL2ra	1.9				
Il10	1.8	3			
Gata3	1.8	10			
Icos	1.5	4			
Cd28	1.4	1			
Jak2	1.3	9			

[a] Results are expressed as fold change in gene expression for genes that were significantly different between treatment groups, $n = 3$ (where $n = 2$ mice) per group, $P < 0.05$.
[b] DRE expression from Table S1b, mouse DREs Sun et al. [14].

increased while Foxp3 expression was decreased in the donor T cells from TCDD-treated mice [50]. Other down-regulated genes included Tnfsf4 (OX40-L), Il13ra, Cd86, Bcl6, Il5, Nfkb1, and Ccl5. Table 3 indicates the presence of DRE sequences in the genes that were altered in the donor T cells from TCDD-treated mice. However, because the cells were analyzed 2 days after TCDD exposure, some of the changes in gene expression may be secondary to changes in other genes that are directly regulated by AHR. While many of the genes that showed increased expression in cells from TCDD-treated mice have been associated with other types of Tregs, the data do not lend themselves to straight-forward interpretation and suggest that AHR activation during $CD4^+$ T cell differentiation induces complex changes in gene expression leading to altered T cell fate.

3.5. *Changes in gene expression in activated $CD4^+$T Cells following AHR activation in vitro*

In order to map the direct consequences of AHR activation on $CD4^+$ T cell differentiation, the author's laboratory has initiated studies to determine the effects of TCDD on gene expression in vitro under different conditions of T cell activation. To identify baseline changes, initial studies have used non-polarizing conditions and stimulation with soluble anti-CD3 and anti-CD28 antibodies to activate purified $CD4^+$ T cells in the presence of 0 or 1 nM TCDD. This concentration of TCDD should be sufficient to fully activate the AHR based on the binding affinity of TCDD ($K_D = 7.1 \times 10^{-12}$ M) but low enough to prevent spurious effects that might result from high concentrations of TCDD [52]. The responsiveness of $CD4^+$ T cells to AHR activation by 1 nM TCDD was validated by significant induction of Cyp1a1 expression at 24–48 h and induction of AHRR at 48 and 72 h of culture. To date, most of the genes that have been analyzed in vitro were selected from the panel of genes that were associated with AHR-induced Tregs at 48 h in the GVH model (Table 4). Even though the conditions of activation are very different, we are interested in discovering the unique effects of AHR activation on gene expression in T cells. The most notable effects of TCDD in vitro were increased expression of the Il12rb2 and Stat4 genes but decreased expression of the downstream targets, Tbet, IFNγ and IL-10 genes. These changes were similar to those seen in the GVH model and suggest that the conditions of T cell activation did not limit the influence of AHR on the IL12R signaling pathway. On the other hand, the lack of changes in expression of other genes may reflect the TH0 stimulating conditions used in this study. Since AHR activation appears to influence expression of genes that are already being actively transcribed, comparative studies using different conditions for T cell activation will be essential to elucidate the full range of genes that are regulated by the AHR in $CD4^+$ T cells. This should prove interesting as well as complex, given the recent finding that T cells activated under TH17-inducing conditions (IL6 + TGFβ or IL21 + TGFβ) express high levels of AHR while activation with either cytokine alone induced only modest AHR message [40,41,53]. It is not yet known if this increase in AHR expression translates to a more sensitive response to AHR ligands since our data show that even the low level of AHR message in T cells cultured under TH0 conditions is sufficient to induce expression of CYP1a1 and AHRR following TCDD exposure.

Table 4 – TCDD-induced changes in gene expression over time in $CD4^+$ T cells activated in vitro with anti-CD3 and anti-CD28 stimuli.[a].

	24 h	36 h	48 h	72 h
Cyp1a1	+18.6	+17.9	+5.1	nt
Ahrr			+4.1	+7.5
Il2				+3.7
Il2ra				
Ctla4	−2.0			
Il12rb2	+1.6	+2.3		nt
Stat4		+2.9		+1.9
Tbet	−3.7			+1.9
CD40Ig				
Tgfb3				
Ifng	−1.7	−2.4		
Foxp3				
Il4				
Il6				
Il10	−3.7		−2.4	
Il17				

[a] Gene expression data are presented as fold-change (TCDD/Veh) based on ΔΔCt relative to b-actin expression in each sample. Expression of all genes listed were measured but only statistically significant ($P < 0.05$) changes are shown. nt = not tested. Data were calculated from the mean response of three independent samples of $CD4^+$ T cell RNA prepared from individual $AHR^{+/+}$ mice at each time point. All genes were expressed within 35 cycles except for IL17 that was expressed at low level in all samples, at or exceeding the 35-cycle cutoff.

4. Immune effects of non-TCDD AHR ligands

4.1. *Halogenated and polycyclic aromatic hydrocarbons*

It is generally accepted that dibenzo-*p*-dioxins, dibenzofurans and biphenyls that are chlorinated in at least 4 non-ortho positions are capable of binding to AHR and inducing similar effects on the immune system as TCDD [1,12]. The major difference between the chemicals is the dose required to induce the effects, which primarily reflects the binding affinity of the chemicals to the AHR and their resistance to metabolism. In contrast, the nonhalogenated polycyclic aromatic hydrocarbons (PAHs) which bind and activate the AHR (e.g., benzo-*a*-pyrene, dimethylbenzanthracene) produce a different pattern of immunotoxic effects that derives from their susceptibility to metabolism and the generation of toxic metabolites, including DNA-reactive diol-epoxides [2–4]. Thus, even though many PAHs are AHR agonists and are immunosuppressive like TCDD, the underlying mechanisms that drive the immune suppression are different and are not considered relevant to the current discussion.

4.2. *Tryptophan metabolites*

Several tryptophan metabolites have been shown to activate AHR in reporter assays [54]. Tryptophan catabolism has also been shown to play a role in immune tolerance via the induction of indoleamine dioxygenase (IDO) or other kynur-

enine pathway enzymes in DCs during infection or inflammation [55]. Originally thought to suppress T cell responses by inducing local depletion of tryptophan needed by the T cells to proliferate, more recent data indicate that tryptophan metabolites themselves act on T cells to induce tolerance [56–59]. It is possible that some of these metabolites bind AHR in $CD4^+$ T cells or in DCs and promote their immunosuppressive effects. Kynurenine (Kyn), for example, activates AHR at concentrations in the 30–50 μM range, and induces a Treg phenotype in cultured T cells at similar concentrations [55]. Kyn concentrations in the 5–50 μM range can be generated by DCs exposed to physiological concentrations of tryptophan [57]. Tranilast® is an immunosuppressive synthetic derivative of the kyn metabolite, 3-hydroxyanthranilic acid, and is used clinically as an antiallergenic drug. In mice, Tranilast® was shown to suppress EAE by a mechanism that appeared to involve Tregs [60]. These effects would be consistent with AHR activation; Tranilast® was shown to activate AHR with an EC50 of ~50 μM (Denison and Kerkvliet, unpublished data).

Another tryptophan-derived product is 6-formylindolo[3,2-b] carbazole (FICZ), a UV-induced photoproduct formed in the skin that binds AHR with very high affinity (K_D = 0.07 nM) [61]. Since UV exposure has been shown to induce systemic immune suppression via the induction of Tregs [62,63], it is possible that FICZ-induced activation of AHR plays a role in this process. However, contrary to expectations, FICZ was recently reported to influence T cells cultured under TH17 conditions resulting in increased proportion of cells that differentiated into TH17 cells and increased production of the inflammatory cytokine, IL-22 [41]. Furthermore, when FICZ was incorporated into the antigenic emulsion used to induce EAE, FICZ significantly enhanced disease development in wild-type but not AHR-deficient mice [40,41]. These responses to FICZ are inconsistent with Treg induction and inconsistent with suppression of TH17 development and EAE that is produced when AHR is activated by TCDD [40]. The most straightforward interpretation for the divergent effects of FICZ is that FICZ was metabolized by AHR-induced enzymes to a non-AHR binding metabolite that was responsible for the induction of IL-22 and exacerbation of EAE. FICZ is known to be rapidly metabolized by AHR-induced Cyp1a1 and Cyp1b1 [64]. On the other hand, Kimura et al. [53] was unable to confirm the effects of FICZ on TH17 cells but instead found that FICZ enhanced Treg induction, consistent with TCDD's effects. The basis for the contradictory results with FICZ remains to be determined. As a precautionary note, multiple tryptophan photoproducts are generated in cell culture media exposed to UV light and these metabolites are capable of activating the AHR, inducing efficient CYP1A in cultured cells and the potential for confounding effects during in vitro studies [65].

4.3. *Leflunamide*

Leflunamide (N-[4-trifluoro-methylphenyl]-5-methylisoxazol-4 carboxaminde) (LF) was identified as an AHR ligand in a large scale screening of pharmaceuticals [66]. In fact, of 147 compounds tested, LF came out at the top of the list for induction of Cyp1A1 activity in vivo. LF is also recognized as an immunoregulatory and anti-inflammatory compound that is used clinically in the treatment of rheumatoid arthritis, and is effective in suppressing the immune response in several animal models of allergic and autoimmune diseases [67]. LF is metabolized to the pharmacologically active product A77 1762 that inhibits de novo pyrimidine synthesis, which preferentially inhibits the proliferation of activated $CD4^+$ T cells [68,69]. In addition, high in vitro concentrations of A77 1762 block NFkB activation by TNFα and inhibit multiple kinases in T cells, among other effects [68,69]. More recently, treatment of mice with LF was linked to the induction of antigen-specific regulatory $CD4^+$ and $CD8^+$ T cells [70]. The role of the AHR in any of these immunosuppressive effects is not currently known. However, studies in the author's laboratory have shown that the inhibition of T cell proliferation by LF or by A77 1762 is not dependent on the AHR, as similar effects were seen in $AHR^{+/+}$ and $AHR^{-/-}$ T cells (Farrer D, Kerkvliet NI, unpublished data). Suppression of proliferation of T cells by the parent compound in $AHR^{-/-}$ T cells further suggests that metabolism of LF to A77 1762 does not depend on AHR-induced enzymes. The role of the AHR in Treg induction by LF remains to be determined.

4.4. *Benzoimidazole derivatives*

Two antiallergenic drugs, 3-[2-(2-phenylethyl) benzoimidazole-4-yl]-3-hydroxypropanoic acid (M50367) and ethyl 3-hydroxy-3-[2-(2-phenylethyl)benzoimidazol-4-yl] propanoate (M50354), are AHR receptor agonists in vivo and in vitro [71,72]. The immunosuppressive effects of M50354 were shown to depend on the AHR as the immune response of $AHR^{-/-}$ mice was not affected by treatment with the drug [71]. Interestingly, the expression of AHR in naive TH cells was significantly up-regulated by co-stimulation with antibodies to the TCR and CD28 at 24 hr, without added cytokines, unlike the recent papers suggesting AHR up-regulation occurs only under TH17 conditions [40,41]. Suppression of TH2 differentiation by M50354 was associated with inhibition of GATA-3 expression. The lack of effect of M50354 on IFNγ production suggested that the TH1 pathway was not altered by the drug. The basis for the selective effects on TH2 cells by this AHR ligand are not clear as activation of the AHR by TCDD potently suppresses both TH1 and TH2 pathways of T cell differentiation.

Recently another low molecular weight, antiallergenic compound, VAF347, was shown to produce its anti-inflammatory effects by activating AHR [73,74]. VAF347 was potent, displacing TCDD from the AHR at a 10 nM concentration. VAF347 also induced Cyp1a1 expression in human monocytes that was augmented when the monocytes were cultured with IL4 and GM-CSF, cytokines that induce the differentiation of monocytes to DCs [73]. Both VAF347 and TCDD suppressed IL-6 production by DCs. Gene chip analysis showed that VAF347 reduced IL-6 gene expression as well as expression of c-myc by 4-fold and thrombospondin-1 by >180-fold. Human T cell proliferation induced by activated DCs was also suppressed by VAF347, and by TCDD as well. These effects of VAF347 were blocked when the cells expressed a transdominant negative form of AHR. As final proof of the involvement of the AHR, allergic lung inflammation induced by OVA challenge of OVA-sensitized mice was suppressed by VAF347 in $AHR^{+/+}$ but not in $AHR^{-/-}$ mice. In other studies, oral administration of a water-soluble derivative of VAF347 (VAG539) was shown to

inhibit the rejection of pancreatic islet allografts in Balb/c mice in association with an increased frequency and survival of $CD4^+$ T cells expressing a $CD25^+Foxp3^+$ Treg phenotype [74]. These Tregs appeared to be induced indirectly via effects on DCs. The chemical structure of VAF347 has not been reported.

4.5. *Flavones*

Several flavones, flavonols and isoflavones have been identified as AHR ligands [64]. Alpha and β-napthoflavones have been used as controls in research studies on AHR effects for many years, with the beta form used as an agonist of AHR and the alpha form as an antagonist or partial agonist of AHR. Genistein, a popularized flavenoid present in soy, and quercetin, a flavenol found in apples, tea and onions, are also AHR ligands [75]. While their affinity for binding the AHR is relatively weak, and many are susceptible to metabolism by AHR-inducible enzymes, their wide occurrence in the diet and their antioxidant, antiproliferative and antiestrogenic activities suggest they might produce physiological effects [64]. In regard to effects on the immune system, genistein has been shown to suppress immune and inflammatory responses in mice [76], and lotions containing genistein reduced inflammation associated with UV exposure [77]. Other studies reported that genistein or other phytoestrogens stimulated various aspects of immune function [78]. Any involvement of the AHR in these effects is not currently known.

4.6. *Bilirubin*

Bilirubin, a product of heme degradation, is considered one of the most likely endogenous ligands of AHR. It competes with TCDD for AHR receptor binding and induces CYP1a1 in hepatoma cells at an EC_{50} of ~30 μM [64]. No longer considered just a toxic by-product that needs to be excreted, the potent antioxidant activity of bilirubin suggests additional roles of physiological significance. Recently, potent effects of bilirubin on the immune system were reported [79]. In vitro, bilirubin significantly inhibited $CD4^+$ T cell responses at multiple steps, including suppressed costimulatory molecule expression. In vivo, treatment of mice with bilirubin suppressed the development of the autoimmune disease EAE, while depletion of endogenous bilirubin significantly exacerbated the disease. These results suggest that endogenous levels of bilirubin may play an immunoregulatory role. A combined protocol of bilirubin, carbon monoxide and heme-oxgenase-1 (HO-1) suppressed the allograft response in mice in conjunction with increased Tgfb and Foxp3 gene expression in the surviving grafts [80]. Interestingly, expression of the gene for HO-1 is up-regulated in activated T cells following TCDD treatment (Kerkvliet, unpublished data). Taken together, regulated expression of AHR could contribute to the potency of bilirubin and other factors associated with heme metabolism to suppress the immune response, a research topic that needs to be explored.

4.7. *Lipoxin A4 (LXA_4)*

LXA_4, another endogenously generated AHR ligand, is the product of arachidonic acid metabolism via the enzyme lipoxygenase. LXA_4 activates AHR in a reporter assay with an EC_{50} in the nanomolar range, competes with AHR for receptor occupancy and stimulates CYP1A1 and CYP1A2 enzyme activity [81]. LXA_4, along with other products of the 5-lipoxygenase pathway, play an important immunomodulatory role by down-regulating inflammatory responses through multiple pathways, including suppression of TNF-induced chemokine production, translocation of NFkB, and pathogen-induced IL-12 production [82,83]. The anti-inflammatory actions of LXA_4 in DCs were shown to depend on Socs-2, a gene with multiple DREs in its promoter that appears to be directly regulated by AHR. The direct involvement of AHR in the induction of Socs-2 in vivo was implicated by the use of $AHR^{-/-}$ mice which showed no increase in Socs-2 in the spleen following challenge with a pathogen-derived antigen that induced Socs in $AHR^{+/+}$ mice [83]. However, the induction of Socs-2 by growth hormone was not altered in $AHR^{-/-}$ mice, suggesting that Socs-2 expression can be induced independent of AHR regulation in other signaling pathways. The possibility that the Socs-2 gene is directly regulated by LXA4-activated AHR is supported by the results of Boverhof et al. [84] who demonstrated direct DRE-dependent regulation of the Socs-2 gene in B cells by TCDD-activated AHR. Taken together, the results strongly implicate LXA_4 as a functionally relevant, endogenous AHR ligand.

5. Immune response of AHR KO mice

If there are functionally relevant endogenous ligands for the AHR that play a physiologically important immunoregulatory role, then $AHR^{-/-}$ mice should show evidence of altered immune functions. Three lines of $AHR^{-/-}$ mice have been studied in terms of their immune system, referred to as Δ1/Δ1 and Δ2/Δ2 strains based on the exon of the AHR that was disrupted, and a third strain developed by Mimura et al. [85], all on a C57Bl/6 background.

Initial reports indicated that the immune system was impaired in Δ1/Δ1 mice based on altered splenic architecture and decreased cellularity with an apparent increased incidence of infection [86]. As the animals aged, they also showed a reduction in the numbers of T and B lymphocytes in the spleen, which was accompanied by splenomegaly due to expanded myeloid cells in the red pulp [87]. Thymocyte differentiation and emigration were not affected in fetal thymi from Δ1/Δ1 mice although total thymocyte numbers were reduced in comparison to wild-type mice [88]. These changes in T and B cell numbers are not consistently reported in all studies with Δ1/Δ1 mice, suggesting that environmental conditions of animal housing might influence the phenotype of $AHR^{-/-}$ mice. Changes in lymphocyte numbers are also not observed in Δ2/Δ2 mice.

The ability of Δ1/Δ1 and Δ2/Δ2 mice to respond to antigenic stimulation was investigated by Vorderstrasse et al. [8]. Two model antigens, allogeneic P815 tumor cells and SRBC, were used in these studies. No significant differences were seen in the alloCTL response or in the antibody response to SRBC in $AHR^{-/-}$ mice of either strain in comparison to the wild-type controls. The ability of $CD4^+$ and $CD8^+$ T cells from Δ2/Δ2$AHR^{-/-}$ and wild-type mice to respond to host alloantigens as donor cells in an

acute GVH model were also unaffected by AHR status in regard to changes in activation phenotype (up-regulation of CD4, down-regulation of CD62L, expression of CD25) [49]. The only significant difference was a small but reproducible increase in cycling of the donor CD8 T cells from Δ2/Δ2AHR$^{-/-}$ mice that sometimes translated into an increase in the number of CD8$^+$ T cells in the spleen of host mice on day 3 [51].

Rodriguez-Sosa et al. [89] reported that ovalbumin (OVA)-immunized Δ1/Δ1 AHR$^{-/-}$ mice produced similar levels of OVA-specific IgG2a, IgG1 and IgG2b antibodies when compared to AHR$^{+/+}$ mice. However the AHR$^{-/-}$ mice had splenomegaly associated with an increased number of B cells. When the splenocytes were cultured in vitro, the cells from AHR$^{-/-}$ mice proliferated normally but produced more IL-12 and IFNγ than cells from AHR$^{+/+}$ mice, while IL-4 production was not altered. In response to Concanavalin A stimulation, cells from AHR$^{-/-}$ mice produced less IL-5 but no change in IL-2 production. These data were the first to suggest that AHR may play an endogenous role in the immune response to down-regulate IL-12 and IFNγ expression.

In another model, Shi et al. [90] reported that Δ2/Δ2 AHR$^{-/-}$ mice were more susceptible to a primary infection with *Listeria monocytogenes* as compared to their heterozygous littermates. An increased CFU in liver and spleen and more severe histopathology in the liver of AHR$^{-/-}$ mice were paralleled by increased serum levels of IL-12 and IL-10 at 24 h post-infection. Serum levels of TNFα, MCP-1, IFNγ and IL-6 were not affected by AHR status. AHR$^{-/-}$ mice were competent to clear the infection, albeit with a delayed kinetics. Interestingly the AHR$^{-/-}$ mice showed enhanced resistance upon reinfection with *L. monocytogenes*. While the number of activated CD4$^+$ memory T cells did not differ based on AHR status, more of the cells from AHR$^{-/-}$ mice were positive for TNFα. The number of activated memory CD8$^+$ T cells was higher in AHR$^{-/-}$ mice. Finally, AHR status did not influence the ability of peritoneal macrophages from infected mice to phagocytose or kill the bacteria, and bone-marrow derived macrophages activated by pretreatment with IFNγ also killed the bacteria equally well whether they expressed the AHR or not.

Lung inflammatory responses were examined in Δ2/Δ2 AHR$^{-/-}$ mice following exposure to cigarette smoke or bacterial endotoxin (LPS) [91]. Smoke-induced neutrophilic inflammation in the lung was more severe in AHR$^{-/-}$ mice compared to AHR$^{+/+}$ or AHR$^{-/+}$ littermates and was associated with increased β-glucuronidase and myeloperoxidase activities. TNFα, IL6, MIP-2, KC and PGE_2 levels were all increased in the bronchoalveolar lavage (BAL) fluid from air-exposed AHR$^{-/-}$ mice compared to AHR$^{+/-}$ mice. TNFα, IL6, MIP-2, and PGE_2 but not KC were also increased in AHR$^{-/-}$ mice after smoke exposure. Inhaled endotoxin also produced more neutrophilic inflammation in AHR$^{-/-}$ mice compared to AHR$^{-/+}$ mice, and when cells from the BAL were cultured with LPS in vitro, the cells from AHR$^{-/-}$ mice produced more TNFα and more IL6. The mechanism for increased cytokine production was postulated to occur as a result of elevated NFkB activity and rapid degradation of Relb that only occurred in AHR$^{-/-}$ mice. Relb has been shown to function as a negative regulator of NFkB activity.

Data from the studies of Negishi et al. [71] comparing vehicle-treated AHR$^{-/-}$ mice to vehicle-treated AHR$^{+/+}$ mice, showed that AHR$^{-/-}$ mice have increased number of peritoneal exudate cells and elevated plasma IgE in response to two ip injections of DNP-Ascaris. Splenocytes from AHR$^{-/-}$ mice also produced more IL-5 in response to in vitro challenge with DNP-Ascaris than cells from AHR$^{+/+}$ mice. An increase in IFNγ was not statistically significant. When naïve CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies, cells from AHR$^{-/-}$ mice produced 2–3-fold higher levels of both IL-4 and IFNγ than cells from AHR$^{+/+}$ mice. Retroviral transfection of AHR led to a TH1 phenotype even in the presence of exogenous IL-4. Analysis of gene expression in stimulated CD4$^+$ T cells showed increased GATA-3 mRNA levels in cells from AHR$^{-/-}$ mice, while the levels of IL4ra and Stat6 mRNA were not different.

Taken together, it is clear that the absence of AHR influences host responses to some infectious diseases and antigenic challenges but not to others, and that the specific changes in the response may be subtle or significant. The specific consequences of AHR deletion appear to vary as the normal response to the insult varies, with different cytokines associated with TH1 and TH2 responses increased under different activating conditions. Overall, the data suggest that the fundamental role of the AHR is to down-regulate immune responses. These observations with AHR$^{-/-}$ mice are consistent with the context-specific suppressive effects of ligand-induced AHR activation on immune functions that have been discussed throughout this chapter.

Some interesting questions for future AHR research include: Are AHR$^{-/-}$ mice more susceptible to autoimmune diseases? Do AHR ligands in the diet reduce or promote susceptibility to immune mediated diseases in the context of AHR status? In humans, do polymorphisms associated with reduced AHR binding affinity predispose to immune mediated diseases? Is the AHR a potential target for drug development that may aid in the treatment of many human diseases, especially in the areas of autoimmunity, allergy or transplant rejection? Answers to these questions will help to define the physiological role of the AHR beyond its current role as a mediator of xenobiotic metabolism.

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