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Title: Investigations of the AhR-induced Treg: Transcriptional Regulation and Suppressive Mechanisms

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Nancy I. Kerkvliet

The aryl hydrocarbon receptor (AhR) has recently been described as a novel therapeutic target, given the potent suppression of multiple immune-mediated diseases following activation by the prototypic ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In the parent-into-F1 graft-versus-host (GVH) model, suppression of the cytotoxic T-lymphocyte (CTL) response is associated with the presence of $CD25^{+}CTLA\text{-}4^{+}IL\text{-}10^{+}Foxp3^{neg}$ regulatory donor $CD4^{+}$ T-cells (AhR-Tregs). These AhR-Treg have a suppressive capacity greater than thymus-derived natural Treg. Therefore it is hypothesized that suppression of the CTL response is mediated by the AhR-Treg. The purpose of these studies was two-fold: 1) to identify mechanisms by which TCDD-activated AhR induces AhR-Tregs and 2) to determine the mechanism by which AhR-Tregs suppress the CTL response. To address the first aim, we hypothesized that the AhR, a transcription factor, may directly influence gene expression during $CD4^{+}$ T-cell activation and differentiation, thereby driving their differentiation into AhR-Treg. T-cell differentiation is dependent upon expression of certain transcription factors and lineage-specific genes for proteins and cytokines. Therefore, gene expression associated with $CD4^{+}$ T-cell activation and/or differentiation was evaluated in $CD4^{+}$ T-cells cultured under different polarizing conditions. Unexpectedly, treatment with TCDD did not alter lineage-specific genes that drive $CD4^{+}$ T-cell differentiation. Apart from the known AhR-regulated genes *Cyp1a1*, *Cyp1b1* and *Ahrr*, only expression of *Il22* was inappropriately up-

regulated across all conditions tested. The increase in *Il22* expression translated to increased IL-22 protein n. Of the 49 genes evaluated there were no other changes in gene expression consistent across polarizing conditions. This suggests that the role of AhR is superimposed upon the cytokine milieu, and therefore may have been obscured by the use of strongly polarizing cytokines. Ultimately, the results cast doubt on the use of *in vitro* conditions to recapitulate *in vivo* events, although the role of IL-22 in AhR biology remains of interest. To address the second part of my hypotheses, the suppressive mechanism(s) of the AhR-Treg were examined, focusing primarily on the increased expression of CTLA-4, CD25 and IL-10 seen on day 2 of the GVH response in previous studies. Expression of CTLA-4 induces the IFNγ - IDO tolerogenic pathway. Expression of CD25, the high-affinity subunit for the IL-2R is associated with regulatory function, through induction of tolerogenic cytokines and sequestration of IL-2. The immunosuppressive cytokine IL-10 is known to inhibit T-cell activation and expansion. Independent blockade of CTLA-4 and IDO function revealed that the AhR-Treg do not appear to use the IDO tolerogenic pathway to mediate suppression. Furthermore, antibody-mediated blockade of neither CD25 nor IL-10 were able to alleviate TCDD-mediated immunosuppression. During these studies we discovered that treatment with TCDD induced Foxp3, the transcription factor for Treg, in donor $CD4^+$ and $CD8^+$ T-cells 15 days after adoptive transfer. Interestingly, the up-regulation of Foxp3 was also seen in un-activated host cells, indicating non-specific regulation of Foxp3 by TCDD-activated AhR. Furthermore, concurrent blockade of CD25 and Foxp3 had no effect on TCDD's ability to suppress the GVH response. In sum, these studies suggest that the AhR-Treg is a novel regulatory cell, with characteristics of a Treg and increased IL-22 production. Regulatory T-cells have a wide repertoire of suppressive mechanisms, including production of TGFβ1 and TGFβ3, sequestration of IL-2 and granzyme B. Understanding the mechanism of suppression

utilized by the AhR-Treg is crucial to develop the AhR as a novel therapeutic target. Future studies will continue to evaluate the potential for the AhR-Treg to use alternative mechanisms to suppress T-dependent immune diseases.

Investigations of the AhR-induced Treg: Transcriptional Regulation and Suppressive Mechanisms

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APPROVED:

_____

Major Professor, representing Toxicology

_____

Head of the Department of Environmental and Molecular Toxicology

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Diana Rohlman, Author

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The various co-authors of the included chapters are listed in alphabetical order, in association with their contributions to the study.

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Sumit Punj	Technical support; critical review of manuscript
Linda B. Steppan	Technical support; critical review of experimental design
Zhen Yu	Technical support; data analysis

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Chapter 1

INTRODUCTION

The Aryl Hydrocarbon receptor

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor first identified in 1976 (Poland *et al*. 1976). The AhR was initially identified as a mediator of xenobiotic metabolism, activated by toxic chemicals such as 3-methylcholanthrene and benzo(a)pyrene (Okey, 2007). However, within the past 10 years, the aryl hydrocarbon receptor (AhR) has emerged as a novel therapeutic target for the treatment of T-cell-mediated diseases, to include autoimmune disease and transplant rejection. As a transcription factor, the AhR resides in the cytoplasm bound to heat shock protein 90 (Hsp90) (Perdew 1988), XAP2 (Meyer *et al.* 1998) and p23 (Kazlauskas *et al.* 1999) (Figure 1). Following activation, the AhR dissociates from these chaperone proteins and translocates to the nucleus, dimerizing with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Hoffman *et al.* 1991; Reyes *et al.* 1992). This complex is capable of binding to dioxin-responsive elements (DRE), and has been shown to influence transcription of AhR-regulated genes (Frericks *et al.* 2008; Kerkvliet 2009; Sun *et al.* 2004) like Cyp1a1, the quintessential marker of AhR activation (Okey *et al.* 1994). The most-studied and well-characterized ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is considered the prototypical AhR ligand. As such, TCDD has been used as a chemical probe to elucidate the role of the AhR in the immune system. Activation of AhR by TCDD results in potent suppression in multiple immune-mediated diseases, including type 1 diabetes, graft-versus-host disease (GVHD) and a murine model of multiple sclerosis (Marshall and Kerkvliet 2010).

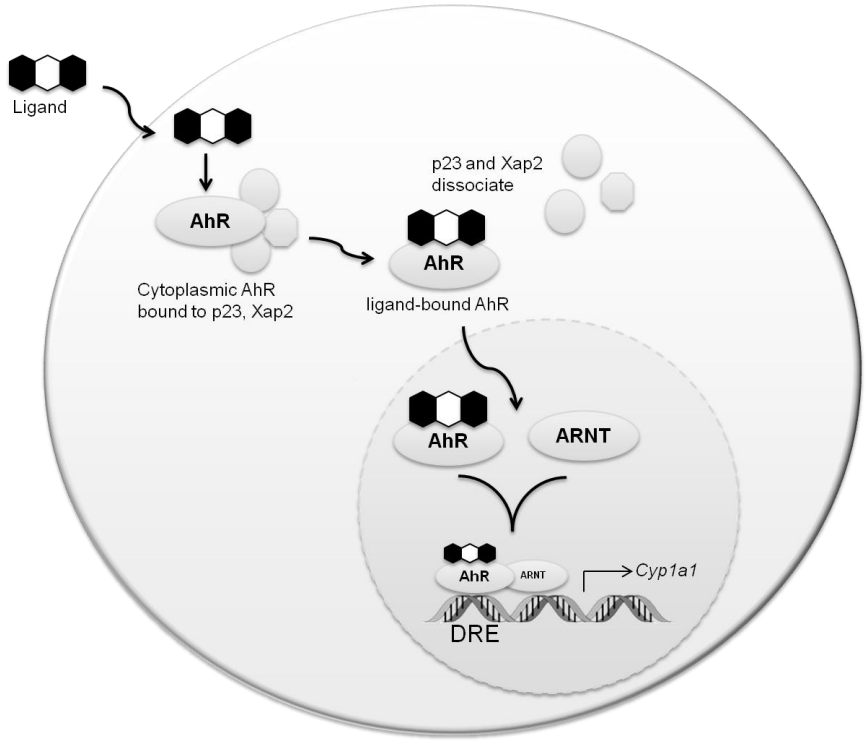

Figure 1 – 1. *Activation of the AhR by TCDD. In the inactivated state, the aryl hydrocarbon receptor (AhR) resides in the cytoplasm, bound to heat shock protein 90 (Hsp90), hepatitis B virus X-association protein (XAP2) and p23. Following TCDD-induced activation, AhR dissociates from these chaperone proteins and translocates to the nucleus, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). This complex is available to bind to dioxin-responsive elements (DRE) within the genome, resulting in transcription of target genes such as Cyp1a1.*

TCDD was never intentionally manufactured, but is a byproduct produced during the manufacturing of chlorinated phenols, such as 2,4,5-trichlorophenol (TCP). TCP contaminated with TCDD was then used to synthesize the herbicide 2,4,5-trichlorophenoxyacetic acid, which has been used as an herbicide (O'Neil 2006). Several other anthropogenic processes lead to the production of TCDD, including the chlorine bleaching of paper pulp and burning of plastics. Besides occupational exposure, humans are exposed to TCDD from medical and municipal waste incinerators and backyard burning (Huwe 2002). However, primary exposure

to the general public is through the diet, due to the bioaccumulation of TCDD in animal fat (Huwe 2002).

In 1997, the International Agency for Research on Cancer classified TCDD as a Group 1 human carcinogen (IARC 1997). Long-term rodent studies identified tumor formation and evidence of carcinogenicity following continuous treatment with TCDD (reviewed in Knerr and Schrenk 2006). However, the classification of TCDD as a human carcinogen has become fairly controversial, as epidemiological data from multiple TCDD-exposed human cohorts do not appear to support TCDD as a human carcinogen (Boffeta *et al.* 2011; Cole *et al.* 2003; Steenland *et al.* 2004).

Despite the controversy regarding TCDD as a human carcinogen, TCDD is known to induce such toxic effects as hepatotoxicity and chloracne in occupationally-exposed humans. Based on animal studies, TCDD toxicity also includes teratogenicity, fetotoxicity, impaired reproductive systems, endometriosis, and wasting syndrome (O'Neil 2006). Within the immune system, TCDD causes thymic involution and suppression of cell-mediated and humoral immunity (Kerkvliet 2002; Kerkvliet and Burleson 1994). Activation of AhR by TCDD suppresses multiple T-cell-mediated animal models of disease, such as graft-versus-host disease (GVHD) (Kerkvliet *et al.* 2002), allergic responses (Fujimaki *et al.* 2002; Luebke *et al.* 2001; Schulz *et al.* 2011), experimental autoimmune encephalomyelitis (EAE) (Quintana *et al.* 2008), colitis (Benson and Shepherd 2011; Takamura *et al.* 2010), experimental autoimmune uveoretinis (EAU) (Zhang *et al.* 2010) and Type 1 diabetes (Kerkvliet *et al.* 2009). However, while TCDD is unlikely to be used clinically, given its associated toxic side-effects, as it is not metabolized, TCDD can be used as a chemical probe to fully elucidate AhR-dependent mechanisms of immune suppression.

In addition to TCDD, many other putative AhR ligands have been described that include both exogenous and endogenous classes of compounds. For example, the carcinogenic compounds benzo(a)pyrene and 3-methylcholanthrene are known exogenous AhR ligands. However, the AhR is a promiscuous receptor, activated by ligands with widely varied structures (Figure 2). Surprisingly, several pharmaceutical drugs currently on the market and in development have been identified as AhR ligands, including the drugs leflunomide, omeprazole and VAF347 (Ettmayer *et al.* 2006; O'Donnell *et al.* 2010; Quattrochi and Tukey 1993), highlighting the potential for designing safe, therapeutic drugs targeting the AhR.

Recently there has been a concerted effort to identify endogenous AhR ligands for two reasons. Firstly, identification of an endogenous ligand would help elucidate the natural function of the AhR beyond that of a receptor involved in xenobiotic metabolism. Secondly, endogenous ligands could more easily be exploited as therapeutics. Research in this area has identified multiple putative endogenous ligands, the majority of which are tryptophan derivatives associated with immunosuppressive or regulatory function (Figure 2). For example, kynurenine induces AhR-dependent regulatory T-cells *in vitro*, while bilirubin suppresses EAE (Liu *et al.* 2008; Mezrich *et al.* 2010). FICZ (6-formylinodol[3,2-b]carbazole), a UV-metabolite of tryptophan, was initially found to exacerbate EAE (Mezrich *et al.* 2010; Quintana *et al.* 2008; Veldhoen *et al.* 2008) , yet recent studies have shown FICZ has a regulatory role in the gut (Qiu *et al.* 2012). Non tryptophan-derived ligands include lipoxin A_4, cyclic AMP (cAMP), indole-3-carbinol (I3C) and ITE (2-(1'***H***-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester) (Bjeldanes *et al.* 1991; Oesch-Bartlomowicz *et al.* 2005; Schaldach *et al.* 1999; Song *et al.* 2002). Of these, lipoxin A_4, I3C, and ITE exert immunosuppressive effects (Li *et al.* 2011; Machado *et al.* 2006; Quintana *et al.* 2010; Sodin-Semrl *et al.* 2000). In summary, the majority of endogenous AhR ligands are

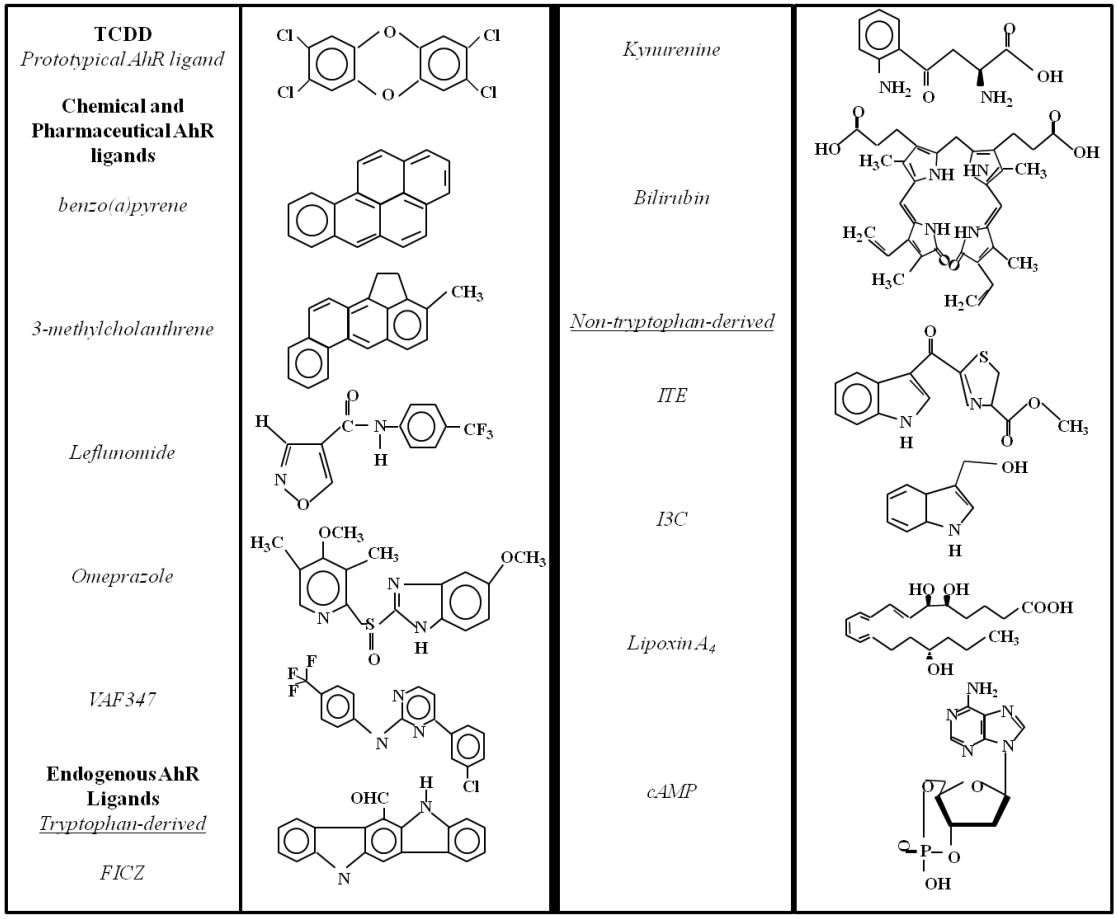

Figure 1 – 2. *Known exogenous and endogenous AhR ligands*

immunomodulatory, yet relative to most of the literature surrounding TCDD, they are poorly characterized. Furthermore, as TCDD is not metabolized, the observed biological effects can be attributed to the parent compound, not metabolites, thereby clearly elucidating the function of AhR in immunity.

Role of AhR in the immune system

Given the broad spectrum of immune diseases that are suppressed following treatment with TCDD, the AhR has been identified as a novel therapeutic target (Marshall and Kerkvliet 2010). Therefore, in an attempt to understand the underlying mechanisms behind AhR-mediated immunosuppression, the role of AhR in immune cell development, differentiation

and function has been heavily researched. The predominant cells of the immune system, the antigen presenting cells, natural killer cells, B-cells and T-cells have all been identified as potential cellular targets of AhR (Kerkvliet 2009) since AhR is present in nearly all cells of the immune system (Lawrence and Kerkvliet 2006). A summary of AhR-mediated effects in the differentiation and function of these cells is discussed below.

Macrophages, dendritic cells

Macrophages and dendritic cells are members of the innate immune system that present processed antigen to T-lymphocytes, thereby triggering an adaptive immune response. The role of AhR in both macrophage and dendritic cell differentiation and function has been a source of ongoing research. For example, activation of AhR by benzo(a)pyrene resulted in inhibition of macrophage differentiation, and increased apoptosis (van Grevenynghe *et al.* 2003; van Grevenynghe *et al.* 2004). In contrast, activation of AhR by TCDD had no effect on apoptosis, nor was the cytotoxic function of the macrophage altered, despite decreased cell numbers (Kimura *et al.* 2009; Mantovani *et al.* 1980; van Grevenynghe *et al.* 2004) indicating that the effect on macrophages is dependent upon the activating ligand, or upon metabolites of the ligand.

AhR-mediated effects on DCs have been characterized in an attempt to understand how DCs may contribute to TCDD-induced suppression of T-cell-mediated immune diseases. Initial studies reported that treatment with TCDD in mice resulted in the loss of DCs from the spleen, possibly due to apoptosis (Ruby *et al.* 2005; Ruby *et al.* 2002; Vorderstrasse and Kerkvliet 2001). However, *in vivo* treatment with TCDD had no effect on late or early apoptotic events (Bankoti *et al.* 2010a). Alternatively, there is evidence that DC travel inappropriately to the draining lymph nodes, based on the increased expression of CCR7, a migration marker, on DC (Bankoti *et al.* 2010a). Furthermore, TCDD-activated AhR appears

to alter the phenotype of DCs, inducing a phenotype reminiscent of a tolerogenic DC. The tolerogenic DC lineage is characterized by expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO), as well as increased expression of the regulatory markers TGFβ and IL-10 (Bankoti *et al.* 2010b; Mezrich *et al.* 2010; Vogel *et al.* 2008). Production of IDO by tolerogenic DCs has been shown to induce CD4+ T-cells to develop as regulatory cells, thereby suppressing proinflammatory responses (Chen *et al.* 2008). Therefore, it has been postulated that activation of AhR in DCs may skew T-cell differentiation toward a regulatory lineage (Mezrich *et al.* 2010; Nguyen *et al.* 2010). Clearly, activation of AhR in the DC leads to phenotypic and functional effects, yet the sum of these results remain unknown and are confounded by the potential for interactions with T-cells. Research in the area of DC-T-cell interactions is ongoing.

Natural Killer (NK) Cells

Natural killer cells, characterized by their cytotoxic function, are conventionally defined as $CD3^{neg}NK1.1^{+}Nkp46^{+}$ cells. Treatment with TCDD *in vivo* had no effect on NK cytotoxic function (Mantovani *et al.* 1980), although the frequency of NKp46 ($NK1.1^{neg}NKp46^{+}$) NK-like cells was diminished (Lee *et al.* 2012). More recently, AhR expression in NK cells was found to regulate production of IL-22, a member of the IL-10 family of cytokines, associated with tolerogenic function (Lee *et al.* 2012; Zenewicz and Flavell 2011). Thus, AhR appears to exert protective effects in the gut environment through production of IL-22 by $NKp46^{+}$ NK-like cells.

B-cells

B-cells differentiate into plasma cells that produce specialized immunoglobulin proteins in response to pathogens. Initial studies revealed that TCDD suppresses the number of antibody producing cells, as well as IgM production (Tucker *et al.* 1986; Vecchi *et al.*

1980). B-cell maturation also appeared to be impaired following treatment with TCDD (Dooley and Holsapple 1988; Luster *et al.* 1988; Sulentic *et al.* 1998; Thurmond *et al.* 2000). Further studies revealed that treatment with TCDD perturbs expression of BCL-6, Blimp-1 and Bach2, genes that are involved in B-cell activation and differentiation (De Abrew *et al.* 2011; North *et al.* 2009, 2010). In addition, TCDD down-regulates the μ gene, which encodes the heavy chain protein for IgM, which might result in the observed suppression of IgM (North *et al.* 2009). These results suggest that TCDD adversely affects B-cell differentiation, maturation and function. Although the consequences of these perturbations have not been widely studied, understanding how TCDD attenuates antibody production remains an important area of research.

T-cells ($CD4^+$ and $CD8^+$)

The $CD4^+$ and $CD8^+$ T-cell lineages comprise the T-lymphocyte population. Kerkvliet and colleagues demonstrated that $CD4^+$ T-cells were direct cellular targets of TCDD (Kerkvliet *et al.* 2002). In a P815 tumor allograft model there was a temporal overlap between administration of TCDD and $CD4^+$ T-cell differentiation and TCDD-mediated immunosuppression (Kerkvliet *et al*. 1996). Specifically, if TCDD was given four or more days after immune challenge, TCDD-mediated suppression was lost (Kerkvliet *et al.* 1996). However, treatment with TCDD within 0-3 days after immune challenge was sufficient to suppress the CTL response (Kerkvliet *et al*. 1996). This time period is coincident with the period of $CD4^+$ T-cell differentiation, a process that is required for the generation of the CTL response, as depletion of CD4+ T-cells prevented a CTL response (Kerkvliet *et al*. 1996). These experiments indicated that TCDD-induced alterations in differentiating $CD4^+$ T-cells were necessary for overall suppression (Kerkvliet *et al.* 1996). Furthermore, expression of

AhR within $CD4^+$ T-cells was required for the immunosuppressive effect of TCDD (Kerkvliet *et al.* 2002) as demonstrated in a parent-into-F1 graft-versus-host disease model (GVHD).

In the GVH model, donor cells from a C57Bl/6 ($H\text{-}2^{b/b}$) mouse are adoptively transferred into a B6D2F1 ($H\text{-}2^{b/d}$) recipient mouse, allowing the responding donor T-cells to be tracked by flow cytometric analysis. In TCDD-treated mice undergoing GVHD, phenotypic analysis of activated $CD4^+$ T-cells revealed the induction of $CD4^+$ T regulatory cells (Tregs) with a suppressive capacity greater than that of a natural Treg (Funatake *et al.* 2005; Marshall *et al.* 2008). Furthermore, induction of this unique $CD4^+$ T-cell leads to an altered $CD8^+$ phenotype with regulatory characteristics (Funatake *et al.* 2008). Similar Tregs have been identified in various other murine models following treatment with TCDD (Marshall and Kerkvliet 2010). As Tregs are capable of negatively regulating immune responses (Chatila 2005), it is plausible that TCDD-mediated immunosuppression is due to the induction of Tregs, thereby suppressing the CTL response. The role of AhR in $CD4^+$ T-cells, and specifically in suppressing T-cell-mediated diseases is also responsible for the focus on the AhR as a therapeutic target.

Role of AhR in T-cell-mediated diseases

Since the discovery of the Th1 and Th2 subsets in 1986 (Mosmann *et al*. 1986), several more $CD4^+$ T-cell subsets have been defined, including Th17, Th22, Tr1 and Tregs (Zhou *et al*. 2009). Each subset is defined by one or more transcription factors and associated cytokines. The transcription factors Tbet, Gata3, RORγ, AhR, cMAF, and Foxp3 are the master regulator genes for Th1, Th2, Th17, Th22, Tr1 and Tregs, respectively (Figure 3).

The cytokines associated with these subsets are IFNγ, IL-4/IL-5, IL-17 and IL-22, with IL-10 expressed by Th2, Tr1 and Treg cells. Several examples of countervailing

regulation exist, such as that between Th1 and Th2 cells, and between Th17 and Tregs (Zhou *et al.* 2008). Many T-cell mediated diseases such as autoimmune diseases, allergic responses and GVHD have been associated with specific $CD4^+$ T-cell subsets. Therefore, characterizing the role of AhR within the various T-cell subsets is necessary to understand the contribution of AhR to those diseases. Currently, it is unknown if TCDD-activated AhR prevents induction of proinflammatory T-cells, or skews the differentiation of said cells towards a regulatory phenotype.

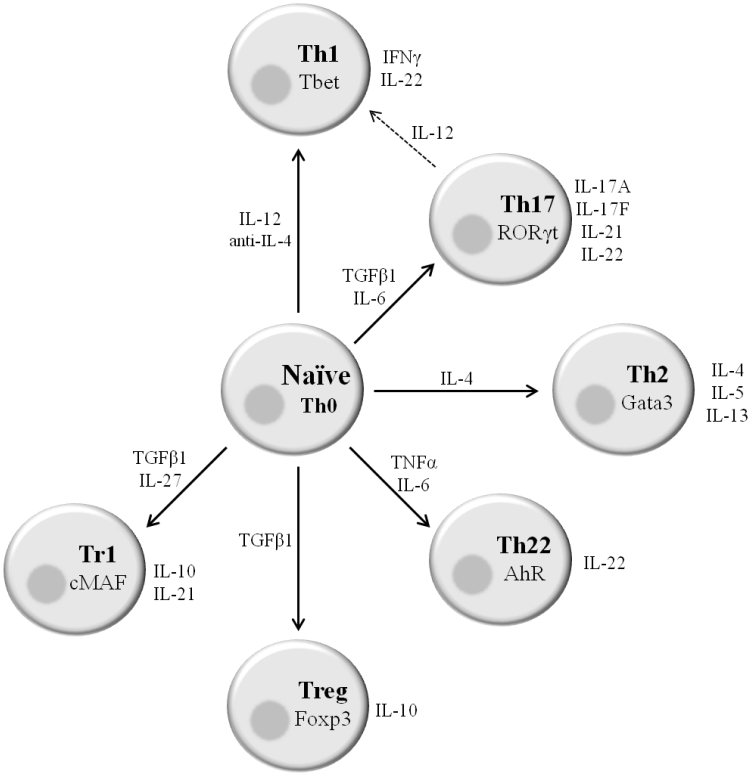

Figure 1 – 3. *Various differentiation pathways of $CD4^+$ T-cells. Th0 = naïve $CD4^+$ T-cell prior to exposure to polarizing cytokines (listed alongside differentiation arrows). All lineages are defined by expression of a specific transcription factor (listed below subset name), and production of characteristic cytokines (listed beside cell).*

Th1 and Th2

Th1 $CD4^+$ T-cells are necessary for the progression of GVHD, Type I diabetes (NOD model), and experimental autoimmune uveoretinitis (EAU), whereas allergic responses are mediated by Th2 cells. Treatment with TCDD severely attenuates the symptoms and mortality seen in these diseases, indicating that Th1 and Th2 cells are sensitive to TCDD-activated AhR (Fujimaki *et al.* 2002; Kerkvliet *et al.* 2002; Kerkvliet *et al.* 2009; Luebke *et al.* 2001; Schulz *et al.* 2011; Zhang *et al.* 2010). For example, in the GVHD model, activation of AhR suppresses the CTL response, while in the NOD model, insulitis and diabetes are averted following continuous treatment with TCDD (Kerkvliet *et al.* 1996; Kerkvliet *et al.* 2002). In Th2 models of house dust mite antigen, atopic dermatitis and peanut allergy, Ig-specific proteins were suppressed by TCDD (Luebke *et al.* 2001; Schulz *et al.* 2011), as were Th2-associated cytokines IL-4 and IL-5 (Fujimaki *et al.* 2002).

Th17

In 2005 a new proinflammatory T-cell subset was discovered, designated Th17 because of the production of IL-17 (Harrington *et al.* 2005). Th17 $CD4^+$ T-cells, often in concert with Th1 cells, mediate EAE and colitis, both immune diseases that are suppressed by TCDD (Benson and Shepherd 2011; Domingues *et al.* 2010; Feng *et al.* 2008; Quintana *et al.* 2008; Takamura *et al.* 2010). Analysis of TCDD-treated animals undergoing EAE found that production of both IFNγ and IL-17 was attenuated (Quintana *et al.* 2008). Furthermore, the percent of $CD4^+$IL-17^+ T-cells, the Th17 phenotype, was also attenuated in TCDD-treated mice (Quintana *et al.* 2008). These results are in direct contrast to results obtained with the UV tryptophan metabolite, FICZ. Treatment with FICZ in the EAE model significantly increased disease onset and symptom severity, while increasing IL-17 production and the expression of Th17 cells (Veldhoen *et al.* 2008). However, FICZ was contained in an

emulsion, preventing the very rapid metabolism known to occur (Wei *et al*. 2000), which may have altered the function of FICZ. Furthermore, a more recent study showed that treatment with FICZ actually protected mice against colitis (Monteleone *et al*. 2011). In sum, activation of AhR appears to suppress Th17-dependent diseases and attenuate Th17 T-cell differentiation.

Th22

In addition to Th1, Th2 and Th17 cells, recent findings have highlighted the role of AhR in the newly described Th22 lineage. The lineage is poorly understood, with both proinflammatory and regulatory functions documented. With AhR as the putative transcription factor, Th22 cells are defined by production of IL-22 (Trifari *et al.* 2009). The production of IL-22 is regulated by AhR, as AhR-deficient animals appear to have diminished levels of IL-22 protein, although transcript levels are unaltered (Qiu *et al.* 2012; Veldhoen *et al.* 2008; Rohlman *et al*. 2012). The role of IL-22 in Th22 cells is unclear, as is the function of Th22 cells. While Th22 cells have been implicated in psoriasis, their contribution is confounded by the continued presence of Th1 cells that also produce IL-22 (Schlaak *et al.* 1994; Yssel and Péne 2009). Currently the biological significance of Th22 cells remains unknown in mediating disease.

Treg

Tregs, which include natural and induced $CD4^{+}CD25^{+}Foxp3^{+}$, $CD4^{+}CD25^{+}Foxp3^{neg}$ and Tr1 regulatory cells are the natural counter to the proinflammatory Th1, Th2 and Th17 $CD4^{+}$ T-cells. The suppression induced by TCDD is reminiscent of the suppression induced by regulatory cells. First described in 2005, the TCDD-induced AhR-dependent $CD4^{+}$ Treg was identified, characterized by the phenotype $CD25^{high}$CTLA-$4^{+}$$GITR^{+}Foxp3^{neg}IL\text{-}10^{+}$ (Funatake *et al.* 2005; Marshall *et al.* 2008). Induction of this phenotype was followed by suppression of

the CTL response in the GVHD model. While initially described as $Foxp3^{neg}$, induction of a $Foxp3^{+}$ $CD4^{+}$ Treg has also been observed following activation of AhR by TCDD (Kerkvliet *et al.* 2009; Kimura *et al.* 2008; Quintana *et al.* 2008). For example, TCDD converted $CD4^{+}Foxp3^{neg}$ T-cells to $CD4^{+}Foxp3^{+}$ T-cells *in vitro* (Kimura *et al.* 2008), while *in vivo* the presence of TCDD-induced $CD4^{+}Foxp3^{+}$ T-cells correlated with the abrogation of symptoms in NOD, EAE and colitis models (Benson and Shepherd 2011; Kerkvliet *et al.* 2009; Quintana *et al.* 2008). Thus, TCDD-activated AhR appears to induce regulatory cells with differential Foxp3 expression, capable of suppressing multiple immune-mediated diseases.

Regardless of Foxp3 expression, TCDD-induced $CD4^{+}$ Tregs are $CD25^{+}$ (Funatake *et al.* 2005; Kerkvliet *et al.* 2009). Recently, the regulatory function of $CD25^{+}$ T-cells has been evaluated in the context of TCDD-activated AhR-mediated immunosuppression. Neutralization of CD25 partially relieved TCDD-mediated suppression in both EAU and a murine model of peanut allergy, suggesting that the TCDD-induced $CD4^{+}CD25^{+}$ Tregs mediate immune suppression (Schulz *et al.* 2011; Zhang *et al.* 2010). However, as the rescue was incomplete, expression of CD25 alone is not sufficient to explain the overall suppression seen following treatment with TCDD. Therefore, while $CD4^{+}$ T-cells may be the direct cellular target of AhR, other accessory cells, influenced by the $CD4^{+}CD25^{+}$ Treg, may be required to mediate suppression.

In addition to the role of AhR in Tregs, AhR is also essential for Tr1 differentiation and function. The transcription factor for Tr1 cells, cMAF, was found to physically associate with AhR (Apetoh *et al.* 2010). The physical association led to transactivation of IL-21 and IL-10 (Apetoh *et al.* 2010). Subsequent *in vivo* analysis revealed that TCDD-induced Tr1 cells were capable of ameliorating the symptoms of EAE, possibly via expression of granzyme B, an immunomodulatory protein capable of killing CTL (Apetoh *et al.* 2010; Gandhi *et al.*

2010). The regulation underlying the choice between an AhR-dependent Treg, or a Tr1 $CD4^+$ T-cell remains unknown, and suggests a secondary form of regulation between the two types of regulatory T-cells.

The sum of the existing research shows that AhR is active and functional in Th1, Th2, Th22, Th17, Treg and Tr1 $CD4^+$ T-cell subsets. Despite these findings, researchers have not been able to determine precisely how TCDD-activated AhR influences T-cell differentiation, nor has a direct link between the AhR-dependent Treg and subsequent TCDD-mediated suppression of the CTL response in GVHD been demonstrated.

Effects of TCDD-activated AhR on $CD4^+$ T-cell gene expression

The immunomodulatory role ascribed to AhR may be based upon transcriptional regulation of immune-related genes during T-cell differentiation. Surprisingly, there are a large number of immune-related genes that contain DRE (Frericks *et al.* 2008; Kerkvliet 2009; Sun *et al.* 2004). Thus, TCDD-activated AhR may bind directly to the DRE in target genes, thereby influencing T-cell differentiation. Furthermore, analysis of transcriptional targets offers the potential to identify signaling networks that may underlie the potent immunosuppression. Identification of these networks would be invaluable for determining the mechanism of AhR-mediated Treg induction as well as in the search for novel therapeutics that target AhR. A list of selected genes shown to be regulated by AhR is provided (Table 1).

Given that AhR is implicated in regulatory T-cell differentiation, a substantial amount of research has evaluated the transcriptional regulation of Treg-associated genes, such as *Foxp3*, *Cd25*, *Ctla4*, *Gzmb*, *Tgfb* and *Il10* (von Boehmer 2005). As stated before, TCDD-activated AhR induced Foxp3 at the level of protein (Kimura *et al.* 2008). However, it was recently discovered that AhR regulates expression of the murine and human Foxp3 gene by

binding directly to the Foxp3 promoter, resulting in increased transcript and protein (Gandhi *et al.* 2010; Quintana *et al.* 2008).

Analysis of gene expression in human $CD4^+$ T-cells following treatment with TCDD identified increased expression of *TGFb1, GZMB* and *IL10* (Gandhi *et al.* 2010). Similarly, expression of *Tgfb1, Cd25, Ctla4* and *Il10* are up-regulated in donor $CD4^+$ T-cells isolated from TCDD-treated host mice undergoing GVHD or EAE (Apetoh *et al.* 2010; Marshall *et al.* 2008). As shown in Table 1-1, genes related to the Tr1 lineage, *Maf, Il10* and *Il21*, have also been identified as either targets of AhR or potential AhR binding partners.

With the clear involvement of AhR in regulatory $CD4^+$ T-cells, it is unsurprising that activation of AhR by TCDD down-regulates genes associated with the pro-inflammatory Th17 and Th1 lineages. In human $CD4^+$ T-cells, the Th17-associated genes *RORC, IL17A* and *IL17F* are significantly down-regulated by TCDD-activated AhR (Gandhi *et al.* 2010). Likewise, the Th1-associated genes *TBX21* and *IFNA1*, the gene encoding IFNγ, are also down-regulated in human $CD4^+$ T-cells following treatment with TCDD (Gandhi *et al.* 2010). Given these results, it is hypothesized that AhR regulation of T-cell differentiation begins at the level of transcription.

While the above lineages have well-defined functions within the immune system, the newly identified Th22 lineage is poorly understood, despite the AhR-dependent induction of IL-22, which has recently garnered much interest. IL-22 is produced by Th1 and Th22 cells as well as innate lymphoid cells (ILC) (Qiu *et al.* 2012; Ramirez *et al.* 2010; Rutz *et al.* 2011; Trifari *et al.* 2009). In ILCs, AhR binds to the Il22 locus, facilitated by the Th17 transcription factor, Rorc (Qiu *et al.* 2012). In the liver, treatment with TCDD also increases IL-22 transcript (Fullerton *et al.* 2012).

Gene	Species-specific Regulation	Associated $CD4^+$ T-cell lineage	Regulation/Function	Reference
TBX21	Human	Th1	In $CD4^+$ T-cells co-cultured with TCDD + TGFβ1, expression of *TBX21* was down-regulated	Gandhi et al, 2010
Ifng/IFNA1	Mouse Human	Th1	Down-regulated in naïve $CD4^+$ human T-cells cultured with TCDD ± TGFβ1	Gandhi et al, 2010
Foxp3	Mouse Zebrafish Human	Treg	AhR binds the *Foxp3* promoter TCDD positively regulates Foxp3 gene expression *in vivo* and *in vitro*	Quintana et al, 2008 Gandhi et al, 2010
IL2	Human	Treg	Naïve $CD4^+$ human T-cells treated with TCDD down-regulated *IL2*.	Gandhi et al, 2010
TGFB1	Human	Treg	Up-regulated in $CD4^+$ T-cells co-cultured with TGFβ1 + TCDD.	Gandhi et al, 2010
Gzmb/GZMB	Mouse Human	Treg	Up-regulated in TCDD-treated $CD4^+$ T-cells and *in vivo*	Gandhi et al, 2010 Marshall et al, 2008
Ctla4	Mouse	Treg	In GVHD, TCDD-treated host animals exhibited a 2.3-fold increase in *Ctla4* expression	Marshall et al, 2008
Cd25	Mouse	Treg	In GVHD, TCDD-treated host animals exhibited a 1.9-fold increase in *CD25* expression	Marshall et al, 2008
Il10	Mouse Human	Treg, Tr1	Up-regulated by TCDD under Tr1 polarizing conditions. Transactivated by the physical association of AhR and cMAF	Marshall et al, 2008 Apetoh et al, 2010 Gandhi et al, 2010
MAF	Mouse Human	Tr1	Physically interacts with AhR to transactivate *Il10* and *Il2*.	Apetoh et al, 2010 Gandhi et al, 2010
Il21	Mouse	Tr1	Transactivated by the physical association of AhR and cMAF	Apetoh et al, 2010 Gandhi et al, 2010
Rorc	Mouse Human	Th17	Expression of RORC decreased in TCDD-treated human $CD4^+$ T-cells RORγt facilitates AhR binding to the *Il22* locus	Gandhi et al, 2010 Qui et al, 2012
Il17a/Il17f	Mouse Human	Th17	Down-regulated in human $CD4^+$ T-cells treated with TCDD	Gandhi et al, 2010
Il22	Mouse Human	Th22, Th17, Th1	Activation of AhR by TCDD results in the up-regulation of *Il22* in the liver and in innate like lymphocytes (ILT) RORγt facilitates AhR binding to the *Il22* locus in ILT	Fullerton et al, 2012 Qui et al, 2012

***Table 1-1**. List of selected AhR-regulated genes in human and mouse associated with $CD4^+$ T-cell differentiation.*

These data suggest that the observed increase in IL-22 protein in Th22 cells is due to transcriptional control of the IL-22 gene. The functional effect of increased IL-22 following TCDD-mediated activation of AhR remains an active area of research.

In sum, AhR exerts significant effects on gene expression under Th1, Treg, Tr1, Th17 and Th22 $CD4^+$ T-cell differentiation. Continued research into AhR regulation of gene expression may help clarify the mechanism by which AhR suppresses such a diverse range of immune responses. The AhR is a promising therapeutic target, but there is still a great deal that must be determined before new drugs can be developed that exploit the AhR. Ultimately, the role of the AhR in the induction of a suppressive Treg must be identified, and the induction of this Treg must be directly linked to the subsequent suppression of disease symptoms, as evidenced in models of GVHD, EAE, colitis and others.

Hypothesis and Objectives

Hypothesis

Activation of the AhR by TCDD induces a unique T-regulatory $CD4^+$ T-cell phenotype that is necessary to suppress symptoms of disease in the GVH model.

First objective

Identify a potential mechanism by which AhR induces a T-regulatory phenotype in $CD4^+$ T-cells by examining AhR-mediated transcriptional perturbations in $CD4^+$ T-cells cultured under Th1, Th17, Treg or Tr1 polarizing conditions.

Second objective

Explore the functional requirement of AhR-Treg-associated phenotypic markers (CD25, CTLA-4, IL-10, Foxp3 and IFNγ) for their contribution to the suppression of the cytotoxic T-lymphocyte response in GVHD.

Chapter 2

Aryl hydrocarbon receptor (AhR)-mediated perturbations in gene expression during early stages of $CD4^+$ T-cell differentiation

Diana Rohlman

Duy Pham

Zhen Yu

Linda B. Steppan

Nancy I. Kerkvliet

Frontiers in Immunology

Science Park PSE-D

CH – 1015 Lausanne

Switzerland

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Abstract: Activation of the aryl hydrocarbon receptor (AhR) by its prototypic ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), mediates potent suppression of T-cell dependent immune responses. The suppressive effects of TCDD occur early during $CD4^+$ T-cell differentiation in the absence of effects on proliferation and have recently been associated with the induction of AhR-dependent regulatory T-cells (Treg). Since AhR functions as a ligand-activated transcription factor, changes in gene expression induced by TCDD during the early stages of $CD4^+$ T-cell differentiation are likely to reflect fundamental mechanisms of AhR action. A custom panel of genes associated with T-cell differentiation was used to query changes in gene expression induced by exposure to 1 nM TCDD. $CD4^+$ T-cells from $AhR^{+/+}$ and $AhR^{-/-}$ mice were cultured with cytokines known to polarize the differentiation of T-cells to various effector lineages. Treatment with TCDD induced expression of *Cyp1a1, Cyp1b1* and *Ahrr* in $CD4^+$ T-cells from $AhR^{+/+}$ mice under all culture conditions, validating the presence and activation of AhR in these cells. The highest levels of AhR activation occurred under Th17 conditions at 24 hours and Tr1 conditions at 48 hours. Unexpectedly, expression levels of most genes associated with early T-cell differentiation were unaltered by AhR activation, including lineage-specific genes that drive $CD4^+$ T-cell polarization. The major exception was AhR-dependent up-regulation of *Il22* that was seen under all culture conditions. Independent of TCDD, AhR down-regulated the expression of *Il17a* and *Rorc* based on increased expression of these genes in AhR-deficient cells across culture conditions. These findings are consistent with a role for AhR in down-regulation of inflammatory immune responses and implicate IL-22 as a potential contributor to the immunosuppressive effects of TCDD.

Introduction

Activation of the transcription factor, aryl hydrocarbon receptor (AhR), by the prototypic ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to exert potent immunosuppressive effects in a variety of T-cell dependent disease models. TCDD suppresses Th1-dependent autoimmune diseases such as Type 1 diabetes in the non-obese diabetic (NOD) mouse (Kerkvliet *et al.* 2009) and experimental autoimmune uveoretinitis (Zhang *et al.* 2010). TCDD also suppresses the Th17-mediated response in both experimental autoimmune encephalomyelitis (EAE) and colitis (Benson and Shepherd 2011; Quintana *et al.* 2008). These potent immunosuppressive effects are also conserved in non-autoimmune disease models. The Th1-driven cytotoxic T-lymphocyte (CTL) and alloantibody responses to allogeneic P815 tumor cells are dose-dependently suppressed by TCDD treatment (Kerkvliet *et al.* 1996), as are CTL and antibody responses to influenza virus infection (Warren *et al.* 2000). Treatment with TCDD also suppresses Th2-dependent cytokine and OVA-specific antibody production (Nohara *et al.* 2002; Shepherd *et al.* 2000), as well as several Th2-dependent allergic diseases including atopic dermatitis (Fujimaki *et al.* 2002), dust mite-induced asthma (Luebke *et al.* 2001) and peanut allergy (Schulz *et al.* 2011).

One of the mechanisms by which TCDD suppresses adaptive immunity occurs via activation of AhR in differentiating $CD4^+$ T-cells. This was demonstrated using an acute parent-into-F1 graft-versus-host (GVH) model wherein the suppression of donor CTL development was contingent upon the donor $CD4^+$ T-cells expressing AhR (Kerkvliet *et al.* 2002). Furthermore, optimal suppression of the CTL response occurred when TCDD was administered within three days of alloantigen stimulation – the time during which donor $CD4^+$ T-cells undergo differentiation (Kerkvliet *et al.* 1996). Phenotypic and functional analysis of the differentiating allospecific donor cells suggested that AhR activation was driving the

development of an immunosuppressive regulatory T-cell (Treg), characterized by a $CD4^{+}CD25^{high}Foxp3^{neg}CTLA\text{-}4^{+}CD62L^{low}IL\text{-}10^{+}$ phenotype (Funatake *et al.* 2005; Marshall *et al.* 2008). Subsequently, TCDD has also been shown to induce $Foxp3^{+}$ Tregs and Tr1 cells *in vitro* (Apetoh *et al.* 2010; Gandhi *et al.* 2010) and to increase the frequency of $Foxp3^{+}$ $CD4^{+}$ T-cells *in vivo* in several models of immune-mediated disease (Benson and Shepherd 2011; Kerkvliet *et al.* 2009; Quintana *et al.* 2008; Schulz *et al.* 2011; Singh *et al.* 2011; Takamura *et al.* 2010; Zhang *et al.* 2010).

Based on its role as a transcription factor, activation of AhR in $CD4^{+}$ T-cells may directly alter $CD4^{+}$ T-cell differentiation by influencing gene expression during early differentiation events. The likelihood of such effects is high given the large number of immune-related genes that contain dioxin response elements (DRE) (Frericks *et al.* 2008; Kerkvliet 2009; Sun *et al.* 2004). In the present studies, we characterized the influence of TCDD-activated AhR on gene expression during $CD4^{+}$ T-cell differentiation under Th0, Th1, Treg, Tr1 and Th17 polarizing conditions. We utilized a custom panel of 48 genes that have been associated with AhR activation, T-cell differentiation and/or Treg induction (Table 1). $CD4^{+}$ T-cells were obtained from $AhR^{+/+}$ and AhR-deficient ($AhR^{-/-}$) mice, allowing us to validate the AhR-dependence of TCDD's effects. In addition, differences in gene expression between vehicle-treated cultures of $AhR^{+/+}$ and $AhR^{-/-}$ $CD4^{+}$ T-cells identified genes that are regulated by AhR during T-cell activation in the absence of an exogenous ligand.

Gene symbol	Gene name	RefSeq		Gene symbol	Gene name	RefSeq
A2br	Adora2b	NM_007413		*Il12b*	IL-12b	NM_008352.2
Actb	Actb	NM_007393.3		*Il12rb2*	IL-12Rβ2	NM_008354.1
Ahr	AhR	NM_013464.4		*Il17a*	IL-17A	NM_010552
Ahrr	AhRR	NM_009644		*Il-21*	IL-21	NM_021782.2
Bach2	Bach2	NM_007521		*Il-21r*	IL-21R	NM_021887.1
Cd27	CD27	NM_001033126.2		*Il22*	IL-22	NM_016971.1
Cd40lg	Tnfsf5	NM_011616.2		*Il27ra*	IL-27Rα	NM_016671.2
Cd69	CD69	NM_001033122		*Il2ra*	IL-2Rα/CD25	NM_008367.2
Ctla4	CTLA4	NM_009843.3		*Maf*	cMAF	NM_001025577.2
Cyp1a1	Cyp1a1	NM_009992.2		*Nfatc2*	Nfatc2	NM_010899.2
Cyp1b1	Cyb1b1	NM_009994.1		*Nfe2l2*	Nfe212	NM_010902
Entpd1	Entpd1/CD39	NM_009848		*Pdcd1*	PD-1	NM_008798.2
Fasl	Tnfsf6	NM_010177.3		*Prdm1*	Prdm1	NM_007548
Foxp3	Foxp3	NM_054039.1		*Rorc*	RORγ	NM_011281.1
Gata3	Gata3	NM_008091.3		*Socs3*	Socs3	NM_007707.2
Gzmb	Gzmb	NM_013542		*Stat4*	Stat4	NM_011487
Hmox1	Hmox1	NM_010442.1		*Tbx21*	Tbx21/Tbet	NM_019507.1
Icos	ICOS	NM_017480.1		*Tgfb1*	TGFβ1	NM_011577.1
Ifng	IFNγ	NM_008337.1		*Tgfb3*	TGFβ3	NM_009368.1
Il2	IL-2	NM_008366.2		*Tgfbr1*	TGFβR1	NM_009370.2
Il4	IL-4	NM_021283.1		*Tgfbr2*	TGFβR2	NM_009371.2
Il6	IL-6	NM_031168.1		*Tnf*	TNFα	NM_013693.1
Il10	IL-10	NM_010548.1		*Tnfrsf4*	Tnfrsf4/OX40	NM_011659
Il10ra	IL-10Ra	NM_008348.2				
Il12a	IL-12a	NM_008351.1				

Table 2-1. *Panel of genes used to evaluate AhR regulation of gene expression in $CD4^+$ T-cells. Genes were chosen based on association with T-cell activation and differentiation, or genes that have been previously identified to be responsive to TCDD in vivo.*

Materials and Methods

Animals - B6.PL-Thy1a/CyJ mice (Thy1.1$^{+/+}$, AhR$^{+/+}$) and B6.129-AhR^{tm1Bra}/J (Thy1.1$^{+/+}$, AhR$^{-/-}$) mice were bred and maintained under specific pathogen-free conditions at the Laboratory Animal Resource Center at Oregon State University (Corvallis, OR). All animal procedures were approved by the Institutional Animal Care and Use Committee.

CD4^{+} T-cell cultures - Spleens were aseptically removed and processed into single-cell suspensions via dissociation between the frosted ends of microscope slides. Red blood cells and dead cells were removed by hypotonic water lysis. CD4^{+} T-cells were isolated by negative selection using a CD4^{+} T-cell isolation kit and an autoMACS separator (Miltenyi Biotec; Auburn, CA). T-cells were cultured in RPMI 1640 media (Invitrogen; Carlsbad, CA), supplemented with 10% fetal bovine serum (HyClone; Logan, UT), 10mM HEPES (Invitrogen), 50µg/ml gentamicin (Invitrogen) and 50 µM 2-β-mercaptoethanol (Sigma; St. Louis, MO). At the time of culture initiation, cells were treated with 1 nM TCDD (dissolved in DMSO) or 0.001% DMSO (vehicle). The 1 nM concentration of TCDD used in these studies was sufficient to induce maximum activation of AhR in T-cells as reflected in expression of known AhR-regulated genes *Cyp1a1*, *Cyp1b1* and *Ahrr* (unpublished data).

CD4^{+} T-cells (1x10^{6} cells/well) were activated with soluble anti-CD3 (0.5 µg/ml; eBioscience) and anti-CD28 (2.5 µg/ml; eBioscience) or plate-bound anti-CD3 (2 µg/ml) and anti-CD28 (2 µg/ml) in a 24-well plate. For Th1 conditions, anti-IL-4 (10 µg/ml) and IL-12 (3 ng/ml) was added to each well. For Treg polarizing conditions, TGFβ1 (3 ng/ml) was added. In addition to TGFβ1, IL-27 (25ng/ml) or IL-6 (15ng/ml) was added for Tr1 or Th17 polarizing conditions, respectively. All reagents for T-cell polarization were purchased from eBioscience. T-cells cultured under Th0 conditions received no exogenous cytokines.

For some genes (*Il22*, *Il17a*, *Rorc*), protein levels were also measured. Whole splenocytes or purified CFSE-labeled $CD4^+$ T-cells were cultured under Th17 conditions. After 48 hours, cells were treated for 6 hours with a Cell Stimulation Cocktail (eBioscience) containing phorbol 12-myristate 13-acetate, ionomycin, Brefeldin A and monensin. The cells were then stained for intracellular protein prior to flow cytometric analysis as described below. Supernatants were collected for ELISA analysis.

RNA isolation and qPCR – $CD4^+$ T-cells were harvested at 24 and 48 hours and flash frozen in liquid nitrogen. Cell pellets were stored at -80°C. RNA was isolated using the RNeasy Mini Kit #74104 (Qiagen; Valencia, CA), with on-column DNase digestion (Qiagen #79254) performed twice to ensure complete elimination of genomic DNA. RNA concentrations and purity were determined using the NanoDrop ND-1000 UV-Vis Spectrophotometer (ThermoScientific). The ReactionReady First Strand cDNA synthesis kit #C-01/C-03 (SA Biosciences, formerly SuperArray; Frederick, MD) was used to synthesize cDNA in a two-step PCR reaction. A minimum of 200ng RNA was used for all reactions. Following reverse transcription, samples were stored at -80°C.

An ABI PRISM 7500 Real-Time PCR system (Applied Biosystems) was used for all qPCR reactions. SYBR Green/ROX qPCR Master Mix (SA Bioscience) was used to prepare a 25µl reaction mix consisting of 12.5µl master mix, cDNA equivalent to 2ng RNA, 1µl primer, and RNase-free H_20 to obtain the final volume. All primers were obtained from SA Biosciences. Sequence accession numbers are shown in Table 1. The qPCR settings were as follows: 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, followed by 1 minute at 60°C. This was followed by a standard dissociation step of 15 seconds at 95°C to generate a melting curve. A no-reverse transcription (NRT) control was included to validate the absence of genomic DNA from all samples.

qPCR Data Analysis – Data were analyzed using ABI 7500 analysis software. Genes that were not detected within 40 cycles received a value of 40. Cycle numbers were then normalized to *Actb* to calculate ΔCt. The data were analyzed as either 1/ΔCt or 1/ΔCt X 100; all other data were presented as fold change. Fold changes were calculated by the following formulas:

$\Delta Ct_{experimental} - \Delta Ct_{control} = \Delta\Delta Ct$

$2^{(-\Delta\Delta Ct)}$ = fold change

When analyzing gene expression across polarizing conditions, fold changes were calculated relative to Th0 as follows:

$\Delta Ct_{(Th1;\ Th17;\ Treg;\ Tr1)} - \Delta Ct_{Th0} = \Delta\Delta Ct$

$2^{(-\Delta\Delta Ct)}$ = fold change

Flow cytometric analysis – The following antibodies were purchased from eBioscience: CD4-PE (GK1.5), IL-22-PE (1H8PWSR), IL-17A-APC (eBio17B7) and RORγ-PE (AFKJS). CD4-APC (GK1.5) and carboxyfluorescein succinimidyl ester (CFSE) were purchased from Invitrogen. Splenocytes or purified CFSE-labeled $CD4^+$ T-cells were resuspended in PAB (PBS, 1% BSA, 0.1% sodium azide), and incubated with PE- or APC-labeled anti-CD4 antibody for ten minutes. After two washes, cells were resuspended in Fixable Viability Dye efluor 780 (eBioscience) for 30 minutes. The BD Cytofix/Cytoperm buffer set or the Foxp3 Staining Buffer set (eBioscience) was used for intracellular staining. Control samples that contained all of the antibodies except the one of interest (fluorescence minus one) were used as negative staining controls. A minimum of 150,000 viable $CD4^+$ T-cells were collected per sample on a Beckman Coulter FC-500 flow cytometer. Compensation and analysis of data were performed in WinList (Verity Software, Version 6.0).

ELISA – Supernatants from purified $CD4^+$ T-cells and splenocyte cultures were collected after 48 hours. IL-22 production was quantified using the IL-22 Ready-Set-Go® ELISA kit from eBioscience, according to manufacturer's instructions.

Statistical analysis – All treatment groups consisted of a minimum of three biological replicates (two mice per replicate), except where indicated. Each biological sample was treated with vehicle or TCDD in separate wells. Differences due to TCDD treatment were determined using a paired Student's t-test. For comparisons between $AhR^{+/+}$ and $AhR^{-/-}$ $CD4^+$ T-cells, or between culture conditions, a non-paired Student's t-test was used. Values were considered statistically significant at $p \leq 0.05$, in conjunction with a fold change of ≥ 2 between treatment groups. For *Il22* analysis across treatments, a linear mixed model was utilized using the Mixed procedure of SAS (v. 9.2).

Results

AhR is present and transcriptionally active during early differentiation of CD4$^+$ T-cells

Ahr message was detected in CD4$^+$ T-cells cultured under all conditions.

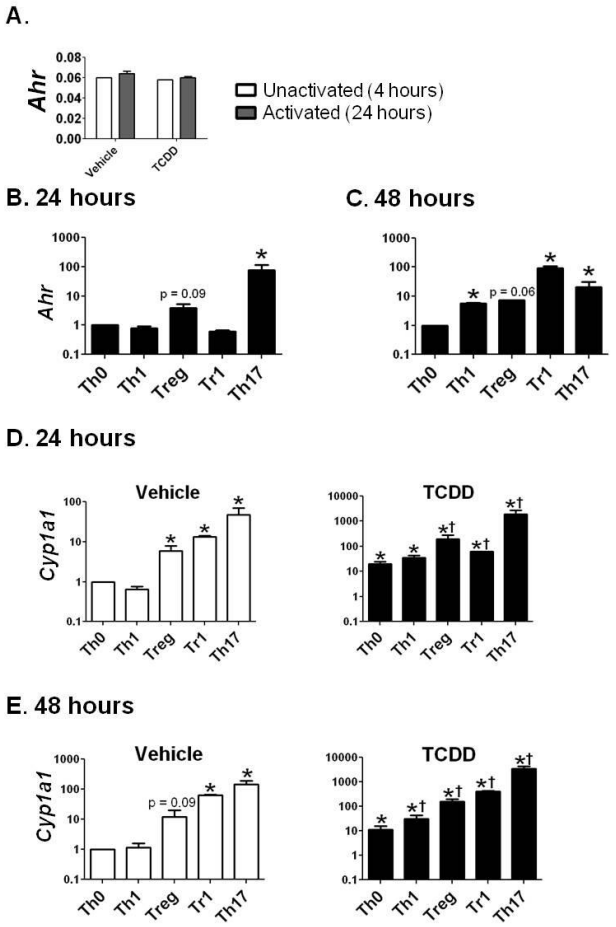

Figure 2-1. *Expression of Ahr and Cyp1a1 in CD4$^+$ T-cells cultured under various conditions. (A) Basal expression of Ahr (1/ΔCt) in CD4$^+$ T-cells cultured for 4 hours without stimulation or 24 hours following stimulation with anti-CD3/anti-CD28. (B, C) Ahr expression in CD4$^+$ T-cells cultured for 24 or 48 hours with polarizing cytokines as described in Materials and Methods. Data are presented as fold change relative to Th0 vehicle conditions at 24 or 48 hours. (D, E) Expression of Cyp1a1 in vehicle- and TCDD-treated CD4$^+$ T-cells cultured for 24 or 48 hours under different polarizing conditions. Data are presented as fold change relative to the vehicle-treated Th0 condition at 24 or 48 hours. *$p \leq 0.05$ relative to either vehicle or TCDD Th0 control within that time point. †$p \leq 0.05$ relative to the TCDD-treated Th0 condition at 24 or 48 hours. Error bars indicate the mean ± SEM; n = 3-4 biological replicates.*

Cells activated for 24 hours in the absence of exogenous cytokines (Th0 conditions) expressed *Ahr* at a level comparable to that of non-activated cells, indicating that expression of *Ahr* is not up-regulated due to T-cell receptor activation alone (Figure 2-1 A). Likewise, neither Th1 nor Tr1 polarizing conditions produced a significant increase in *Ahr* expression. In contrast, Treg conditions induced a 4-fold increase and Th17 conditions induced a 78-fold increase in *Ahr* expression at 24 hours (Figure 2-1 B).

At 48 hours, *Ahr* expression under Th0 conditions was equivalent to the level of expression seen at 24 hours, while expression was up-regulated by 5.6-fold under Th1 conditions and 7.4-fold under Treg polarizing conditions (Figure 2-1 C). Expression of *Ahr* declined under Th17 conditions but remained significantly up-regulated (21-fold) compared to Th0 conditions. *Ahr* expression was highest at 48 hours under Tr1 conditions with a fold-change of 92 relative to Th0 conditions.

	24 hours			**48 hours**		
	Average ΔCt‡		Fold change	Average ΔCt		Fold Change
	VEH	TCDD	TCDD/VEH	VEH	TCDD	TCDD/VEH
Th0	11.2	12.4	-2.3†	12.2	13.4	-2.4†
Th1	12.0	11.2	1.7	10.7	11.1	-1.3
Treg	9.8	10.8	-2.0*	8.0	9.4	-2.8†
Tr1	12.3	12.8	-1.5	6.7	6.9	-1.2
Th17	4.9	5.3	-1.4	9.0	9.9	-2.0
‡Average cycle number of 3-4 biological replicates, corrected to β-actin. †NSD; *$p \leq 0.05$						

***Table 2-2**. Influence of TCDD on Ahr expression in CD4$^+$ T-cells cultured under different polarizing conditions.*

To determine if the level of *Ahr* message present under different culture conditions was indicative of AhR protein, the ability of TCDD to increase expression of known AhR-

regulated genes was examined. Addition of TCDD *per se* did not influence *Ahr* expression except for a small increase that was noted under Treg conditions at 24 hours (Table 2-2). In contrast, addition of TCDD significantly up-regulated *Cyp1a1* expression under all conditions tested (Figure 2-2 D, E), with good correlation between the levels of expression of *Cyp1a1* and *Ahr* at both 24 and 48 hours (Figure 2). A similar correlation was observed between expression levels of *Ahr* and expression levels of the AhR-regulated genes *Cyp1b1* and *Ahrr* (Figure 2-2). These results indicate that sufficient AhR is expressed in $CD4^+$ T-cells cultured under any condition to be able to respond to 1 nM TCDD and alter expression of AhR-regulated genes.

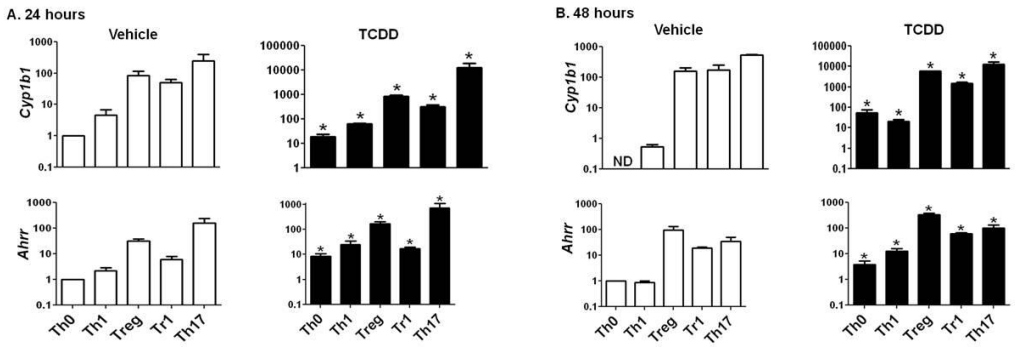

***Figure 2-2**. Expression of Cyp1b1 and Ahrr in $AhR^{+/+}$ $CD4^+$ T-cells treated with vehicle or TCDD. Data are presented as fold change calculated relative to Th0 vehicle at (A) 24 or (B) 48 hours. Expression of Cyp1b1 at 48 hours in Th0 vehicle was greater than 40 cycles; a value of 40 was used to calculate fold changes. Error bars represent the mean ± SEM; n = 3-4 biological replicates. * $p \leq 0.05$ relative to the vehicle control. ND = not detected.*

While TCDD induced relatively high levels of *Cyp1a1*, low levels of *Cyp1a1* were also seen in the absence of an exogenous ligand in $CD4^+$ T-cells cultured under Treg, Tr1 and Th17 polarizing conditions at both 24 and 48 hours (Figure 2-1 D, E). These results suggest that the cytokine milieu either contains or is capable of inducing the formation of an AhR ligand in the responding T-cells. Interestingly very low but detectable *Cyp1a1* expression was

also seen in AhR-deficient cells especially at 48 hours under all polarizing conditions (Figure 2-3). Addition of TCDD did not up-regulate this expression, validating the absence of functional AhR protein in $AhR^{-/-}$ cells (Figure 2-3), but implicating an AhR-independent pathway for *Cyp1a1* expression.

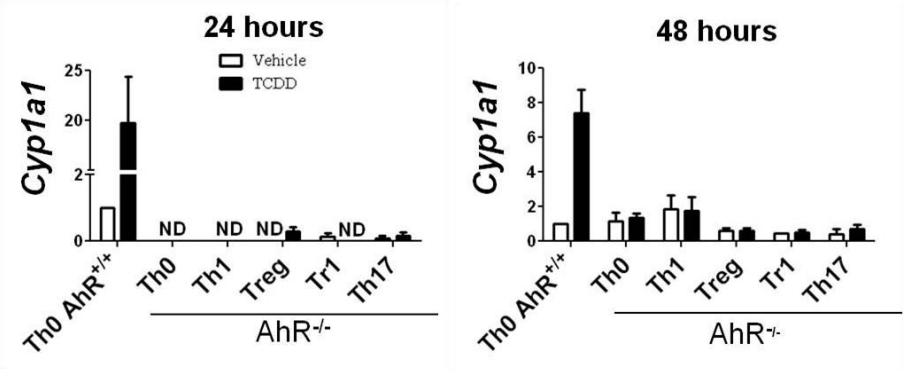

Figure 2-3. *Expression of Cyp1a1 in $AhR^{-/-}$ $CD4^+$ T-cells. Data are presented as fold change relative to $AhR^{+/+}$ $CD4^+$ T-cells cultured under vehicle-treated Th0 conditions for (A) 24 hours or (B) 48 hours. Error bars indicate the mean ± SEM; n = 3-4 biological replicates, except for 24 hour $AhR^{-/-}$ Treg and Th17 conditions, and 48 hour $AhR^{-/-}$ Treg and Tr1 conditions, where n = 2. *$p \leq 0.05$; ND = not detected within 40 cycles.*

Effect of AhR activation by TCDD on expression of genes involved in $CD4^+$ T-cell differentiation

The pattern of gene expression observed under the various polarizing conditions was consistent with early differentiation events specific to different $CD4^+$ T-cell subsets (Figure 2-4) (Hermann-Kleiter and Baier 2010; Zhou *et al.* 2009). Under Th1 polarizing conditions, the transcription factor *Tbet* was up-regulated at 48 hours, and the associated cytokine *Ifng* was up-regulated at 24 and 48 hours when compared to expression levels in non-polarized (Th0) cells. The up-regulation of these Th1-associated genes was concurrent with the down-regulation of *Gata3*, also consistent with Th1 differentiation. Similarly, the transcription factor

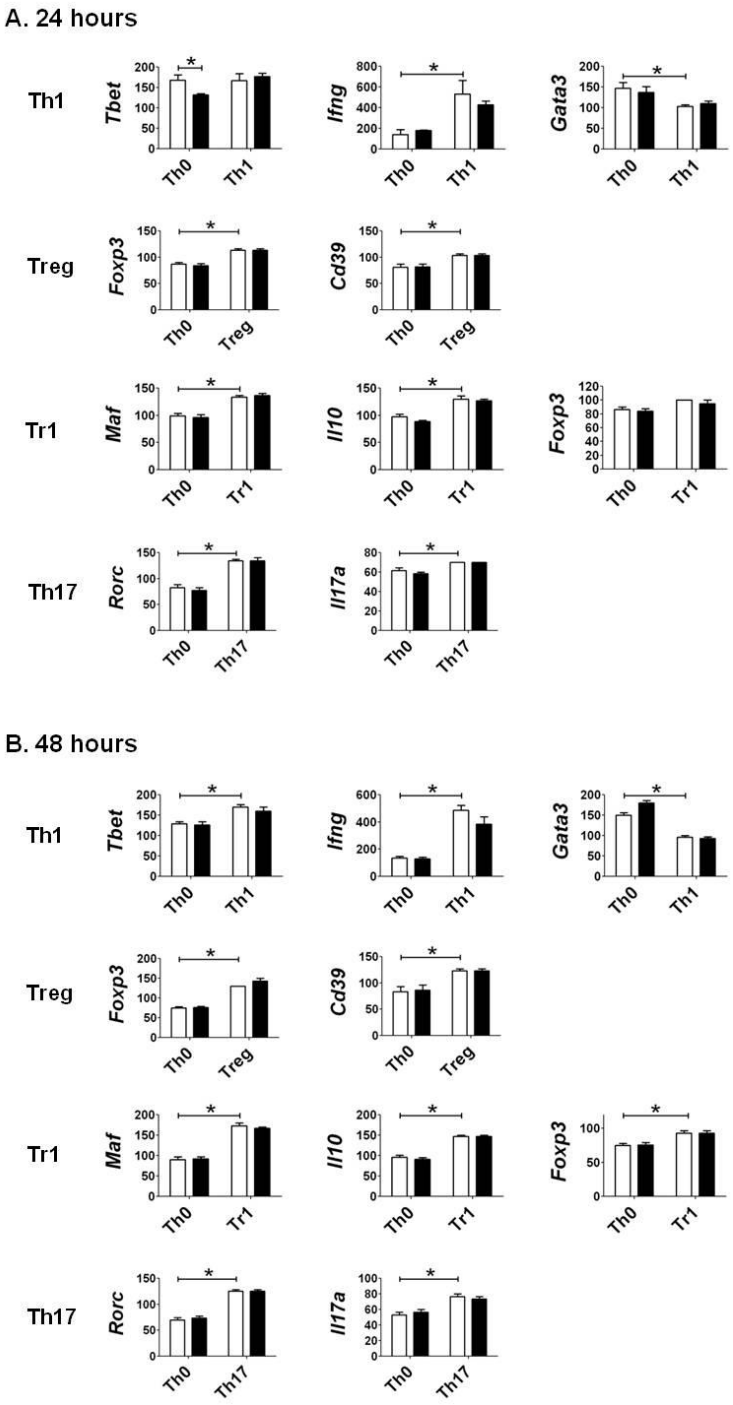

Figure 2-4. *Expression of genes associated with specific polarization in CD4+ T-cells treated with vehicle (white bar) or TCDD (black bar) for (A) 24 or (B) 48 hours. Data are presented as 1/ΔCt X 100. Gene expression under Th0 conditions (no polarizing cytokines) is presented for comparison with gene expression under specific polarizing conditions as described in Materials and Methods. Error bars represent the mean ± SEM; n = 3-4 biological replicates. *p ≤ 0.05.*

for T-regulatory cells, *Foxp3* and the associated Treg marker *Cd39* were up-regulated under Treg polarizing conditions, while Tr1 polarizing conditions induced the expression of *Maf* and *Il10*. *Foxp3* expression was also up-regulated under Tr1 polarizing conditions at 48 hours, albeit to a lesser extent than that seen under Treg polarizing conditions. As expected Th17 polarizing conditions produced increased expression of *Rorc* and *Il17* at 24 and 48 hours.

Surprisingly, activation of AhR by TCDD did not significantly alter expression of any of the master regulator genes associated with $CD4^+$ T-cell polarization or other genes associated with polarization (Figure 2-4). In fact, expression of only one gene in the panel of genes tested, *Il22*, was clearly altered by treatment with TCDD, and this effect was seen under all conditions except Tr1 (Table 2-3).

Polarizing condition	**$AhR^{+/+}$ TCDD/VEH**		**$AhR^{-/-}$ TCDD/VEH**	
	24 hours	48 hours	24 hours	48 hours
	2.5	3.8	1.3	-1.8
Th1	2.3	13.7	-1.4	3.1
Treg	2.2	6.4	1.5	1.0
Tr1	-1.1	1.7	-1.9	1.4
Th17	4.6	17.6	-1.1	-1.2
TCDD effect (p-value) excluding Tr1 conditions	0.03	<0.001	0.88	0.82

Table 2-3. *Influence of TCDD on Il22 expression in $AhR^{+/+}$ and $AhR^{-/-}$ $CD4^+$ T-cells activated under different polarizing conditions. Data are presented as fold change in TCDD-treated cells relative to vehicle-treated controls at 24 or 48 hours. Data represent 3-4 biological replicates, except for $AhR^{-/-}$ cells at 24-hour Treg and Th17 and 48-hour Treg conditions, where n = 2. Data were analyzed for significant TCDD-dependent effects across culture conditions, excluding Tr1.*

When analyzed over all conditions except Tr1, expression of *Il22* was significantly increased in the presence of TCDD at both 24 ($p = 0.03$) and 48 hours ($p < 0.001$). The level of *Il22*

expression was highest under Th1 conditions in vehicle-treated cells, and addition of TCDD further increased expression (Figure 2-5), while the greatest induction of *Il22* expression by TCDD (17.6-fold) occurred under Th17 conditions at 48 hours (Table 3). The minimal expression of *Il22* under Tr1 conditions was surprising given the high level of *Ahr* expressed in these cells at 48 hours and suggested that the presence of IL-27 as a polarizing cytokine inhibits *Il22* transcription. In fact, Rutz *et al*. (2011) recently showed that the induction of cMaf by IL-27 results in downstream repression of *Il22*. Analysis of $AhR^{-/-}$ $CD4^+$ T-cells showed no significant increase in *Il22* expression upon treatment with TCDD at either 24 or 48 hours under any polarizing condition, verifying that the up-regulation of *Il22* by TCDD was dependent on AhR activation (Table 2-3).

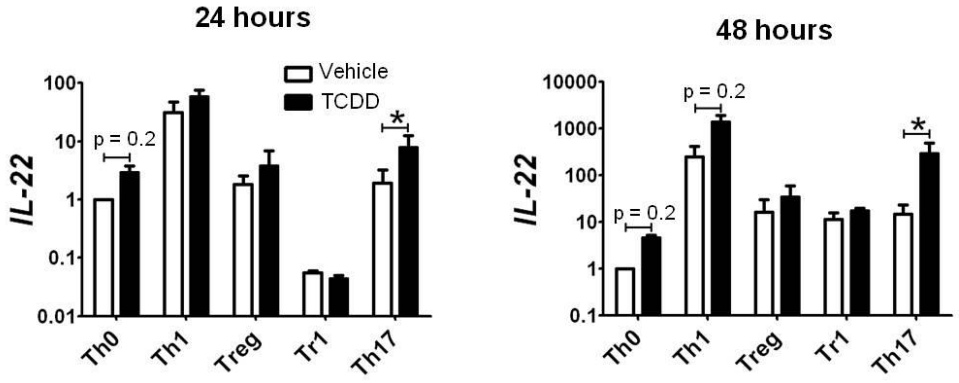

Figure 2-5. *Influence of TCDD on Il22 expression under various polarizing conditions. Data are presented as fold change relative to expression in Th0 vehicle controls at 24 or 48 hours. Error bars represent the mean ± SEM; n = 3-4 biological replicates. *p ≤ 0.05.*

To determine if AhR-mediated regulation of *Il22* was conserved at the level of protein, purified $CD4^+$ T-cells or whole splenocytes were cultured with or without TCDD for 48 hours under Th17 polarizing conditions. IL-22 was measured in $CD4^+$ T-cells and in culture supernatants. As shown in Figure 2-6A, a small population of $CD4^+$ T-cells was found to express IL-22 in vehicle-treated cultures. Addition of TCDD increased the percentage of $IL\text{-}22^+$ cells ($p = 0.06$) and the amount of IL-22 produced on a per cell basis ($p = 0.08$) as

measured by the median fluorescence intensity (MFI). IL-22 was also detected in supernatants from cultures of purified $CD4^+$ T-cells. TCDD-treated samples had an average of 28 pg/ml compared to <8 pg/ml (the reporting limit) in vehicle-treated cultures (Figure 2-6 B). Likewise, when whole splenocytes were cultured, treatment with TCDD increased the frequency of $IL\text{-}22^+$ $CD4^+$ T-cells ($p = 0.05$) and the MFI ($p < 0.001$) (Figure 5C).

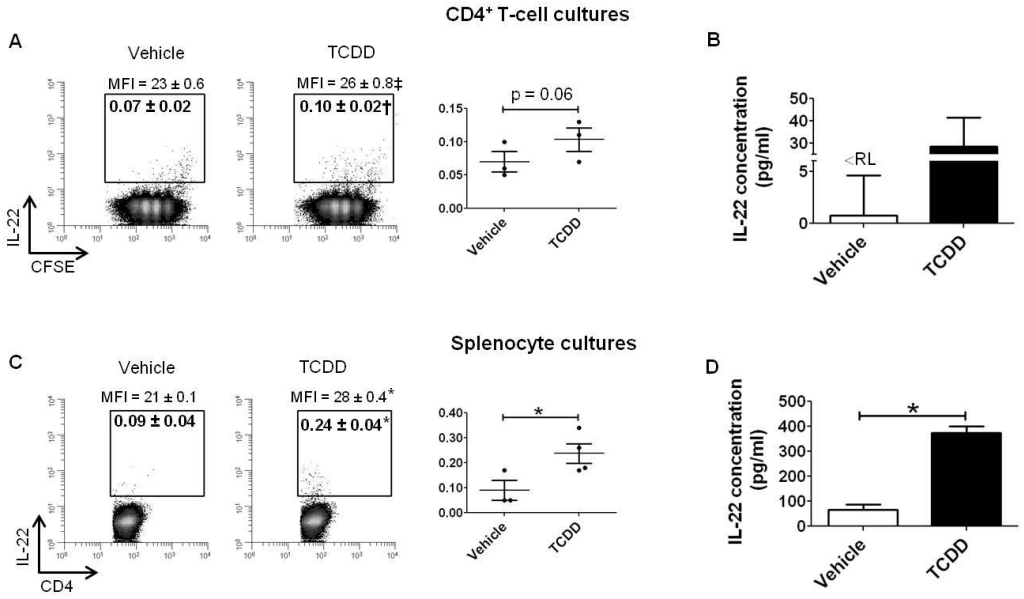

***Figure 2-6**. Influence of TCDD on IL-22 protein in $CD4^+$ T-cells cultured under Th17 polarizing conditions for 48 hours. Data shown are gated on viable $CD4^+$ T-cells. (A) $CD4^+$ T-cells were purified and labeled with CFSE prior to activation. MFI = median fluorescence intensity. Individual biological replicates are represented graphically. Histograms shown are representative of 3-4 biological replicates. (B) Concentration of IL-22 in supernatant from purified $CD4^+$ T-cell cultures. <RL = below reporting limit. (C) Whole spleen cell suspensions were activated and cultured under Th17 polarizing conditions, as in A. (D) Concentration of IL-22 in supernatant from whole splenocyte cultures. Error bars represent the mean ± SEM; n = 3-4 biological replicates. †$p = 0.06$; ‡$p = 0.08$; *$p \leq 0.05$.*

Significantly more IL-22 was found in supernatants from whole splenocytes consistent with a previous report (Alam *et al*., 2010). Treatment with TCDD significantly increased the amount of IL-22 present ($p = <0.001$) from 65 pg/ml in vehicle-treated cultures

to 374 pg/ml in TCDD-treated cultures (Figure 2-6 D).Other statistically significant changes in gene expression induced by TCDD in differentiating $CD4^+$ T-cells are summarized in Table 2-4. Increased expression of *Ctla4* (2.3-fold), *Cd40lg* (2.2-fold) and *Il12rb2* (2.9-fold) was associated with TCDD treatment at 24 hours under Th1 conditions. *Il12rb2* was also increased (7.1-fold) by TCDD under Th17 conditions at 48 hours. Under Treg conditions, only *Stat4* expression was altered by TCDD and it was down-regulated (-2.7-fold), while no changes were seen under Tr1 conditions. Under Th0 conditions, TCDD significantly suppressed expression of *Tbet* (2.7-fold) and *Il17a* (2.1-fold).

Polarizing condition	**24 hours** TCDD/Vehicle			**48 hours** TCDD/Vehicle		
	Gene	Fold change	p-value	Gene	Fold change	p-value
Th0	*Il17a* *Tbet*	-2.1 -2.7	0.00 0.05	No significant changes		
Th1	*Il12rb2* *Ctla4* *Cd40lg*	2.9 2.3 2.2	0.06 0.02 0.02	No significant changes		
Treg	No significant changes			*Stat4*	-2.7	0.01
Tr1	No significant changes			No significant changes		
Th17	No significant changes			*Il12rb2*	7.1	0.03

Table 2-4*. Summary of changes in expression of immune related genes induced by TCDD under different polarizing conditions. Data are presented as fold change in TCDD-treated cells relative to vehicle-treated controls at 24 or 48 hours. None of the changes shown were observed in $AhR^{-/-}$ $CD4^+$ T-cells exposed to TCDD. Data represent 3-4 biological replicates.*

Influence of AhR deficiency on gene expression in activated $CD4^+$ T-cells

Significant changes in gene expression were observed between vehicle-treated $AhR^{+/+}$ and $AhR^{-/-}$ $CD4^+$ T-cells cultured under different polarizing conditions (Figure 2-7). The most

significant effect of AhR deficiency was increased expression of *Il17a* at both 24 and 48 hours under all culture conditions (Figure 2-7A). The greatest up-regulation of *Il17a* was noted under Treg conditions, with a 53-fold increase, whereas 16- and 4-fold changes were observed under Th17 and Tr1 conditions, respectively, at 24 hours, with a similar trend for Th1 and Th0 conditions. At 48 hours, *Il17a* expression was significantly up-regulated in $AhR^{-/-}$ $CD4^{+}$ T-cells under Th1, Treg, and Tr1 conditions.

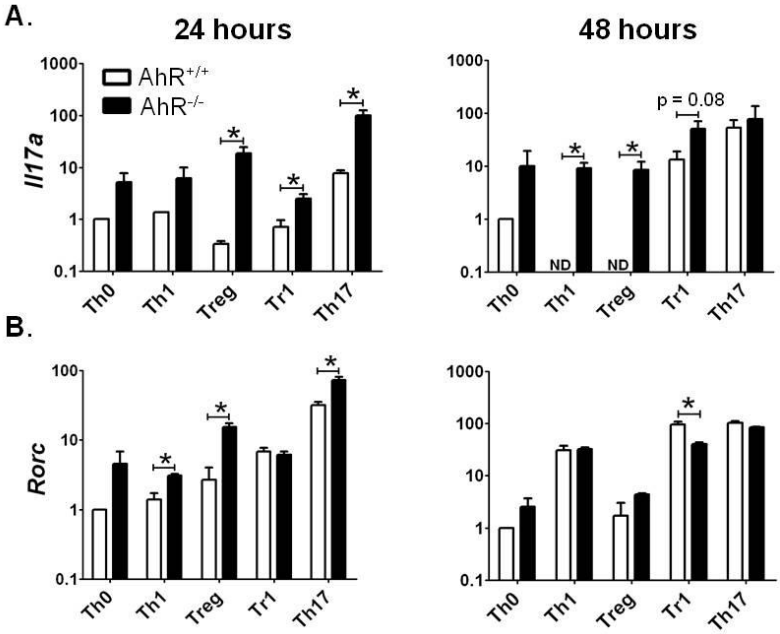

***Figure 2-7**. Influence of AhR deficiency on expression of (A) Il17a or (B) Rorc in $AhR^{+/+}$ or $AhR^{-/-}$ $CD4^{+}$ T-cells cultured under different polarizing conditions for 24 or 48 hours. Data are expressed as fold change relative to $AhR^{+/+}$ T-cells cultured under vehicle-treated Th0 conditions. Error bars represent the mean ± SEM; n = 3 biological replicates, except for 24 or 48 hour $AhR^{-/-}$ Treg conditions, and 24 hour Th17 conditions, where n = 2. *p ≤ 0.05. ND = not detected within 40 cycles.*

AhR deficiency also resulted in increased expression of *Rorc*, the master transcription factor for Th17 differentiation (Figure 2-7B). At 24 hours, $AhR^{-/-}$ T-cells expressed a 7-fold increase in *Rorc* under Treg conditions, and a 2-fold increase under Th1 and Th17 conditions. *Rorc* expression was also increased in $AhR^{-/-}$ cells under Th0 conditions (p = 0.06), while

expression was unaltered under Tr1 polarizing conditions. AhR regulation of *Rorc* was no longer seen at 48 hours, with the exception of Tr1 conditions, where *Rorc* was down-regulated 2-fold in $AhR^{-/-}$ $CD4^{+}$ T-cells.

To determine if the increase in expression of *Il17a* and *Rorc* was observed at the protein level, IL-17A and RORγ were measured by flow cytometry in cells cultured under Th17 conditions for 48 hours. As shown in Figure 2-8A, 1.9% of the $AhR^{+/+}$ $CD4^{+}$ T-cells expressed IL-17A compared to 1.0% of the $AhR^{-/-}$ $CD4^{+}$ T-cells ($p = 0.05$). MFI values were also lower in $AhR^{-/-}$ cells ($p = 0.1$).

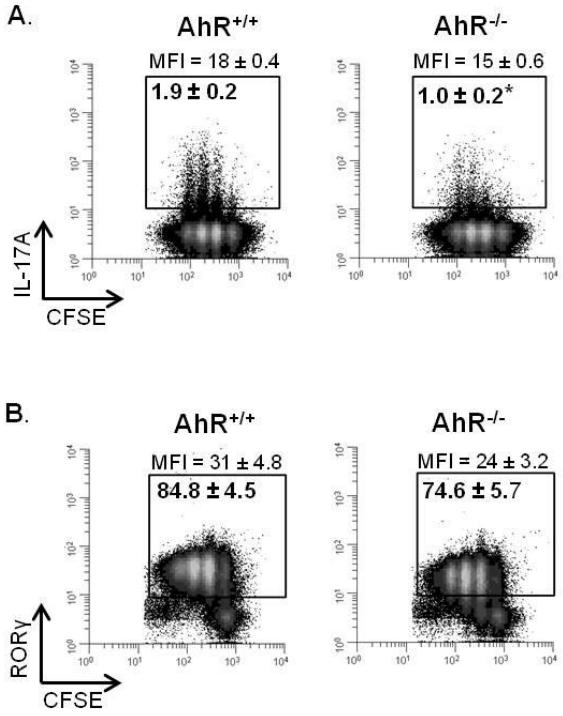

***Figure 2-8**. Influence of AhR deficiency on (A) IL-17A or (B) RORγ protein in $AhR^{+/+}$ or $AhR^{-/-}$ $CD4^{+}$ T-cells cultured under Th17 polarizing conditions for 48 hours. Data shown are gated on viable $CD4^{+}$ T-cells. MFI = median fluorescence intensity. Histograms shown are representative of 3-4 biological replicates. *$p \leq 0.05$. Changes in RORγ expression were not statistically significant.*

Similarly, a smaller percentage of $AhR^{-/-}$ $CD4^{+}$ T-cells stained positive for RORγ compared to $AhR^{+/+}$ $CD4^{+}$ T-cells, but the changes were not statistically significant ($p = 0.2$)

(Figure 2-8B). The basis for the lack of correlation between gene and protein expression is not known but may be related to the transient nature of the up-regulation of *Il17a* and *Rorc* expression seen in AhR-deficient cells. Under Th17 conditions, this effect was most pronounced at 24 hours but gone by 48 hours. As expected, there was no effect of AhR status on $CD4^+$ T-cell proliferation, assessed by CFSE dilution.

AhR deficiency had no significant effect on expression of *Il22* or *Foxp3* under any of the polarizing conditions tested (Figure 2-9). *Tgfb3* and *Gzmb* were up-regulated under Treg and Th0 conditions and *Il6* was up-regulated under Treg and Tr1 conditions in AhR-deficient cells, while other changes were unique to specific culture conditions (Table 5).

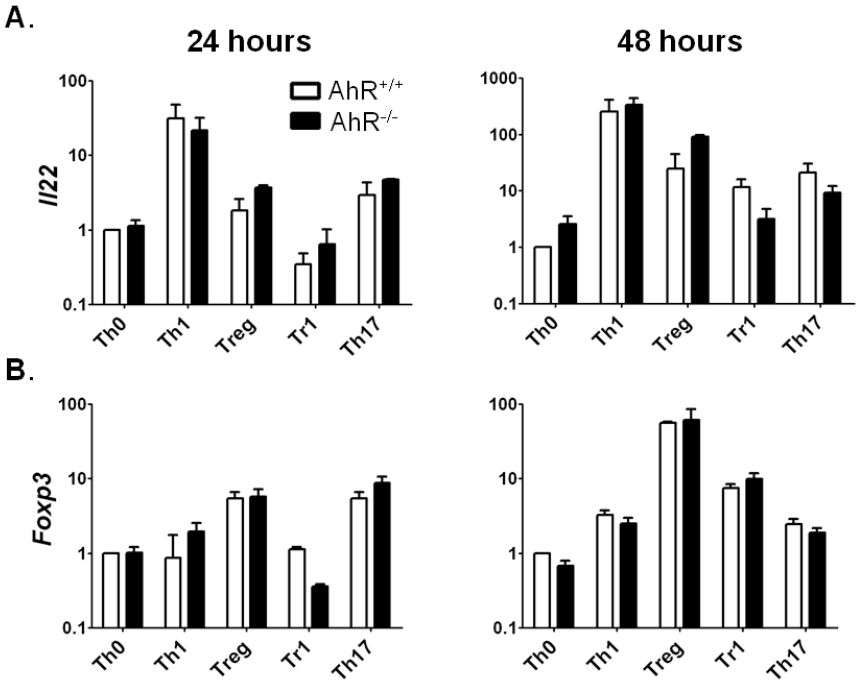

***Figure 2-9**. Expression of Il22 and Foxp3 in $AhR^{+/+}$ and $AhR^{-/-}$ $CD4^+$ T-cells cultured under different polarizing conditions. Data are presented as fold change calculated relative to $AhR^{+/+}$ T-cells cultured under vehicle-treated Th0 conditions. Expression of (A) Il22 and (B) Foxp3 in $AhR^{+/+}$ and $AhR^{-/-}$ cells at 24 and 48 hours. Error bars represent the mean ± SEM; n = 3 biological replicates except for 24 and 48 hour $AhR^{-/-}$ Treg conditions, and 24 hour Th17 polarizing conditions, where n = 2. *p ≤ 0.05.*

In general, the results suggest that AhR deficiency in $CD4^+$ T-cells favors increased expression of genes associated with inflammation, consistent with the pro-inflammatory phenotype of AhR-deficient mice.

Polarizing condition	**24 hours** $AhR^{-/-}$ / $AhR^{+/+}$			**48 hours** $AhR^{-/-}$ / $AhR^{+/+}$		
	Gene	Fold change	p-value	Gene	Fold change	p-value
Th0	*Nfatc2* *Cd27*	2.9 -2.2	0.04 0.05	*Tgfb3* *Gzmb* *Cd69*	6.0 2.4 -2.1	0.01 0.02 0.02
Th1	*Ox40* *Cd25*	2.6 2.3	0.01 0.05	No significant changes		
Treg	*Il2*	6.0	0.02	*Ifng* *Gzmb* *Tgfb3* *Il6* *Cd25* *Cd69* *Stat4*	3.9 3.6 3.1 3.0 2.1 -3.0 -5.8	0.04 0.01 0.05 0.05 0.05 0.02 0.03
Tr1	No significant changes			*Il6* *Tgfbr1* *Adora2b*	3.4 2.1 2.2	0.04 0.01 0.00
Th17	No significant changes			*Il2*	-5.0	0.01

Table 2 -5. *Summary of changes in immune-related genes related to AhR deficiency in CD4+ T-cells cultured under different polarizing conditions. Data are presented as fold change in $AhR^{-/-}$ $CD4^+$ T-cells relative to $AhR^{+/+}$ $CD4^+$ T-cells at 24 or 48 hours. Data represent 3-4 biological replicates, except for $AhR^{-/-}$ cells at 24-hour Treg and Th17 and 48-hour Treg conditions where n = 2.*

Discussion

The AhR is a ligand-activated transcription factor that has been shown to play a role in $CD4^+$ T-cell differentiation, yet little is known about the changes in gene expression that occur in these cells following AhR activation. Previous studies have examined gene expression in donor $CD4^+$ T-cells isolated from the spleen of TCDD-treated mice during a GVH response, however the identification of direct gene targets was confounded by the presence of contaminating host cells that also express AhR (Marshall *et al.* 2008). Identification of gene targets has become more important as recent reports indicate that there may be ligand-specific effects of AhR activation on $CD4^+$ T-cell differentiation (Quintana *et al.* 2008; Veldhoen *et al.* 2008). Furthermore, the initial report of highly increased AhR expression in $CD4^+$ T-cells cultured under Th17 conditions (Kimura *et al.* 2008; Veldhoen *et al.* 2008) led to an early misconception that AhR was functional only in Th17 cells in the mouse (Ho and Steinman 2008; Ramirez *et al.* 2010; Stockinger 2009; Veldhoen *et al.* 2008). However, more recently, AhR was shown to be significantly up-regulated in mouse and human $CD4^+$ T-cells under Tr1 conditions as well (Apetoh *et al.* 2010; Gandhi *et al.* 2010). Given that TCDD suppresses Th1-, Th2- and Th17-mediated responses *in vivo*, it implicates a role for AhR in the differentiation of many T-cell subsets. The present studies were designed to determine if genes involved in $CD4^+$ T-cell differentiation are selectively regulated by AhR when the cells are activated in the presence of TCDD under a variety of Th-polarizing culture conditions.

The results of our studies show that *Ahr* is expressed in $CD4^+$ T-cells under all conditions tested, including un-activated cells, while elevated levels of *Ahr* are seen under Treg, Tr1 and Th17 conditions. The highest level of *Ahr* expression was seen under Th17 conditions at 24 hours and Tr1 conditions at 48 hours. Functional AhR protein was also

present under all culture conditions based on the ability of TCDD to induce the expression of known AhR-regulated genes (*Cyp1a1*, *Cyp1b1* and *Ahrr*). The level of induced gene expression was generally proportional to the amount of *Ahr* present with the highest induction seen under Th17 conditions. Since many putative endogenous AhR ligands have anti-inflammatory functions, it suggests that up-regulation of AhR may be a protective response during highly inflammatory Th17 conditions, allowing low-affinity endogenous AhR ligands to down-regulate the response, consistent with the effects seen with TCDD. Examples of low-affinity ligands with immunosuppressive activity include lipoxin A_4, bilirubin, and kynurenine (Liu *et al.* 2008; Mezrich *et al.* 2010; Schaldach *et al.* 1999; Sinal and Bend 1997). Indeed, bilirubin and kynurenine have been shown to suppress EAE and induce $CD4^+Foxp3^+$ T-cells, respectively (Liu *et al.* 2008; Mezrich *et al.* 2010). Elevated levels of AhR in $CD4^+$ T-cells undergoing Tr1 and Treg differentiation would also facilitate AhR signaling to promote the development of these anti-inflammatory cells.

TCDD did not appear to alter early polarization of activated $CD4^+$ T-cells despite strong activation of AhR. Both vehicle- and TCDD-treated cells showed the expected increase in expression of *Tbet*, *Foxp3*, *Maf* and *Rorc* under Th1, Treg, Tr1 and Th17 polarizing conditions, respectively. Likewise, the expression of other genes that are associated with specific polarizing conditions was unaffected by TCDD, including *Ifng*, *Cd39*, *Il10* and *Il17a*. The lack of effect of TCDD on *Foxp3* expression was somewhat surprising since TCDD has been previously reported to directly regulate Foxp3 expression *in vitro* and increase the generation of $Foxp3^+$ cells (Kimura *et al.* 2008; Quintana *et al.* 2008). However, the lack of effect of TCDD on *Foxp3* expression is consistent with the $Foxp3^{neg}$ Treg phenotype induced by TCDD *in vivo* (Funatake *et al.* 2005; Marshall *et al.* 2008).

Only a few of the other genes in our 48-gene panel showed altered expression as a result of the presence of TCDD during $CD4^+$ T-cell activation. *Ctla4* expression was increased by TCDD under Th1 conditions, a change that is consistent with the AhR-dependent increase in expression of CTLA-4 on donor $CD4^+$ T-cells *in vivo* during the GVH response (Funatake *et al.* 2005). Likewise, increased expression of *Il12rb2* under both Th1 and Th17 conditions is consistent with the increased responsiveness of donor $CD4^+$ T-cells from TCDD-treated mice to IL-12, resulting in enhanced STAT4 phosphorylation (Marshall *et al.* 2008). Under Th0 conditions, TCDD also suppressed the expression of *Tbet* and *Il17a*, providing a mechanism to promote Treg differentiation at the expense of effector Th1 or Th17 cells.

The most noteworthy effect of TCDD in $CD4^+$ T-cells was the AhR-dependent increase in expression of *Il22* that was seen across all culture conditions except Tr1. AhR-mediated up-regulation of *Il22* was also observed at the protein level in $CD4^+$ T-cells cultured under Th17 conditions. AhR signaling has been implicated in IL-22 production in previous studies (Ramirez *et al.* 2010; Rutz *et al.* 2011; Veldhoen *et al.* 2008). Additionally, Notch signaling was shown to create AhR ligands that enhance IL-22 production (Alam *et al.* 2010). Based on the fact that IL-22 can be produced in the absence of IL-17A or IFNγ, a unique Th22 subset of $CD4^+$ T-cells has been proposed (Trifari *et al.* 2009). The putative transcription factor for this Th22 subset is the AhR (Trifari *et al.* 2009). IL-22 has complex functions and has been shown to produce both inflammatory (psoriasis, rheumatoid arthritis) and protective (irritable bowel disease) responses (Zenewicz and Flavell 2011). Enhanced production of IL-22 by $CD4^+$ T-cells following AhR activation by TCDD may play a role in the immunoregulatory effects of TCDD.

Apart from its role in mediating effects of exogenous ligands such as TCDD, the consequences of AhR activation via endogenous ligands is also of interest. Although *in vivo*

studies indicate that $AhR^{-/-}$ mice generate normal adaptive immune responses to model antigens (Vorderstrasse *et al.* 2001) and that $AhR^{-/-}$ donor $CD4^+$ T-cells differentiate normally during an acute GVH response (Funatake *et al.* 2005; Kerkvliet *et al.* 2002), AhR-deficient mice have been reported to express a hypersensitive phenotype in experimental colitis, and following challenge with either LPS or cigarette smoke (Furumatsu *et al.* 2011; Sekine *et al.* 2009; Thatcher *et al.* 2007). Since several putative endogenous AhR ligands have been associated with the induction of immunosuppressive Tregs, the absence of AhR could impair Treg differentiation resulting in hyper-inflammation. Several pro-inflammatory genes were up-regulated in AhR deficient cells at 48 hours under Treg polarizing conditions including *Ifng, Gzmb, Tgfb3,* and *Il6* suggesting that the absence of AhR signaling in $CD4^+$ T-cells alters Treg differentiation. Also consistent with a hypersensitive phenotype, $CD4^+$ T-cells from $AhR^{-/-}$ mice showed a transient increase in expression of genes associated with Th17 differentiation, *Rorc* and *Il17a,* which was seen under Th1, Treg, Tr1 and Th17 conditions at 24 hours. However, neither transcript nor protein levels were affected at 48 hours when measured under Th17 conditions. These results suggest that the impairment of Th17 differentiation in the absence of AhR (Kimura *et al.* 2008; Veldhoen *et al.* 2009) results from post-transcriptional events. Similarly, whereas Kimura *et al.* (2008) reported that fewer $Foxp3^+$ cells were generated when $AhR^{-/-}$ cells were cultured under Treg conditions, AhR deficiency did not influence *Foxp3* expression under any of the conditions tested in our studies.

Given the emerging role of AhR in IL-22 production and the significant up-regulation of *Il22* by TCDD, we were surprised to find that AhR deficiency did not impair expression of the *Il22* gene. This finding was in direct contrast to the results of Veldhoen *et al*., (2008) who first reported that IL-22 expression was absent in AhR-deficient cells (Veldhoen *et al.* 2008) .

One possible explanation for these divergent findings may derive from the type of media used for T- cell culture. All of our studies utilized RPMI 1640 whereas Veldhoen *et al*., (2008) may have used a specific medium (IMDM) that contains high levels of aromatic amino acids including tryptophan, a source of several AhR ligands (Veldhoen *et al.* 2009). If IMDM was used, the expression of *Il22* may have been up-regulated in $AhR^{+/+}$ cells due to the presence of endogenous AhR ligands, while AhR-deficient cells would be unable to respond. We saw this effect when we compared IL-22 expression in Th17-polarized cultures of $AhR^{+/+}$ and $AhR^{-/-}$ $CD4^+$ T cells treated with TCDD. Expression of *Il22* was more than 50-fold higher in $AhR^{+/+}$ cells at 48 hours (unpublished observations). These data suggest that AhR is not needed for constitutive *Il22* expression.

In summary, strong activation of AhR by TCDD in differentiating $CD4^+$ T-cells failed to influence the expression of numerous genes associated with T-cell activation and differentiation. Since several of these genes have been shown to be altered by TCDD in differentiating $CD4^+$ T-cells *in vivo*, the results cast doubt on the ability of *in vitro* conditions to recapitulate *in vivo* events. This could be due to lack of accessory cell signals not mimicked by antibodies to CD3 and CD28, or to the presence of high levels of polarizing cytokines that might dominate the signaling pathways. Other than known AhR-regulated genes (*Cyp1a1, Cyp1b1, Ahrr*), the only immune-related gene that was clearly regulated by AhR in $CD4^+$ T-cells was *Il22*, a gene that has been recently associated with the AhR pathway. The possible role that IL-22 plays in the immunoregulatory effects of TCDD awaits future studies.

Chapter 3

Suppression of acute graft-vs-host (GVH) response by TCDD is independent of the CTLA-4-IFNγ-IDO pathway.

Diana Rohlman

Sumit Punj

Jamie Pennington

Sam Bradford

Nancy I. Kerkvliet

Toxicological Sciences

Oxford University Press

2001 Evans Road

Cary, NC 27513

USA

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ABSTRACT

Activation of the aryl hydrocarbon receptor (AhR) by its prototypic ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), induces potent suppression of an acute graft-vs-host (GVH) response and prevents GVHD. Suppression is associated with an early increase in a regulatory population of donor $CD4^+$ T-cells that is Foxp3-negative but expresses high levels of CD25 and CTLA-4. However, a direct link between these AhR-induced Tregs (AhR-Tregs) and suppression of GVHD remains to be shown. CTLA-4 is a negative regulator of T-cell responses and is associated with the induction of tolerogenic DC that produce indoleamine 2,3-dioxygenase (IDO). We hypothesized that AhR-Tregs mediate suppression via their enhanced expression of CTLA-4 which, in turn, induces IFNγ and IDO in host DC. Subsequent depletion of tryptophan by IDO leads to termination of the donor T-cell response prior to development of effector CTL. Here we show that despite increased expression of *Ifng, Irf3, Irf7, Ido1* and *Ido2* in the lymph nodes of TCDD-treated host mice, inhibition of IDO enzyme activity by 1-methyl-tryptophan was unable to relieve TCDD-mediated suppression of the GVH response. Furthermore, treatment with an anti-CTLA-4 antibody that blocks CTLA-4 signaling was also unable to alleviate TCDD-mediated suppression. Alternatively, we investigated the possibility that donor-derived AhR-Tregs produce IFNγ to suppress effector CTL development. However, suppression of GVHD by TCDD was not affected by the use of *Ifng*-deficient donor cells. Together these results indicate that neither over-expression of CTLA-4 nor production of IFNγ by AhR-Tregs plays a major role in the manifestation of their immunosuppressive function *in vivo*.

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is known to mediate potent immunosuppression upon activation by the prototypic ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Marshall and Kerkvliet 2010). The ability of TCDD to suppress the development of several autoimmune diseases in association with an increase in the frequency of $Foxp3^+$ Tregs has sparked an interest in the AhR as a therapeutic target (Benson and Shepherd 2011; Kerkvliet *et al.* 2009; Quintana *et al.* 2008; Zhang *et al.* 2010). Direct activation of AhR in $CD4^+$ T-cells also induces Foxp3-negative Tregs (AhR-Tregs). These AhR-Tregs were first described in an acute parent-into-F1 graft-vs-host (GVH) model, and were characterized by their high levels of CD25 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) and potent *in vitro* suppressive activity (Funatake *et al.* 2005). Additional studies have shown that AhR-Tregs express higher levels of several genes that have been associated with Treg function such as IL-10, granzyme B, and CD39 (Marshall *et al*. 2008). These AhR-Tregs are postulated to mediate suppression of the alloCTL response and protection from GVHD in TCDD-treated animals. However, the mechanisms of suppression used by AhR-Tregs remain poorly characterized.

CTLA-4 is an inhibitory receptor that shares 30% homology with CD28, a co-stimulatory receptor on T-cells. Reaching maximal expression 48 hours after T-cell activation (Walunas *et al.* 1994), CTLA-4 competes with CD28 for the shared ligands CD80 and CD86, expressed on dendritic cells (DC). As CTLA-4 has a higher affinity for these ligands relative to CD28, CTLA-4 may prevent CD28 signaling (Alegre *et al.* 2001). Therefore, while engagement of CD28 promotes T-cell proliferation and differentiation, engagement of CTLA-4 attenuates T-cell responses. Animals deficient for CTLA-4 succumb to a severe T-dependent

lymphoproliferative disease, resulting in death at 3-4 weeks of age (Alegre *et al.* 2001) underscoring the necessity for CTLA-4 in T-cell regulation.

In addition to T-cell regulation, CTLA-4 can also influence DC function by interaction with CD80/86. This interaction can induce a tolerogenic phenotype through the induction of IFNγ and indoleamine 2,3-dioxygenase (IDO) (Munn *et al.* 2004). IDO catalyzes the rate-limiting step of tryptophan degradation to kynurenine, resulting in depleted tryptophan levels and suppression of T-cell responses (Mellor and Munn 2004). Interestingly, TCDD has been shown to alter IDO expression and function. For example, increased expression of the genes *Ido1* and *Ido2* has been observed in several DC subsets cultured with TCDD *in vitro*, including DC derived from the human U937 monocytic cell line, murine bone-marrow derived DC and plasmacytoid DC (pDC) (Bankoti *et al.* 2010; Benson and Shepherd 2011; Mezrich *et al.* 2010; Vogel *et al.* 2008). This process appeared to be dependent on AhR, as use of BMDC from $AhR^{-/-}$ mice, or use of an AhR antagonist prevented the TCDD-dependent increase in IDO expression (Bankoti *et al.* 2010; Vogel *et al.* 2008). IDO enzyme activity was also increased by TCDD in U937-derived DC (Vogel *et al.* 2008). In other studies, IDO was implicated in the induction of $Foxp3^{+}$ Tregs by kynurenine, another known AhR ligand (Mezrich *et al.* 2010; Nguyen *et al.* 2010). In one study, AhR in the DC was required for Treg induction (Nguyen *et al.* 2010), while in the other, AhR expression in the $CD4^{+}$ T-cell was necessary (Mezrich *et al.* 2010).

The studies reported here have addressed the hypothesis that the TCDD-mediated increase in CTLA-4 expression on donor $CD4^{+}$ T-cells induces IFNγ and ultimately IDO in host DCs, thereby suppressing the allospecific CTL response (see Figure 3-1). To address this hypothesis, we examined the effect of TCDD on the CTL response while independently blocking CTLA-4 and inhibiting IDO enzyme activity.

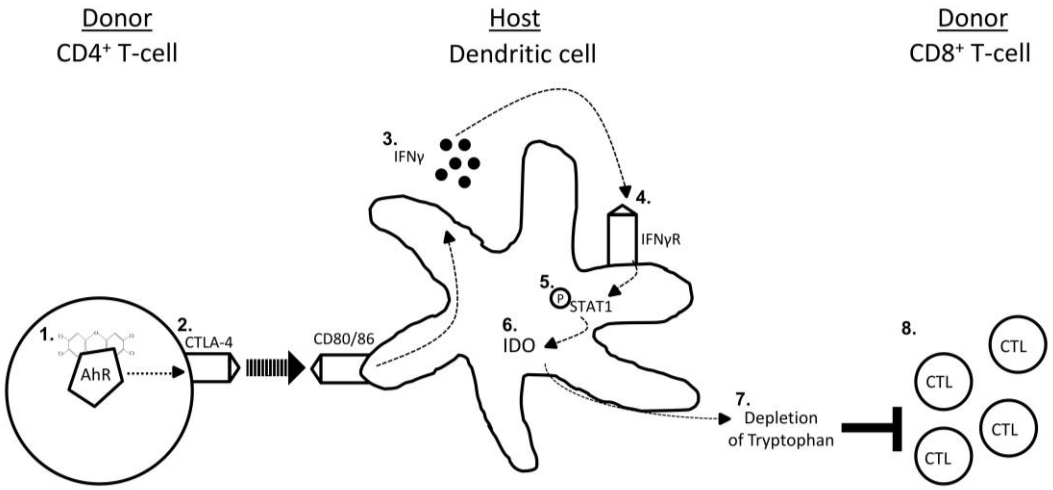

Figure 3-1. *Depiction of TCDD-induced CTLA-4 on AhR-Treg activating the tolerogenic IDO pathway and suppressing the acute GVH response. AhR is activated by TCDD in donor CD4$^+$ T-cells (1). CTLA-4 is up-regulated in an AhR-dependent manner in donor CD4$^+$ T-cells and ligates CD80/86 on host DC (2). The activated DC produces IFNγ (3). IFNγ feeds back in an autocrine manner through the IFNγ receptor (IFNγR) (4). IFNγ signaling through the IFNγR phosphorylates STAT1 (5). Phosphorylated STAT1 directly induces IDO (6), which catalyzes the rate-limiting step of $_L$-tryptophan metabolism to $_L$-kynurenine, depleting local tryptophan levels (7). Lack of tryptophan suppresses the development of effector CTL (8).*

MATERIALS AND METHODS

Animals

B6 (H-$2^{b/b}$), B6.129s7-*Ifng*^{tm1Ts}/J (*Ifng*$^{-/-}$, H-$2^{b/b}$) and B6D2F1 (H-$2^{b/d}$) mice were purchased from the Jackson Laboratory. B6.PL-Thy1a/CyJ (Thy1.1^{+}, H-$2^{b/b}$) mice (originally purchased from the Jackson Laboratory) were maintained as a breeding colony on-site. Animals were housed at the specific pathogen-free Laboratory Animals Resource Center. All experimental procedures and treatments were approved by the Institutional Animal Care and Use Committee at Oregon State University.

TCDD preparation

TCDD (Cambridge Isotope Laboratories; 99% purity) was dissolved in anisole (J.T. Baker), and further diluted in peanut oil. Host B6D2F1 mice were given 15 µg/kg of TCDD or vehicle control following adoptive transfer of donor cells on day 0.

Preparation of donor cells

Spleens and lymph nodes from donor C57Bl/6 (H-$2^{b/b}$) mice were aseptically removed and processed into single-cell suspensions by pressing the organs through a 70 µm nylon mesh cell strainer (BD Falcon). Red blood cells were removed via hypotonic water lysis. For experiments involving injection of whole splenocytes, $CD4^{+}$ and $CD8^{+}$ T-cell purity was determined by FACS analysis. Splenocyte suspensions were resuspended in injection buffer (HBSS, 1.5mM HEPES, 50µg/ml gentamicin). A maximum of $4x10^{7}$ donor T-cells were injected by tail vein injection into B6D2F1 (H-$2^{b/d}$) hosts.

Where noted, $CD4^{+}$ and $CD8^{+}$ T-cells were isolated from donor splenocytes using a negative selection PanT kit with autoMACS magnetic isolation (Miltenyi autoMACS). Purity

was determined by FACS, and was routinely above 90%. In some experiments the donor T-cells were labeled with 2μM CFSE (Molecular Probes) prior to adoptive transfer.

Anti-CTLA-4 treatment

LEAF™ purified anti-mouse CD152 (CTLA-4) (9H10) and LEAF™ purified Syrian hamster IgG isotype control (SHG-1) were obtained from BioLegend. Following adoptive transfer, host mice received 100 μg of either anti-CTLA-4 antibody or isotype control via intraperitoneal injection. Injections were repeated on days 1 and 2 after adoptive transfer. A similar treatment regimen resulted in increased proliferation of antigen-specific T-cells following immunization with vacc-HA (Sotomayor *et al.* 1999).

1-MT treatment

1-methyl-tryptophan (1-MT) was prepared as previously described (Saxena *et al.* 2007). Briefly, 1-MT was dissolved in 0.05N NaOH to a concentration of 20mM. The solution was adjusted to pH 7 with concentrated HCl. To achieve a final concentration of 2 mg/ml, the 1-MT solution was diluted in autoclaved, distilled water, sweetened with 1 packet aspartame per 2 liters of water. Beginning on day -3, all animals received sweetened water *ad libitum*. 1-MT supplemented water was administered in light-protected water bottles and given *ad libitum* to mice a day prior to adoptive transfer. Control animals received sweetened water. Water bottles were checked daily to ensure mice were consuming approximately 4ml (8mg) of 1-MT daily.

Quantification of IFNγ production

Splenocytes were cultured in RPMI 1640 medium containing 10% FBS, 50 μg/ml gentamicin, 10 mM HEPES and 50 μM βME. The cultures were incubated overnight (~18 hours) at 37°C and 5% CO_2 and supernatants collected for ELISA analysis. IFNγ was detected using the IFNγ Ready-Set-Go® ELISA kit from eBioscience.

Flow cytometry

Host splenocytes were processed as described for donor cell preparation. Following red blood cell lysis, splenocytes were aliquoted into 96-well V-bottom plates (Corning) and washed twice with PAB (PBS, 1% BSA, 0.1% sodium azide). Samples were resuspended in rat IgG (Jackson ImmunoResearch) and incubated on ice prior to surface staining. For detection of donor cells, cells were stained with mAbs to Thy1.1 (OX-7 or HIS51; BD PharMingen or eBioscience) or H-2D^d (KH95; BioLegend) in conjunction with either CD4 (GK1.5) or CD8 (53-6.7) (BD PharMingen). mAbs to CD25 (PC61.5), CD19 (eBio1D3) and CTLA-4 (UC10-4B9) were purchased from eBioscience. For detection of CTLA-4, samples were resuspended in fixable viability dye efluor 780 (eBioscience) for 20 minutes following surface staining for CD4, CD8, Thy1.1, H-2D^d, CD25, and CD19. Intracellular staining was performed following fixation and permeabilization protocols from BD Biosciences.

Fluorescence minus one (FMO) samples, in which all antibodies except the one of interest are included were used as staining controls. A minimum of 10,000 donor CD4$^+$ events or 1x10^6 total cells were collected per sample on a Beckman Coulter FC-500 flow cytometer. Data was compensated and analyzed using WinList (Verity Software, Version 6.0).

RNA extraction and qPCR

RNA was isolated from pooled axial, brachial and cervical lymph nodes of host mice using the RNeasy Mini Kit #74104 (Qiagen), with the on-column DNase digestion repeated twice to ensure removal of genomic DNA. RNA integrity was assessed by using a Bioanalyzer 2100 (Agilent). Reverse transcription was performed using the Superscript III first strand synthesis supermix (Invitrogen), following the manufacturer's instructions. For all qPCR reactions, SYBR Green/Rox qPCR Master Mix (SA Bioscience) was mixed with 10ng cDNA per reaction, plus the appropriate primers. An ABI PRISM 7500 Real-Time PCR system (Applied Biosystems) was used for all qPCR reactions. Primers for *Ido1* (NM_008324.1), *Ido2* (NM_145949.2), and *Ifng* (NM_008337.1) were obtained from SA Biosciences, and used according to the manufacturer's instructions. All other primer sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank). Forward and reverse primers were purchased from Invitrogen and validated. The PrimerBank ID codes were as follows: *E2-2*: 1001888a1; *Irf3*: 8393627a1; *Irf7*: 8567364a1; *Lag3*: 6678654a1; *Siglech*: 30520121a1; *Tlr7*: 18875360a1; *Tlr9*: 13626030a1. Data were analyzed using 7500 Software (v2.0.1) (Applied Biosystems).

Quantification of IDO enzyme activity

Splenocytes ($1x10^7$) were cultured in 0.5ml 1X HBSS without phenol red (Sigma), containing 100µM $_L$-tryptophan (Alfa Aesar, CAS #73-22-3), and incubated at 37°C, 5% $C0_2$ as previously described (Hwu *et al.* 2000). After 4 hours, the supernatant was removed and stored at -80°C for analysis of $_L$-kynurenine. A standard curve of $_L$-kynurenine (Sigma Aldrich, CAS #2922-83-0) (0.1nM - 10µM) was prepared and run in tandem with the experimental samples. An Agilent SB-C18 RRHD 1.8µm (2.1mm x 150mm) column was used

for UPLC analysis on a Shimdazu Nexera Liquid Chromatograph, using the following eluents, H_20 + 0.1% formic acid (solvent A) and acetonitrile (solvent B). The gradient elution was 5% B for 2 minutes, followed by 90% B for 4 minutes, and 5% B for 2 minutes for a total duration of 8 minutes. The eluents were directed to an ABSciex Triple TOF 5600 for MS analysis of $_L$-kynurenine.

Statistical analysis

All results are presented as the mean ± SEM of biological replicates (n = 3 – 5). For comparisons between two treatment groups, students *t* tests were used with statistical significance shown as with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) indicates statistical significance. Where indicated, the Mixed procedure with the Satterthwaite option was performed in SAS (version 9.3).

RESULTS

Activation of AhR by TCDD increases expression of CTLA-4, IFNγ and IDO

Prior studies have shown that activation of AhR by TCDD during an acute GVH response induces a Treg phenotype ($CD25^+CTLA\text{-}4^+$) in alloresponding donor $CD4^+$ T-cells (Funatake *et al*. 2005). Based on additional studies that have linked TCDD with increased expression of IDO under various conditions, we hypothesized that increased expression of CTLA-4 on these AhR-Tregs drives IDO production in the DC via induction of IFNγ. Secretion of excessive IDO then leads to depletion of tryptophan and suppression of the differentiation of effector $CD8^+$ CTL (Figure 3-1). To test this hypothesis, we first determined if the increased expression of CTLA-4, known to occur on day 2 of the GVH response, was also observed on day 3. At the same time we evaluated the expression levels of genes for *Ifng, Ido1* and *Ido2*. *Ido2* is a paralog of *Ido1* (Ball *et al.* 2007) that has previously been shown to be up-regulated by TCDD (Vogel *et al.* 2008). Because IDO production has been associated with plasmacytoid DCs (pDC), we also analyzed the expression of other genes associated with these cells, including *E2-2, Tlr7, Siglech, Tlr9, Lag3, Irf3* and *Irf7* to determine if additional functions of the pDC may be affected by TCDD *in vivo* (Matta *et al.* 2010).

The gating strategy for identifying donor $CD4^+$ T-cells in host spleen by flow cytometry is shown in Figure 3-2A. The percentage and number of donor $CD4^+CD25^+CTLA\text{-}4^+$ cells were significantly increased in TCDD-treated animals relative to vehicle on day 2 and 3 of the GVH response (Figure 3-2B, C). The expression of CD25 was also significantly increased on a per cell basis (MFI) on day 2, however this difference was lost on day 3 as the expression level of CD25 declined in both treatment groups (Figure 3-2D). In contrast, the MFI of CTLA-4 was significantly increased by TCDD on both days (Figure 3-2D).

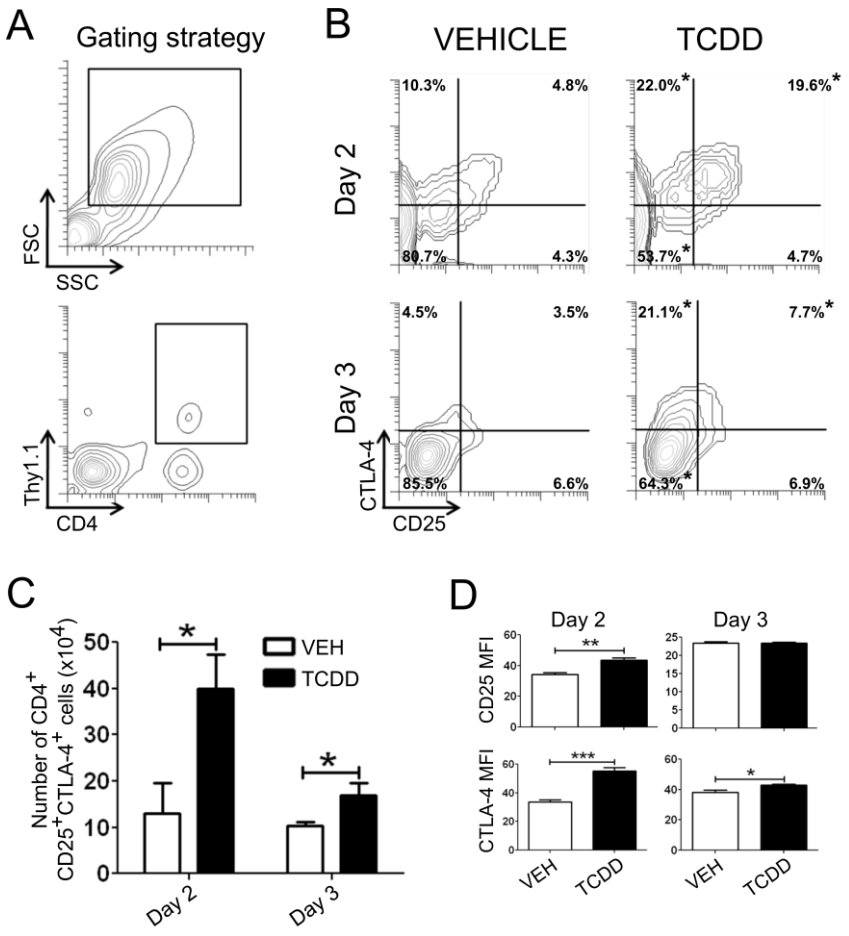

Figure 3-2. *Influence of TCDD on CD25 and CTLA-4 expression in donor CD4$^+$ T-cells on day 2 or 3 of the acute GVH response. GVHD was initiated by adoptive transfer of purified donor CD4$^+$ and CD8$^+$ T-cells. (A) Using the FSC vs SSC profile lymphocytes were identified. Donor CD4$^+$ T-cells were identified by CFSE dilution (day 2) or the congenic marker Thy1.1 (day 3). (B) Flow cytometric analysis of CD25 and CTLA-4 expression on donor CD4$^+$ T-cells in vehicle- or TCDD-treated animals 2 or 3 days after adoptive transfer. (C) Total number of donor CD4$^+$ T-cells expressing CD25 and CTLA-4. (D) Median fluorescence intensity of CD25 and CTLA-4 in donor CD4$^+$ T-cells. Statistical differences, calculated by Student's t-test, are denoted by different letters. Error bars represent the mean ± SEM, n = 4-5 biological replicates.*

Expression levels of *Ifng*, *Irf3*, *Irf7* and *Ido2* were significantly up-regulated in the lymph nodes of TCDD-treated host mice on day 2 (Table 1). On day 3, both *Ido1* and *Ido2*

expression levels were increased over 10-fold in TCDD-treated mice, along with increased expression of *Ifng* and *Irf7*.

Gene	**Fold change** TCDD/VEH	
	Day 2	*Day 3*
Cyp1a1	208*	300*
Ido1	1.6	12.6*
Ido2	3.0*	10.7*
Ifng	3.0†	1.9
Irf3	1.9*	0.8
Irf7	2.6†	2.7*

***Table 3-1**. Influence of TCDD on expression of genes associated with the CTLA-4 – IFNγ – IDO signaling pathway. Expression of Cyp1a1 was queried as an indicator of AhR activation. Genes associated with pDC were also evaluated, to include E2-2, Siglech, Lag3, Irf3, Irf7, Tlr7, Tlr9. RNA was isolated from the cervical, brachial and axial lymph nodes from host mice on days 2 or 3 of the GVH response and used to query gene expression. Fold change ($2^{-\Delta\Delta Ct}$) was calculated relative to vehicle (VEH. n = 4-5 biological replicates. Student's t-test was used to calculate significance. †p = 0.1, *p <0.05.*

IFNγ protein levels were also significantly increased by TCDD as measured in supernatants of host splenocytes on day 3 (Figure 3-3). On the other hand, the level of secreted $_L$-kynurenine in day 3 supernatants was not significantly different between vehicle- and TCDD-treated cultures, suggesting that the overall enzymatic activity of IDO was not affected by TCDD.

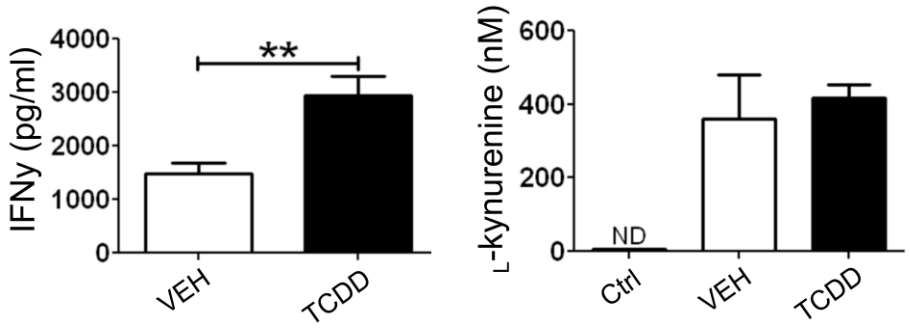

Figure 3- 3*. Effect of TCDD on IFNγ production and IDO enzyme activity. On day 3 of the GVH response, host splenocytes were isolated and cultured overnight for analysis of IFNγ levels in the supernatant using an IFNγ Ready-Set-Go® ELISA (eBioscience). Host splenocytes were also prepared for quantification of IDO enzyme activity on day 3. Splenocytes were resuspended in 1X HBSS without phenol red and cultured with excess tryptophan (100μM) for 4 hours. The concentration of* $_L$*-kynurenine was quantified using LC-MS as a measure of IDO enzyme function. CTRL = background level of* $_L$*-kynurenine in 1X HBSS alone. Error bars represent the mean ± SEM, n = 4-5 biological replicates. Student's t-test, was used to determine significant differences between treatments. *p < 0.05 and **p < 0.01. ND = not detected.*

Role of IDO in TCDD-mediated suppression

Although no difference in IDO activity was observed in spleen cell culture supernatants from vehicle- and TCDD-treated mice, the bulk measurement may not reveal differences in IDO activity at the interface of DC-T-cell interaction. To directly evaluate the functional role of IDO in TCDD-mediated suppression of the GVH response, host mice were given 1-MT, a pharmacological inhibitor of IDO's enzymatic activity, in the drinking water beginning 2 days before injection of donor cells and continuing for 15 days thereafter (Figure 3-4A). Water consumption per cage was monitored daily to estimate intake of 1-MT. The average dose of 1-MT in vehicle- treated mice was 277 mg/kg/day compared to 324 mg/kg/day in TCDD-treated mice, both within the range of 1-MT doses shown to be effective at suppressing T-cell responses (Kwidzinski *et al.* 2005; Sakurai *et al.* 2002; Uyttenhove *et al.* 2003).

The effect of 1-MT on the GVH response was monitored by body weight loss and donor $CD8^+$ T-cell engraftment in the spleen of host mice 15 days after adoptive transfer. Donor $CD8^+$ T-cells were identified by the absence of $H\text{-}2D^d$ expression, and CTL activity was estimated based on the percentage of $CD19^+$ host B-cells in the spleen. As summarized in Figure 3-4, vehicle-treated mice lost a significant amount of body weight beginning on day 9 of the GVH response, and treatment with 1-MT did not influence this loss.

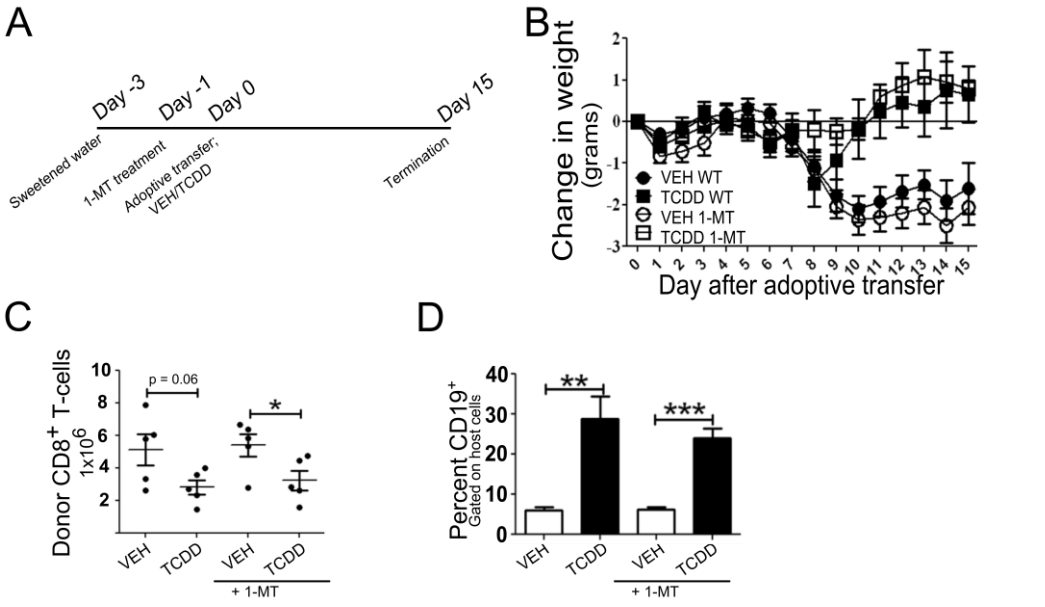

Figure 3- 4. *Effect of inhibition of IDO enzyme activity on suppression of the GVH response by TCDD. (A) Schematic of experimental design. Three days prior to adoptive transfer of donor splenocytes, mice were given autoclaved, distilled water sweetened with aspartame. On day -1, treatment with 1-MT, a pharmacological inhibitor of IDO, was begun. (B) Water consumption was tracked daily by cage. Water consumption per animal was determined and used to calculate the average dose (mg/kg) of 1-MT consumed. (C) Change in host body weight following adoptive transfer. (D) Using flow cytometry, the percent of $H\text{-}2D^d$ negative donor cells was determined and used to calculate the total number of donor cells from the total number of splenocytes. The percent of $H\text{-}2D^d negCD8^+$ T-cells was multiplied by the number of donor cells to calculate the total number of engrafted donor $CD8^+$ T-cells in the spleen of host animals. (E) CTL function was assessed as a measure of host B-cell depletion. Host B-cells were identified by co-expression of $H\text{-}2D^d$ and CD19. Error bars represent the mean $\pm$ SEM, n = 5 biological replicates. Treatment-related differences were determined using Student's t-test. $*p < 0.05$, $***p < 0.001$.*

In agreement with previous studies (Kerkvliet *et al.* 2002), TCDD treatment prevented the loss of body weight associated with the GVH response, and this was also unaffected by 1-MT. Donor $CD8^+$ T-cell engraftment was reduced in TCDD-treated mice and the increased percentage of host B-cells in the spleen of TCDD-treated mice validated suppression of the allo-CTL response. Treatment with 1-MT did not affect donor cell engraftment or CTL activity in either vehicle- or TCDD-treated mice. The absence of effects of 1-MT on these endpoints suggests that increased IDO activity is not a primary mechanism for suppression of the GVH response by TCDD. However, this conclusion must be tempered by the lack of an effect of 1-MT on the GVH response in vehicle-treated mice as well.

Functional requirement for CTLA-4 in TCDD-mediated immunosuppression

While the lack of effect of 1-MT on TCDD-induced suppression did not support the CTLA-4- IFNγ-IDO hypothesis, CTLA-4 can also mediate suppression of T-cell responses by pathways independent of IDO. To determine if increased CTLA-4 expression was directly involved in the suppression of the GVH response by TCDD, we tested whether blockade of CTLA-4 could prevent TCDD-induced suppression of GVHD. Host mice were treated with anti-CTLA-4 antibody on days 0, 1 and 2 relative to donor T-cell transfer (Figure 3-5A), and GVHD pathology was assessed on day 11 by analyzing donor $CD8^+$ T-cell engraftment and host $CD19^+$ B-cell depletion. Relative to vehicle-treated animals, treatment with TCDD suppressed donor T-cell engraftment and preserved the host B-cell population as expected. Anti-CTLA-4 treatment had no effect on GVHD pathology in either vehicle- or TCDD-treated animals (Figure 3-5B, C). These results suggest that enhanced expression of CTLA-4 on donor $CD4^+$ T-cells following treatment with TCDD does not play a functional role in the suppression of GVHD by TCDD.

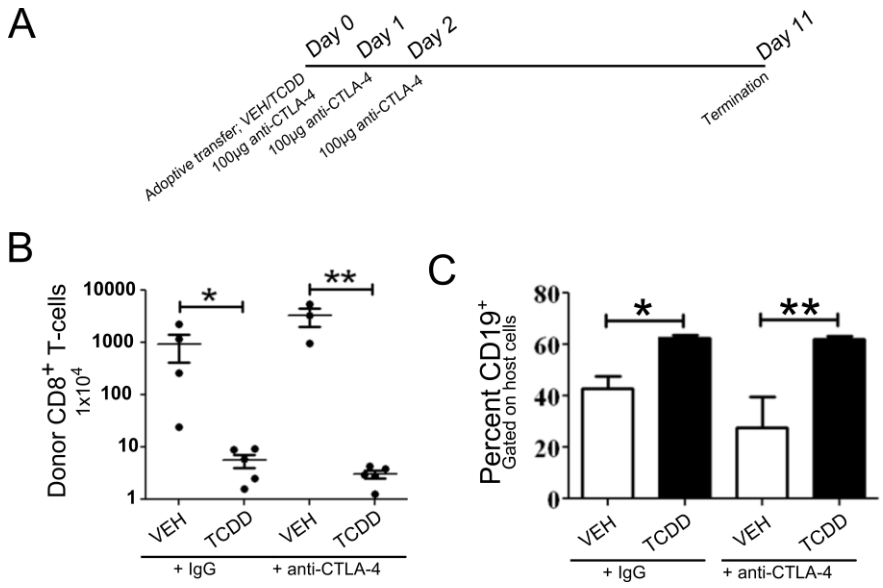

Figure 3- 5*. Effect of blocking CTLA-4 during donor CD4$^+$ T-cell differentiation on suppression of GVHD by TCDD. (A) Schematic of experimental design. 100µg of anti-CTLA-4 or control IgG were administered on day 0, 1 and 2 following adoptive transfer of purified donor T-cells and treatment with either vehicle or TCDD. (B) Using flow cytometry, the percent of Thy1.1$^+$ donor cells was determined and used to calculate the total number of donor cells from the total number of splenocytes. The percent of Thy1.1$^+$CD8$^+$ T-cells was multiplied by the number of donor cells to calculate the total number of engrafted donor CD8$^+$ T-cells in the spleen of host animals. (C) CTL function was assessed as a function of host B-cell depletion. Host B-cells were tracked by expression of CD19. Error bars represent the mean ± SEM, n = 3-5 biological replicates. For statistical analysis, the Mixed procedure with the Satterthwaite option was used. *p < 0.05, ** p < 0.01.*

Effect of donor-derived IFNγ in TCDD-mediated immunosuppression

Since neither IDO nor CTLA-4 appeared to be required for TCDD-mediated immunosuppression, a potential independent role for IFNγ was investigated. Based on the fact that nTregs from IFNγ-deficient animals have attenuated suppressive function (Koenecke *et al.* 2012; Markees *et al.* 1998; Sawitzki *et al.* 2005; Wei *et al.* 2010), it is possible that AhR-Tregs also utilize IFNγ to mediate suppression of the CTL response. If true, lack of IFNγ in the donor cells should result in evidence of GVHD in TCDD-treated mice. To directly evaluate the role of donor-derived IFNγ, host mice were injected with donor splenocytes

obtained from C57Bl/6 (WT) or IFNγ-deficient (*Ifng*$^{-/-}$) mice, which were then treated with vehicle or TCDD.

Vehicle-treated host mice that received *Ifng*$^{-/-}$ donor cells exhibited early-onset and more severe body weight loss (Figure 3-6A) consistent with previous results (Ellison *et al.* 1998). Mice that received *Ifng*$^{-/-}$ donor cells also had significantly fewer splenocytes than mice with WT donor cells (Figure 3-6B) yet a much higher proportion of B-cells ($p<0.001$) (Figure 3-6C). These results are consistent with a prior study showing a selective loss of Fas-mediated killing of host B-cells that is promoted by IFNγ (Puliaev *et al.* 2004). We also found that donor CD4^{+} and CD8^{+} T-cell engraftment was significantly reduced when the donor cells lacked IFNγ (Figure 3-6E - F). This was not due to a difference in the number of T-cells injected in the initial donor cell inoculum (Figure 3-6G).

Treatment of host mice with TCDD prevented body weight loss (Figure 3-6A), decrease in splenocyte numbers (Figure 3-6B) and donor T-cell engraftment (Figure 3-6E-F) associated with GVHD, independent of the *Ifng* status of the donor cells. These results indicate that AhR-Tregs do not use IFNγ for suppression of the GVH response.

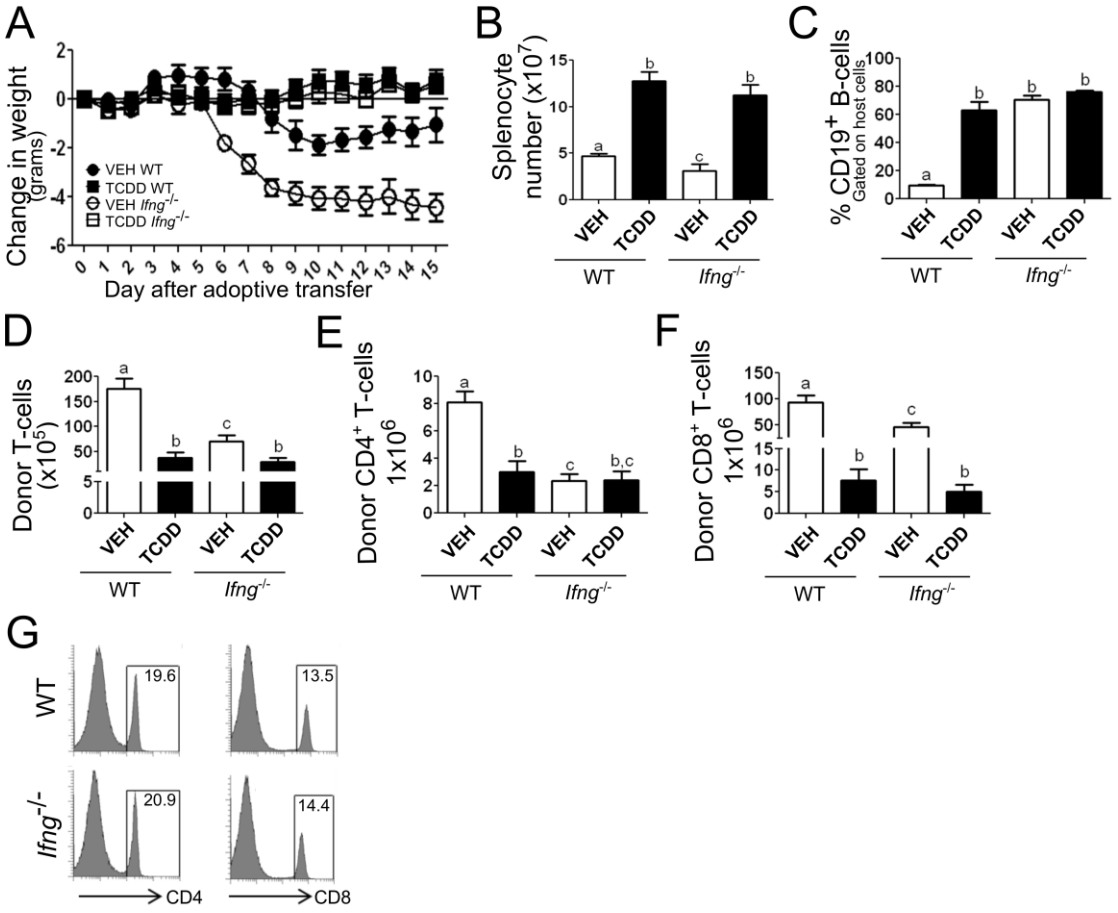

Figure 3-6. *Absence of IFNγ in donor cells does not affect suppression of the CTL response by TCDD. (A) Change in host body weight following adoptive transfer. (B) (C) Total splenocyte numbers on day* $^{+}$*15. (C) Percent of CD19*$^{+}$ *B-cells remaining in the host (H-2D*$^{d+}$*) fraction. (D) Total number of donor (H-2D*$^{d\text{-}neg}$*) T-cells present in the spleen of host animals. Number of donor (E) CD4*$^{+}$ *or (F) CD8*$^{+}$ *T-cells in the spleen of host animals. (G) Percent of CD4*$^{+}$ *and CD8*$^{+}$ *T-cells in the donor inocula prior to adaptive transfer of splenocytes on day 0. Significant differences between treatment groups were calculated using Student's t-test ($p < 0.05$) and are denoted by different alphabetical characters. Error bars represent the mean ± SEM, n = 5 biological replicates.*

DISCUSSION

Activation of AhR by TCDD during an acute GVH response induces Tregs with a suppressive capacity greater than that of natural Treg (Marshall *et al.* 2008). These AhR-Tregs do not express Foxp3, but express high levels of other Treg-associated markers such as CD25 and CTLA-4 (Funatake *et al.* 2005), suggesting that AhR functions as an autonomous ligand-dependent transcription factor for induction of Tregs. However, the mechanism by which AhR-Tregs mediate suppression of immune function remains unclear. The studies presented here were designed to test the hypothesis that the enhanced expression of CTLA-4 induced by TCDD on donor $CD4^+$ T-cells activates the IDO pathway, resulting in suppression of the CTL response (see Figure 3-1). However, while treatment with TCDD significantly up-regulated expression of *Ifng*, *Ido1* and *Ido2* in the lymph nodes and increased production of IFNγ in spleen, blockade of CTLA-4 or IDO enzyme activity did not alleviate TCDD's suppressive effects. Furthermore the use of IFNγ-deficient donor cells did not influence suppression suggesting that AhR-Tregs do not rely on IFNγ for their immunosuppressive activity.

We initially tested the role of IDO by blocking its catalytic activity with the pharmacological inhibitor 1-MT. However, treatment with 1-MT had no effect on the day-15 GVH response in either control or TCDD-treated animals. The lack of effect of 1-MT in control animals was initially surprising since IDO-deficient animals were reported to show increased GVHD-associated mortality (Jasperson *et al*. 2008). However, the enhanced mortality in IDO-deficient mice was not due to excessive T-cell activation but rather to lack of up-regulation of IDO in gut epithelial cells that reduced inflammatory injury in the colon. Taken together with our results, IDO and its associated depletion of tryptophan do not appear to play a prominent role in the regulation of T-cell responses in GVHD.

The inability of 1-MT to alter suppression of the GVH response in TCDD-treated animals indicates that the enzymatic activity of IDO is not involved in the suppressive mechanism of AhR-Tregs. This was surprising given the consistent up-regulation of IDO by TCDD in this and other studies (Bankoti *et al*. 2010; Vogel *et al*. 2008). However, IDO has recently been shown to have signaling properties that are distinct from its catalytic function and are unaffected by 1-MT (Pallotta *et al.* 2011). In this study, TGFβ-conditioned pDCs were shown to use phosphorylated IDO as a signal transducer that activated p52-RelB to promote TGFβ production, which in turn promoted the induction of $Foxp3^+$ Tregs. This IDO signaling pathway could be involved in the generation of $Foxp3^+$ Tregs by TCDD-activated AhR that has been reported in several different autoimmune disease models (Benson and Shepherd 2011; Kerkvliet *et al*. 2009; Quintana *et al*. 2008). Interestingly, we have recently observed that treatment with TCDD induces Foxp3 expression in both donor and host $CD4^+$ T cells on day 15 of the GVH response (Rohlman *et al.* manuscript in preparation). However, when the induction of $Foxp3^+$ expression was blocked, it did not affect suppression of the GVH response by TCDD. Thus, while IDO may play a role as a signaling molecule to up-regulate Foxp3 expression, these $Foxp3^+$ T-cells are not involved in TCDD-mediated suppression of acute GVH responses.

Since IDO has been associated primarily with a subset of tolerogenic pDC, we looked at expression of several genes associated with pDC phenotype and function in the lymph nodes of TCDD-treated host mice. Of the genes examined, expression of *Irf3* and *Irf7* were significantly increased by TCDD. *Irf3* and *Irf7* code for transcription factors involved in regulation of expression of type I IFNs (IFNα/β) (Honda *et al.* 2005). TCDD was previously shown to increase *Irf3* expression in cultured U937 cells, and the mechanism was shown to be AhR- and RelB-dependent (Vogel *et al*. 2007). Increased production of type 1 IFNs by pDC is

a down-stream consequence of IDO signaling (Palotta *et al*. 2011), suggesting that increased expression of *Irf3* and *Ido* may be linked in TCDD-treated pDC. Although type I IFNs have long been known to play critical roles in virus defense, they have recently been shown to promote the development of $Foxp3^+$ T-cells and enhance their suppressive function (Gonzãlez-Navajas *et al*. 2012). The involvement of this pathway in AhR-mediated immunoregulation remains to be shown.

Apart from altered interactions with host DCs, up-regulation of CTLA-4 on T-cells could play a direct role in suppressing effector T-cell activation and/or proliferation by out-competing CD28 signaling. However, when host mice were treated with an antibody that neutralizes the function of both the full-length and soluble isoforms of CTLA-4 (Ward *et al*. 2013), there was no effect on the suppression of the GVH response by TCDD. There was also no effect of antibody treatment on the GVH response in vehicle-treated mice. A similar treatment regimen in a murine tumor model resulted in increased expansion of antigen-specific T-cells in response to HA-expressing tumor cells but did not prevent tolerance to tumor antigen (Sotomayor *et al.* 1999). In addition, although the same anti-CTLA-4 antibody has been shown to exacerbate symptoms of disease in colitis, diabetes and EAE (Luhder *et al.* 1998; Perrin *et al.* 1996; Watanabe *et al.* 2008), these studies used repeated dosing throughout the course of disease development. Since we specifically targeted CTLA-4 blockade to the first three days of the GVH response, during the window of susceptibility to TCDD and coincident with AhR-Treg differentiation, any effects of CTLA-4 blockade on the CTL effector phase of the immune response would have been spared. In fact, the half-life of the anti-CTLA-4 mAb (9H10) that we used has been shown to be 3-4 days (James P. Allison, personal communication). This explanation is consistent with previous studies showing that antibody blockade of CTLA-4 in regulatory T-cells alone was insufficient to enhance anti-

tumor responses, whereas blockade of CTLA-4 in effector cells increased antitumor activity (Peggs *et al*. 2009). Thus, the inability of CTLA-4 blockade to affect TCDD-mediated suppression of GVHD indicates that increased expression of CTLA-4 on AhR-Treg is not involved in their suppressive function.

The apparent lack of involvement of CTLA-4 and IDO in TCDD-mediated suppression of GVHD led us to consider alternate pathways that would address the increase in IFNγ seen in TCDD-treated mice (Figure 3). Based on recent studies that demonstrated an autocrine role for IFNγ in the suppressive mechanism of nTreg in Th1-mediated diseases (Beeston *et al.* 2010; Koenecke *et al.* 2012), we hypothesized that AhR-Tregs produce IFNγ to mediate suppression of GVHD. *Ifng*$^{-/-}$ donor cells were used to initiate GVHD and the effects on the CTL response in the presence or absence of TCDD were examined. In vehicle-treated mice, GVHD was exacerbated by the lack of IFNγ in the donor cells, in agreement with prior reports (Ellison *et al.* 1998; Koenecke *et al.* 2012; Yang *et al.* 1998). Splenocyte numbers were decreased, but percent of B-cells was not diminished. This phenomenon has been attributed to the loss of IFNγ-induced Fas expression on host B-cells, preventing their selective targeting for FasL-mediated killing by CTL (Puliaev *et al.* 2004). Non-Fas-mediated pathways of cytotoxicity (ie., perforin) were not affected by the lack of IFNγ (Puliaev *et al.* 2004). However our data indicate that donor T-cell engraftment is altered by the lack of IFNγ, since significantly fewer $CD4^+$ and $CD8^+$ donor T-cells were present in the spleen of mice receiving *Ifng*$^{-/-}$ donor cells. This is surprising, given their exacerbated symptoms of GVHD. The exacerbation of GVHD in mice that received *Ifng*$^{-/-}$ donor cells has been attributed to loss of suppressive activity of nTreg (Koenecke *et al.* 2012). Unlike the nTreg, AhR-Tregs apparently do not use IFNγ as a suppressive mediator, since TCDD-treated animals did not lose weight and showed minimal evidence of a CTL response.

In summary, we found no evidence to support the hypothesis that AhR-Tregs induced by TCDD during a GVH response rely on the tolerogenic IDO pathway, CTLA-4 *per se* or IFNγ for suppression of the GVH response. This is in contrast to previous studies implicating IDO in Treg induction by several AhR ligands including TCDD (Mezrich *et al.* 2010; Nguyen *et al.* 2010; Vogel *et al.* 2008). However these studies have focused on the expression of Foxp3 as a marker of Treg induction, which may arise from direct AhR-dependent induction of IDO in pDC to drive $Foxp3^{+}$ Treg expansion. The present study also confirms *Irf3* and identifies *Irf7* as potential AhR targets for induction of $Foxp3^{+}$ Tregs. However, these $Foxp3^{+}$ Tregs induced by TCDD are distinct from the $Foxp3^{neg}$ Tregs that emerge early in the GVH response and are dependent on AhR expression in the $CD4^{+}$ T-cell itself (Funatake *et al.* 2005). These AhR-Tregs share characteristics with Tr1-like Tregs including increased IL-10 and granzyme B expression (Gandhi *et al.* 2010; Marshall *et al.* 2008). The mechanisms of suppression used by AhR-Tregs *in vivo* remain to be defined.

Chapter 4

The role of CD25 and IL-10 signaling in TCDD-mediated immunosuppression

Diana Rohlman

Nancy Kerkvliet

Abstract: The aryl hydrocarbon receptor (AhR) has been identified as a novel therapeutic target for T-cell-mediated diseases. Activation of AhR by the prototypic ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) results in potent suppression of several murine disease models, including graft-versus-host disease (GVHD). Coincident with suppression, TCDD induces a $CD25^{+}CTLA\text{-}4^{+}Foxp3^{neg}IL\text{-}10^{+}$ regulatory T-cell within the donor $CD4^{+}$ and $CD8^{+}$ T-cell populations. These AhR-induced Treg (AhR-Treg) are postulated to mediate suppression of the CTL response in GVHD. Given the very high expression of CD25, and the increased production of IL-10, we hypothesized that AhR-Treg utilize either CD25 function or IL-10 cytokine production to mediate suppression of the GVH response. Using antibody blockades, we independently neutralized CD25 and IL-10 function, evaluating the role of these proteins in the TCDD-mediated suppression of the cytotoxic T-lymphocyte (CTL) response in animals undergoing GVHD. However, TCDD-induced suppression of the CTL response was unaffected by independent blockade of either IL-10 or CD25. Despite the $Foxp3^{neg}$ phenotype of the AhR-Treg on day 2, treatment with TCDD induced Foxp3 expression in donor T-cells on day 15 of the response. Interestingly, Foxp3 expression was also increase in host T-cells on day 15. However, Foxp3 was not involved in TCDD-mediated immunosuppression, as blockade of CD25 concurrently blocked Foxp3 expression, with no effect on the TCDD-mediated suppression of the GVH response. Taken together, TCDD-mediated suppression of the GVH response appears to function independently of IL-10, CD25 and Foxp3. Also TCDD appears to up-regulate Foxp3 expression in both $CD4^{+}$ and $CD8^{+}$ T-cells of host and donor origin. The delayed nature of this increase (day 15) suggests that the induction of Foxp3 is indirect, perhaps via induction of IDO in agreement with previous studies.

INTRODUCTION

Activation of the aryl hydrocarbon receptor (AhR) by the prototypic ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) results in potent immunosuppression in multiple T-cell-dependent murine disease models, including graft-versus-host disease (GVHD) and autoimmune disease (Marshall and Kerkvliet 2010). Given the broad spectrum of suppression observed, the AhR is a novel therapeutic target, necessitating research that will elucidate the mechanism of action underlying the abrogation of disease symptoms.

The Kerkvliet lab was the first to identify and characterize AhR-dependent $CD4^+CD25^+CTLA\text{-}4^+Foxp3^{neg}IL\text{-}10^+$ Treg (AhR-Treg) in the murine parent-into-F1 GVHD model (Funatake *et al.* 2005; Marshall *et al.* 2008). The AhR-Treg population is associated with decreased donor cell engraftment and suppression of the cytotoxic T-lymphocyte (CTL) response (Funatake *et al.* 2005; Kerkvliet *et al.* 2002). These AhR-Treg are induced, rather than expanded from an existing Treg population, as depletion of the naturally occurring $CD4^+CD25^+$ T-cells from the donor inoculum did not prevent induction of the AhR-Treg $CD4^+CD25^+$ phenotype (Funatake *et al.* 2005). Activation of AhR by TCDD induces the differentiation of naïve, allo-responding T-cells into $CD4^+$ and $CD8^+$ Tregs that appear to block the differentiation of effector Th and CTL (Funatake *et al*. 2005; Funatake *et al*. 2008).Relative to natural Treg, the $CD4^+$ AhR-Treg has a greater suppressive capacity, although the mechanism of suppression remains unclear. AhR-Treg are notable for their very high expression of CD25, and CD25 has been suggested to mediate the suppressive effects caused by TCDD-activated AhR (Schulz *et al*. 2011; Zhang *et al*. 2010).

$CD4^+CD25^+$ Treg have been shown to play an essential role in regulating T-cell responses via sequestration of IL-2 and production of IL-10 and TGFβ1 (von Boehmer 2005). CD25 (IL-2Rα) is the high affinity subunit of the heterotrimeric IL-2 receptor (IL-2R),

comprised of CD122 (IL-2Rβ) and the common γ_c chain. Signaling of IL-2 through the IL-2R results in phosphorylation of STAT5, driving Treg differentiation (Antov *et al.* 2003). Animals deficient for either IL-2 or the IL-2R suffer from a severe lymphoproliferative disorder, and are deficient for $CD4^+CD25^+$ T-cells (Almeida *et al.* 2002; Malek *et al.* 2008; Papiernik *et al.* 1998). Addition of $CD4^+CD25^+$ T-cells prevents this lymphoproliferative disorder. We have previously shown that excess IL-2 was able to partially recapitulate the $CD25^{high}$ AhR-Treg phenotype (Funatake 2006). Furthermore, the increase in CD25 expression resulted in functional IL-2R signaling, as assessed by increased STAT5 phosphorylation (Funatake 2006). Recently, the TCDD-mediated suppression of experimental autoimmune uveoretinitis (EAU) and the Th2-dependent peanut allergy response was alleviated by the neutralization of CD25, offering the first direct evidence that TCDD-induced $CD4^+CD25^+$ Tregs are required to mediate immunosuppression (Schulz *et al.* 2011; Zhang *et al.* 2010). Antibody-mediated blockade of CD25 abrogated suppression of clinical and pathological symptoms in EAU (Zhang *et al.* 2010) and partially reversed suppression of the Th2-mediated model of murine peanut allergy (Schulz *et al.* 2011). Therefore, expression of CD25 on the AhR-Treg may be required for suppression of the CTL response in GVHD.

The potential role of IL-10 in TCDD-mediated immunosuppression is also of interest. IL-10 belongs to the IL-10 family of cytokines, which includes IL-19, IL-20, IL-22, IL-24 and IL-26, although only IL-10 is considered necessary for Treg function (Ouyang *et al.* 2011). IL-10 is thought to attenuate T-cell responses by directly inhibiting IL-12 and IL-23 production, cytokines that are necessary for the pro-inflammatory subsets Th1 and Th17 (Ouyang *et al.* 2011). Perhaps unsurprisingly, $Il10^{-/-}$ animals spontaneously develop $CD4^+$ T-cell dependent colitis, indicating a link between IL-10 and regulation of $CD4^+$ Treg (Kuhn *et al.* 1993). Furthermore, there is substantial evidence to indicate TCDD influences IL-10 production.

Analysis of donor T-cells and splenocytes from TCDD-treated recipient animals undergoing GVHD revealed increased levels of IL-10 mRNA and protein (Marshall *et al.* 2008). More recently, the physical association between TCDD-activated AhR and the transcription factor for Tr1 cells, cMAF, was shown to directly regulation expression of IL-10 (Apetoh *et al.* 2010).

The studies described herein have evaluated the possibility that the TCDD-mediated increase in either CD25 expression on donor $CD4^+$ T-cells or the increased IL-10 production may influence suppression of the allospecific CTL response. To address this hypothesis, we independently blocked IL-10 and CD25 while evaluating the TCDD-mediated effect on the CTL response.

Materials and Methods

Animals

C57BL/6 (H-$2^{b/b}$) and B6D2F1 (H-$2^{b/d}$) mice were purchased from the Jackson Laboratory. Animals were housed at the specific pathogen-free Laboratory Animals Resource Center on-site. The Institutional Animal Care and Use Committee at Oregon State University approved all experimental procedures and treatments.

TCDD preparation

TCDD (Cambridge Isotope Laboratories; 99% purity) was dissolved in anisole (J.T. Baker), and further diluted in peanut oil. A solution of peanut oil diluted with anisole was used as a vehicle control. Following adoptive transfer of donor splenocytes, host B6D2F1 mice received either 15µg/kg of TCDD or vehicle control by gavage.

Preparation of donor cells

Spleens and lymph nodes from donor C57Bl/6 (H-$2^{b/b}$) mice were aseptically removed and processed into single-cell suspensions by pressing the organs through a 70µm nylon mesh cell strainer (BD Falcon). Red blood cells were removed via hypotonic water lysis. $CD4^+$ and $CD8^+$ T-cell purity was determined by FACS, with purity routinely above 90%. Splenocyte suspensions were resuspended in injection buffer (HBSS, 1.5mM HEPES, 50µg/ml gentamicin). A maximum of $4x10^7$ donor T-cells were injected by tail vein injection into B6D2F1 recipients.

Antibody treatments

For blockade of IL-10, recipient mice were treated with anti-mouse IL-10 (JES5-2A5) FG purified mAb (eBioscience). On day 0 and day 1, animals received either 250 µg of anti-IL-10, or a sham treatment with PBS via intraperitoneal injection. For blockade of CD25, purified anti-mouse CD25 (PC61.5) was purchased from eBioscience. On day 1, recipient mice received either a sham treatment with PBS, or 250 µg of anti-CD25 neutralizing antibody via intraperitoneal injection.

Flow cytometry

Host splenocytes were processed as described for donor cell preparation. Following red blood cell lysis, samples were resuspended and aliquoted into 96-well V-bottom plates (Corning). Samples were washed twice with a PAB solution (PBS, 1% BSA, 0.1% sodium azide) and resuspended in rat IgG (Jackson ImmunoResearch) to block Fc receptors prior to surface staining. Samples were stained with a mAb to H-2D^{d} (34-2-12; BioLegend) to identify between host and donor cells, in conjunction with mAbs to CD4 (RM4-5) and CD8 (5H10; 53-6.7), purchased from Invitrogen and eBioscience. Furthermore, mAb to CD19 (eBio1D3), CD122 (TM-b1) and CD25 (PC61.5; 7D4) were purchased from eBioscience, while CD69 (H1.2F3) was purchased from BioLegend.

Donor cell viability was determined using Fixable viability dye efluor 780 (eBioscience) following surface stain and prior to fixation and permeabilization. Intranuclear staining for Foxp3 (FJK-16s) was performed following manufacturers' instructions (eBioscience). Staining controls consisted of fluorescence minus one (FMO) samples, wherein all the antibodies except the one of interest are included. All samples were collected on a Beckman Coulter FC-500 flow cytometer. A minimum of 10,000 events of interest or a total

of $1x10^6$ cells were collected per sample. Data was compensated and analyzed using WinList (Verity Software, Version 6.0).

Statistical analysis

All results are presented as the mean ± SEM of biological replicates (n = 3 – 5). Statistical significance was calculated using a student's t test where $p \leq 0.05$.

Results

Involvement of IL-10 signaling in TCDD-mediated immunosuppression

In the GVHD model, IL-10 production from donor T-cells has been shown to mediate protection (Tawara *et al.* 2012). To directly determine if IL-10 is necessary for the TCDD-mediated suppression of the CTL response, vehicle- or TCDD-treated host mice received 250 μg of an IL-10 neutralizing antibody at the time of adoptive transfer (day 0), and again one day later. Control animals received a sham injection of PBS. The time period of treatment is reflective of the time at which AhR activation induces the AhR-Treg phenotype.

Characteristic of GVHD, control animals exhibited significant weight loss and diminished splenocyte numbers irrespective of antibody treatment, 15 days after adoptive transfer (Figure 4-1A, B) (Kerkvliet *et al.* 2002). Vehicle-treated animals exhibited significant donor cell engraftment, determined by the percent of donor $CD8^+$ T-cells in the host spleen (Figure 4-1C). Treatment with the anti-IL-10 mAb resulted in increased donor $CD8^+$ T-cell engraftment relative to control animals receiving sham treatment, demonstrating the efficacy of the anti-IL-10 antibody treatment.

In addition to donor $CD8^+$ T-cell engraftment, we examined the effector CTL response. The cytolytic activity of the donor CTL was assessed as a function of host $CD19^+$ B-cell depletion. An inverse relationship exists between CTL activity and host B-cells, wherein functional CTL will eradicate host B-cells. Irrespective of anti-IL-10 mAb treatment, vehicle-treated animals showed a significantly reduced host B-cell population, indicating a functional CTL response (Figure 4-1D). In contrast, TCDD-treated animals maintained body weight and displayed increased numbers of total splenocytes relative to vehicle-treated animals. The percent of donor $CD8^+$ T-cells was greatly reduced, indicating a suppressed CTL response. Relative to control animals, the host B-cell population was preserved, also indicative of a

suppressed CTL response. However, there was no evidence of an anti-IL-10 treatment effect on either donor $CD8^+$ T-cell engraftment or the CTL response in TCDD-treated mice (Figure 4-1), suggesting that increased IL-10 production is not responsible for TCDD-mediated immunosuppression.

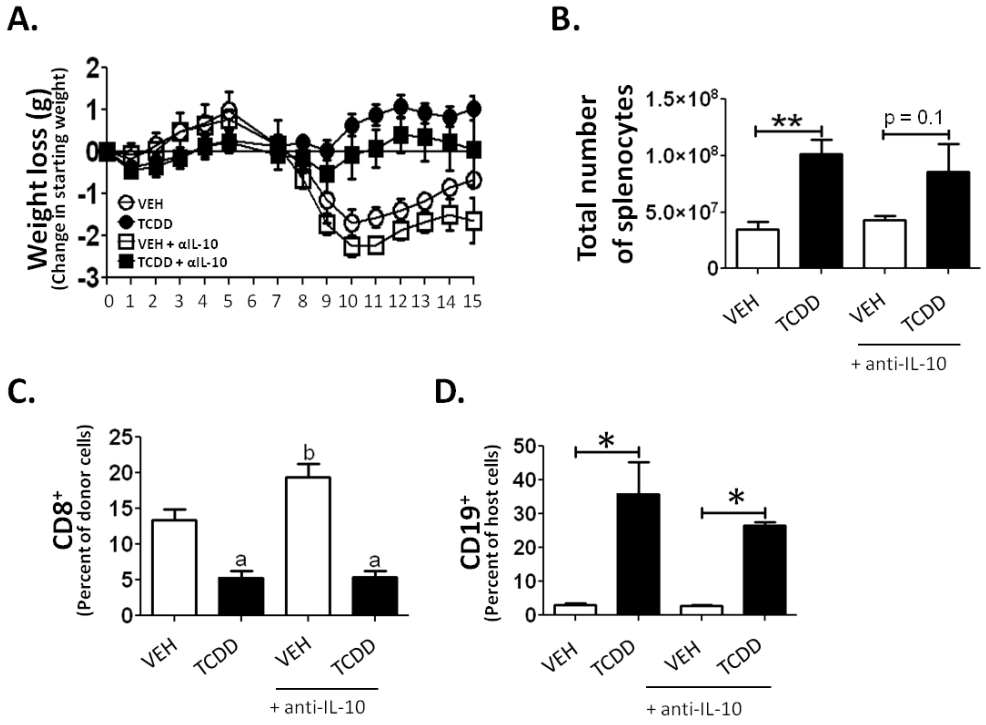

Figure 4-1. *Blockade of IL-10 does not alleviate TCDD-mediated immunosuppression. Host animals received 15 µg/kg TCDD or vehicle at the time of adoptive transfer. On day 0 and day 1 recipient mice received 250 µg of anti-IL-10 mAb via intraperitoneal injection for a total dose of 500 µg. (A) Changes in weight were monitored throughout the experiment. (B) The total number of splenocytes, a mixture of host and donor cells, was enumerated 15 days after adoptive transfer. (C) Percent of donor $CD8^+$ T-cells in host spleen. Significance is denoted by alphabetical letters, with similar letters indicating no significant difference between values. (D) The cytolytic activity of the donor $CD8^+$ T-cells was assessed as a function of remaining host B-cells. Host B-cells were identified as H-$2D^{d+}CD19^+$. Statistical significance was calculated with a student's t-test, where $p \leq 0.05$. Data are representative of 5 biological replicates per treatment group. Error bars represent the mean ± SEM.*

TCDD-mediated induction of CD25$^+$ T-cells during the GVH response

The role of CD25 in suppression of the CTL response by TCDD was tested using an approach similar to that used to test the role of IL-10, using an antibody to neutralize CD25 function. We first tracked the TCDD-mediated increase in CD25 expression early in the response (day 2) and later in the response (day 15). As previously shown, treatment with TCDD induced a CD4$^+$CD25^{high} T-cell phenotype on day 2 (Figure 4-2A). Furthermore, the level of CD25 expression was increased on a per cell basis (MFI) (Funatake *et al.* 2005).

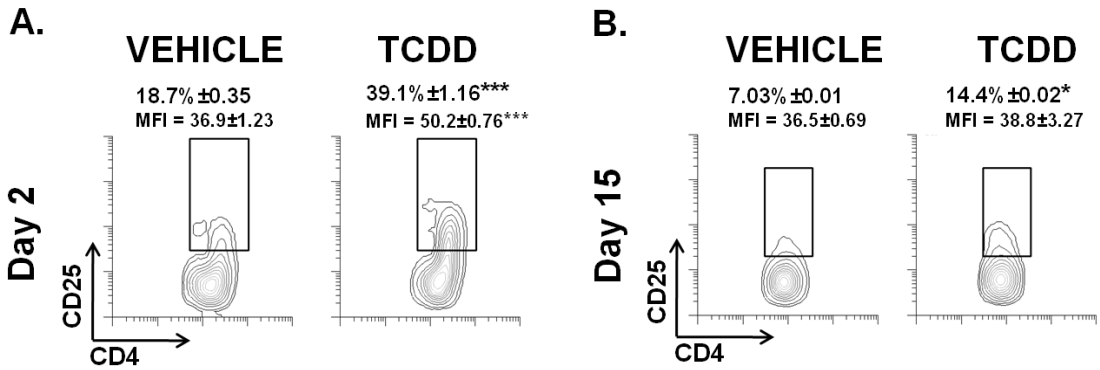

***Figure 4-2**. Activation of AhR by TCDD induces a CD4$^+$CD25$^+$ Treg phenotype. Donor splenocytes were adoptively transferred into B6D2F1 hosts concurrent with TCDD treatment. (A) Donor CD4$^+$ T-cells were analyzed 2 days after adoptive transfer for expression of CD25. Histograms are gated on Thy1.1$^+$CD4$^+$ T-cells, where Thy1.1 was used to identify donor cells. Histograms are representative of 3 biological replicates. MFI = median fluorescence intensity of CD25 expression. (B) Donor cells were analyzed 15 days after adoptive transfer. Histograms are gated on H-2D^{dneg}CD4$^+$ to identify the donor T-cells. Histograms are representative of 5 biological replicates. Statistical significance was calculated with a student's t-test, where *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. †$p = 0.1$*

Despite the decrease in the number of donor cells from the spleen beginning on day 4 (Funatake *et al.* 2005), the CD4$^+$CD25^{high} Treg phenotype was lost by day 15 of the GVH response, as measured by analysis of CD25 MFI (Figure 4-2B). However, there was increased expression of donor CD4$^+$CD25$^+$ T-cells present in TCDD-treated hosts relative to vehicle (Figure 4 – 2). Therefore, treatment with TCDD appears to induce CD25 expression in CD4$^+$

T-cells that persists through the GVH response although the $CD25^{high}$ sub-population appears to be transient.

Effect of CD25 blockade on CTL function and phenotype

The increase in CD25 expression on day 2 has previously been shown to result in increased CD25 signaling, as assessed by STAT5 phosphorylation (Funatake 2006). CD4+CD25+ Treg have been shown to utilize multiple suppressive pathways through functional expression of CD25, including induction of TGFβ1, or suppression of IL-2 in responder cells (Shevach 2002). To determine if the AhR-Treg uses CD25 expression or signaling to mediate suppression of GVHD, we blocked CD25 signaling with a neutralizing antibody, administered one day after adoptive transfer. Control animals received sham treatments with PBS.

All vehicle-treated animals exhibited symptoms characteristic of GVHD, with significant weight loss, diminished splenocyte numbers, and increased donor $CD8^+$ T-cell engraftment (Figure 4-3A – C). The host $CD19^+$ B-cell population was also significantly depleted in vehicle-treated animals, indicating the engrafted donor $CD8^+$ T-cells were functional CTL (Figure 4-3D). In contrast to previous reports (Schulz *et al*. 2011; Zhang *et al*. 2010), treatment with anti-CD25 had no effect on any of these parameters. Therefore, we later validated the efficacy of the anti-CD25 mAb by evaluating the expected decrease in Foxp3 expression. Expression of Foxp3 in donor $CD4^+$ T-cells was significantly decreased following treatment with the anti-Cd25 mAb (Figure 4 – 4), thereby validating the efficacy of the antibody.

As expected, treatment with TCDD preserved the number of splenocytes and the host B-cell population, while attenuating donor $CD8^+$ T-cell engraftment. Surprisingly, blockade of

CD25 had no effect on T-cell engraftment or CTL activity. Therefore, despite the significant up-regulation of CD25 on $CD4^+$ T-cells on day 2 and day 15, there does not appear to be a functional requirement or contribution for CD25 expression in the TCDD-mediated immunosuppression of murine GVHD.

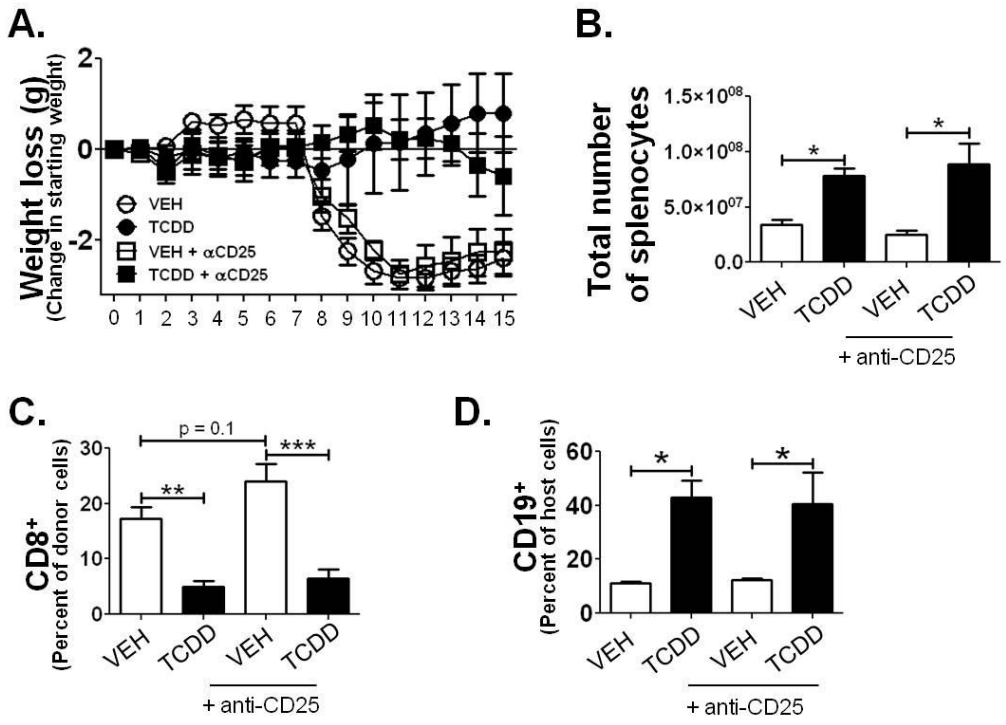

Figure 4-3. *Blockade of CD25 does not alleviate TCDD-mediated immunosuppression. Host animals received 15 µg/kg TCDD or vehicle at the time of adoptive transfer. One day after adoptive transfer recipient mice received 250 µg of anti-CD25 mAb via intraperitoneal injection. (A) Changes in weight were monitored throughout the experiment. (B) The total number of splenocytes, a mixture of host and donor cells, was enumerated 15 days after adoptive transfer. (C) Percent of donor $CD8^+$ T-cells in host spleen. (D) The cytolytic activity of the donor $CD8^+$ T-cells was assessed as a function of remaining host B-cells. Host B-cells were identified as H-$2D^{d+}CD19^+$. Data are representative of 5 biological replicates per treatment group. Error bars represent the mean ± SEM. Statistical significance was calculated with a student's t-test, where *$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$.*

Involvement of Foxp3$^+$ T-cells in TCDD-mediated immunosuppression

In our seminal 2005 publication (Funatake *et al.* 2005), we described a $CD4^+CD25^{high}IL\text{-}10^+$ Treg phenotype that was Foxp3-negative, in apparent contrast to other reports describing the TCDD-mediated regulation of Foxp3 via binding to dioxin responsive elements within the promoter of Foxp3 (Kerkvliet *et al.* 2009; Kimura *et al.* 2008; Quintana *et al.* 2008; Veldhoen *et al.* 2008). However, in the present study, when we examined Foxp3 expression to validate our anti-CD25 treatment regimen, we identified increased Foxp3 frequency and expression in both the donor $CD4^+$ and $CD8^+$ T-cells of TCDD-treated animals on day 15 of the GVH response (Figure 4-4). In sum, this data agrees with our previous data, showing clear evidence that AhR induces a novel Treg (Funatake *et al*. 2005), which later acquires Foxp3 expression, and is not indicative of expansion from a preexisting Treg population, as has been suggested by others (Stockinger 2009).

Despite the increase in Foxp3, the concurrent suppression of both $CD25^+$ and $Foxp3^+$ T-cells had no effect on CTL function and phenotype (Figure 4-3). Thus, while TCDD induces a $Foxp3^+$ subset in both $CD4^+$ and $CD8^+$ donor T-cells, this subset is not required for TCDD-mediated suppression of the CTL response, indicating that the induction of Foxp3 is not specific to activated, allo-specific T-cells. To test this theory, we examined Foxp3 expression in un-activated $CD4^+$ host cells. By day 15, the expression of Foxp3 in TCDD-treated animals was significantly higher relative to vehicle, in both percent and total number (Figure 4-5). This suggests that while activated AhR regulates Foxp3, the regulation is not specific to regulatory cells and regulatory function.

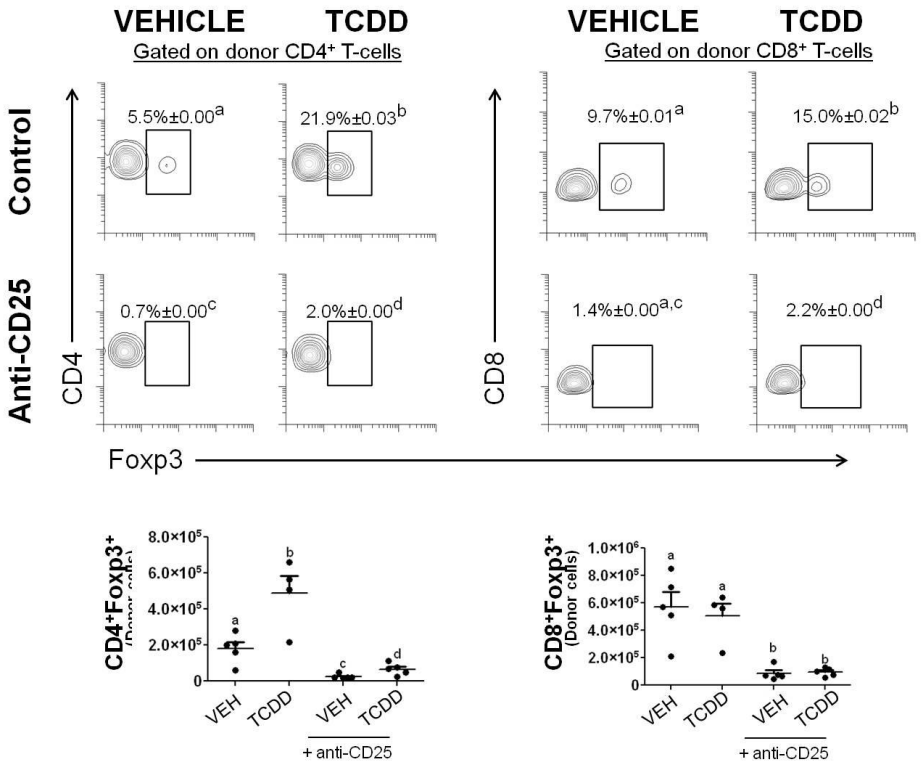

***Figure 4-4**. Treatment with TCDD induces a Foxp3$^+$ population in donor cells that is suppressed upon treatment with an anti-CD25 mAb. Histograms are gated on H-2D^{dneg}CD4$^+$ or H-2D^{dneg}CD8$^+$ T-cells to identify the donor T-cell populations in the spleen of recipient animals on day 15. Histograms are representative of 5 biological replicates. Total numbers of H-2D^{dneg}CD4$^+$Foxp3$^+$ and H-2D^{dneg}CD8$^+$Foxp3$^+$ donor cells were determined by multiplying the percent positive cells by the total number of donor cells in the host spleen. Statistical differences ($p \leq 0.05$) are denoted by different alphabetical letters, with similar letters indicating no statistical difference between values. Error bars represent the mean ± SEM.*

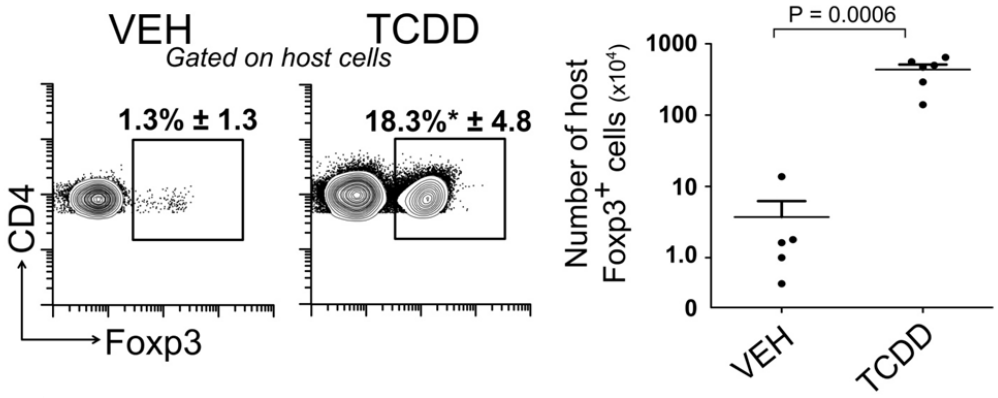

Figure 4 – 5*. Treatment with TCDD induces a Foxp3$^+$ population in host CD4$^+$ T-cells 15 days after adoptive transfer. Histograms are gated on H-2D^{d+}CD4$^+$ T-cells to identify the host T-cell populations in the spleen of recipient animals on day 15. Histograms are representative of 5 biological replicates. Total numbers of H-2D^{d+}CD4$^+$Foxp3$^+$ host cells were determined by multiplying the percent positive cells by the total number of host cells in the host spleen. Significance was determined with a student's t-test, with *p<0.05. Error bars represent the mean ± SEM.*

Discussion

Following the discovery that TCDD induces AhR-dependent Tregs during the GVH response, similar discoveries were made in other T-dependent disease models (Marshall and Kerkvliet 2010). Interestingly, both $Foxp3^{+}$ and $Foxp3^{neg}$ AhR-Treg have been described, confusing the requirement for Foxp3 expression in AhR-mediated immunosuppression (Kerkvliet *et al*. 2009; Quintana *et al*. 2008; Vogel *et al*. 2008), although it has been established that AhR regulates Foxp3 expression (Quintana *et al*. 2008; Quintana *et al*. 2010). Given the $Foxp3^{neg}$ phenotype of the AhR-Treg on day 2 of the GVH response, we have focused primarily on this phenotype in the search to identify the mechanism of suppression utilized by these regulatory cells. Specifically we focused on CD25 and IL-10, both increased by TCDD treatment (Funatake *et al*. 2005; Gandhi *et al*. 2010; Marshall *et al*. 2008) for their potential contributions to TCDD-mediated suppression of the CTL response.

We were initially interested in IL-10 as a soluble factor released from AhR-dependent $CD4^{+}CD25^{high}$ Treg, which would then mediate the subsequent suppression of the CTL response. However, while vehicle-treated animals displayed increased CTL engraftment following blockade of IL-10, validating the treatment regimen, no effect was observed in TCDD-treated animals. Initially considered a Treg-associated cytokine, IL-10 has recently been identified as a hallmark cytokine of Tr1 regulatory cells. Tr1 cells are defined by the transcription factors AhR and cMAF, and have been shown to suppress EAE (Apetoh *et al*. 2010). AhR and cMAF physically associate to transactivate both IL-21 and IL-10 (Apetoh *et al*. 2010). However, despite the documented effect of activated AhR on IL-10 production, Tr1 cells appeared to utilize granzyme B to mediate suppression (Gandhi *et al*. 2010). Therefore, the lack of IL-10 involvement in TCDD-mediated immunosuppression is perhaps unsurprising.

We next evaluated the role of CD25 in TCDD-mediated suppression of the CTL response. As previous reports have identified a role for CD25 in TCDD-mediated immunosuppression (Schulz *et al*. 2011; Zhang *et al*. 2010), we were surprised to see that blockade of CD25 had no effect on weight loss, donor cell engraftment or CTL activity in TCDD-treated animals. In sum, blockade of CD25 was unable to reverse the suppressive effect of TCDD-activated AhR. This is in direct contrast to the results obtained by Schulz *et al*. (2011), who reported a partial alleviation of TCDD-mediated immunosuppression in the Th2-mediated murine peanut allergy model. Similarly, Zhang *et al*. (2010) found that blockade of CD25 and Foxp3 by the same anti-CD25 mAb significantly reversed the protection of TCDD in EAU, as assessed by clinical and pathological scores. To ensure the lack of effect noted in our studies was not due to incomplete CD25 neutralization, we assessed expression of Foxp3 within the donor T-cells, ostensibly evaluating the effect of the anti-CD25 antibody on Foxp3 expression by natural Treg. Animals that received the anti-CD25 mAb had significantly attenuated levels of Foxp3, thereby validating the treatment regimen. Given these results, we conclude that within the acute, parent-into-F1 GVHD model used herein, neither CD25 nor Foxp3 expression is required for TCDD-mediated suppression of the CTL response. The disparities between our study and those previously reported include differences in dosing regimens for both TCDD and the anti-CD25 mAb. Our dosing regimen was specifically limited to the period of T cell differentiation, and used a total of 300 μg anti-CD25 mAb. In contrast, Shulz *et al*. (2011) used a total dose of 1.2 mg anti-CD25 mAb administered over 18 days, while Zhang *et al*. (2010) used a 200-fold higher amount of anti-CD25 mAb. Thus, the discrepancies between our results and those of others may be reflective of different dosing regimens. However, the discrepancies between the results presented herein and those reported previously (Shulz *et al*. 2011; Zhang *et al*. 2010) may also suggest that

TCDD-activated AhR has the potential to induce Tregs with differential function dependent upon the environment. We have previously shown that the preexisting cytokine milieu has strong effects on TCDD-mediated perturbations during $CD4^+$ T-cell differentiation (Rohlman *et al*. 2012). GVHD is a Th1-mediated disease, whereas EAU is mediated by a complex interplay between Th1 and Th17 cells (Feng *et al*. 2011), and Th2 cells mediate peanut allergy. Therefore, the different disease environments may alter the suppressive mechanism of the AhR-dependent Treg. However, as functional analysis of TCDD-induced Tregs from different murine models has not been conducted, we can only speculate that TCDD-activated AhR induces Tregs that adapt to the existing disease state.

Previous work in our lab characterized the TCDD-induced Treg to be Foxp3-negative on day 2 of the GVH response (Funatake *et al*. 2005). However, Foxp3 is up-regulated by day 15, thereby reconciling our data with earlier reports that have shown Foxp3 to be regulated by TCDD-activated AhR (Kimura *et al*. 2008; Quintana *et al*. 2008; Veldhoen *et al*. 2008). The immunosuppressive effect of TCDD was at one point thought to be due to the death of effector cells, thereby leading to an artificial increase in natural $Foxp3^+$ Tregs (Stockinger 2009). However, our results argue against the preservation of an existing population, as the $CD4^+CD25^{high}$ Treg are initially Foxp3-negative. Furthermore, we have previously shown that TCDD-activated AhR induces Treg, as depletion of $CD4^+CD25^+$ T-cells from the donor cell inoculum did not prevent induction of $CD4^+CD25^{high}$ Treg by day 2 of the GVHD response (Funatake *et al*. 2005). Of interest, the increase in Foxp3 was not specific to activated, allo-specific donor $CD4^+$ T-cells, as host $CD4^+$ T-cells from TCDD-treated animals also exhibited increased frequency and expression of Foxp3. Therefore, the regulation of Foxp3 expression appears to be nonspecific, resulting in increased Foxp3 expression in activated and un-activated T-cells. Therefore, our results, taken with previous results, suggest that TCDD-

activated AhR induces a regulatory T-cell that acquires Foxp3 at a later date, but which maintains a suppressive mechanism independent of Foxp3, as concurrent neutralization of CD25 and Foxp3 was unable to rescue TCDD-mediated immunosuppression.

In sum, IL-10, CD25 and Foxp3 do not appear to be required for TCDD-mediated immunosuppression in the GVH response. Future work will evaluate alternative suppressive mechanisms in an attempt to identify the direct effect of the AhR-Treg on the overall suppression of the GVH response.

Chapter 5

CONCLUSIONS

The discovery of TCDD-induced Tregs in 2005 (Funatake *et al*. 2005) initiated interest in the AhR beyond the realm of traditional immunotoxicology to the broader discipline of T-cell immunology. Within the last decade, significant progress has been made in improving our understanding of the cellular and molecular events that occur as a consequence of AhR activation in T-cells. Despite these advances, the signaling events that precede AhR-dependent Treg induction, as well as the immunomodulatory mechanisms used by these Tregs, remain uncharacterized. Therefore, the focus of the studies described herein was to determine the role of AhR in $CD4^+$ T-cell differentiation, as well as to evaluate the mechanism of suppression associated with AhR-induced Tregs.

As a transcription factor, the AhR may perturb $CD4^+$ T-cell polarization leading to direct induction of Treg. Therefore initial studies targeted the signaling events that occur during $CD4^+$ T-cell activation and differentiation. Surprisingly, the signaling events required for differentiation of Th1, Th17, Th22, Treg and Tr1 were unaffected by TCDD-activated AhR. However, *Il22* was identified as a direct target of AhR, and the increase in IL-22 transcript translated to increased IL-22 protein. These data support the recent finding that AhR is involved in Th22 $CD4^+$ T-cell differentiation (Ramirez *et al*. 2010; Trifari *et al*. 2009). Production of IL-22 has both pro-inflammatory and protective functions, as reviewed in Zenwicz and Flavell (2011). In the context of GVHD, adoptive transfer of IL-22-deficient T-cells exacerbated GVHD mortality (Hanash *et al*. 2012). The role of TCDD-induced IL-22 in the subsequent suppression of GVHD remains an area of interest.

In addition to the up-regulation of IL-22, known AhR-regulated genes *Cyp1a1*, *Cyp1b1*, *Arnt* and *Ahrr* were also up-regulated by TCDD *in vitro*, although the overall lack of effect of TCDD-activated AhR on genes associated with $CD4^+$ T-cell activation and differentiation suggest that the effect of TCDD-activated AhR is superimposed upon the signaling events that drive T-cell differentiation. In this scenario, the role of AhR might be directed by the preexisting cytokine milieu or by the master regulator genes. Such a scenario has been demonstrated previously, revealing that Treg are capable of adapting to different environmental cues (Murphy and Stockinger 2010; Wing and Sakaguchi 2012). As the *in vitro* system relied upon exogenous and controlled cytokine additions, such a system may not accurately represent the environment found in animals during an immune response. Recent studies in the Kerkvliet laboratory utilizing microarray analysis of purified $CD4^+$ T-cells sorted from animals undergoing GVHD may be able to elucidate signaling pathways perturbed by TCDD-activated AhR during $CD4^+$ T-cell differentiation.

In addition to understanding the molecular mechanisms leading to induction of the AhR-induced Treg, understanding the suppressive mechanism(s) used by the AhR-Treg remains of great interest. Currently, there is no direct evidence to conclude that the AhR-dependent Treg is necessary for suppression of the CTL response in the Th1-mediated GVHD model. Initially characterized as $CD4^+CD25^{high}$CTLA-$4^+Foxp3^{neg}IL\text{-}10^+$, the AhR-dependent Treg identified in GVHD have a suppressive capacity greater than that of natural Treg (Funatake *et al.* 2005; Marshall *et al.* 2008). In an attempt to identify the suppressive function of the AhR-dependent Treg, phenotypic components of the Treg were systematically blocked to determine the functional requirement of those proteins in the suppression of the CTL response. Targets of interest were CTLA-4, IFNγ, IDO, IL-10 and CD25, identified in a microarray analysis of donor T-cells from TCDD-treated host animals, and *in vivo*, up-

regulated in both transcript and protein in TCDD-treated animals (Funatake *et al.* 2005; Koenecke *et al.* 2012; Marshall *et al.* 2008; Vogel *et al.* 2008) Furthermore, these markers are inherently associated with Treg phenotype and function (von Boehmer 2005). Using antibody blockades, pharmacological inhibitors and transgenic donor animals, the expression or function of CTLA-4, IFNγ, IDO or IL-10 was attenuated or ablated. However, analysis of the resultant GVHD response by examination of body weight and donor cell engraftment revealed that these proteins are not required for TCDD-mediated immunosuppression.

During the course of these studies, it was revealed that treatment with TCDD appeared to induce Foxp3 expression in donor T-cells on day 15 in agreement with previous reports (Benson and Shepherd 2011; Kerkvliet *et al.* 2009; Kimura *et al.* 2008; Quintana *et al.* 2008; Singh *et al.* 2011; Vogel *et al.* 2008; Zhang *et al.* 2010). This was surprising, as the AhR-Treg is initially defined to be Foxp3-negative, suggesting the AhR-Treg acquires Foxp3 expression at a later date. However, concurrent blockade of CD25 and Foxp3 had no effect on the GVHD response in TCDD-treated animals, suggesting neither CD25 nor Foxp3 are required for TCDD-mediated immunosuppression. These results were unexpected, as CD25 and Foxp3 have not only been shown to be highly up-regulated in TCDD-treated animals, but also have known roles in immunosuppression (Funatake *et al.* 2005; Quintana *et al.* 2008; von Boehmer 2005). Furthermore, previous studies have shown that the concurrent neutralization of CD25 and Foxp3 resulted in a partial alleviation of TCDD-mediated immunosuppression (Schulz *et al.* 2011; Zhang *et al.* 2010), in direct contrast to the results presented herein. However, those disease models were dependent upon Th2 and Th17 cells, versus the requirement for Th1 cells in the GVHD model. The discrepant results suggest the AhR-dependent Treg has significant flexibility in function, potentially altering the suppressive mechanism in response to the existing disease environment.

Recent evidence supports inherent flexibility in $CD4^+$ Treg. For example, in Th1-mediated diseases, Treg express the Th1 transcription factor, Tbet, in response to the Th1-associated cytokine IFNγ (Koch *et al.* 2009). Furthermore, the expression of Tbet was required for the Treg to regulate Th1-mediated disease (Koch *et al.* 2009). Additionally, Treg express GATA-3, the Th2 transcription factor, under Th2 conditions (Wang *et al.* 2011), and Treg that mediate suppression of Th17-dependent diseases express Stat3, a Th17-associated transcription factor (Chaudhry *et al.* 2009). Therefore, the AhR-induced Treg may have a flexible program, capable of responding to the disease environment and thereby adapting the suppressive function (Murphy and Stockinger 2010; Wing and Sakaguchi 2012). From the studies described herein, we know that TCDD-activated AhR does not perturb the expected pattern of gene expression during T-cell differentiation, resulting in expression of Th-associated cytokines and transcription factors. Therefore, the AhR-dependent Treg may respond to the underlying signaling events that occur during $CD4^+$ T-cell differentiation, initiating a specific suppressive mechanism capable of suppressing disease symptoms. This is supported by the recent findings highlighting different mechanisms for TCDD-activated AhR to mediate suppression, to include CD25-dependent, IDO-dependent and Granzyme-B-dependent mechanisms (Gandhi *et al.* 2010; Mezrich *et al.* 2010; Schulz *et al.* 2011; Zhang *et al.* 2010). Therefore, identification of a specific AhR-dependent pathway in Th2- or Th17-mediated diseases may not be applicable to the pathway used in a Th1-mediated disease, such as GVHD.

In sum, the studies presented here describe a unique AhR-dependent Treg induced during the GVH response that does not appear to use a mechanism of suppression involving CTLA-4, IFNγ, IDO, CD25, IL-10 or Foxp3. The apparent ability of the AhR-induced Treg, to adapt to various disease states strengthens the characterization of the AhR as a therapeutic

target. Future work should examine the requirement for Th1-associated transcription factors and cytokines in the TCDD-mediated suppression of the Th1-mediated disease GVHD, to examine the interplay between environmental factors, such as cytokines and transcription factors, and TCDD-activated AhR. Additionally, understanding the differential methods of suppression employed by AhR-dependent Treg within different *in vivo* disease models will be necessary to fully understand the role of AhR in immunity and suppression of immune-mediated diseases.

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APPENDIX

Appendix A

Effect of TCDD on allo-specific $CD4^+$ T-cells activated under normoxic or hypoxic conditions

Diana Rohlman

Jamie Pennington

Sumit Punj

Charles S. Bradford

Nancy I. Kerkvliet

Abstract

Following activation by the prototypic ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the aryl hydrocarbon receptor (AhR) translocates to the nucleus and binds with the aryl hydrocarbon receptor nuclear translocator (ARNT), also known as hypoxia-inducible factor 1β (HIF-1β). In the acute graft-versus-host (GVH) model, suppression of the allo-specific T-cell response is dependent upon expression of AhR within the donor $CD4^+$ and $CD8^+$ T-cell subsets. Following activation of AhR, TCDD induces a characteristic T-regulatory (Treg) T-cell phenotype with suppressive function, although the mechanism of induction remains unknown. To date, no *in vitro* assay has been capable of replicating the *in vivo* Treg phenotype. Multiple factors, such as insufficient tryptophan levels, the cytokine environment and accessory cell involvement have been suggested as the crucial missing link to developing an *in vitro* assay. However, the impact of differing oxygen concentrations between *in vivo* and *in vitro* conditions has not been evaluated. *In vivo*, oxygen tension in the spleen is measured at 2.5% O_2, significantly lower than the 20% O_2 used under typical *in vitro* culture conditions. Under such low O_2, HIF-1, a heterodimer consisting of HIF-1α and ARNT, is up-regulated. The possibility exists that activated AhR sequesters ARNT, disrupting HIF-1 function and inducing $CD4^+$ T-regulatory cells in the absence of negative effects on proliferation, T-cell activation and viability. In an *in vitro* semi-allogeneic co-culture model, treatment with 10nM TCDD inhibits IL-17A and induces IL-22 under hypoxic conditions, without adverse effects on $CD4^+$ T-cell activation, proliferation or viability.

Introduction

Upon activation by the prototypic ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the aryl hydrocarbon receptor (AhR) translocates to the nucleus and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). Long considered an environmental contaminant, TCDD is a potent immunosuppressant, efficiently suppressing multiple immune-mediated disease, including autoimmune diseases such as Type 1 diabetes, experimental autoimmune encephalomyelitis (EAE) and experimental colitis (Benson and Shepherd 2011; Kerkvliet *et al.* 2009; Quintana *et al.* 2008; Singh *et al.* 2011; Takamura *et al.* 2010). Suppression is concurrent with the induction of a regulatory T-cell subset (Treg) (Benson and Shepherd 2011; Kerkvliet *et al.* 2009; Quintana *et al.* 2008; Takamura *et al.* 2010).

The potent immunosuppression observed with TCDD has been extensively studied in the murine graft-versus-host (GVH) model, wherein donor T-cells ($H\text{-}2^{b/b}$) are injected into B6D2F1 ($H\text{-}2^{b/d}$) host mice treated with either vehicle or 15μg/kg TCDD. In vehicle-treated animals, the donor cells develop into cytotoxic T-lymphocytes (CTL) and destroy host cells (Kerkvliet *et al.* 2002). However, in TCDD-treated animals, the donor cells develop a Treg phenotype characterized by high levels of CD25, CTLA-4 and IL-10, with low CD62L expression, that precedes suppression of the CTL response (Funatake *et al.* 2005; Marshall *et al.* 2008). To date, no *in vitro* assay has been designed that is capable of replicating the *in vivo* phenotype, thus confounding detailed mechanistic studies of the TCDD-mediated Treg induction.

There are multiple possibilities underlying the inability to recapitulate the *in vivo* phenotype, including the absence of necessary cytokines, inadequate tryptophan levels, TCDD

toxicity, and the requirement of accessory cells, such as dendritic cells. Another factor to consider is the O_2 concentration under which cells are cultured. Typically, splenocytes are cultured at 5% $C0_2$, 37°C in humidified conditions. This is not representative of conditions in the spleen, the lymphoid organ often used to generate $CD4^+$ T-cells for culture. Direct, *in vivo* measurements of oxygen tension in the murine spleen reveal a hypoxic environment, with 2.5% oxygen and 5% $C0_2$ (Caldwell *et al.* 2001).

Under conditions of low O_2, the hypoxia inducible factor-1 (HIF-1) is induced. Multiple roles for HIF-1 have been identified, including roles in embryogenesis, tumor progression, cell proliferation and viability, and angiogenesis, amongst others (Semenza 2000). HIF-1 is composed of two subunits, HIF-1α and HIF-1β, the latter of which is also known as ARNT (Semenza 2007). While HIF-1β is constitutively expressed, the HIF-1α subunit is regulated by O_2 (Semenza 2007). Under low O_2 levels, HIF-1α protein accumulates and dimerizes to HIF-1β; the resulting complex binds to hypoxia response elements in target genes (Semenza 2007).

The recent publication implicating HIF-1 regulation of the Th17/Treg balance reveals a clearly defined mechanism wherein HIF-1 induces transcription of RORγt, the master transcription factor for the Th17 lineage (Dang *et al*. 2011). Induction of RORγt induces IL-17A, IL-17F and IL-23R (Dang *et al*. 2011). Furthermore, HIF-1 skews the balance towards Th17 by mediating proteasomal degradation of Foxp3, the master transcription factor for Tregs (Dang *et al*. 2011). $CD4^+$ T-cells deficient for HIF-1α were deficient for IL-17A/F and IL-23R (Dang *et al*. 2011). Additionally, HIF-$1\alpha^{-/-}$ mice are protected against EAE, along with concomitant increases in the $Foxp3^+$ Treg population (Dang *et al*. 2011).

ARNT is shared by both AhR and HIF-1α as a dimerization partner, suggesting that high levels of AhR may sequester ARNT, disrupting HIF-1 function. Transcriptional analysis

of differentiating $CD4^+$ T-cells reveals very high levels of *Ahr* under Th17, Treg and Tr1 $CD4^+$ T-cells at 24 and 48 hours after activation (Rohlman *et al*. 2012; Kimura *et al*. 2008; Veldhoen *et al*. 2008). As such, the potential for AhR to sequester ARNT under these conditions is high.

Multiple Th17-mediated diseases, such as EAE and colitis are suppressed following activation of AhR by TCDD, suggesting that Th17 differentiation is attenuated (Benson and Shepherd 2011; Quintana *et al*. 2008; Singh *et al*. 2011; Takamura *et al*. 2010). Additionally, suppression is concurrent with increased Treg cell frequencies, similar to the profile exhibited with disrupted HIF-1 function (Semenza 2007).

To address the possibility that TCDD-activated AhR perturbs HIF-1 function, resulting in a Treg phenotype, we developed an *in vitro* semi-allogeneic co-culture system wherein naïve ($CD4^+CD62L^+$) T-cells were titrated into culture with semi-allogeneic $CD11c^+$ DCs. The effect of TCDD on the activation, proliferation and differentiation of $CD4^+$ T-cells under normoxia (20% O_2) and hypoxia (2.5% O_2) was evaluated.

Materials and Methods

Animals

C57Bl/6 (H-$2^{b/b}$) and B6D2F1 (H-$2^{b/d}$) mice were obtained from Jackson Laboratories, and housed in the pathogen-free lab Laboratory Animal Resource Center at Oregon State University, Corvallis OR, 97331. Food and water were provided *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Isolation of $CD4^+CD62L^+$ cells

Spleens were harvested from C57Bl/6 mice and processed into a single-cell suspension by pressing the tissues through a 70µm nylon mesh sieve (Falcon). Red blood cells were removed via hypotonic water lysis. $CD4^+CD62L^+$ T-cells were then isolated using the $CD4^+CD62L^+$ II kit (Miltenyi), following manufacturer's instructions. Purity was routinely ≥85%. Following isolation, cells were stained with CFSE (Invitrogen) and titrated into culture with purified $CD11c^+$ DCs.

Isolation of $CD11c^+$ DCs

Spleens were harvested from B6D2F1 mice, minced into small fragments and incubated at room temperature, with rocking, for 30 minutes in Spleen Dissociation Medium (StemCell, Vancouver, BC, Canada). The spleen suspension was incubated for an additional 5 minutes following addition of 10mM EDTA, then pressed through a 70µm nylon mesh sieve (Falcon). A dense BSA gradient was created by layering 106g BSA over a solution containing 186ml PBS, 29ml 1N NaOH and 65ml water, and incubating at 4°C overnight. The density was corrected by adding BSA or PBS to ensure a refractive index of 1.384-1.385, corresponding to a density of 1.080g/ml. Cells were resuspended in the BSA gradient, and

1.5ml of RPMI 1640 (Invitrogen) was layered over the top in a 15ml Corex tube. The tubes were centrifuged at 9,500g for 15 minutes, 4°C, using slow acceleration/brake settings. Low density splenocytes (B-cells, macrophages, dendritic cells, lymphocytes) were collected from the interface. $CD11c^+$ cells were isolated using the EasySep® Mouse CD11c Positive Selection Kit (StemCell), following manufacturers' instructions. The purity of the $CD11c^+$ cells was routinely ≥85%.

CD4:DC co-cultures

DC's were titrated into $CD4^+CD62L^+$ cultures at a 20:1 CD4:DC ratio in a 96-well V-bottom plate (Corning) and cultured under either normoxic or hypoxic conditions as described, for 3 days.

Normoxic and hypoxic culture conditions

Cells cultured under normoxia were incubated at 37°C, 5% $C0_2$, 20% 0_2, 75% N_2 in a humidified incubator (Caron). Cells cultured under hypoxia were incubated at 37°C, 5% $C0_2$, 2.5% 0_2 and 92.5% N_2 in a humidifed InVivo 300 hypoxia chamber (Ruskinn). Under normoxic conditions, cells were cultured in RPMI 1640 (Invitrogen), containing 10% FBS, 10mM HEPES, 50µg/ml gentamicin and 50µM 2-ME. For cultures under hypoxia, culture media was deficient for 2-ME, as cells grown at 2.5% O_2 plus 2-ME were found to have a significant decline in the total number of cells and attenuated T-cell differentiation (Caldwell *et al*. 2001).

Flow cytometry

After three days in culture, supernatants were collected and CD4:DC aggregates were dissociated by re-suspending the cells in 200µl of 0.5mM EDTA and incubating for an additional 7 minutes. The cells were washed twice with PBS containing 10% BSA and 5% sodium azide (PAB), and incubated with rat IgG for 10 minutes. mAbs to CD62L (MEL-14) and CD25 (PC61.5) were purchased from BD Biosciences and eBioscience, respectively. Following surface staining, cells were resuspended in fixable viability dye (eBioscience) for 20 minutes, and washed twice prior to analysis.

Control samples that contained all of the antibodies except the one of interest (fluorescence minus one) were used as negative staining controls. A minimum of 300,000 events were collected per sample on a Beckman Coulter FC-500 flow cytometer. Compensation and analysis of data were performed in WinList (Verity Software, Version 6.0).

Cytokine analysis

Supernatants were collected and stored at -80°C. Production of IL-10 and IL-22 was analyzed using the Ready-Set-Go® ELISA kits (eBioscience), following manufacturer's instructions. Data were analyzed on a Spectra MAX 250 (Molecular Devices). Production of IL-6 and IL-17A was measured using the IL-17A and IL-6 FlowCytomix Simplex kits (eBioscience). Data were analyzed using FlowCytomixPro software (v2.4, eBioscience).

Statistical analysis

To compare between treatment groups (vehicle versus TCDD), a student's t-test was used for statistical analysis, where n = 3-6 technical replicates.

Results

Development of an *in-vitro* semi-allogeneic co-culture assay

We first developed an *in vitro* assay designed to mimic the activation events occurring in the GVH model. CFSE-labeled $CD4^+$ ($H\text{-}2^{b/b}$) T-cells were titrated into culture with purified $CD11c^+$ DCs ($H\text{-}2^{b/d}$), resulting in activation of allo-specific $CD4^+$ T-cells. Proliferation and activation of $CD4^+$ T-cells was assessed by CFSE dilution, and expression of the activation markers CD25 and CD62L.

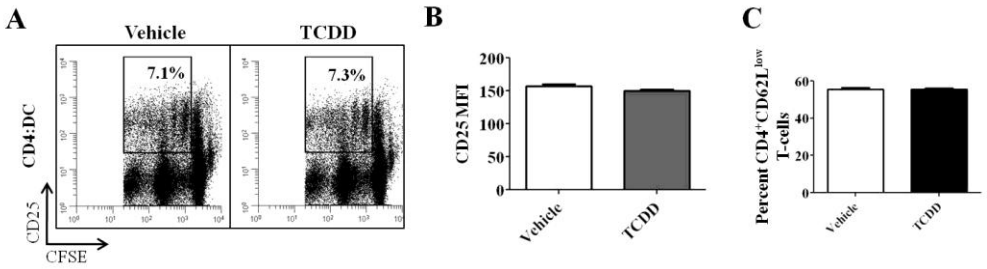

Figure A-1*. Effect of TCDD on the activation of allo-specific $CD4^+$ T-cells. $CD4^+CD62L^+$ T-cells were stained with CFSE prior to culture with purified $CD11c^+$ DCs at a 20:1 CD4:DC ratio. At culture initiation, cells were treated with either vehicle or 10nM TCDD. After 3 days in culture, cells were harvested and stained for expression of CD25. Histograms are gated on CFSE+ T-cells, and are representative of n = 3 technical replicates. (A) The frequency of allo-specific T-cells was measured by the expression of CD25. Only actively dividing cells, identified by CFSE dilution, were analyzed. (B) The median fluorescent intensity of CD25 expression on allo-specific $CD4^+$ T-cells. (C) The percent of $CFSE^+CD62L^{low}$ T-cells. n = 3 technical replicates. Error bars represent the mean ± SEM.*

In both vehicle- and TCDD-treated co-cultures, approximately 7% of the $CD4^+$ T-cells were actively dividing in response to allogeneic stimulation, as assessed by CFSE dilution and concurrent expression of CD25, indicating $CD4^+$ T-cell activation was unaffected (Figure A-1A). Similarly, $CD4^+$ T-cell proliferation was unaffected.

In the GVH model, treatment with TCDD induces a $CD25^{high}$ population indicative of a Treg phenotype, in addition to low expression of CD62L (Funatake *et al.* 2005). In this

model system, treatment with 10nM TCDD did not induce a $CD25^{high}$ population, as measured by the median fluorescent intensity (Figure A-1B), nor alter the $CD62L^{low}$ population (Figure A-1C). Similarly, the $CD62L^{low}$ population and frequency were unaffected, indicating that this assay, conducted under normal O_2 conditions, was unable to replicate the TCDD-induced phenotype observed *in vivo* (Funatake *et al.* 2005). Furthermore, while semi-allogeneic DC activated $CD4^+$ T-cells, they were insufficient to induce the Treg phenotype. Therefore, the potential for TCDD-activated AhR to sequester ARNT and disrupt HIF-1 function under low O_2 conditions was examined by culturing CD4:DC co-cultures under hypoxic conditions.

Effect of TCDD on activated, allo-specific $CD4^+$ T-cells under hypoxic conditions

CD4:DC co-cultures were cultured under normoxic (20% O_2) or hypoxic (2.5% O_2) conditions in tandem for three days. $CD4^+$ T-cells were identified by expression of CFSE, and gated on viable events. Analysis of total $CD4^+CD25^+$ T-cells revealed that a hypoxic environment increased the total percent of $CD4^+CD25^+$ T-cells, although this was independent of treatment (Figure A-2A). The percent of allo-specific, actively proliferating $CD4^+CD25^+$ T-cells was unaltered by low O_2 conditions (Figure A-2A, see inset bars). Additionally, treatment with TCDD did not induce a $CD25^{high}$ population (Figure A-2B). Thus, hypoxic conditions were unable to recapitulate the $CD25^{high}$ TCDD-induced phenotype.

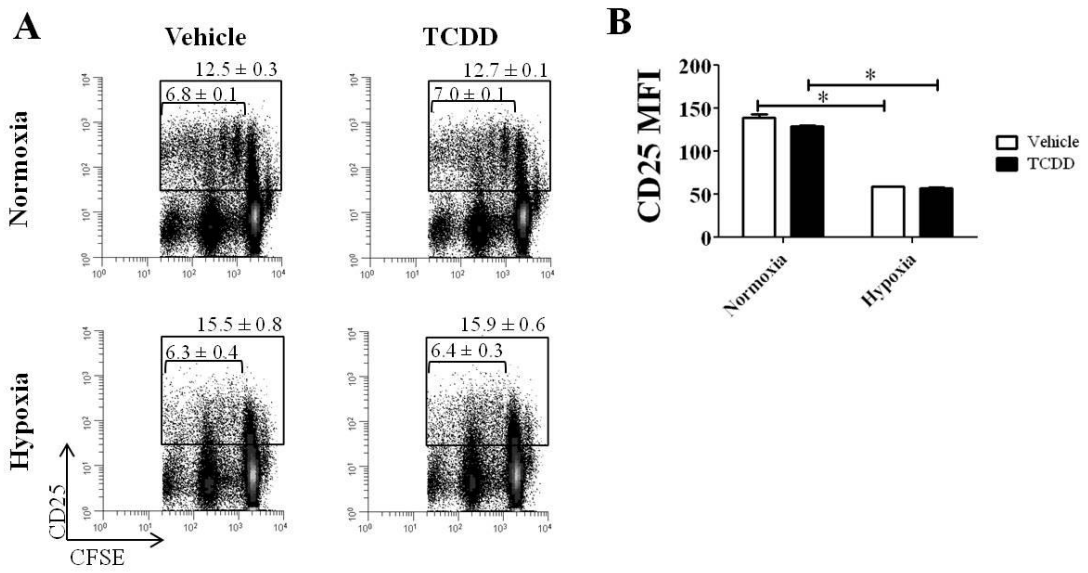

Figure A-2*. CD25 expression on CD4$^+$ T-cells cultured under normoxia or hypoxia with or without TCDD. CD4:DC co-cultures were cultured as described, and incubated under normoxic or hypoxic conditions in tandem for three days. Histograms are gated on CFSE+ T-cells, and are representative of n = 3 technical replicates. (A) Frequency of CFSE+CD25+ T-cells cultured under normoxia or hypoxia. Events within the boxed region are all the CFSE$^+$CD25$^+$ cells, while the inset bars indicate the percent of CFSE$^+$CD25$^+$ undergoing active division, based on CFSE dilution. (B) Median fluorescent intensity of CD25 for all CFSE$^+$CD25$^+$ T-cells. Significance was calculated with a student's t-test, where *p ≤ 0.05, n = 3. Error bars represent the mean ± SEM.*

TCDD alters cytokine production under conditions of low oxygen

Under hypoxic conditions, HIF-1 induces transcription of RORγt, leading to induction of the CD4$^+$ Th17 effector lineage (Dang *et al.* 2011). Th17 cells are induced in the presence of TGFb1 and IL-6, and express high levels of IL-17, and IL-22 (Veldhoen *et al.* 2008). In contrast, fully differentiated CD4$^+$ Tregs express IL-10. We investigated production of IL-6, IL-10, IL-17 and IL-22 in the supernatant following three days of culture under either normoxic or hypoxic culture conditions to determine if T-cell differentiation was perturbed.

Under normoxic conditions, treatment with TCDD had no effect on production of IL-6, IL-10 or IL-17 (IL-22 was undetected) (Figure A-3). Production of all four cytokines was

significantly increased under hypoxic conditions. Of note, IL-6 production declined (p = 0.2) and IL-17 production was significantly decreased (p = 0.04) in TCDD-treated co-cultures. IL-22 production was significantly increased in TCDD-treated cultures under hypoxia, although production of IL-10 was unaltered.

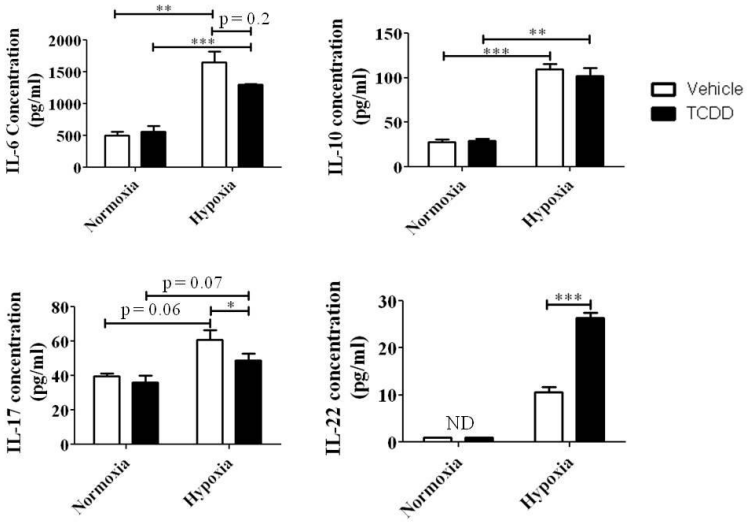

Figure A-3*. Effect of TCDD and hypoxia on cytokine production. CD4:DC co-cultures were harvested after three days of culture under normoxic or hypoxic conditions, and supernatants harvested. IL-6 and IL-17A production was measured using the FlowCytomix kit, while IL-10 and IL-22 were measured using the Ready-Set-Go® ELISA kits, both from eBioscience. Signficance was calculated with a student's t-test, n = 3-6 technical replicates. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Error bars represent the mean ± SEM.*

Effect of TCDD on $CD4^+$ T-cell proliferation and viability under normoxic and hypoxic conditions

Recently, the immunosuppressive effect of TCDD was attributed to the purported toxicity of TCDD, causing apoptosis in effector cells and creating an artificial expansion of Tregs (Stockinger 2009). Additionally, HIF-1 targets genes involved in cell proliferation and viability. Thus, we evaluated the effect of TCDD on proliferation and viability under conditions of differential O_2 concentrations (Semenza 2000).

Under normoxic conditions, cultures containing 10nM TCDD exhibited a mild yet significant decrease in overall culture viability (Figure A-4A) and total $CD4^+$ T-cell viability ($p < 0.05$) (Figure A-4B).

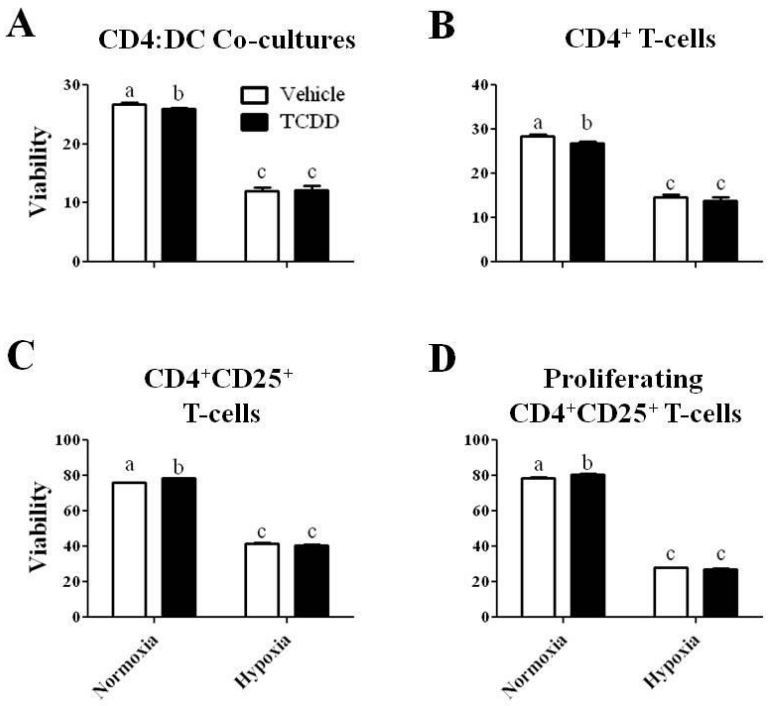

Figure A-4*. Viability of CD4:DC co-cultures after three days cultured under normoxic or hypoxic conditions. At culture initiation, co-cultures were treated with either vehicle or 10nM TCDD. (A) Total viability of CD4:DC co-cultures. (B) $CD4^+$ T-cells were identified by CFSE dilution, and viability assessed. (C) $CD4^+$ T-cells were identified as in B, and viability of activated ($CD25^+$) cells was examined. (D) Viability of actively dividing, $CD4^+CD25^+$ T-cells. Significance calculated with a student's t-test, where n = 3 technical replicates. Different letters indicate a significant difference ($p \leq 0.05$) between treatments. Error bars represent the mean ± SEM.*

However, analysis of allo-specific $CD4^+CD25^+$ T-cells revealed a minor increase in viability of TCDD-treated cultures ($p = 0.01$) (Figure A-4C). This effect was also seen upon analysis of actively proliferating $CD4^+CD25^+$ T-cells ($p = 0.01$) (Figure A-4D). CD4:DC co-cultures incubated under hypoxic conditions displayed significantly decreased viability relative to that

of cultures incubated under normoxic conditions (Figure A-4). However, treatment with TCDD had no effect on T-cell viability under hypoxic conditions.

Finally we tested the ability of TCDD to attenuate $CD4^+$ T-cell proliferation. Prior to culture, $CD4^+$ T-cells were stained with CFSE, and proliferation was assessed by CFSE dilution three days after culture initiation. Relative to normoxic conditions, incubation under low O_2 attenuated $CD4^+$ T-cell proliferation (Figure A-5). However, addition of 10nM TCDD at culture initiation had no effect on $CD4^+$ T-cell proliferation under either normoxic or hypoxic conditions. In summary, treatment with TCDD did not attenuate $CD4^+$ T-cell proliferation, nor result in decreased viability of activated, allo-specific $CD4^+$ T-cells

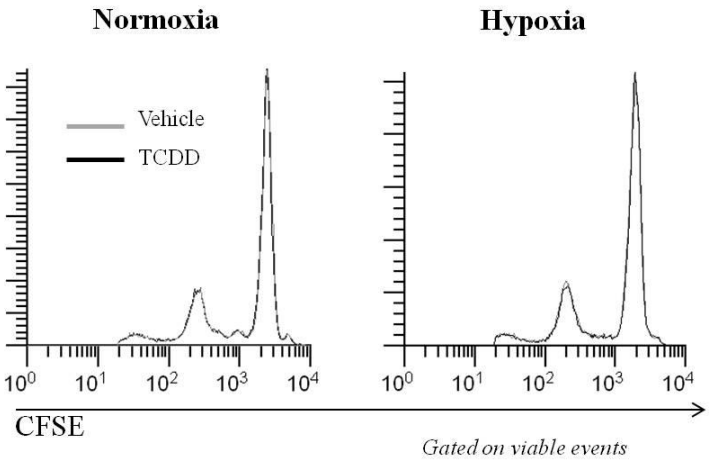

Figure A-5*. Effect of TCDD on the proliferation of semi-allogeneic $CD4^+$ T-cells cultured under normoxic or hypoxic conditions. Histograms are gated on viable $CFSE^+$ cells, and are representative of 3 technical replicates.*

Discussion

Continuing research efforts to identify the role of AhR in mediating $CD4^+$ T-cell differentiation have focused heavily on the Th17/Treg balance. Multiple *in vivo* models demonstrate the suppressive function concomitant with activation of AhR, (Marshall and Kerkvliet 2010), including Th17-mediated diseases such as EAE and colitis (Benson and Shepherd 2011; Quintana *et al.* 2008; Singh *et al.* 2011; Takamura *et al.* 2010). In many instances, suppression is linked with the induction of a regulatory $CD4^+$ T-cell (Apetoh *et al.* 2010; Funatake *et al.* 2005; Marshall *et al.* 2008). However, *in vitro*, addition of TCDD alone to purified $CD4^+$ T-cells is insufficient to replicate the *in vivo* phenotype.

To evaluate the potential for AhR to sequester ARNT, thereby perturbing HIF-1 function and suppressing Th17 differentiation, semi-allogeneic CD4:DC co-cultures were incubated under normoxic or hypoxic conditions. The inclusion of accessory cells (DC) was sufficient to activate $CD4^+$ T-cells, but did not induce the $CD4^+CD25^{high}$ Treg population, thus prompting evaluation of hypoxia as a critical factor. Treatment with TCDD did not induce the characteristic $CD4^+CD25^{high}$ population first described in the GVH model under either normoxic or hypoxic conditions (Funatake *et al*. 2005). However, under hypoxic conditions, IL-17A was significantly suppressed relative to vehicle-treated co-cultures, and IL-6 production was decreased, suggesting that Th17 differentiation was impaired. Production of IL-10, a Treg-associated cytokine, was not increased. However, this may be reflective of the early time point, capturing the $CD4^+$ T-cells in the transitory state between Th17 and Treg lineage determination.

Of great interest, treatment with TCDD significantly up-regulated IL-22, a cytokine recently linked to the newly discovered Th22 lineage, of which AhR is the putative transcription factor (Trifari *et al*. 2009). AhR mediated regulation of IL-22 has been

demonstrated before, at both the level of transcript and protein (Ramirez *et al.* 2010; Trifari *et al.* 2009; Veldhoen *et al.* 2009; Veldhoen *et al.* 2008) (Rohlman *et al*. 2012). Indeed, i*n vitro* analysis of differentiating $CD4^+$ T-cells revealed TCDD up-regulated IL-22 gene expression as early as 24 hours, and this translated to an increase in IL-22 protein, under normal O_2 conditions (Rohlman *et al*. 2012). Surprisingly levels of IL-22 were undetectable under normoxic conditions, although this may be due to the method of activation; less than 10% of cells respond to semi-allogeneic antigen presenting cells, while other methods use anti-CD3 and anti-CD28, resulting in polyclonal activation. Under hypoxia, expression of all cytokines was increased, allowing us to detect a difference in IL-22 following treatment with TCDD, despite the low number of activated cells. Currently, the role of IL-22 remains conflicted, as IL-22 signaling has been found to exacerbate diseases such as psoriasis, and ameliorate diseases such as irritable bowel disease (Zenewicz and Flavell 2011). It remains unknown if the TCDD-mediated increase in IL-22 is required for the potent immunosuppression seen in multiple *in vivo* models (Marshall and Kerkvliet, 2010).

Importantly, culture of CD4:DC co-cultures under hypoxia resulted in significantly enhanced production of IL-6, IL-10, IL-17A and IL-22, suggesting that traditional *in vitro* assays performed under normoxia may underestimate cytokine production. Thus, translational studies attempting to link *in vitro* data to *in vivo* results may be confounded by underrepresentation of cytokine expression.

While the TCDD-mediated induction of $CD4^+CD25^+$ Tregs has been shown in multiple *in vivo* models, and induction of $CD4^+Foxp3^+$ Tregs has been shown both *in vitro* and *in vivo*, concern has been raised that the observed induction is no more than a reflection of TCDD-mediated apoptosis of effector $CD4^+$ T-cell populations (Stockinger 2009). The area of TCDD-mediated apoptosis remains controversial, with both Fas-dependent and Fas-

independent mechanisms of TCDD-mediated apoptosis reported (Camacho *et al.* 2002; Funatake *et al.* 2004). Furthermore, while *in vitro* screens have shown that treatment with TCDD induced apoptosis, this was dependent upon a 24 hour culture period, as freshly isolated cells showed no difference in apoptotic levels relative to controls (Pryputniewicz *et al.* 1998). We saw no evidence of attenuated $CD4^+$ T-cell activation, proliferation or decreased viability following treatment with TCDD under either normoxic or hypoxic culture conditions. This agrees with other reports, wherein allo-specific $CD4^+$ T-cells proliferate normally in response to alloantigen (Funatake *et al.* 2004; Funatake *et al.* 2005). Indeed, TCDD has been linked to increased T-cell proliferation, as assessed by the *in vivo* incorporation of ^{3}H-TdR (Neumann *et al.* 1993). Additionally, Shepherd et al. (2000) used the DO11.10 transgenic mouse model to further elucidate the effect of TCDD on antigen-specific cells, and reported that treatment with TCDD had no effect on the clonal expansion of OVA-specific T-cells (Shepherd *et al.* 2000). Thus, the observed increase in Tregs does not appear to be due to targeted apoptosis of effector $CD4^+$ T-cells, but rather to the *de novo* induction of the subset by TCDD-activated AhR.

In summary, activation of AhR by TCDD under hypoxic conditions appears to suppress Th17 differentiation, indicating that HIF-1 function was perturbed. The role of HIF-1 in TCDD-mediated immunosuppression requires further studies to understand the interplay between AhR, ARNT and HIF-1α, as well as the functional consequences of increased IL-22.

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