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Consequences of Aryl hydrocarbon receptor activation in dendritic cells

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CONSEQUENCES OF ARYL HYDROCARBON RECEPTOR ACTIVATION IN DENDRITIC CELLS

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Chairperson: Dr. David M. Shepherd

Abstract

TCDD (dioxin) causes immunosuppression via activation of the Aryl hydrocarbon receptor (AhR). Dendritic cells (DCs), the professional antigen-presenting cells in the immune system, are adversely affected by TCDD. However, limited information exists regarding the effects of AhR activation on DCs. We evaluated the consequences of AhR activation by TCDD on both steady-state and inflammatory DCs using *in vivo* and *in vitro* approaches, respectively. We hypothesized that AhR activation alters DC homeostasis and differentiation leading to generation of immunosuppression. C57Bl/6 mice gavaged with an immunosuppressive dose of TCDD (15 μg/kg) displayed decreased frequency and number of splenic $CD11c^{high}$ DCs on day 7. Moreover, TCDD induced a selective loss of the $CD11c^{high}CD8\alpha^{-}33D1^{+}$ splenic DCs subset, specialized at activating $CD4^{+}$ T cells, but not the regulatory $CD11c^{high}CD8\alpha^{+}DEC205^{+}$ splenic DCs. Additionally, TCDD increased the expression of CD86 and CD54, while decreasing the frequency of CD11a and MHC II on the splenic $CD11c^{high}$ DCs. Although TCDD did not alter the number and frequency of $CD11c^{low}$ splenic DCs, it decreased their MHC II and CD11a expression. The loss of $CD11c^{high}$ DC was independent of an apoptotic event but involved a CCR7-mediated migratory event. Popliteal and brachial lymph node (PBLNs) $CD11c^{+}$ cells displayed elevated levels of MHC II and CD40, but not DC loss following TCDD exposure. To examine the effects of TCDD on inflammatory DCs, BMDCs were generated in the presence of GM-CSF and vehicle or TCDD. TCDD decreased CD11c expression but increased MHC II, CD86 and CD25 expression on these BMDCs. These effects were AhR-dependent but not exclusively DRE-mediated. Additionally, TCDD modulated antigen uptake and increased LPS- and CpG-induced IL-6 and TNF-α levels but decreased nitric oxide production by the BMDCs. TCDD downregulated LPS- and CpG-induced p65 levels and induced a trend towards upregulation of RelB levels in BMDCs. Despite the induction of suppressive mediators IDO1, IDO2 and TGFβ3, TCDD-BMDCs failed to suppress T cell activation *in vivo* or induce the generation of adaptive T-regs *in vitro*. Collectively, our data suggest that AhR activation disrupts DC homeostasis, modulates DC differentiation, TLR responsiveness and induces a regulatory phenotype, effects that may underlie TCDD-induced immunosuppression.

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CHAPTER 1

INTRODUCTION

This study describes the effect of Aryl hydrocarbon receptor (AhR) activation on the fate and function of dendritic cells (DCs). The introductory chapter will: 1) broadly describe the immune system with a central focus on DCs and T cells 2) provide detailed information on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the AhR, and lastly 3) describe TCDD immunotoxicity.

The Immune system

Following entry of a pathogenic organism in our body, the immune system is activated to limit the pathogenic insult to the host. The immune system achieves this function in a series of coordinated steps carried out by two specialized branches referred to as the innate and the adaptive immune system. Although the central purpose of both is to protect the host, the innate and the adaptive immune system differ in their mode of action as described below.

Innate Immune System: The innate immune system constitutes the first line of defense against pathogenic insult and encompasses anatomical, physiological, phagocytic, and inflammatory barriers. The anatomical barrier consists of skin, epithelial layers and mucous membranes, which form an immediate physical barrier to most infectious agents preventing their direct entry into the body. The physiological barrier includes fatty acids, enzymes such as lysozyme, and antibacterial peptides that further prevent pathogen establishment. Additionally, the normal flora of the body secretes factors that are detrimental to pathogens

and prevent colonization. Pathogens that escape these initial barriers, encounter specialized cells of the innate immune system, which consists of neutrophils, macrophages, natural killer cells, eosinophils and dendritic cells (DCs). These may phagocytose the pathogen thereby killing it intracellularly. Alternately, certain innate immune cells may also process and present portions of the pathogen to adaptive immune cells such as T cells and B cells. In this capacity, macrophages and DCs are referred as antigen presenting cells (APCs). These cells express cell surface proteins as well as secrete soluble inflammatory mediators that serve to attract and stimulate the adaptive immune cells at the site of injury as well as the draining lymph nodes.

Antigen presenting cells: Antigen presenting cells function to detect pathogen in the periphery, process and present it to the lymphocytes in the draining lymph nodes and subsequently activate the adaptive immune system. In addition to the ubiquitously expressed major histocompatibiliy antigen I (MHC I), APCs express MHC II and B7 molecules also known as CD80 (B7-1) and CD86 (B7-2) (Bryant and Ploegh, 2004). Effective activation of T cell by an APC is described using the three-signal hypothesis. As shown in Figure 1-1, three signals are necessary for complete activation of T cell by APCs. Signal one comprises delivery of the MHC bound antigen to the T cell receptor (TCR). Signal two constitutes the binding of B7 co-stimulatory signals to cognate CD28 receptors on T cells. Secretion of soluble mediators, such as cytokines from activated APCs constitutes signal three. The nature of the secreted cytokine in turn governs T cell polarization as discussed later in this chapter (Goldsby, 2003). Appropriate and timely delivery

Figure 1-1

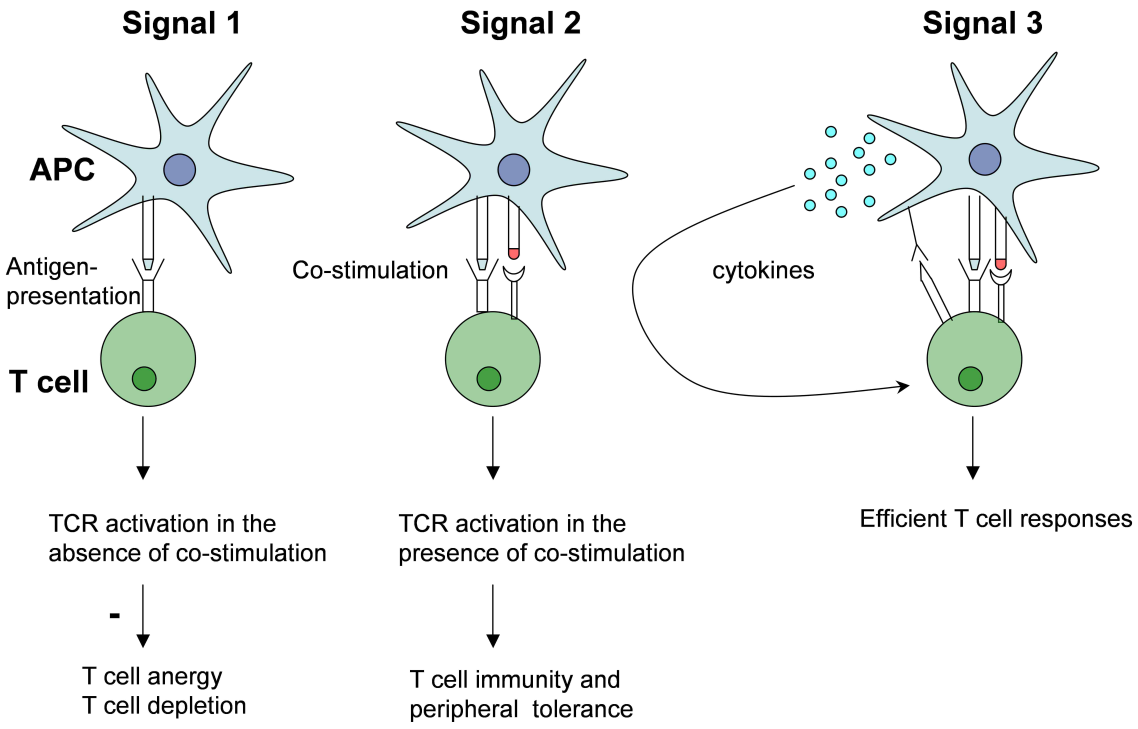


Figure 1-1. Effective activation of a T cell by an APC is dependent on three signals. Signal 1 is the interaction of MHC bound Ag with the T cell receptor. Binding of costimulatory molecules expressed on DC with cognate molecules on the T cells generates signal 2. APCs also secrete cytokines that contribute to T cell polarization, and this interaction constitutes signal 3.The lack of these signals leads to T cell anergy, depletion or tolerance. Adapted from *Cools et al., 2007.*

of the three signals is required for a sucessful T cell response. A lack of these signals can lead to anergy, deletion or tolerance. Anergy refers to a state in which a T cell is inactivated and hyporesponsive. For instance, delivery of signal 1 in the absence of costimulation can lead to T cell anergy. Additionally, maturational state of an APC also influences the development of a T cell response as discussed in the following section.

Dendritic cells: Amongst the existing varieties of APCs, DCs are considered the most potent APCs that execute both innate and adaptive functions. DCs constitute part of the first line of defense against invading pathogens and play a critical role in the generation of effective innate immunity to pathogenic challenge. Crucial functions of DCs include their ability to take up antigens, secrete cytokines, and recognize unique pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). The PRRs such as the toll like receptors (TLRs) encompasses membrane-bound receptors such as TLR4, and intracellular receptors such as TLR9. As shown in Figure 1-2, TLR4 recognizes lipopolysaacharide (LPS), whereas TLR9 specifically recognizes cytosine phosphate guanine (CpG) motif. TLR stimulation activates DCs leading to the secretion of proinflammatory cytokines such as TNF-α and IL-6, which further modulate the developing immune response by orchestrating T cell polarization.

In addition, DCs also regulate adaptive immunity and are considered to be the most potent for initiating activation of naïve T cells. Concomitant with the

constitutively high expression of MHC II, DCs also express T cell costimulatory molecules such as CD80 and CD86 (Greenwald *et al.*, 2005). Following encounter with pathogen or host-derived inflammatory products such as cytokines, immature DCs are induced to mature. This maturation is associated with upregulation of chemokine receptors such as CCR7 resulting in migration to the lymph nodes, where subsequent interactions with T cells lead to generation of adaptive immunity (Sanchez-Sanchez *et al.*, 2006). As shown in Figure 1-1, activation of a T cell by a DC requires three signals. In the most traditional form of antigen (Ag) presentation, DCs uptake antigen and present short, Ag-derived peptides, bound to MHC to the T cell. Intracellulary-derived Ag such as viral products are usually presented via MHC I molecules to the TCR of $CD8^+$ T cells, and exogenously-derived antigen such as bacterial products are presented via MHC II to the TCR of $CD4^+$ T cells (Cresswell, 2005). Following receptor engagement, costimulatory molecules on DCs such as CD80 and CD86 bind and activate surface receptors expressed on T cells such as CD28 (Lenschow *et al.*, 1996). Other accessory molecules such as CD54:CD11a and CD40:CD154 also play important roles in T cell activation (Croft and Dubey, 1997). Final differentiation of a T cell into an effector cell is mediated by cytokines secreted by the DCs and other immune and non-immune cells. Therefore, full T cell activation and generation of an immunogenic response is dependent on appropriate and timely presentation of three distinct signals.

Figure 1-2

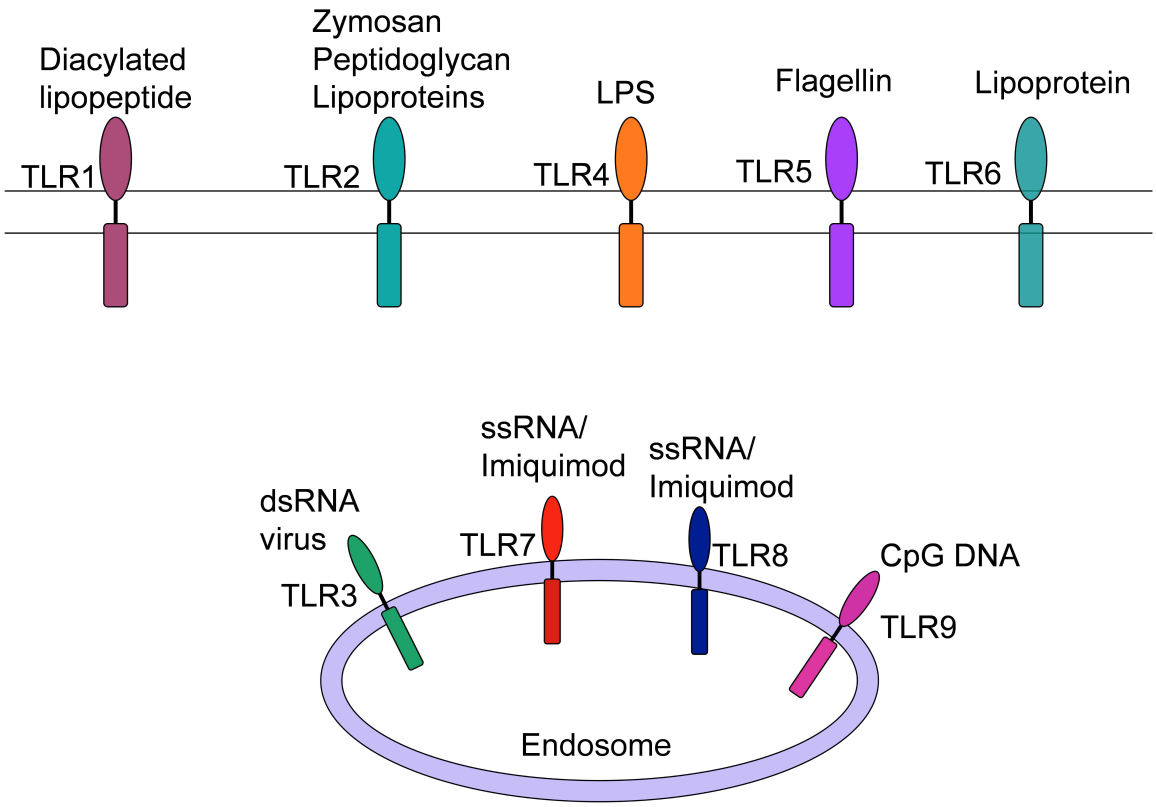


Figure 1-2. TLRs and their ligands: Dendritic cells express both surface bound and intracellular TLRs. Binding of ligands to their respective TLR activates a signaling cascade leading to induction of downstream transcription factors and drives the activation and maturation of DCs.

An accumulating body of evidence suggests that DCs also function to generate and maintain Ag-specific unresponsiveness in central and peripheral immune tissues (Steinman *et al.*, 2003). Such DCs are referred to as tolerogenic or regulatory DCs (DCregs). There are multiple ways in which DCs can induce T cell tolerance. For instance, T cells are tolerized when their TCR engages peptide-MHC complexes on an immature DC (Forster and Lieberam, 1996; Kurts *et al.*, 1996). Alternately, numerous studies have shown that Ag presentation in the absence of co-stimulation leads to T cell anergy (Adler *et al.*, 1998; Schwartz, 2003; Cools *et al.*, 2007). Similarly, DCs expressing low levels of MHC Class II and costimulatory molecules are also inefficient at inducing T cell activation (Lutz *et al.*, 2000). Recently, DCregs have been characterized by the expression of maturation markers and the immune-inhibitory enzyme indoleamine 2,3 dioxygenase (IDO) (Mellor and Munn, 2004; Popov and Schultze, 2008). IDO enzymatically degrades the essential amino acid tryptophan generating metabolites known as kynurenines which block T-cell proliferation resulting in T cell apoptosis (Grohmann *et al.*, 2003). In addtion to expression of IDO, interaction of CD86 on a DC with CTLA4 expressed on T cell is also shown suppress T cell responses.

DCs are a heterogeneous immune cell population (Vremec *et al.*, 2000; Shortman and Liu, 2002). Initial attempts to classify DCs focused on the idea that there were two distinct DC lineages: myeloid DCs and lymphoid DCs. It has

become increasingly evident, however, that multiple DCs subsets exist beyond this. Currently, DCs are classified into three distinct cell types: pre DCs, conventional DCs and inflammatory DCs (Table 1-1) (Shortman and Naik, 2007). Pre DCs such as plasmacytoid DCs (pDCs) and monocytes develop into DCs following an inflammatory or microbial stimulus (Shortman and Naik, 2007). Conventional DCs can be further categorized into migratory DCs and lymphoid-tissue-resident DCs. Lymphoid-resident DCs as the name suggests, reside in the lymphoid tissue and exhibit no migratory capacity. In the spleen, these DCs are further classified into 2 subtypes based on the presence of the marker CD8α, and are referred to as CD8α^+DEC205$^-$and CD8α^-33D1$^+$. The aforementioned splenic DC subsets differ in their anatomic location and functional properties. CD8α^+DEC205$^+$ DCs reside in the T cell zone and specialize in the uptake of dying cells (Dudziak *et al.*, 2007). More recently, a Treg-inducing function has been ascribed to these DCs (Yamazaki *et al.*, 2008). In contrast, CD8α^-33D1$^+$ DCs found in the marginal zone specialize in CD4$^+$ T cell activation (Dudziak *et al.*, 2007). In addition, inflammatory DCs constitute a novel DC population that are absent in the steady-state and only appear following an inflammatory insult. These DCs, characterized by copious production of TNF-α and inducible NO synthase (iNOS), are derived *in vitro* using GM-CSF/IL4 (Xu *et al.*, 2007).

Even with such heterogeneity, DCs constitute a very limited pool of the total immune cell population and the number of DCs obtainable from an animal is extremely limited. Thus, various strategies using bone marrow precursors have emerged to generate large number of DCs in culture. DC populations may be

Table 1-1: Different types of dendritic cells. Adapted from (Shortman and Naik, 2007)

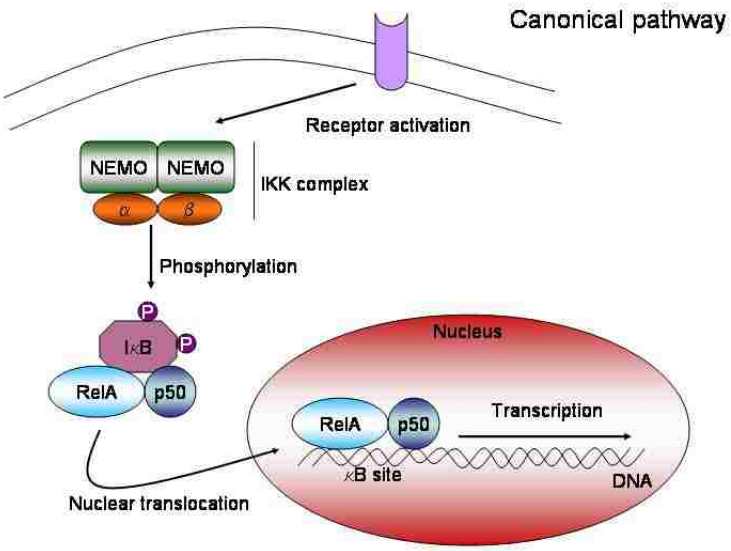
Types of dendritic cells
Pre-dendritic cells Exhibit capacity to develop into DCs Examples: pDCs, monocytes
Conventional dendritic cells ***Migratory dendritic cells*** Migrate to the lymphoid areas following Ag capture from the periphery Examples: Langerhan cells, dermal DCs ***Lymphoid-tissue resident dendritic cells*** Do not migrate to the lymphoid organ Spend their life span in one lymphoid organ Examples: $CD4^+CD8^-$cDCs, $CD4^-CD8^-$ cDCs and $CD4^-CD8^+$ cDCs.
Inflammatory dendritic cells Absent in the steady-state Appear following inflammatory insult Examples: Tip DCs

generated *in vitro* using the growth factors Granulocyte Macrophage Colony stimulating factor (GM-CSF) and FMS-like tyrosine kinase 3 ligand (Flt3-L). GM-CSF is widely used for development and expansion of inflammatory DCs *in vitro.* These inflammatory DCs *in vitro* are characterized by production of TNF-α and inducible nitric-oxide-synthase (Tip DCs)(Xu *et al.*, 2007). Flt3-L, on the other hand, generates steady-state pDCs and cDCs. Additionally, studies from several labs have demonstrated that a single injection of the hematopoietic cytokine, Flt3-L enhanced the production of both myeloid and lymphoid DCs that encompass both pDCs and cDCs (Maraskovsky *et al.*, 1996; Pulendran *et al.*, 1999; Brawand *et al.*, 2002; Gilliet *et al.*, 2002). The importance of this cytokine in promoting the development of DCs was demonstrated by studies demonstrating that Flt3 L KO mice displayed reduced number of pDCs, cDCs and NK cells.

In addition to the aforementioned growth factors, DC development is dependent on the nuclear transcription factor NF-kB, which mediates the expression of numerous genes involved in cellular processes such as apoptosis, proliferation and inflammation. The mammalian NF-kB transcription family is composed of five distinct members: p50, p52, p65 (RelA), c Rel and Relb (Moynagh, 2005; Hoffmann *et al.*, 2006). In unstimulated DCs, NF-kB members reside in the cytosol in an inactive form via interactions with inhibitory IkB proteins. NF-kB signaling ensues in two distinct pathways referred to as the canonical signaling pathway and the non-canonical signaling pathway (Figure 1-

3). The canonical signali

Figure 1-3 **A.**



B.

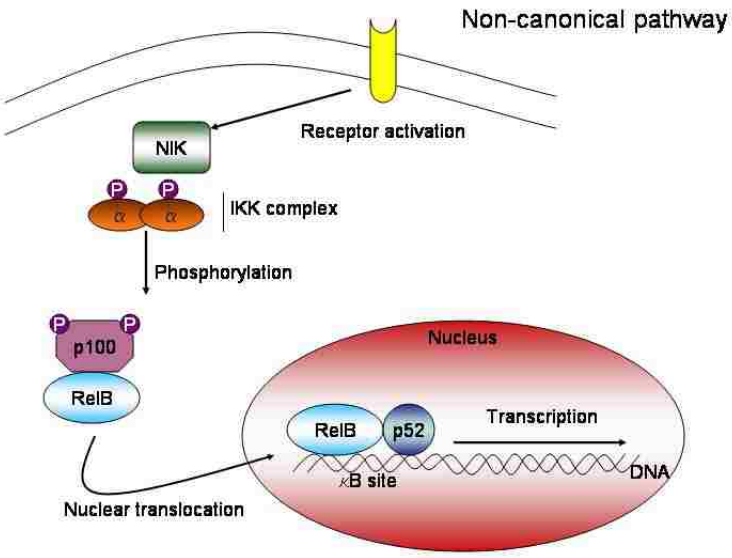


Figure 1-3. Overview of canonical and non-canonical NF-kB signaling pathways A) The canonical NF-kB signaling pathway involves binding of a ligand to a receptor leading to activation of IKK complex. The IKK complex degrades IkB leading to translocation of active NF-kB components Rel A and p50 into the nucleus, where they initiate transcription of genes. **B)** The non-canonical pathway involves binding of a ligand to a receptor, ultimately leading to activation of the IKK complex. The IKK complex functions to release the active NF-kB components RelB/p52 into the nucleus, where they initiate transcription of genes regulated by kB sites. Figure adapted from *www.abcam.com*.

complex and is initiated following binding of a ligand to a cell surface receptor such as TLRs. This leads to recruitment of adaptors such as TRAF to the cytoplasmic domain of the receptor followed by recruitment of the IKK complex that phosphorylate and degrades the IkB inhibitor, thus releasing the active NF-kB members (p65-p50). This heterodimeric complex then translocates into the nucleus and activates transcription of target genes. The non-canonical signaling on the other hand involves activation of p100-RelB complex following phosphorylation of p100 through IKK. Although distinct, crosstalk between the Canonical and non-canonical NF-kB signaling pathway has been shown to regulate inflammatory and developmental signaling (Basak and Hoffmann, 2008).

Adaptive Immune System: The non-specific nature of the innate immune system is often inadequate for the complete clearance of pathogens. In these instances, specificity by the immune system is conferred by the adaptive immune system as discussed below. The adaptive immune system includes effector cells including B and T lymphocytes, which recognize and respond to pathogenic insult in an an Ag-specific manner. The Ag-specific nature of the adaptive immune system imparts specificity to the immune system and also generates immunological memory (Charles A. Janeway, 2005).

T cells: T cells are hematopoietically-derived cells which originate in the bone marrow and reach full maturation in the thymus, where the process of positive and negative selection directs their development into two functional types: $CD4^+$ and $CD8^+$ T cells. The T cells express the T cell receptor (TCR) that recognises and responds to processed antigenic peptides. Full activation of a T cell requires

the delivery of three signals by an APC as described previously in Figure 1-1. $CD4^+$ T cells commonly referred to as T helper cells (Th) aid the orchestration of adaptive immune responses by contributing to the activation of B cells and $CD8^+$ T cells and the generation of humoral and cell-mediated immune responses, respectively. Initial studies divided a mature $CD4^+$ T cells into two distinct cell types referred to as Th1 and Th2 based on their cytokine profile and effector functions (Mosmann *et al.*, 1986). Th1 cells provide immunity to a variety of intracellular pathogens and produce cytokines such as IFN-γ, lymphotoxin-α (LT-α) and IL-2 (Mosmann and Coffman, 1989a; Mosmann and Coffman, 1989b; Zhu and Paul, 2008). Th1 cells express the transcription factor T-bet and require IFN-γ and IL-12 for their differentiation (Szabo *et al.*, 2000; Zhu and Paul, 2008). In contrast, Th2 cells participate in host defense against extracellular parasites and produce cytokines IL-4, IL-5, IL-10, IL-9 and IL-13 (Mosmann and Coffman, 1989b; Paul and Seder, 1994; Zhu and Paul, 2008). Th1 cells express the transcription factor GATA-3 and require IL-4 and IL-2 for differentiation (Kurata *et al.*, 1999; Zhu *et al.*, 2001). Extensive studies in the past decade have identified 2 novel Th subsets, in addition to these classic Th cell subtypes referred to as the Tregs and Th17 cells

Two distinct types of regulatory T cell have been described: natural T regs that arise in the thymus and inducible/adaptive Tregs, that form from $CD4^+CD25^-Foxp3^-$ precursors in the periphery (Shevach, 2006; Sakaguchi *et al.*, 2008; Yamazaki and Steinman, 2009). Regulatory T cells (T-regs), characterized by CD25 and Foxp3 expression as well as IL-10 and TGF β, play a critical role in

maintenance of self-tolerance, as well as inflammatory responses.

The most recent addition to the Th family is the Th17 subset, characterized by expression of the transcription factor RORγT and secretion of the cytokine IL-17 (IL-17a, IL-17f) and IL-22 (Yao *et al.*, 1995; Ivanov *et al.*, 2006). Th17 cells function in the clearance of extracellular bacteria and have been implicated in the generation of autoimmunity (Weaver *et al.*, 2006; Zhu and Paul, 2008).

Lastly $CD8^+$ T cells, also referred to as cytotoxic T lymphocytes (CTLs), recognize an antigen in an MHC I-restricted manner. Their prime effector function is to detect and kill virally-infected cells, tumor cells and allogenic cells that differ in MHC Class I expression. Following its activation, $CD8^+$ T cells employ a series of effector mechanisms to achieve immunity. These range from inducing extrinsic apoptotic pathways to secretion of perforin and granzymes (Charles A. Janeway, 2005).

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a member of the family of chemicals referred to as halogenated aromatic hydrocarbons (HAHs) (Figure 1-4). Other members of this family include structurally-related chemicals such as dibenzofurans (PCDFs), biphenyl (PCBs) and napthlenes. Amongst these chemicals, TCDD is most potent at inducing toxicity and is considered as the prototypical HAHs. TCDD is generated and released into the environment as a by-product of various industrial activities such as waste incineration, metal smelting and burning of chlorine-containing organic chemicals such as plastics

Figure 1-4

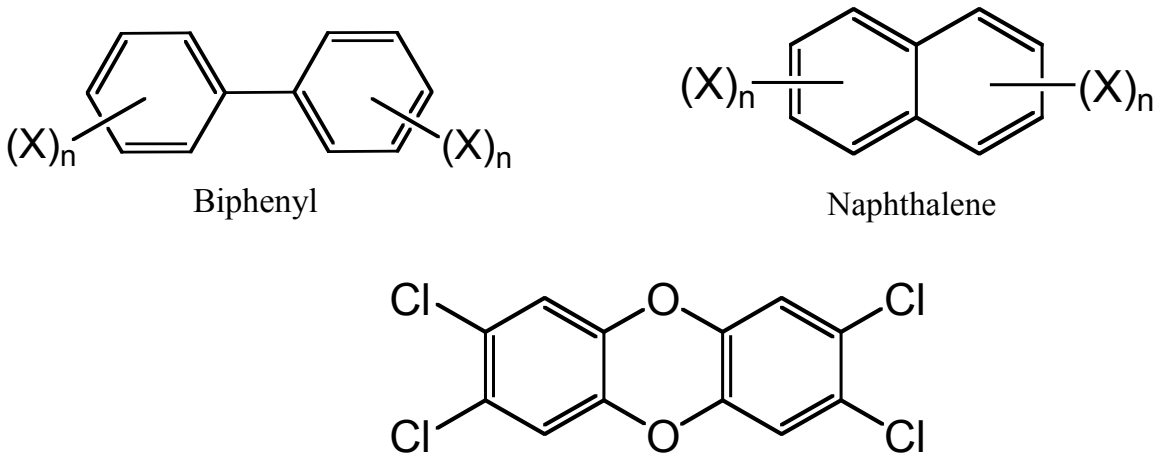


Figure 1-4. Chemical structures of various halogenated aromatic hydrocarbons

(Birnbaum, 1994). Additionally, unintentional releases of TCDD into the environment have also occurred in the past. For instance, in Seveso an explosion released around 300-1200 kg of TCDD into the environment (Dickson and Buzik, 1993). TCDD has a molecular weight of 322 and occurs as white crystalline solid. A relatively stable compound, TCDD is resistant to breakdown by acids, hydrolysis and heat (Webseter and Commoner 1994). However, TCDD degrades slowly via photodegradation. As TCDD and related HAHs are lipophilic in nature, they exhibit a propensity to accumulate in fatty tissues and concentrate up the food chain from plants to human (Schecter *et al.*, 1994a; Schecter *et al.*, 1994b). The half-life of TCDD ranges from 7 to over 10 years in humans (Aylward *et al.*, 2005). Human exposure to TCDD results from diet, inhalation and skin contact. Of these, dietary intake constitutes by far the most important route of exposure and accounts for greater than 90% of dioxin exposure via fatty foods such as fish and dairy products (Brouwer *et al.*, 1998).

Aryl hydrocarbon receptor

The Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the basic-helix-loop–helix (bHLH) family that mediates the toxic manifestations of TCDD and dioxin-like chemicals (Schmidt and Bradfield, 1996). AhR activation by TCDD has been implicated in the initiation of variety of biochemical and toxic responses such as chloracne, developmental and reproductive toxicity, carcinogenesis and immune suppression (Bock, 1994). Cloning of the AhR revealed important information regarding its structure and function (Whitlock, 1993). In the cytosol, the receptor exists as part of a protein

complex with 2 heat shock proteins (hsp90) and XAP 2 (Carver *et al.*, 1994; Meyer *et al.*, 1998). In the classical schematic of AhR signaling depicted in Figure 1-5, binding of the ligand to the cytosolic receptor induces a conformational change leading to dissociation of hsp90 and XAP2 from the receptor. This is followed by association of the ligand-bound receptor with the helix-loop-helix type nuclear transcription factor, AhR nuclear translocator (Arnt) (Reyes *et al.*, 1992). Next, the AhR-ARNT heterodimer binds specific sequences termed dioxin responsive elements (DREs) in DNA leading to activation of AhR The core consensus sequence within the DRE has been identified as 5'GCGTG-3'. In addition to Cyp1A1, which harbors nine DREs in its 5' promoter region, other genes involved in growth, differentiation and homeostasis such as c-fos and c-jun (Hankinson, 1995; Safe *et al.*, 1998; Nebert *et al.*, 2000). Binding of the AhR-ARNT heterodimer to DREs also activates transcription of the Aryl hydrocarbon receptor repressor (AhRR), which competes with AhR for formation of the heterodimeric complex with Arnt and binding to DREs in target genes. The AhRR initiates a feedback mechanism regulating the activity of the AhR (Mimura *et al.*, 1999; Watanabe *et al.*, 2001; Karchner *et al.*, 2002). In addition, AhR is regulated by protein degradation by ubiquitination mediated by the 26 S proteosome (Ma and Baldwin, 2000; Ma *et al.*, 2000).

Non-DRE mediated AhR signaling: Although binding of activated AhR to DREs mediates TCDD toxicity in many physiological systems, an accumulating body of evidence shows that activated AhR interacts with other signaling pathways

Figure 1-5

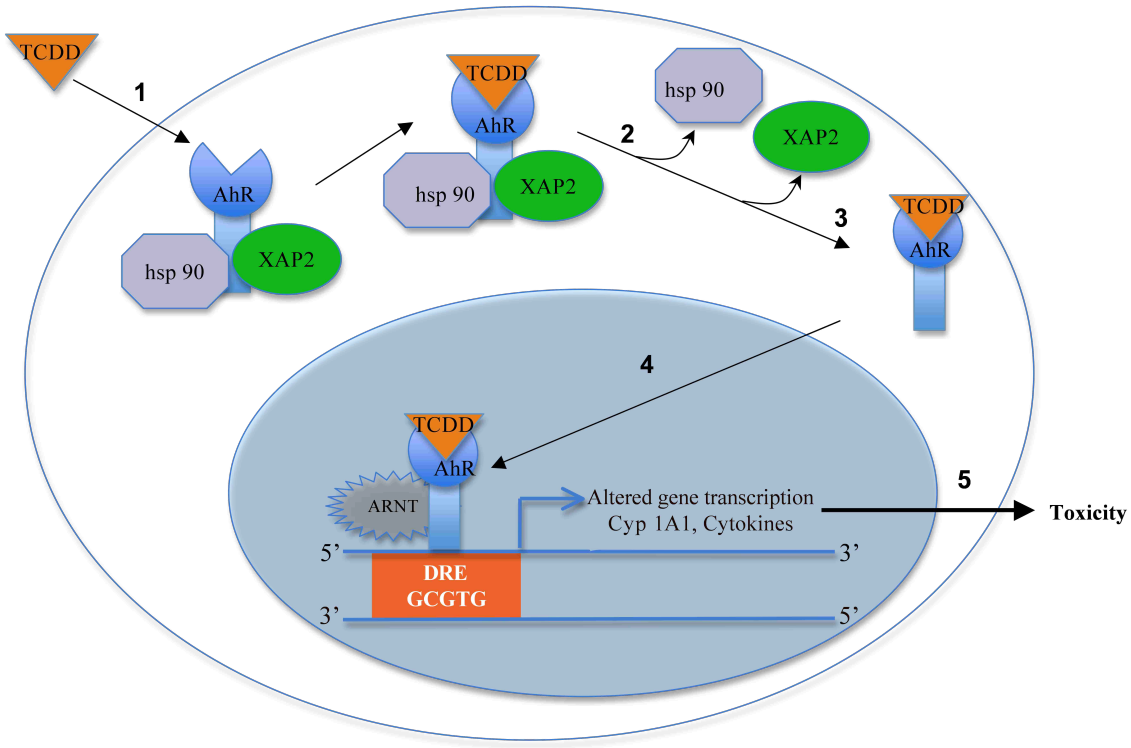


Figure 1-5. The classical AhR signaling is shown. (1) The prototypical AhR ligand TCDD enters into the cell via passive diffusion and binds the AhR with high affinity (2) Ligand binding causes dissociation of hsp90 and XAP2 from the receptor. (3) Ligand-bound AhR translocates into the nucleus and interacts with the AhR nuclear translocator (ARNT). (4) The heterodimeric AhR-ARNT complex binds specific DNA sequences known as Dioxin response elements (DREs), leading to altered gene transcription of target genes (5) Toxicities are manifested in several physiological systems due to AhR activation regulated gene battery (Schmidt and Bradfield, 1996; Whitlock, 1999).

resulting in cell cycle arrest, inhibition of cellular proliferation, anti-estrogenic effects, and modulation of inflammatory responses. Non-DRE mediated AhR signaling pathways include interactions between the AhR and retinoblastoma protein (Rb), E2F1, Estrogen receptor (ER), MAP kinases and NF-kB members.

Two distinct models have been proposed to describe the inhibitory effect of TCDD on cellular proliferation (Marlowe and Puga, 2005; Puga *et al.*, 2009). One model involves AhR-Rb interactions and the other supports the AhR-mediated induction of cyclin-dependent kinase inhibitors (CDKs) (Puga *et al.*, 2009). The AhR associates with hypophosphorylated Rb and prevents its phosphorylayion in the G1 stage, which represses the E2F-dependent gene expression, thus inhibiting progression through the cell cycle (Ge and Elferink, 1998; Puga *et al.*, 2000). (Puga *et al.*, 2000; Puga *et al.*, 2009). An alternate explaination for the induction of G1 arrest by TCDD is the induction of cyclin dependent kinase inhibitors such as $p27^{kip}$. Huang and Elferlink demonstrated in TCDD-induced AhR-mediated G_1 arrest to be partially regulated by direct AhR transcriptional activity in rat hepatoma cells. The same study also provided evidence that AhR-mediated G1 arrest required the Arnt protein (Huang and Elferink, 2005).

Another non-DRE event implicated in mediating TCDD toxicity is the activation of c-src. Binding of the AhR ligand to the receptor liberates the AhR from the Hsp complex leading to activation of c-src. The c-src then activates c-ras proteins and the MAP kinases pathway, ultimately activating nuclear transcription factors such as AP1. In this context, TCDD induces p38 and

ERK1/2 activation in an AhR-independent manner resulting in enhanced TNF-α production (Park *et al.*, 2005).

Similar to the AhR, the Estrogen receptor (ER) is a ligand-activated transcription factor and several studies have demonstrated AhR-ER crosstalk. Activated AhR exerts antiestrogenic effect by inhibiting ER activity. For instance, TCDD represses the induction of ER target genes such as pS2, cathepsin D, c-fos and cyclin D1 (Beischlag *et al.*, 2008). Several hypothesis emerged to explain the repression of these ER target genes. The first explanation proposed enhanced E2 metabolism mediated by TCDD-induced expression of Cyp1A1 and Cyp1B1 (Spink *et al.*, 1990; Spink *et al.*, 1992). An alternate mechanism proposed TCDD-mediated activation of proteosomes leading to increased ER degradation (Wormke *et al.*, 2003). Studies have also shown activated AhR to redirect ER from ER target genes to AhR target genes, suggesting that AhR regulates ERα protein levels and subsequently influence the estrogenic response (Matthews *et al.*, 2005). Additionally, recent studies have described the AhR to function as a ligand-dependent ubiquitin ligase promoting the degradation of ERα and modulating steroid receptor function (Ohtake *et al.*, 2009).

Recently, evidence has emerged implicating crosstalk between the AhR and NF-kB (Ruby *et al.*, 2002; Vogel and Matsumura, 2008). More importantly, physical interactions between NF-kB signaling members such as p65 and RelB with the AhR contribute to immune suppression and altered inflammatory responses (Camacho *et al.*, 2005; Vogel *et al.*, 2007a; Vogel *et al.*, 2007b; Vogel and Matsumura, 2008). *In vitro* AhR activation inhibited p65 nuclear

translocation, which was implicated in altered DC survival contributing to immune suppression. TCDD-induced modulation in BMDC differentiation was associated with decreased RelB levels in BMDCs (Lee *et al.*, 2007). More recently, TCDD-mediated induction of immune inhibitory enzyme IDO was suggested to require both AhR and NF-kB component, RelB (Vogel and Matsumura, 2009). Activation of the AhR by TCDD elicits active inflammation in different organs. TCDD-induced inflammatory responses have been associated with an increased production of proinflammatory mediators including TNF-α, IL8, IL-1β and cycloxygenase 2 (Cox2)(Vogel and Matsumura, 2009). NF-kB proteins play a critical role in regulating inflammatory responses and interact with the AhR. In an attempt to elucidate the consequences of RelA/AhR or RelB/AhR interactions on inflammatory responses, Vogel et al proposed that activated AhR facilitates the function of RelB, both in the induction as well as resolution of inflammation. Interaction of the AhR with RelA, on the other hand, dampened the action of RelA on inflammatory responses (Vogel and Matsumura, 2009). Taken together, these studies highlight a critical role of the AhR in the regulation of cellular inflammation via interactions with the NF-kB signaling pathway.

TCDD-induced toxicity in animals: Exposure to TCDD generates a spectrum of adverse health effects in multiple animals species (Birnbaum and Tuomisto, 2000). Reported TCDD toxicity, mediated by AhR activation in animals ranges from induction of cachexia, disruption of endocrine pathways, chloracne, hepatotoxicity, teratogenesis to immune suppression. A cascade of common toxic effects of TCDD is observed in all animals following exposure. TCDD-

induced lethality that occurs in weeks is associated with wasting syndrome, characterized by severe weight loss concomitant with reduction in both muscle and adipose tissue (Seefeld *et al.*, 1984; Max and Silbergeld, 1987). On a similar note, thymic atrophy is also another common manifestation of TCDD toxicity and is characterized by lymphocyte depletion in the thymic cortex (Staples *et al.*, 1998). TCDD-induced toxicity is also associated with severe a skin condition characterized by hyperkeratotic and hyperproliferative skin response known as Chloracne (Birnbaum and Tuomisto, 2000). This acute skin condition has been reported in cows, horses, ears of rabbit and also on the skin of hairless mice (Birnbaum and Tuomisto, 2000). Hepatotoxicity is reported in rats and rabbits following TCDD exposure. Reproductive effects from TCDD exposure have been documented in mice, rats and monkeys where TCDD is demonstrated to reduce litter size, fertility and uterine weights (Kociba *et al.*, 1976; Barsotti *et al.*, 1979; Umbreit *et al.*, 1987). TCDD exposure has been associated with induction of developmental syndrome in mice characterized by fetal thymic atrophy, hydronephrosis and cleft palate (Couture *et al.*, 1990a; Couture *et al.*, 1990b; Birnbaum and Tuomisto, 2000). In chickens, TCDD affects the cardiovascular system. Additionally, TCDD also targets the nervous system; however, mechanisms underlying neurotoxic effects of TCDD remain poorly investigated (Birnbaum and Tuomisto, 2000). Studies have also identified TCDD as a complete carcinogen in lab animals. Exposure to TCDD at increasing doses induces cancer in experimental animals and is associated with development of tumors in the liver, thyroid, respiratory tract and other organs.

The severity of TCDD toxicity is dependent on multiple factors such as the animal species, strain, age and gender (Neubert, 1997). Differential sensitivity of animal species to TCDD is attributed to polymorphisms in the Ah locus (Takashi Moriguchi 2003). For example, a 10-fold difference in susceptibility to TCDD has been reported between C57Bl/6 (sensitive) and DBA/2 (resistant) strain of mice (Dolwick *et al.*, 1993; Ema *et al.*, 1994a; Ema *et al.*, 1994b). C57Bl/6 and Balb/c express the dominant responsive allele, Ahr^{b-1} and Ahr^{b-2}, respectively when compared to the less responsive strain DBA/2 bearing the recessive non-responsive allele designated Ahr^{d} (Poland and Grover 1990). Similarly, Long Evans (sensitive) and Han/Wistar rats (resistant) differ in a magnitude of >1000 fold in terms of their susceptibility to dioxins due to a mutation in the Trans activation domain of the AhR (Takashi Moriguchi 2003).

AhR knockout, mutant and transgenic mice: Generation of AhR knockout and mutant mice has played an instrumental role in dissecting the AhR-mediated toxicities from those arising from alternative pathways (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996). AhR knockout mice ($AhR^{-/-}$) were first created using gene targeting strategies that inactivated the murine AhR gene by homologous recombination (Schmidt *et al.*, 1996). The resulting $AhR^{-/-}$ mice were viable, yet displayed developmental defects in the liver (Schmidt *et al.*, 1996). Furthermore, $AhR^{-/-}$ mice were protected against a spectrum of TCDD toxicities ranging from hepatomegaly, thymus atrophy and also immunotoxicity (Fernandez-Salguero *et al.*, 1995; Thurmond *et al.*, 2000). Studies have demonstrated a lack of TCDD effect in AhR KO even at a high dose TCDD

(2000μg/kg). Also, no TCDD-induced lesions were observed in the kidney, heart, pancreas, spleen, lymph nodes and uterus in $AhR^{-/-}$ mice following TCDD exposure, suggesting that the pathologic changes induced by TCDD were AhR dependent.

Mice harboring a mutation in the AhR nuclear localization ($AhR^{nls/nls}$) were also generated to determine whether nuclear DRE-mediated events are essential for the generation of toxic and developmental effects following AhR activation (Bunger *et al.*, 2003). These mice express the mutant AhR that is capable of ligand binding but is unable to translocate to the nucleus and bind the DREs. $AhR^{nls/nls}$ mice also display defects in liver development and are resistant to TCDD-induced thymic involution, hepatomegaly and cleft palate formation (Bunger *et al.*, 2003). Bradfield et al., subsequently developed AhR mutant mice designated $AhR^{dbd/dbd}$ that are capable of herterodimerizing with ARNT, but incapable of binding DREs (Bunger 2008). Thus, despite nuclear translocation, $AhR^{dbd/dbd}$ mice displayed no manifestation of TCDD-induced toxic effects including, cleft palate, hydronephrosis, hepatomegaly and thymic involution (Perdew, 2008). To assess the importance of ARNT heterodimerization events in dioxin-induced toxicities, Walisser et al., generated a hypomorphic or low-expressing Arnt allele, designated Arnt fx^{neo}. Similar to the $AhR^{-/-}$ mice, ARNT hypomorphs displayed a patent ductus venousus establishing that ARNT heterodimerization constituted an essential aspect of AhR developmental signaling.

A major manifestation of TCDD immunotoxcitiy in animals is thymic involution, characterized by decreased $CD4^+CD8^+$ T cell numbers and skewing of thymocyte differentiation to single positive $CD8^+$T cells(Staples *et al.*, 1998; Nohara *et al.*, 2000). To understand the role of AhR in T cells and its contribution in TCDD-induced immuotoxicity, Nohara and colleagues generated transgenic mice that expressed a constitutively active mutant of AhR (CA-AhR) in T lineage cells (Nohara *et al.*, 2005). These transgenic mice displayed decreased numbers of thymocytes and an increased percentage of $CD8^+$ T cells, suggesting that AhR activation in T lineage cell was directly responsible for the thymocyte loss and altered differentiation.

Lastly, to assess the functioning of human AhR (hAhR), Moriguchi et al., created AhR-humanized mice, possessing hAHR instead of mouse AhR. Decreased responsiveness to TCDD was observed in mice harboring hAhR as compared to DBA/2 AhR. For instance, TCDD induced embryonic hydronephrosis but no cleft palate in maternally-exposed homozygous hAHR fetuses. Creation of these transgenic mice provides a novel tool not only to better understand the function of the human AhR but also predict TCDD toxicities in humans (Moriguchi *et al.*, 2003).

TCDD toxicity in humans: Much of what is known today about TCDD toxicity comes from populations accidentally exposed to TCDD, workers occupationally exposed to TCDD or populations living close to toxic waste dumps. Since the first reported incidence of human exposure to TCDD due to a trichlorophenol reactor explosion in Nitro, West Virginia, several other accidental exposures have been

reported. These global incidents of human exposure include but are not limited to PCB-contaminated rice oil consumption in Japan and Taiwan, an explosion at a trichlorophenol manufacturing plant in Seveso, Italy, and contamination of a resident community with tainted waste oil in Missouri (Sweeney and Mocarelli, 2000; Kerkvliet, 2002). Because TCDD has a long half-life in humans and accumulates at a high rate in the body, manifestation of TCDD-induced toxicities occurs after prolonged low level, daily exposure. Thus, human exposure to dioxin is associated with chronic, long-term adverse health effects. Studies have linked TCDD exposure with symptoms such as chloracne, hyper pigmentation, metabolic alteration, weight loss, immune suppression, neuropathy, endocrine dysfunction and perturbations in the gastrointestinal systems. For example, temporary hepatomegaly observed in individuals exposed to dioxin in the Seveso incident was associated with no concomitant elevation in hepatic enzymes (Sweeney and Mocarelli, 2000). Acute exposure to TCDD is documented to transiently upregulate liver enzymes such as AST, ALT and gamma glutamyl transferase (GGT). No association of TCDD to altering cholesterol levels was found in studies that involved Seveso residents and Vietnam veterans (Sweeney and Mocarelli, 2000). Similarly, no effect on thyroid function was demonstrated in studies involving production workers (Suskind and Hertzberg, 1984; Ott *et al.*, 1994; Calvert *et al.*, 1999).

In stark contrast to the variable reports on the non-cancer toxic endpoints of dioxins, the carcinogenic potential of TCDD is well accepted within the scientific community. Epidemiological studies have linked TCDD exposure with

soft-tissue sarcomas, respiratory system tumors and other cancers including Non Hogkins lymphoma (NHL), in populations exposed accidentally to high concentrations of TCDD (Fingerhut *et al.*, 1991). Based on carcinogenicity data in animal models and epidemiological data, TCDD is listed as a human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1997). However, the mechanisms underlying the human carcinogenic potential of TCDD need further investigation (Cole *et al.*, 2003; Steenland *et al.*, 2004).

In addition, immunological effects of dioxin exposure are also documented in humans. While some studies have described no clinical signs of immune suppression in exposed cohorts, others have documented alterations in serum IgA, IgG and lymphocyte populations and antinuclear antibodies in exposed individuals (Ott *et al.*, 1994). For instance a study conducted on 40 Missouri residents whose adipose TCDD levels ranged from 20pg/g to over 430 pg/g, no clinical evidence of immune suppression was detected (Webb *et al.*, 1989). Children exposed to TCDD in the Seveso accident, revealed increased serum complement levels, peripheral blood lymphocytes and increased lymphoproliferative responses with no adverse clinical effects (Tognoni and Bonaccorsi 1982). Increased serum IgA levels were detected in US Air Force personnel exposed to herbicides (Wolfe *et al.*, 1990). An assessment of immunologic spectrum of the Vietnam War veterans exposed to Agent Orange contaminated with TCDD showed decreased plasma IgG and increased IgE levels in the veterans compared to age-matched healthy controls. Following activation of their peripheral blood mononuclear cells with polyclonal T cell

activators, decreased levels of IFN-γ and increased levels of IL-4 and IL-10 were detected in the veteran group (Kim *et al.*, 2003). Taken together, these studies demonstrate that TCDD exposure results in dysregulated B and T cell responses eventually leading to perturbation of immune homeostasis.

Using an *in vitro* model, several investigators have examined the direct toxic effects of TCDD on various human leukocyte populations. Neubert et al reported direct effects of TCDD on T cells and B cell proliferation. Both the percentage and the number of $CD4^+$ and B cells activated with PWM decreased following activation with 10^{-12} and 10-^{14} M, TCDD concentration respectively (Neubert *et al.*, 1991). Subsequently, Lang et al., reported no direct effect of TCDD on human lymphocytes treated with 10^{-7}-10^{-14} M TCDD in the absence of stimulation with PMW or anti-CD3. An increase in Cyp1A1 activity, with no effect on lymphocyte proliferation led the authors to conclude that the immunotoxic effects do not correlate with Cyp1A1 induction (Lang *et al.*, 1994). Additionally, a study assessing Cyp1A1 induction in human blood lymphocytes in 10 non-smoking females showed that concentrations of TCDD necessary to elicit *in vitro* induction of Cyp1A1 were much higher than human blood levels in vivo. These studies concluded that the measurement of Cyp1A1 gene expression levels was not a suitable marker for assessing human immunotoxicity (van Duursen *et al.*, 2005).

Taken together, a defined relationship between exposure and impaired immune status remains unestablished. However, with such varying reports regarding the effects of TCDD on human health, continued surveillance of

exposed populations and research is warranted to fully understand the long-term effects of dioxin exposure. The lack of consistent effects on humans have been attributed to several factors such as lack of proper exposure documentation, small study populations, genetic variation between human populations and also insensitive immunological assays. Therefore, future prospective research examining the immunotoxic effects of TCDD is warranted. Understanding the mechanism of action of TCDD in laboratory animals first, will provide a stronger platform for assessing human health risks. Additionally, it will also allow identification and development of a novel biomarker that can be applied to human studies. Nevertheless, considering the spectrum of adverse health effects generated by TCDD, industries have taken measures to minimize release of dioxin into the environment.

Animal studies have shown that TCDD toxicities are mediated via the Aryl hydrocarbon receptor (AhR). Additional studies are needed to completely understand the physiological role of human AhR as well as its role in TCDD toxicity. Additionally, using laboratory animals, there is a continuous effort to elucidate AhR-regulated pathways in mediating TCDD toxicities as discussed in the following sections.

TCDD Immunotoxicity

The immune system has been identified as a sensitive target of TCDD (Luster *et al.*, 1989). Exposure of laboratory rodents to very low doses of TCDD (1<μg/kg body weight) suppresses the generation of adaptive immunity and increases the susceptibility to infectious diseases (Kerkvliet et al., 1994). TCDD primarily exerts its toxic effects by binding and activation of the AhR. The AhR is expressed in several immune cell populations including macrophages, T cells and B cells (Crawford *et al.*, 1997). Both the innate and adaptive immune systems are sensitive to AhR activation by TCDD.

Effects of TCDD on the innate immune system: AhR activation by TCDD affects innate cell populations such as neutrophils, natural killer cells, macrophages and DCs (Kerkvliet, 2009). In various experimental models, TCDD-treatment has been linked with neutrophilia. Injection of caesin or sheep RBCs in TCDD-treated mice resulted in peritoneal and pulmonary neutrophilia (Ackermann *et al.*, 1989; Kerkvliet and Oughton, 1993). Neutrophilia was also reported in the spleen in allograft response to P815 tumor cells and in the lung following flu infection (Choi *et al.*, 2003; Teske *et al.*, 2005). In the context of flu infection, neutrophilia occurred independent of classical chemoattracants such as macrophage inflammatory protein (MIP)-Ia, MIP-2, IL-6, lipopolysaacharide-induced CXC chemokine (LIX) or C5a, but rather AhR expressing cells in the lung were implicated (Teske *et al.*, 2008). Although, the mechanisms underlying TCDD-induced neutrophila are not completely understood, AhR has been demonstrated to be an essential component. Several conflicting studies

documented the effects of AhR activation in NK cells. For example, the cytolytic activity of NK cells has been shown to be increased, decreased or unaffected (Lawrence 2007). Adverse effects of AhR activation on macrophages have similarly been documented. Although AhR activation does not influence either macrophage phagocytosis, oxidative burst, or tumor cytolytic function, increased production of proinflammatory cytokines such as TNF-α and IL-1 have been identified as mechanism underlying TCDD-induced inflammation (Moos *et al.*, 1997) (Lawrence 2007). This increase in TNF-α production in human macrophages was found to be dependent on the AhR-EGFR-Erk pathway (Cheon *et al.*, 2007) Moreover, AhR activation in macrophages induces transcriptional gene expression changes via either the CaM kinase or the AhR-RelB pathway. TCDD-treatment upregulates mRNA levels for Cyp1b1, IL1b, IL8, Ccl1, β7-integrin and Ahrr in primary human macrophages, in a CaM kinase-dependent manner (Monteiro *et al.*, 2008). In other studies, AhR-RelB interactions were implicated in TCDD-induced expression of B cells activating factor (Baff), B cell chemoattractant (Blc), chemokine (C-Cmotif) ligand 1 (Ccl1) and Interferon γ response factor (Ifr) in a human macrophage cell line (Vogel *et al.*, 2007b). Interestingly, these AhR/RelB interactions did not require ARNT demonstrating the role of non-DRE-mediated effects on TCDD induced inflammatory responses.

DCs express the AhR constitutively and several studies suggest that defects in DCs following AhR activation contribute to immune suppression. Decreased numbers of splenic DCs, as well as increased expression of MHC II,

CD54, CD40, and CD24 was observed in TCDD-treated mice (Vorderstrasse and Kerkvliet, 2001). DCs from TCDD-treated mice exhibited higher T cell proliferative response in a mixed lymphocyte reaction and also produced higher levels of IL-12. Despite this, TCDD did not alter the ability of DCs to phagocytose latex beads or present Keyhole Limpet Hemocyanin (KLH) to KLH-specific T cells suggesting that processing and presentation function were unaffected by TCDD (Vorderstrasse *et al.*, 2003). Later studies also demonstrated the sensitivity of *in vitro* generated DCs to TCDD. TCDD enhanced TNF-α-induced DC maturation and Fas-mediated apoptotic death (Ruby *et al.*, 2005). Microarray analysis revealed that TCDD increased expression of several genes including Fadd, Dff40, Ox40l and caspase 9 in purified DCs, suggesting their involvement in induction of DC apoptosis following AhR activation (Ruby *et al.*, 2005). Mechanistically, several investigators have implicated non-DRE mediated events in the generation of DC dysfunction following TCDD exposure. In this context, interactions of the AhR with NF-kB signaling pathway have been reported as a mechanism underlying TCDD-induced alterations in DCs. TCDD was shown to suppress TNF-α- and CD40-induced NF-kB/Rel activation *in vitro* (Ruby *et al.*, 2002). In a separate study, Lee et al., demonstrated TCDD altered the differentiation of bone marrow-derived dendritic cells (BMDCs), an effect that was attributed to down regulation of RelB (Lee *et al.*, 2007). These studies suggest that TCDD-induced alterations in NF-kB signaling contributes to defects in DCs, leading to immune suppression.

Effects on adaptive immunity: AhR activation by TCDD induces profound suppression of the adaptive immune system. Both B and T cells constitutively express low levels of AhR and require activation to enhance its expression (Lawrence *et al*., 1996; Crawford *et al*., 1997; Marcus *et al*., 1998)

B cells: Evidence that B cells are directly affected by TCDD is provided by studies conducted by several labs that show modulation of B cell responses *in vitro* by TCDD. Kramer et al., linked TCDD-induced kinase activity and modulation of intracellular Ca^{2+} levels in B cell to altered B cell responses (Kramer *et al.*, 1987). Studies by Luster et al demonstrated for the first time that TCDD inhibits B cell differentiation into plasma cells in response to TNP-LPS (Luster *et al.*, 1988). TCDD-induced alterations in Ca^{2+} levels was also linked with TCDD-induced suppression of surface Ig, but not CD40-mediated antibody production (Karras *et al.*, 1996). In other studies, TCDD inhibited μ gene expression and IgM secretion in a CH12.LX B-cell line (Sulentic 2000). This inhibition of LPS-stimulated IgM secretion was not noted in the BCL-1 B-cell line that lacks the AhR (Sulentic 1998). Decreased levels of the human B cell differentiation marker, CD19, following TCDD exposure were linked to competition between the AhR and B-lineage–specific activator protein (BSAP) (Masten and Shiverick, 1995). The suppression of CD19, a key molecule involved in signaling through the BCR, could underlie suppression of antibody responses by TCDD (Kerkvliet, 2002).

$CD4^+$ and $CD8^+$ T cells*:* TCDD triggers thymic atrophy and suppresses the generation of antigen-specific T cell responses *in vivo* (Kerkvliet, 2002). TCDD

decreased both $CD4^+$ and $CD8^+$ T cell function, however debate continues as to whether T cells are direct or indirect targets of TCDD. While some reports suggest that T cells are not directly affected by TCDD due to lack of AhR binding to the consensus DRE in T cells, (Lawrence *et al.*, 1996), other investigators suggest a direct AhR-dependent effect of TCDD in both $CD4^+$ and $CD8^+$ T cells (Kerkvliet *et al.*, 2002). $CD4^+$ T cell differentiate into Th17, Th1, Th2 or Treg subsets. The differentiation into each of these specific subsets is dependent on the expression of transcription factors such as ROR-γT, GATA and Foxp3 that are shown to express multiple DREs, respectively (Kerkvliet, 2009). This observation suggests that the AhR may play a prominent role in specifying T cell differentiation. In a murine graft-versus-host (GvH) model, TCDD promotes Treg differentiation in donor $CD4^+$ T cells, as defined by their increased expression of CD25, CTLA-4 and GITR (Funatake *et al.*, 2005). The same study also demonstrated that TCDD did not merely expand a natural Treg population in the donor cells as depletion of natural Tregs from donor population prior to injection into F1 host did not affect the AhR-mediated generation of Tregs (Funatake *et al.*, 2005). Interestingly, Foxp3 expression was not detected in AhR-dependent $CD4^+CD25^+$ cells, highlighting the AhR as a novel transcription factor for adaptive Tregs. Subsequent studies demonstrated AhR-dependent modulation of numerous genes in TCDD-Tregs including TGFβ-3, CCr4, Bcl3, CTLA-4, IL-10, GATA-3, Icos, CD28 and genes involved in IL-12 signaling pathway. Downregulated genes included Tnfsf4 (OX40-L), IL13ra, CD86, Bcl6, IL5, NFkb1 and Ccl5 (Marshall *et al.*, 2008). Recently, ligand-specific AhR activation in T

cells has been shown to generate Tregs or Th17 cells. These studies demonstrated AhR activation by TCDD or natural AhR ligand formylindolo[3,2-b]carbazole (FICZ) to generate Treg or Th17 cells, respectively (Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). Cumulatively, these findings suggest that AhR activation during $CD4^+$ T cell differentiation alters gene expression contributing to altered T cell fate.

In addition to studies on $CD4^+$ T cells, several investigators have assessed the effects of AhR activation on $CD8^+$ T cell function using the P815 tumor allograft model and the influenza model (Kerkvliet, 2002; Lawrence *et al.*, 2006). Using the P815 tumor model, Kerkvliet et al., showed that TCDD did not affect the normal expansion and activation of $CD8^+$ T cells, but affected their ability to differentiate into functional cytotoxic T lymphocytes (CTLs). The $CD8^+$ T cells in TCDD-treated mice initially displayed normal production of IL-2 and IFNγ that diminished on day 5-6 as compared to vehicle-treated control mice (Kerkvliet *et al.*, 1996). Various hypothesis emerged to describe the observed suppression of CTL response such as the induction of TCDD-induced apoptosis and/or anergy of $CD8^+$ T cells (Prell and Kerkvliet, 1997; Prell *et al.*, 2000). Suppression of the CTL response in this model was also linked to $CD4^+$ T cells. Since TCDD-treated mice produced higher IL-2 on day 4 and 5 of the P815 response, it was hypothesized that TCDD-induced excess production of IL-2 by $CD4^+$ T cells leading to their premature deletion. Such deletion deprived the $CD8^+$ T cells of cognate $CD4^+$ T cell help, preventing CTL expansion (Kerkvliet, 2002). Further support for this hypothesis arrived from studies by Jeon and Esser demonstrating

the existence of 3 DREs in the distal region of mouse IL 2 promoter, suggesting that TCDD-induced excess IL-2 production by $CD4^+$ T may lead to activation-induced cell death (Jeon and Esser, 2000).

In addition to the P815 tumor model, $CD8^+$ T cell function following AhR activation has been assessed using a murine model of influenza A infection. TCDD suppressed clonal expansion and differentiation of influenza virus-specific $CD8^+$ T cells during primary infection, decreased the number of virus-specific $CD8^+$ T cells secreting IFNγ, and decreased the number but not the response of virus-specific memory $CD8^+$ T cells (Head and Lawrence, 2009). TCDD treatment also decreased the number of $CD4^+$ T cells required for antibody isotype switching and maintenance of anti-viral $CD8^+$ T cell memory. Interestingly, recent reports have linked AhR activation in $CD8^+$ T cells with $CD8^+$ Treg development as defined by increased CD25 expression and suppressed cytolytic activity (Funatake *et al.*, 2008). However, AhR expression in $CD4^+$ T cells was essential for the generation of $CD8^+$ T regs Overall, these results suggest that AhR-mediated events impact anti-viral immunity, decreasing the priming of $CD8^+$ T cells.

AhR activation by ligands other than TCDD: As previously described, the AhR mediates the toxic manifestation of TCDD and TCDD-like chemicals found in the environment. However, the AhR is an evolutionarily-conserved receptor suggesting it's physiological function may extend beyond mediating toxicities of environmental pollutants such as TCDD. Corroborating this idea are studies describing AhR to negatively regulate LPS-induced inflammatory responses in

macrophages, demonstrating its physiologic role in inflammation (Kimura *et al*., 2009). More recently, numerous novel natural AhR ligands ranging from dietary components such as flavones to bilirubin have been described (Table1-2). Although several investigators have attempted to assess the impact of AhR activation by natural ligands, very little is known about the character of these natural AhR ligands and their role in AhR signaling. Characterizing the effects of natural AhR ligands and their specific mechanisms in immune cells may provide insights into the physiological functions of the AhR receptor in the immune system and help devise strategies by which AhR activation can be therapeutically exploited.

Table 1-2: The diverse range of natural and non-TCDD AhR ligands.

Natural and non-TCDD AhR ligands		
Parent Category	**Examples**	**Function**
Flavones	α-napthaflavone	• AhR antagonist (Zhang *et al.*, 2003)
	β-napthoflavone	• AhR agonist (Zhang *et al.*, 2003)
	Genistein	• Suppresses immune and inflammatory responses (Verdrengh *et al.*, 2003) • Decreases UV-induced inflammation(Widyarini *et al.*, 2001)
Indole-containing molecules	2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE)	• AhR agonist but does does not induce TCDD-induced toxicity such as cleft palate.
	6-formylindolo(3,2-b) carbazole	• Induces Th17 differentiation (Veldhoen et al., 2008) • Enhances EAE development
Benzoimidazole derivatives	M50367	• AhR receptor agonist (Morales *et al.*, 2008)
	M50354	• Suppresses Th2 differentitaion (Negishi *et al.*, 2005)
	VAF347	• Anti-inflammatory and anti-allergenic(Hauben *et al.*, 2008; Lawrence *et al.*, 2008)
	VAG539	• Inhibits rejection of pancreatic islet allografts in Balb/c mice associated with increased frequency and survival of $CD4^+$ $CD25^+$Foxp3 T-reg phenotype.(Hauben *et al.*, 2008)
Bilirubin		• Inhibits CD4 T cell function in vitro(Liu *et al.*, 2008; Kerkvliet, 2009). • Suppresses EAE development in vivo(Liu *et al.*, 2008)
Leflunamide		• Anti-inflammatory compound used in treatment of rheumatoid arthritis.(Herrmann *et al.*, 2000) • Metabolized into A771762, that inhibits denovo pyrimidine synthesis and inhibits T cell proliferation (Williamson *et al.*, 1995; Dimitrova *et al.*, 2002) • Induces antigen-specific regulatory CD4+ and CD8+ T cells (Weigmann *et al.*, 2006).

HYPOTHESIS

Dendritic cells (DCs) are the primary APCs involved in the activation of naïve T cells and constitutively express the AhR receptor. DCs are sensitive to TCDD; however limited information exists on the effects of AhR activation in these cells. Defects in T cell activation following AhR activation have been linked to AhR-induced defects in DCs. Previous studies demonstrated loss of splenic DCs following TCDD-exposure, as well as increased expression of CD54, MHC II and CD40 (Vorderstrasse and Kerkvliet, 2001; Vorderstrasse *et al.*, 2003). Interestingly, TCDD did not modulate the ability of DCs to process and present antigen (Vorderstrasse *et al.*, 2003). Ruby et al., also reported direct effects of TCDD on the maturation and survival of murine, GM-CSF-derived BMDCs. In these studies, TNF-α-induced DC maturation and Fas-mediated apoptotic death was enhanced by TCDD (Ruby *et al.*, 2005). Preliminary data from our laboratory demonstrated AhR activation by TCDD enhanced DC deletion and generation of defective T cell responses. TCDD-mediated loss or deletion of antigen-bearing DCs in the lymph node of mice that received Ag-specific OT II cells was found to be dependent on AhR expression. In addition, DCs from TCDD-treated mice produced less IL-12 and IL-18 compared to vehicle-treated mice. Cumulatively, these studies demonstrate that DCs are direct targets of TCDD-induced toxicity mediated by AhR activation. These TCDD-induced changes in DCs may dysregulate an immune response and underlie immune suppression.

Because DCs play a key role in generation of successful T cell-mediated immunity, clearly defining the potential effects of persistent environmental

pollutants such as TCDD on their fate and function is important. The overall goal purpose of this study is to provide in depth characterization of the consequences of AhR activation on murine DCs. The central hypothesis is that ***AhR activation modulates DC fate and function, thereby contributing to TCDD-mediated immune suppression***. The following specific aims using *in vivo* and *in vitro* approaches were designed to address this hypothesis:

Aim 1. To evaluate the effects of TCDD on the fate of naïve DCs. (Chapter 2) Previous studies conducted by Vorderstrasse and coworkers provided an initial characterization of the effects of TCDD on murine DCs. In these studies, TCDD induced a decrease in $CD11c^+$ DCs and also affected the expression of key surface costimulatory molecules in an AhR-mediated fashion (Vorderstrasse and Kerkvliet, 2001; Vorderstrasse *et al.*, 2003). In a separate study, TCDD was reported to enhance the TNF-α-induced maturation of BMDCs rendering them more sensitive to CD95-mediated apoptosis (Ruby et al., 2005). However, the effects of TCDD on specific murine DC subsets remains to be defined and the mechanisms underlying the loss of naïve splenic DCs remain to be elucidated. ***We hypothesized that TCDD alters DC homeostasis, resulting in the loss of DCs in naïve mice***.

To perform this investigation, C57Bl/6 mice were gavaged with vehicle or an immunosuppressive dose of TCDD (15μg/kg). Spleen and lymph nodes were harvested and assessed for TCDD-induced modulation of DC differentiation markers. Furthermore, effects of TCDD on the fate of functionally distinct splenic $CD11c^{high}$ DC subsets, the $CD11c^{high}$ $CD8\alpha^+$ $DEC205^+$ and the $CD11c^{high}$ $CD8\alpha^-$

33D1^{+} cells were assessed. Since TCDD selectively, decreased splenic CD11c^{high} DC, we evaluated the role of apoptotic and/or migratory events in mediating this loss of splenic CD11c^{high} DCs. The extrinsic apoptotic pathway was assessed using Fas deficient (lpr/lpr) and FasL deficient (gld/gld) deficient mice. Alternatively, TCDD-induced migration was evaluated via induction of CCR7, a prominent chemokine receptor involved in the migration and trafficking of DCs.

Aim 2. To examine the effects of TCDD on the innate and adaptive immune function of murine BMDCs and assess whether the effects are mediated exclusively via Dioxin response elements (DREs) (Chapter 3)

Dendritic cells are hematopoietically-derived cells which can be generated *in vitro* from murine bone marrow cells using the growth factor, GM-CSF. Generation of bone marrow-derived dendritic cells (BMDCs) allows a rapid, convenient assessment of DCs following exposure to TCDD. Many investigators have attempted to recapitulate the observed *in vivo* effects of TCDD exposure on DCs in an *in vitro* setting. For instance, studies conducted by Ruby et al., showed that TCDD-treatment of BMDCs enhanced TNF-α-induced maturation, and also augmented the CD95-mediated death of BMDCs (Ruby et al., 2004). More recently, Lee et al., demonstrated TCDD-induced down regulation of RelB mRNA levels in GM-CSF derived BMDCs (conventional DCs) (Lee *et al.*, 2007). However, the effects of TCDD on a homogenous, pure population of DCs remains to be characterized. Additionally, several investigators have linked AhR activation by TCDD with induction of Tregs and Treg inducing mediators such as

IDO1, IDO2 and TGF-β3. Despite these studies, it remains to be determined whether TCDD affects the normal development and function of DCs. Additionally, it is unknown whether AhR activation induces regulatory DCs that could contribute to Treg generation. Under the second specific aim TCDD-induced phenotypic and functional alterations in conventional DCs (GM-CSF derived) will be examined. ***We hypothesize that TCDD alters BMDC differentiation and generates a regulatory phenotype.***

To perform this study, inflammatory BMDCs from C57Bl/6 mice were generated using GM-CSF, in the presence of vehicle (DMSO) or TCDD. TCDD-induced modulation in BMDC differentiation markers, antigen-uptake and TLR responsiveness was assessed. Furthermore, NF-kB activation profile in these BMDC cultures was assessed using TransAM NF-κB colorimetric assays. The functional ability of vehicle-or TCDD-treated BMDCs to interact with $CD4^+$ T cells in an antigen-specific fashion and influence the development of an adaptive immune response was tested using an adoptive transfer model. To delineate the role of the activated AhR in the fate and development of conventional DCs *in vitro*, we generated BMDCs from the bone marrow of $AhR^{-/-}$ and $AhR^{nls/nls}$ and $AhR^{dbd/dbd}$ mice. Bone marrow cells from these mutant mice were cultured in presence of GM-CSF and vehicle or TCDD (10nM) for 6 days. Non-adherent cells were harvested and evaluated for TCDD effects on phenotypic DC markers. Alteration in DC phenotype was compared to naïve C57Bl/6 mice expressing high-affinity AhR. Additionally, mRNA levels of suppressive T-reg inducing mediators such as Indoleamine 2,3-dioxygenase (IDO) and TGF-β3 were

evaluated in BMDCs.

Aim 3: To examine whether AhR-activated BMDCs contribute to the generation of regulatory T cells (Chapter 4) Results from the previous specific aim 2 demonstrated TCDD-induced regulatory phenotype in DCs characterized by increased IDO1, IDO2 and TGF-β3 mRNA levels. We therefore, assessed the effects of these regulatory DCs on the generation of T regs. ***We hypothesized that TCDD-treated BMDCs, displaying a regulatory phenotype induce adaptive Tregs***.

To perform this investigation, ova-peptide loaded, vehicle- or TCDD – treated BMDCs were cocultured with antigen-specific $CD4^+CD25^-$ T cells. On day 4, cells were harvested and evaluated. Additionally, an adoptive transfer model examining the role of LPS-activated TCDD BMDCs in suppressing T cell activation was employed.

REFERENCES

Ackermann, M. F., Gasiewicz, T. A., Lamm, K. R., Germolec, D. R., and Luster, M. I. (1989). Selective inhibition of polymorphonuclear neutrophil activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology and applied pharmacology* **101**, 470-480.

Adler, A. J., Marsh, D. W., Yochum, G. S., Guzzo, J. L., Nigam, A., Nelson, W. G., and Pardoll, D. M. (1998). CD4+ T cell tolerance to parenchymal self-antigens requires presentation by bone marrow-derived antigen-presenting cells. *The Journal of experimental medicine* **187**, 1555-1564.

Aylward, L. L., Brunet, R. C., Carrier, G., Hays, S. M., Cushing, C. A., Needham, L. L., Patterson, D. G., Jr., Gerthoux, P. M., Brambilla, P., and Mocarelli, P. (2005). Concentration-dependent TCDD elimination kinetics in humans: toxicokinetic modeling for moderately to highly exposed adults from Seveso, Italy, and Vienna, Austria, and impact on dose estimates for the NIOSH cohort. *J Expo Anal Environ Epidemiol* **15**, 51-65.

Barsotti, D. A., Abrahamson, L. J., and Allen, J. R. (1979). Hormonal alterations in female rhesus monkeys fed a diet containing 2, 3,7,8-tetrachlorodibenzo-p-dioxin. *Bulletin of environmental contamination and toxicology* **21**, 463-469.

Basak, S., and Hoffmann, A. (2008). Crosstalk via the NF-kappaB signaling system. *Cytokine & growth factor reviews* **19**, 187-197.

Beischlag, T. V., Luis Morales, J., Hollingshead, B. D., and Perdew, G. H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression. *Critical reviews in eukaryotic gene expression* **18**, 207-250.

Birnbaum, L. S. (1994). The mechanism of dioxin toxicity: relationship to risk assessment. *Environmental health perspectives* **102 Suppl 9**, 157-167.

Birnbaum, L. S., and Tuomisto, J. (2000). Non-carcinogenic effects of TCDD in animals. *Food additives and contaminants* **17**, 275-288.

Bock, K. W. (1994). Aryl hydrocarbon or dioxin receptor: biologic and toxic responses. *Reviews of physiology, biochemistry and pharmacology* **125**, 1-42.

Brawand, P., Fitzpatrick, D. R., Greenfield, B. W., Brasel, K., Maliszewski, C. R., and De Smedt, T. (2002). Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. *J Immunol* **169**, 6711-6719.

Brouwer, A., Ahlborg, U. G., van Leeuwen, F. X., and Feeley, M. M. (1998). Report of the WHO working group on the assessment of health risks for human infants from exposure to PCDDs, PCDFs and PCBs. *Chemosphere* **37**, 1627-1643.

Bryant, P., and Ploegh, H. (2004). Class II MHC peptide loading by the professionals. *Current opinion in immunology* **16**, 96-102.

Bunger, M. K., Moran, S. M., Glover, E., Thomae, T. L., Lahvis, G. P., Lin, B. C., and Bradfield, C. A. (2003). Resistance to 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity and abnormal liver development in mice carrying a mutation

in the nuclear localization sequence of the aryl hydrocarbon receptor. *The Journal of biological chemistry* **278**, 17767-17774.

Calvert, G. M., Sweeney, M. H., Deddens, J., and Wall, D. K. (1999). Evaluation of diabetes mellitus, serum glucose, and thyroid function among United States workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Occupational and environmental medicine* **56**, 270-276.

Camacho, I. A., Singh, N., Hegde, V. L., Nagarkatti, M., and Nagarkatti, P. S. (2005). Treatment of mice with 2,3,7,8-tetrachlorodibenzo-p-dioxin leads to aryl hydrocarbon receptor-dependent nuclear translocation of NF-kappaB and expression of Fas ligand in thymic stromal cells and consequent apoptosis in T cells. *J Immunol* **175**, 90-103.

Carver, L. A., Jackiw, V., and Bradfield, C. A. (1994). The 90-kDa heat shock protein is essential for Ah receptor signaling in a yeast expression system. *The Journal of biological chemistry* **269**, 30109-30112.

Charles A. Janeway, P. T., Mark Walport, Mark J. Schlomick (2005). *Immunobiology*. Garland Science Publishing, New York

Cheon, H., Woo, Y. S., Lee, J. Y., Kim, H. S., Kim, H. J., Cho, S., Won, N. H., and Sohn, J. (2007). Signaling pathway for 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced TNF-alpha production in differentiated THP-1 human macrophages. *Experimental & molecular medicine* **39**, 524-534.

Choi, J. Y., Oughton, J. A., and Kerkvliet, N. I. (2003). Functional alterations in CD11b(+)Gr-1(+) cells in mice injected with allogeneic tumor cells and treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *International immunopharmacology* **3**, 553-570.

Cole, P., Trichopoulos, D., Pastides, H., Starr, T., and Mandel, J. S. (2003). Dioxin and cancer: a critical review. *Regul Toxicol Pharmacol* **38**, 378-388.

Cools, N., Ponsaerts, P., Van Tendeloo, V. F., and Berneman, Z. N. (2007). Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. *Journal of leukocyte biology* **82**, 1365-1374.

Couture, L. A., Abbott, B. D., and Birnbaum, L. S. (1990a). A critical review of the developmental toxicity and teratogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin: recent advances toward understanding the mechanism. *Teratology* **42**, 619-627.

Couture, L. A., Harris, M. W., and Birnbaum, L. S. (1990b). Characterization of the peak period of sensitivity for the induction of hydronephrosis in C57BL/6N mice following exposure to 2,3,7, 8-tetrachlorodibenzo-p-dioxin. *Fundam Appl Toxicol* **15**, 142-150.

Crawford, R. B., Holsapple, M. P., and Kaminski, N. E. (1997). Leukocyte activation induces aryl hydrocarbon receptor up-regulation, DNA binding, and increased Cyp1a1 expression in the absence of exogenous ligand. *Molecular pharmacology* **52**, 921-927.

Cresswell, P. (2005). Antigen processing and presentation. *Immunological reviews* **207**, 5-7.

Croft, M., and Dubey, C. (1997). Accessory molecule and costimulation requirements for CD4 T cell response. *Critical reviews in immunology* **17**, 89-118.

Dickson, L. C., and Buzik, S. C. (1993). Health risks of "dioxins": a review of environmental and toxicological considerations. *Veterinary and human toxicology* **35**, 68-77.

Dimitrova, P., Skapenko, A., Herrmann, M. L., Schleyerbach, R., Kalden, J. R., and Schulze-Koops, H. (2002). Restriction of de novo pyrimidine biosynthesis inhibits Th1 cell activation and promotes Th2 cell differentiation. *J Immunol* **169**, 3392-3399.

Dolwick, K. M., Schmidt, J. V., Carver, L. A., Swanson, H. I., and Bradfield, C. A. (1993). Cloning and expression of a human Ah receptor cDNA. *Molecular pharmacology* **44**, 911-917.

Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M., and Nussenzweig, M. C. (2007). Differential antigen processing by dendritic cell subsets in vivo. *Science (New York, N.Y* **315**, 107-111.

Ema, M., Matsushita, N., Sogawa, K., Ariyama, T., Inazawa, J., Nemoto, T., Ota, M., Oshimura, M., and Fujii-Kuriyama, Y. (1994a). Human arylhydrocarbon receptor: functional expression and chromosomal assignment to 7p21. *Journal of biochemistry* **116**, 845-851.

Ema, M., Ohe, N., Suzuki, M., Mimura, J., Sogawa, K., Ikawa, S., and Fujii-Kuriyama, Y. (1994b). Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors. *The Journal of biological chemistry* **269**, 27337-27343.

Fernandez-Salguero, P., Pineau, T., Hilbert, D. M., McPhail, T., Lee, S. S., Kimura, S., Nebert, D. W., Rudikoff, S., Ward, J. M., and Gonzalez, F. J. (1995). Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science (New York, N.Y* **268**, 722-726.

Fingerhut, M. A., Halperin, W. E., Marlow, D. A., Piacitelli, L. A., Honchar, P. A., Sweeney, M. H., Greife, A. L., Dill, P. A., Steenland, K., and Suruda, A. J. (1991). Cancer mortality in workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *The New England journal of medicine* **324**, 212-218.

Forster, I., and Lieberam, I. (1996). Peripheral tolerance of CD4 T cells following local activation in adolescent mice. *European journal of immunology* **26**, 3194-3202.

Funatake, C. J., Marshall, N. B., and Kerkvliet, N. I. (2008). 2,3,7,8-Tetrachlorodibenzo-p-dioxin alters the differentiation of alloreactive CD8+ T cells toward a regulatory T cell phenotype by a mechanism that is dependent on aryl hydrocarbon receptor in CD4+ T cells. *Journal of immunotoxicology* **5**, 81-91.

Funatake, C. J., Marshall, N. B., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2005). Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin generates a population of CD4+ CD25+ cells with characteristics of regulatory T cells. *J Immunol* **175**, 4184-4188.

Ge, N. L., and Elferink, C. J. (1998). A direct interaction between the aryl hydrocarbon receptor and retinoblastoma protein. Linking dioxin signaling to the cell cycle. *The Journal of biological chemistry* **273**, 22708-22713.

Gilliet, M., Boonstra, A., Paturel, C., Antonenko, S., Xu, X. L., Trinchieri, G., O'Garra, A., and Liu, Y. J. (2002). The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *The Journal of experimental medicine* **195**, 953-958.

Goldsby, R., Kindt, T.J., Osbourne, B.A. and Kuby, J. (2003). *Immunology*. W.H.Freeman and Company, New York.

Greenwald, R. J., Freeman, G. J., and Sharpe, A. H. (2005). The B7 family revisited. *Annual review of immunology* **23**, 515-548.

Grohmann, U., Fallarino, F., and Puccetti, P. (2003). Tolerance, DCs and tryptophan: much ado about IDO. *Trends in immunology* **24**, 242-248.

Hankinson, O. (1995). The aryl hydrocarbon receptor complex. *Annual review of pharmacology and toxicology* **35**, 307-340.

Hauben, E., Gregori, S., Draghici, E., Migliavacca, B., Olivieri, S., Woisetschlager, M., and Roncarolo, M. G. (2008). Activation of the aryl hydrocarbon receptor promotes allograft-specific tolerance through direct and dendritic cell-mediated effects on regulatory T cells. *Blood* **112**, 1214-1222.

Head, J. L., and Lawrence, B. P. (2009). The aryl hydrocarbon receptor is a modulator of anti-viral immunity. *Biochemical pharmacology* **77**, 642-653.

Herrmann, M. L., Schleyerbach, R., and Kirschbaum, B. J. (2000). Leflunomide: an immunomodulatory drug for the treatment of rheumatoid arthritis and other autoimmune diseases. *Immunopharmacology* **47**, 273-289.

Hoffmann, A., Natoli, G., and Ghosh, G. (2006). Transcriptional regulation via the NF-kappaB signaling module. *Oncogene* **25**, 6706-6716.

Huang, G., and Elferink, C. J. (2005). Multiple mechanisms are involved in Ah receptor-mediated cell cycle arrest. *Molecular pharmacology* **67**, 88-96.

IARC (1997). IARC Monograpgs on the evaluation of carcinogenic risks to humans. *Polychlorinated dibenzo-para-dioxins and polychlorinated dibenzofurans* **69**.

Ivanov, II, McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J., and Littman, D. R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* **126**, 1121-1133.

Jeon, M. S., and Esser, C. (2000). The murine IL-2 promoter contains distal regulatory elements responsive to the Ah receptor, a member of the evolutionarily conserved bHLH-PAS transcription factor family. *J Immunol* **165**, 6975-6983.

Karchner, S. I., Franks, D. G., Powell, W. H., and Hahn, M. E. (2002). Regulatory interactions among three members of the vertebrate aryl hydrocarbon receptor family: AHR repressor, AHR1, and AHR2. *The Journal of biological chemistry* **277**, 6949-6959.

Karras, J. G., Morris, D. L., Matulka, R. A., Kramer, C. M., and Holsapple, M. P. (1996). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) elevates basal B-cell intracellular calcium concentration and suppresses surface Ig- but not CD40-induced antibody secretion. *Toxicology and applied pharmacology* **137**, 275-284.

Kerkvliet, N. I. (2002). Recent advances in understanding the mechanisms of TCDD immunotoxicity. *International immunopharmacology* **2**, 277-291.

Kerkvliet, N. I. (2009). AHR-mediated immunomodulation: the role of altered gene transcription. *Biochemical pharmacology* **77**, 746-760.

Kerkvliet, N. I., Baecher-Steppan, L., Shepherd, D. M., Oughton, J. A., Vorderstrasse, B. A., and DeKrey, G. K. (1996). Inhibition of TC-1 cytokine production, effector cytotoxic T lymphocyte development and alloantibody production by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Immunol* **157**, 2310-2319.

Kerkvliet, N. I., and Oughton, J. A. (1993). Acute inflammatory response to sheep red blood cell challenge in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): phenotypic and functional analysis of peritoneal exudate cells. *Toxicology and applied pharmacology* **119**, 248-257.

Kerkvliet, N. I., Shepherd, D. M., and Baecher-Steppan, L. (2002). T lymphocytes are direct, aryl hydrocarbon receptor (AhR)-dependent targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): AhR expression in both CD4+ and CD8+ T cells is necessary for full suppression of a cytotoxic T lymphocyte response by TCDD. *Toxicology and applied pharmacology* **185**, 146-152.

Kim, H. A., Kim, E. M., Park, Y. C., Yu, J. Y., Hong, S. K., Jeon, S. H., Park, K. L., Hur, S. J., and Heo, Y. (2003). Immunotoxicological effects of Agent Orange exposure to the Vietnam War Korean veterans. *Industrial health* **41**, 158-166.

Kimura, A., Naka, T., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., Fujii-Kuriyama, Y., and Kishimoto, T. (2009b). Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *The Journal of experimental medicine*.

Kociba, R. J., Keeler, P. A., Park, C. N., and Gehring, P. J. (1976). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): results of a 13-week oral toxicity study in rats. *Toxicology and applied pharmacology* **35**, 553-574.

Kramer, C. M., Johnson, K. W., Dooley, R. K., and Holsapple, M. P. (1987). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) enhances antibody production and protein kinase activity in murine B cells. *Biochemical and biophysical research communications* **145**, 25-33.

Kurata, H., Lee, H. J., O'Garra, A., and Arai, N. (1999). Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity* **11**, 677-688.

Kurts, C., Heath, W. R., Carbone, F. R., Allison, J., Miller, J. F., and Kosaka, H. (1996). Constitutive class I-restricted exogenous presentation of self antigens in vivo. *The Journal of experimental medicine* **184**, 923-930.

Lang, D. S., Becker, S., Clark, G. C., Devlin, R. B., and Koren, H. S. (1994). Lack of direct immunosuppressive effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin

(TCDD) on human peripheral blood lymphocyte subsets in vitro. *Archives of toxicology* **68**, 296-302.

Lawrence , B. a. K. N. (2007). Immune modulation by TCDD and related polyhalogneated aromatic hydrocarbons. In *Immunotoxicology and Immunopharmacology* (R. H. a. I. k. R. Luebke, Ed.), pp. 239-258. CRC Press.

Lawrence, B. P., Denison, M. S., Novak, H., Vorderstrasse, B. A., Harrer, N., Neruda, W., Reichel, C., and Woisetschlager, M. (2008). Activation of the aryl hydrocarbon receptor is essential for mediating the anti-inflammatory effects of a novel low-molecular-weight compound. *Blood* **112**, 1158-1165.

Lawrence, B. P., Leid, M., and Kerkvliet, N. I. (1996). Distribution and behavior of the Ah receptor in murine T lymphocytes. *Toxicology and applied pharmacology* **138**, 275-284.

Lawrence, B. P., Roberts, A. D., Neumiller, J. J., Cundiff, J. A., and Woodland, D. L. (2006). Aryl hydrocarbon receptor activation impairs the priming but not the recall of influenza virus-specific CD8+ T cells in the lung. *J Immunol* **177**, 5819-5828.

Lee, J. A., Hwang, J. A., Sung, H. N., Jeon, C. H., Gill, B. C., Youn, H. J., and Park, J. H. (2007). 2,3,7,8-Tetrachlorodibenzo-p-dioxin modulates functional differentiation of mouse bone marrow-derived dendritic cells Downregulation of RelB by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Lett* **173**, 31-40.

Lenschow, D. J., Walunas, T. L., and Bluestone, J. A. (1996). CD28/B7 system of T cell costimulation. *Annual review of immunology* **14**, 233-258.

Liu, Y., Li, P., Lu, J., Xiong, W., Oger, J., Tetzlaff, W., and Cynader, M. (2008). Bilirubin possesses powerful immunomodulatory activity and suppresses experimental autoimmune encephalomyelitis. *J Immunol* **181**, 1887-1897.

Luster, M. I., Ackermann, M. F., Germolec, D. R., and Rosenthal, G. J. (1989). Perturbations of the immune system by xenobiotics. *Environ Health Perspect* **81**, 157-162.

Luster, M. I., Germolec, D. R., Clark, G., Wiegand, G., and Rosenthal, G. J. (1988). Selective effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and corticosteroid on in vitro lymphocyte maturation. *J Immunol* **140**, 928-935.

Lutz, M. B., Kukutsch, N. A., Menges, M., Rossner, S., and Schuler, G. (2000). Culture of bone marrow cells in GM-CSF plus high doses of lipopolysaccharide generates exclusively immature dendritic cells which induce alloantigen-specific CD4 T cell anergy in vitro. *European journal of immunology* **30**, 1048-1052.

Ma, Q., and Baldwin, K. T. (2000). 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway. Role of the transcription activaton and DNA binding of AhR. *The Journal of biological chemistry* **275**, 8432-8438.

Ma, Q., Renzelli, A. J., Baldwin, K. T., and Antonini, J. M. (2000). Superinduction of CYP1A1 gene expression. Regulation of 2,3,7, 8-tetrachlorodibenzo-p-dioxin-induced degradation of Ah receptor by cycloheximide. *The Journal of biological chemistry* **275**, 12676-12683.

Maraskovsky, E., Brasel, K., Teepe, M., Roux, E. R., Lyman, S. D., Shortman, K., and McKenna, H. J. (1996). Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *The Journal of experimental medicine* **184**, 1953-1962.

Marcus, R. S., Holsapple, M. P., and Kaminski, N. E. (1998). Lipopolysaccharide activation of murine splenocytes and splenic B cells increased the expression of aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator. *The Journal of pharmacology and experimental therapeutics* **287**, 1113-1118.

Marlowe, J. L., and Puga, A. (2005). Aryl hydrocarbon receptor, cell cycle regulation, toxicity, and tumorigenesis. *Journal of cellular biochemistry* **96**, 1174-1184.

Marshall, N. B., Vorachek, W. R., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2008). Functional characterization and gene expression analysis of CD4+ CD25+ regulatory T cells generated in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Immunol* **181**, 2382-2391.

Masten, S. A., and Shiverick, K. T. (1995). The Ah receptor recognizes DNA binding sites for the B cell transcription factor, BSAP: a possible mechanism for dioxin-mediated alteration of CD19 gene expression in human B lymphocytes. *Biochemical and biophysical research communications* **212**, 27-34.

Matthews, J., Wihlen, B., Thomsen, J., and Gustafsson, J. A. (2005). Aryl hydrocarbon receptor-mediated transcription: ligand-dependent recruitment of estrogen receptor alpha to 2,3,7,8-tetrachlorodibenzo-p-dioxin-responsive promoters. *Molecular and cellular biology* **25**, 5317-5328.

Max, S. R., and Silbergeld, E. K. (1987). Skeletal muscle glucocorticoid receptor and glutamine synthetase activity in the wasting syndrome in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology and applied pharmacology* **87**, 523-527.

Meyer, B. K., Pray-Grant, M. G., Vanden Heuvel, J. P., and Perdew, G. H. (1998). Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. *Molecular and cellular biology* **18**, 978-988.

Mimura, J., Ema, M., Sogawa, K., and Fujii-Kuriyama, Y. (1999). Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. *Genes Dev* **13**, 20-25.

Monteiro, P., Gilot, D., Le Ferrec, E., Rauch, C., Lagadic-Gossmann, D., and Fardel, O. (2008). Dioxin-mediated up-regulation of aryl hydrocarbon receptor target genes is dependent on the calcium/calmodulin/CaMKIalpha pathway. *Molecular pharmacology* **73**, 769-777.

Moos, A. B., Oughton, J. A., and Kerkvliet, N. I. (1997). The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on tumor necrosis factor (TNF) production by peritoneal cells. *Toxicology letters* **90**, 145-153.

Morales, J. L., Krzeminski, J., Amin, S., and Perdew, G. H. (2008). Characterization of the antiallergic drugs 3-[2-(2-phenylethyl) benzoimidazole-4-yl]-3-hydroxypropanoic acid and ethyl 3-hydroxy-3-[2-(2-phenylethyl)benzoimidazol-4-yl]propanoate as full aryl hydrocarbon receptor agonists. *Chemical research in toxicology* **21**, 472-482.

Moriguchi, T., Motohashi, H., Hosoya, T., Nakajima, O., Takahashi, S., Ohsako, S., Aoki, Y., Nishimura, N., Tohyama, C., Fujii-Kuriyama, Y., and Yamamoto, M. (2003). Distinct response to dioxin in an arylhydrocarbon receptor (AHR)-humanized mouse. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 5652-5657.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348-2357.

Mosmann, T. R., and Coffman, R. L. (1989a). Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Advances in immunology* **46**, 111-147.

Mosmann, T. R., and Coffman, R. L. (1989b). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual review of immunology* **7**, 145-173.

Moynagh, P. N. (2005). The NF-kappaB pathway. *Journal of cell science* **118**, 4589-4592.

Nebert, D. W., Roe, A. L., Dieter, M. Z., Solis, W. A., Yang, Y., and Dalton, T. P. (2000). Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochemical pharmacology* **59**, 65-85.

Negishi, T., Kato, Y., Ooneda, O., Mimura, J., Takada, T., Mochizuki, H., Yamamoto, M., Fujii-Kuriyama, Y., and Furusako, S. (2005). Effects of aryl hydrocarbon receptor signaling on the modulation of TH1/TH2 balance. *J Immunol* **175**, 7348-7356.

Neubert, D. (1997). Reflections on the assessment of the toxicity of "dioxins" for humans, using data from experimental and epidemiological studies. *Teratogenesis, carcinogenesis, and mutagenesis* **17**, 157-215.

Neubert, R., Jacob-Muller, U., Helge, H., Stahlmann, R., and Neubert, D. (1991). Polyhalogenated dibenzo-p-dioxins and dibenzofurans and the immune system. 2. In vitro effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on lymphocytes of venous blood from man and a non-human primate (Callithrix jacchus). *Archives of toxicology* **65**, 213-219.

Nohara, K., Pan, X., Tsukumo, S., Hida, A., Ito, T., Nagai, H., Inouye, K., Motohashi, H., Yamamoto, M., Fujii-Kuriyama, Y., and Tohyama, C. (2005). Constitutively active aryl hydrocarbon receptor expressed specifically in T-lineage cells causes thymus involution and suppresses

the immunization-induced increase in splenocytes. *J Immunol* **174**, 2770-2777.

Nohara, K., Ushio, H., Tsukumo, S., Kobayashi, T., Kijima, M., Tohyama, C., and Fujimaki, H. (2000). Alterations of thymocyte development, thymic emigrants and peripheral T cell population in rats exposed to 2,3,7, 8-tetrachlorodibenzo-p-dioxin. *Toxicology* **145**, 227-235.

Ohtake, F., Fujii-Kuriyama, Y., and Kato, S. (2009). AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions. *Biochemical pharmacology* **77**, 474-484.

Ott, M. G., Zober, A., and Germann, C. (1994). Laboratory results for selected target organs in 138 individuals occupationally exposed to TCDD. *Chemosphere* **29**, 2423-2437.

Park, S. J., Yoon, W. K., Kim, H. J., Son, H. Y., Cho, S. W., Jeong, K. S., Kim, T. H., Kim, S. H., Kim, S. R., and Ryu, S. Y. (2005). 2,3,7,8-Tetrachlorodibenzo-p-dioxin activates ERK and p38 mitogen-activated protein kinases in RAW 264.7 cells. *Anticancer research* **25**, 2831-2836.

Paul, W. E., and Seder, R. A. (1994). Lymphocyte responses and cytokines. *Cell* **76**, 241-251.

Perdew, G. H. (2008). Ah receptor binding to its cognate response element is required for dioxin-mediated toxicity. *Toxicol Sci* **106**, 301-303.

Prell, R. A., Dearstyne, E., Steppan, L. G., Vella, A. T., and Kerkvliet, N. I. (2000). CTL hyporesponsiveness induced by 2,3,7, 8-tetrachlorodibenzo-p-dioxin: role of cytokines and apoptosis. *Toxicology and applied pharmacology* **166**, 214-221.

Prell, R. A., and Kerkvliet, N. I. (1997). Involvement of altered B7 expression in dioxin immunotoxicity: B7 transfection restores the CTL but not the autoantibody response to the P815 mastocytoma. *J Immunol* **158**, 2695-2703.

Puga, A., Barnes, S. J., Dalton, T. P., Chang, C., Knudsen, E. S., and Maier, M. A. (2000). Aromatic hydrocarbon receptor interaction with the retinoblastoma protein potentiates repression of E2F-dependent transcription and cell cycle arrest. *The Journal of biological chemistry* **275**, 2943-2950.

Puga, A., Ma, C., and Marlowe, J. L. (2009). The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. *Biochemical pharmacology* **77**, 713-722.

Pulendran, B., Smith, J. L., Caspary, G., Brasel, K., Pettit, D., Maraskovsky, E., and Maliszewski, C. R. (1999). Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 1036-1041.

Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., Caccamo, M., Oukka, M., and Weiner, H. L. (2008). Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* **453**, 65-71.

Reyes, H., Reisz-Porszasz, S., and Hankinson, O. (1992). Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science (New York, N.Y* **256**, 1193-1195.

Ruby, C. E., Funatake, C. J., and Kerkvliet, N. I. (2005). 2,3,7,8 Tetrachlorodibenzo-p-Dioxin (TCDD) Directly Enhances the Maturation and Apoptosis of Dendritic Cells In Vitro. *Journal of immunotoxicology* **1**, 159-166.

Ruby, C. E., Leid, M., and Kerkvliet, N. I. (2002). 2,3,7,8-Tetrachlorodibenzo-p-dioxin suppresses tumor necrosis factor-alpha and anti-CD40-induced activation of NF-kappaB/Rel in dendritic cells: p50 homodimer activation is not affected. *Molecular pharmacology* **62**, 722-728.

Safe, S., Wang, F., Porter, W., Duan, R., and McDougal, A. (1998). Ah receptor agonists as endocrine disruptors: antiestrogenic activity and mechanisms. *Toxicology letters* **102-103**, 343-347.

Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008). Regulatory T cells and immune tolerance. *Cell* **133**, 775-787.

Sanchez-Sanchez, N., Riol-Blanco, L., and Rodriguez-Fernandez, J. L. (2006). The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *J Immunol* **176**, 5153-5159.

Schecter, A., Startin, J., Wright, C., Kelly, M., Papke, O., Lis, A., Ball, M., and Olson, J. (1994a). Dioxins in U.S. food and estimated daily intake. *Chemosphere* **29**, 2261-2265.

Schecter, A., Startin, J., Wright, C., Kelly, M., Papke, O., Lis, A., Ball, M., and Olson, J. R. (1994b). Congener-specific levels of dioxins and dibenzofurans in U.S. food and estimated daily dioxin toxic equivalent intake. *Environmental health perspectives* **102**, 962-966.

Schmidt, J. V., and Bradfield, C. A. (1996). Ah receptor signaling pathways. *Annual review of cell and developmental biology* **12**, 55-89.

Schmidt, J. V., Su, G. H., Reddy, J. K., Simon, M. C., and Bradfield, C. A. (1996). Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 6731-6736.

Schwartz, R. H. (2003). T cell anergy. *Annual review of immunology* **21**, 305-334.

Seefeld, M. D., Corbett, S. W., Keesey, R. E., and Peterson, R. E. (1984). Characterization of the wasting syndrome in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology and applied pharmacology* **73**, 311-322.

Shevach, E. M. (2006). From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* **25**, 195-201.

Shortman, K., and Liu, Y. J. (2002). Mouse and human dendritic cell subtypes. *Nature reviews* **2**, 151-161.

Shortman, K., and Naik, S. H. (2007). Steady-state and inflammatory dendritic-cell development. *Nature reviews* **7**, 19-30.

Spink, D. C., Eugster, H. P., Lincoln, D. W., 2nd, Schuetz, J. D., Schuetz, E. G., Johnson, J. A., Kaminsky, L. S., and Gierthy, J. F. (1992). 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1A1: a

comparison of the activities induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in MCF-7 cells with those from heterologous expression of the cDNA. *Archives of biochemistry and biophysics* **293**, 342-348.

Spink, D. C., Lincoln, D. W., 2nd, Dickerman, H. W., and Gierthy, J. F. (1990). 2,3,7,8-Tetrachlorodibenzo-p-dioxin causes an extensive alteration of 17 beta-estradiol metabolism in MCF-7 breast tumor cells. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 6917-6921.

Staples, J. E., Murante, F. G., Fiore, N. C., Gasiewicz, T. A., and Silverstone, A. E. (1998). Thymic alterations induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin are strictly dependent on aryl hydrocarbon receptor activation in hemopoietic cells. *J Immunol* **160**, 3844-3854.

Steenland, K., Bertazzi, P., Baccarelli, A., and Kogevinas, M. (2004). Dioxin revisited: developments since the 1997 IARC classification of dioxin as a human carcinogen. *Environmental health perspectives* **112**, 1265-1268.

Steinman, R. M., Hawiger, D., and Nussenzweig, M. C. (2003). Tolerogenic dendritic cells. *Annu Rev Immunol* **21**, 685-711.

Sulentic, C. E., Holsapple, M. P., and Kaminski, N. E. (1998). Aryl hydrocarbon receptor-dependent suppression by 2,3,7, 8-tetrachlorodibenzo-p-dioxin of IgM secretion in activated B cells. *Molecular pharmacology* **53**, 623-629.

Sulentic, C. E., Holsapple, M. P., and Kaminski, N. E. (2000). Putative link between transcriptional regulation of IgM expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin and the aryl hydrocarbon receptor/dioxin-responsive enhancer signaling pathway. *The Journal of pharmacology and experimental therapeutics* **295**, 705-716.

Sulentic, C. E., Zhang, W., Na, Y. J., and Kaminski, N. E. (2004). 2,3,7,8-tetrachlorodibenzo-p-dioxin, an exogenous modulator of the 3'alpha immunoglobulin heavy chain enhancer in the CH12.LX mouse cell line. *The Journal of pharmacology and experimental therapeutics* **309**, 71-78.

Suskind, R. R., and Hertzberg, V. S. (1984). Human health effects of 2,4,5-T and its toxic contaminants. *Jama* **251**, 2372-2380.

Sweeney, M. H., and Mocarelli, P. (2000). Human health effects after exposure to 2,3,7,8-TCDD. *Food additives and contaminants* **17**, 303-316.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., and Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**, 655-669.

Teske, S., Bohn, A. A., Hogaboam, J. P., and Lawrence, B. P. (2008). Aryl hydrocarbon receptor targets pathways extrinsic to bone marrow cells to enhance neutrophil recruitment during influenza virus infection. *Toxicol Sci* **102**, 89-99.

Teske, S., Bohn, A. A., Regal, J. F., Neumiller, J. J., and Lawrence, B. P. (2005). Activation of the aryl hydrocarbon receptor increases pulmonary neutrophilia and diminishes host resistance to influenza A virus. *American journal of physiology* **289**, L111-124.

Thurmond, T. S., Staples, J. E., Silverstone, A. E., and Gasiewicz, T. A. (2000). The aryl hydrocarbon receptor has a role in the in vivo maturation of

murine bone marrow B lymphocytes and their response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology and applied pharmacology* **165**, 227-236.

Umbreit, T. H., Hesse, E. J., and Gallo, M. A. (1987). Reproductive toxicity in female mice of dioxin-contaminated soils from a 2,4,5-trichlorophenoxyacetic acid manufacturing site. *Archives of environmental contamination and toxicology* **16**, 461-466.

van Duursen, M. B., Sanderson, J. T., and van den Berg, M. (2005). Cytochrome P450 1A1 and 1B1 in human blood lymphocytes are not suitable as biomarkers of exposure to dioxin-like compounds: polymorphisms and interindividual variation in expression and inducibility. *Toxicol Sci* **85**, 703-712.

Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J. C., and Stockinger, B. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* **453**, 106-109.

Verdrengh, M., Jonsson, I. M., Holmdahl, R., and Tarkowski, A. (2003). Genistein as an anti-inflammatory agent. *Inflamm Res* **52**, 341-346.

Vogel, C. F., and Matsumura, F. (2008). A new cross-talk between the aryl hydrocarbon receptor and RelB, a member of the NF-kappaB family. *Biochemical pharmacology*.

Vogel, C. F., and Matsumura, F. (2009). A new cross-talk between the aryl hydrocarbon receptor and RelB, a member of the NF-kappaB family. *Biochemical pharmacology* **77**, 734-745.

Vogel, C. F., Sciullo, E., Li, W., Wong, P., Lazennec, G., and Matsumura, F. (2007a). RelB, a new partner of aryl hydrocarbon receptor-mediated transcription. *Molecular endocrinology (Baltimore, Md* **21**, 2941-2955.

Vogel, C. F., Sciullo, E., and Matsumura, F. (2007b). Involvement of RelB in aryl hydrocarbon receptor-mediated induction of chemokines. *Biochemical and biophysical research communications* **363**, 722-726.

Vorderstrasse, B. A., Dearstyne, E. A., and Kerkvliet, N. I. (2003). Influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the antigen-presenting activity of dendritic cells. *Toxicol Sci* **72**, 103-112.

Vorderstrasse, B. A., and Kerkvliet, N. I. (2001). 2,3,7,8-Tetrachlorodibenzo-p-dioxin affects the number and function of murine splenic dendritic cells and their expression of accessory molecules. *Toxicology and applied pharmacology* **171**, 117-125.

Vremec, D., Pooley, J., Hochrein, H., Wu, L., and Shortman, K. (2000). CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* **164**, 2978-2986.

Watanabe, T., Imoto, I., Kosugi, Y., Fukuda, Y., Mimura, J., Fujii, Y., Isaka, K., Takayama, M., Sato, A., and Inazawa, J. (2001). Human arylhydrocarbon receptor repressor (AHRR) gene: genomic structure and analysis of polymorphism in endometriosis. *J Hum Genet* **46**, 342-346.

Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M., and Murphy, K. M. (2006). Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* **24**, 677-688.

Webb, K. B., Evans, R. G., Knutsen, A. P., Roodman, S. T., Roberts, D. W., Schramm, W. F., Gibson, B. B., Andrews, J. S., Jr., Needham, L. L., and Patterson, D. G. (1989). Medical evaluation of subjects with known body levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Journal of toxicology and environmental health* **28**, 183-193.

Weigmann, B., Jarman, E. R., Sudowe, S., Bros, M., Knop, J., and Reske-Kunz, A. B. (2006). Induction of regulatory T cells by leflunomide in a murine model of contact allergen sensitivity. *The Journal of investigative dermatology* **126**, 1524-1533.

Whitlock, J. P., Jr (1993). Mechanistic aspects of dioxin action. *Chemical research in toxicology* **6**, 754-763.

Whitlock, J. P., Jr. (1999). Induction of cytochrome P4501A1. *Annual review of pharmacology and toxicology* **39**, 103-125.

Widyarini, S., Spinks, N., Husband, A. J., and Reeve, V. E. (2001). Isoflavonoid compounds from red clover (Trifolium pratense) protect from inflammation and immune suppression induced by UV radiation. *Photochemistry and photobiology* **74**, 465-470.

Williamson, R. A., Yea, C. M., Robson, P. A., Curnock, A. P., Gadher, S., Hambleton, A. B., Woodward, K., Bruneau, J. M., Hambleton, P., Moss, D., Thomson, T. A., Spinella-Jaegle, S., Morand, P., Courtin, O., Sautes, C., Westwood, R., Hercend, T., Kuo, E. A., and Ruuth, E. (1995). Dihydroorotate dehydrogenase is a high affinity binding protein for A77 1726 and mediator of a range of biological effects of the immunomodulatory compound. *The Journal of biological chemistry* **270**, 22467-22472.

Wolfe, W. H., Michalek, J. E., Miner, J. C., Rahe, A., Silva, J., Thomas, W. F., Grubbs, W. D., Lustik, M. B., Karrison, T. G., Roegner, R. H., and et al. (1990). Health status of Air Force veterans occupationally exposed to herbicides in Vietnam. I. Physical health. *Jama* **264**, 1824-1831.

Wormke, M., Stoner, M., Saville, B., Walker, K., Abdelrahim, M., Burghardt, R., and Safe, S. (2003). The aryl hydrocarbon receptor mediates degradation of estrogen receptor alpha through activation of proteasomes. *Molecular and cellular biology* **23**, 1843-1855.

Xu, Y., Zhan, Y., Lew, A. M., Naik, S. H., and Kershaw, M. H. (2007). Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol* **179**, 7577-7584.

Yamazaki, S., Dudziak, D., Heidkamp, G. F., Fiorese, C., Bonito, A. J., Inaba, K., Nussenzweig, M. C., and Steinman, R. M. (2008). CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J Immunol* **181**, 6923-6933.

Yamazaki, S., and Steinman, R. M. (2009). Dendritic cells as controllers of antigen-specific Foxp3+ regulatory T cells. *J Dermatol Sci* **54**, 69-75.

Yao, Z., Fanslow, W. C., Seldin, M. F., Rousseau, A. M., Painter, S. L., Comeau, M. R., Cohen, J. I., and Spriggs, M. K. (1995). Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* **3**, 811-821.

Zhang, S., Qin, C., and Safe, S. H. (2003). Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context. *Environmental health perspectives* **111**, 1877-1882.

Zhu, J., Guo, L., Watson, C. J., Hu-Li, J., and Paul, W. E. (2001). Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. *J Immunol* **166**, 7276-7281.

Zhu, J., and Paul, W. E. (2008). CD4 T cells: fates, functions, and faults. *Blood* **112**, 1557-1569.

CHAPTER 2

Effects of TCDD on the fate of naïve dendritic cells

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ABSTRACT

The environmental contaminant, TCDD (dioxin), causes immune suppression via activation of the Aryl hydrocarbon receptor (AhR). Dendritic cells (DCs), the professional antigen-presenting cells in the immune system, are adversely affected by TCDD. We hypothesize that TCDD alters DC homeostasis, resulting in DC loss in naïve mice. To test this hypothesis, C57Bl/6 mice were gavaged with either vehicle or TCDD (15 μg/kg). Consistent with previous reports, TCDD exposure decreased the frequency and number of splenic $CD11c^{high}$ DCs on day 7, when compared to vehicle. TCDD increased the expression of CD86 and CD54, while decreasing the frequency of CD11a and MHC Class II on the splenic $CD11c^{high}$ DCs. The frequency of CD40 on $CD11c^{high}$ DCs was increased by TCDD. Moreover, TCDD selectively decreased the $CD11c^{high}CD8\alpha^{-}33D1^{+}$ splenic DCs subset specialized at activating $CD4^{+}$ T cells but did not affect the regulatory $CD11c^{high}CD8\alpha^{+}DEC205^{+}$ splenic DC subset. TCDD did not alter the number and frequency of $CD11c^{low}$ splenic DCs, but decreased their MHC II and CD11a expression. The splenic $CD11c^{high}$ DC loss was independent of Fas-mediated apoptosis. Furthermore, $CD11c^{high}$ DCs displayed no apoptosis as defined by Annexin V/7-AAD staining. Instead, increased CCR7 expression on $CD11c^{high}$ DCs suggested involvement of a migratory event. Popliteal and brachial lymph node (PBLNs) $CD11c^{+}$ cells displayed elevated levels of MHC II and CD40 but not DC loss following TCDD exposure. Collectively, this study demonstrates the presence of a TCDD-

sensitive splenic DC subpopulation in naïve mice, suggesting that TCDD may induce suppression of T cell-mediated immunity by disrupting DC homeostasis.

Key words: Aryl hydrocarbon receptor; dendritic cells; immunosuppression; TCDD; apoptosis; CCR7

Abbreviations: AhR, Aryl hydrocarbon receptor; cells; Ag, antigen; APC, antigen presenting cell; CCR7, Chemokine receptor 7; CCL19, EBI1 ligand chemokine/MIP-3β; CCL21, secondary lymphoid tissue chemokine [SLC]/6Ckine; DCs, dendritic cells; IKDC, Interferon producing killer dendritic cell; PAMPs, pathogen-associated molecular patterns; PBLN, popliteal and brachial lymph nodes; pDC, plasmacytoid dendritic cell; TCDD, 2,3,7,8 tetrachlorodibenzo-p-dioxin.

INTRODUCTION

The immune system has been identified as a sensitive target of 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) (Luster *et al.*, 1989). Exposure of laboratory rodents to very low doses of TCDD suppresses the generation of adaptive immunity and increases the susceptibility to infectious diseases (Kerkvliet and Burleson, 1994). TCDD primarily exerts its toxic effects by binding and activation of the Aryl hydrocarbon receptor (AhR) (Fernandez-Salguero *et al*., 1996; Perdew, 2008).

Dendritic cells (DC) are the most potent antigen presenting cells and the primary immune cells responsible for the activation of naive T cells. DCs populate most tissues in mice and can be identified by the expression of the integrin, CD11c. DCs progress through multiple differentiation stages based on their activation status. Immature DCs are characterized by expression of low levels of CD11c, and of co-stimulatory molecules such as MHC II, CD86 and CD54, but are very efficient at the phagocytosis of antigen. Following activation, immature DCs migrate to the draining lymph nodes, undergo maturation and activate naïve T cells leading to generation of antigen-specific, T cell-mediated immune responses. Mature DCs are $CD11c^{high}$ but exhibit a decreased capacity to internalize antigens. The activation, maturation and migration of DCs to T cell-rich areas such as lymph nodes can be triggered by phagocytic uptake of antigens or exposure to pathogen-associated molecular patterns (PAMPs) such as LPS (Winzler *et al.*, 1997a; Winzler *et al.*, 1997b; Banchereau *et al.*, 2001). Migration of DCs to lymph nodes is mediated by chemokine receptors such as

CCR7 (Gunn, 2003; MartIn-Fontecha *et al.*, 2003; Randolph *et al.*, 2005). Deficiency of CCR7 or its primary ligands CCL19 or CCL21, results in abnormal lymph node development and dysfunctional leukocyte trafficking (Forster *et al.*, 1999; Gunn *et al.*, 1999; Mori *et al.*, 2001; MartIn-Fontecha *et al.*, 2003).

DCs exist in a variety of subsets specialized in mediating distinct effector functions. In the murine spleen, two distinct subpopulations of DCs have been identified phenotypically by the expression of CD11c: $CD11c^{high}$ and $CD11c^{low}$ cells. $CD11c^{low}$ DCs have been characterized to be immature DCs whereas the $CD11c^{high}$ DCs are considered mature. Recently, two functionally distinct $CD11c^{high}$ DC subsets in the spleen were identified based on their expression of $CD11c^{high}CD8\alpha^{+}DEC205^{+}$ and $CD11c^{high}CD8\alpha^{-}33D1^{+}$ (Dudziak *et al.*, 2007). $CD8\alpha^{+}DEC205^{+}$ DCs are specialized at cross-presentation of antigens to $CD8^{+}$ T cells and at inducing $Foxp3^{+}$ regulatory T cells (Dudziak *et al.*, 2007; Yamazaki *et al.*, 2008). On the other hand, $CD8\alpha^{-}33D1^{+}$ DCs are more effective at the uptake of exogenous Ags and subsequent activation of $CD4^{+}$ T cells.

DCs are sensitive to TCDD, however, limited information exists on the effects of AhR activation in these cells. Because DCs play a key role in the generation of successful T cell-mediated immunity, it is important to define the potential effects of persistent environmental pollutants such as TCDD on their fate and function. Studies conducted by Vorderstrasse and coworkers provided an initial characterization of the effects of TCDD on naïve murine DCs. In these studies, TCDD induced a decrease in $CD11c^{+}$ DCs and also affected the expression of key surface co-stimulatory molecules in an AhR-mediated fashion

(Vorderstrasse and Kerkvliet, 2001; Vorderstrasse *et al.*, 2003). In a separate study, TCDD was reported to enhance the TNF-α-induced maturation of bone marrow-derived dendritic cells (BMDCs) rendering them more sensitive to CD95-mediated apoptosis (Ruby *et al.*, 2005). However, the effects of TCDD on specific murine DC subsets remains to be defined and the mechanisms underlying the loss of naïve splenic DCs remain to be elucidated. In the present study, we have characterized the fate of distinct DC subsets in the spleen and lymph nodes of TCDD-treated naïve mice. In addition, we have explored potential mechanisms responsible for TCDD-induced disruption of DC homeostasis in unimmunized mice.

MATERIALS AND METHODS:

Animals. 6-8 week old C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, ML) and maintained in accordance with UM IACUC standards. Animals were provided rodent chow and tap water ad libitum. Breeding pairs of AhR knockout mice ($AhR^{-/-}$) were obtained from Dr. Paige Lawrence (University of Rochester) and subsequently bred and maintained at UM. $C57Bl/6^{gld/gld}$ mice (B6Smn.C3-*Fasl*^{gld}*)* possessing a natural mutation in the Fas Ligand gene, originally purchased from Jackson Laboratory (Bar Harbor, ML) were generously provided by Dr. Andrij Holian University of Montana). $C57Bl/6^{lpr/lpr}$ mice (B6.MRL-***Fas***^{lpr}**)** possessing a natural mutation in the Fas gene were obtained from Jackson Laboratory (Bar Harbor, ML). For all experiments, animals were euthanized by CO_2 overdose followed by cervical dislocation.

TCDD exposure*.* TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) was obtained from Cambridge Isotope laboratories Inc., (Woburn MA), dissolved in anisole and diluted in peanut oil. Control mice received vehicle consisting of comparable doses of anisole and peanut oil. For all experiments, mice were gavaged once with vehicle or an immunosuppressive dose of TCDD (15 μg/kg) (Kerkvliet *et al.*, 1996). Animals were sacrificed either on day 5 or day 7 after treatment and immune tissues harvested for subsequent analyses.

Preparation of Splenocytes. Spleens were harvested and processed as previously described (Shepherd *et al.*, 2001). Briefly, spleens were processed between the frosted ends of slides, erythrocytes were depleted by hypotonic lysis and cells were washed and resuspended in cold HBBS, 5% FBS, supplied with

20 mM hepes, 50 μg/ml gentamycin and 1.5mM sodium pyruvate (referred to as cHBSS).

Preparation of Popliteal and brachial lymph nodes (PBLNs). Popliteal and Brachial lymph nodes were harvested 5 or 7 days after vehicle or TCDD treatment. Lymph node tissue was processed using the end of a 1 ml syringe and cell strainer in 5 ml of cHBSS. The cell suspension was centrifuged at RT at 1200rpm for 10 minutes. The supernatant was discarded and cells resuspended in 1 ml of cHBSS.

Flow Cytometry. Immune cells were stained for FACS analyses as previously described (Shepherd *et al.*, 2001). Rat, hamster or mouse IgG block was added to aliquots of prepared leukocytes for 10 minutes on ice to prevent non-specific binding. Splenocytes were stained for 10 minutes on ice with monoclonal antibodies (mAb) from Pharmingen (San Diego, CA): CD11c (HL3), CD11b (M1/70), B220 (RA3.6B2), Ly6C (AL-21), CD11a (2D7), MHC II (2G9), CD86 (GL1) and CD40 (3/23); Miltenyi Biotec (CA): DEC205 (NLDC-145); Biolegend (San Diego, CA): CD11c (N418), CCR7 (4B12), CD8 (53-6.7), CD54 (YN1/1.7.4); and eBioscience (San Diego, CA): 33D1. Similarly, PBLNs were stained for 10 minutes on ice with mAbs to CD11c (N418), CD11a (2D7), MHC II (2 G9), CD86 (GL1), CD40 (3/23) and CCR7 (4B12). All mAbs used were titrated for optimal concentration and appropriately-labeled, isotype-matched mAbs were used as controls. Acquisition of 100,000-300,000 events from freshly prepared cells was performed using a FACS Aria flow cytometer (BD biosciences) and analyzed by BD FACS Diva software (Version 4.0).

For detection of apoptosis, immune cells were stained with Annexin V and 7AAD as per the manufacturer's instructions (BD Pharmingen). After surface staining for CD11c, cells were resuspended in 1 X Annexin binding buffer, incubated with Annexin-V FITC for 15 minutes at room temperature in the dark, followed by addition of 7AAD for 10 minutes and analyzed as described above.

For sorting $CD11c^{high}$ and $CD11c^{low}$ splenic DCs, mice were injected i.p. once daily for l0 consecutive days with 20μg human recombinant Flt3L (Peprotech Inc., New Jersey) as previously described (Maraskovsky *et al.*, 1997; Pulendran *et al.*, 1997). Mice were harvested on day 11, splenocytes were prepared, stained with CD11c and purified using anti-PE beads from Miltenyi Biotec (CA). Cells were sorted in sterile sorting buffer using FACS Aria flow cytometer for $CD11c^{high}$ (>92% purity) and $CD11c^{low}$ populations (>86% purity).

Western blotting. Lysates were prepared from FACS-sorted splenic $CD11c^{high}$ and $CD11c^{low}$ DCs. Protein concentrations from cell lysates were determined using the BCA assay (Pierce, IL). Protein lysates (17μg per lane) were loaded on a 4-12 % SDS PAGE gel (Invitrogen, CA) and transferred onto PVDF membrane (Biorad, CA). Anti-AhR antibody (Biomol, PA) was used to specifically detect murine AhR^b at a dilution of 1:5000 at 4°C overnight. After washes in TBST (Tris-buffered saline Tween-20), horseradish peroxidase conjugated goat anti-rabbit IgG (Jackson Immuno research laboratories) was used at a dilution of 1:1000 for 1 hour at room temperature. The membrane was washed and incubated in enhanced chemiluminiscence reagent (ECL: Amersham, New Jersey). Membranes were visualized and analyzed using Fuji Film Image Reader

(Las 3000, Version 2). Similiarly, the membrane was also probed for β-actin using anti-β actin antibody (Abcam, MA) and horseradish peroxidase conjugated rabbit anti-mouse IgG (Southern biotech, Alabama) as the secondary antibody.

Statistical analysis. Results are presented as the mean ± SEM of 6 mice unless otherwise indicated. For all experiments, an unpaired t test was used to compare the mean of the vehicle-treated group to the mean of the TCDD-treated group. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

DC subpopulations in the peripheral immune tissues. Based on the expression of CD11c**,** the murine marker for DCs, splenocytes and PBLNs differ in their DC composition. Two distinct subpopulation of DCs, referred to as $CD11c^{high}$ and $CD11c^{low}$ exist in the mouse spleen (Figure 2-1). In contrast, the peripheral lymph nodes contain a uniform $CD11c^{+}$ population with no discernible $CD11c^{high}$ and $CD11c^{low}$ DCs (Figure 2-1). DCs representing an immature phenotype, display low levels of co-stimulatory molecules such as MHC II and decreased capacity to activate naïve T cells. On the other hand, mature APCs mostly display increased levels of costimulatory molecules and are highly efficient at T cell stimulation (Sato and Fujita, 2007).

Effects of TCDD on naïve splenic DC subpopulations. To assess the potential sensitivity of DC subsets to TCDD, AhR protein levels were evaluated in purified $CD11c^{high}$ and $CD11c^{low}$ splenic DCs. As shown in Figure 2-1B, both splenic DC subpopulations constitutively express comparable levels of AhR. Consistent with previous studies, TCDD exposure caused a decline in the percentage and number of $CD11c^{high}$ DCs recovered from the spleen on day 7 (Figure 2-2A+2B) (Vorderstrasse and Kerkvliet, 2001). In the majority of experiments, TCDD had no effect on the percentage or number of splenic $CD11c^{low}$ DCs on 7 (Figure 2-2C+2D).

Costimulatory molecule expression on naïve splenic DCs from TCDD-treated mice. Expression of co-stimulatory molecules such as MHC II, CD86, CD40 and CD11a reflect the activation and/or maturational status of DCs.

Figure 2-1

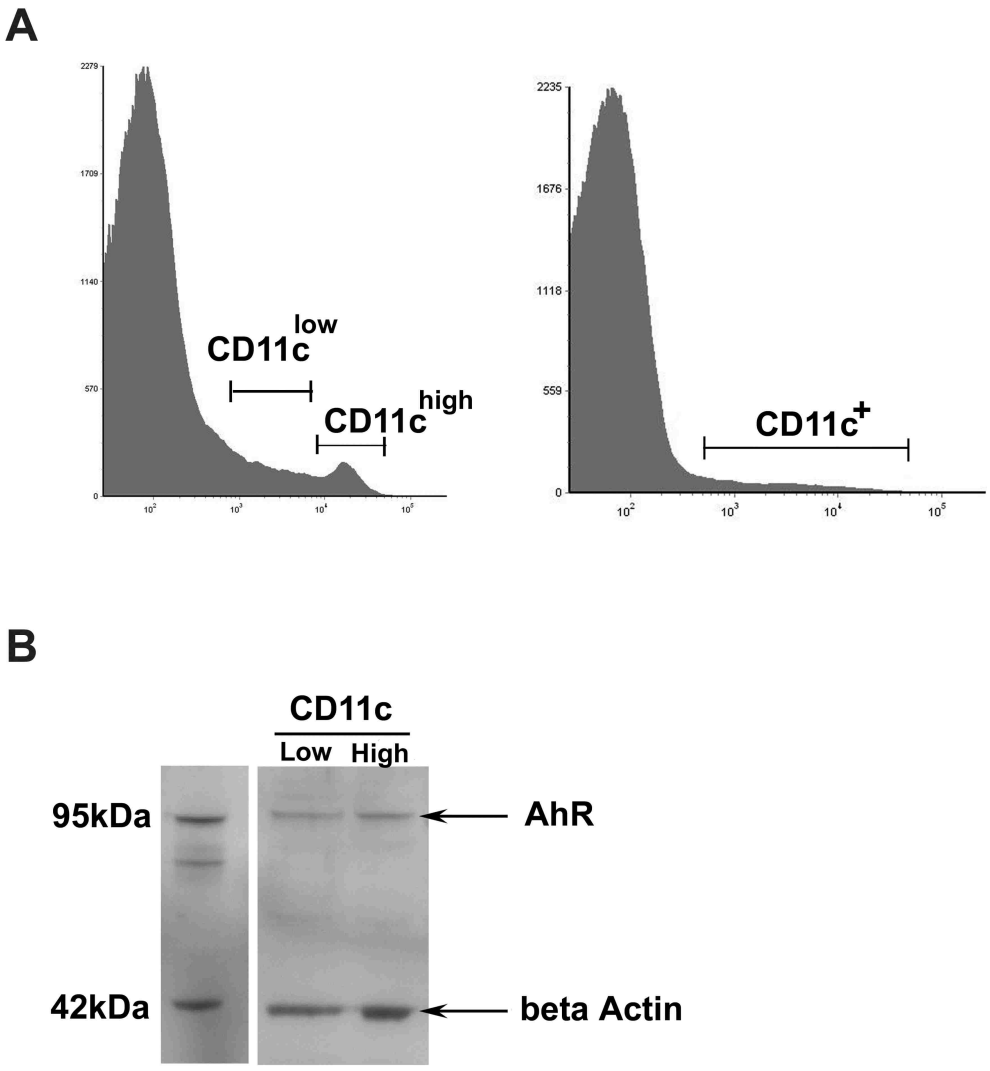

Figure 2-1. (A) CD11c expression on DCs from peripheral immune tissues. Histograms represent two distinct populations of dendritic cells in unimmunized mice, CD11c^{high} and CD11c ^{low} cells in the spleen or lymph nodes. **(B) AhR protein levels in splenic CD11c^{high} and CD11c^{low} DCs.** Lysates were prepared from Flt3L-treated C57Bl/6 mice. Spleens were harvested on day 11 and DC lysates were prepared from FACS-sorted CD11c^{high} and CD11c^{low} DCs. Purified, non-adherent bone marrow-derived dendritic cells (BMDCs) were used as a positive control for the expression of AhR. Whole cell lysates were evaluated by Western blotting for AhR and β-actin as described in the *Materials and Methods.*

Figure 2-2

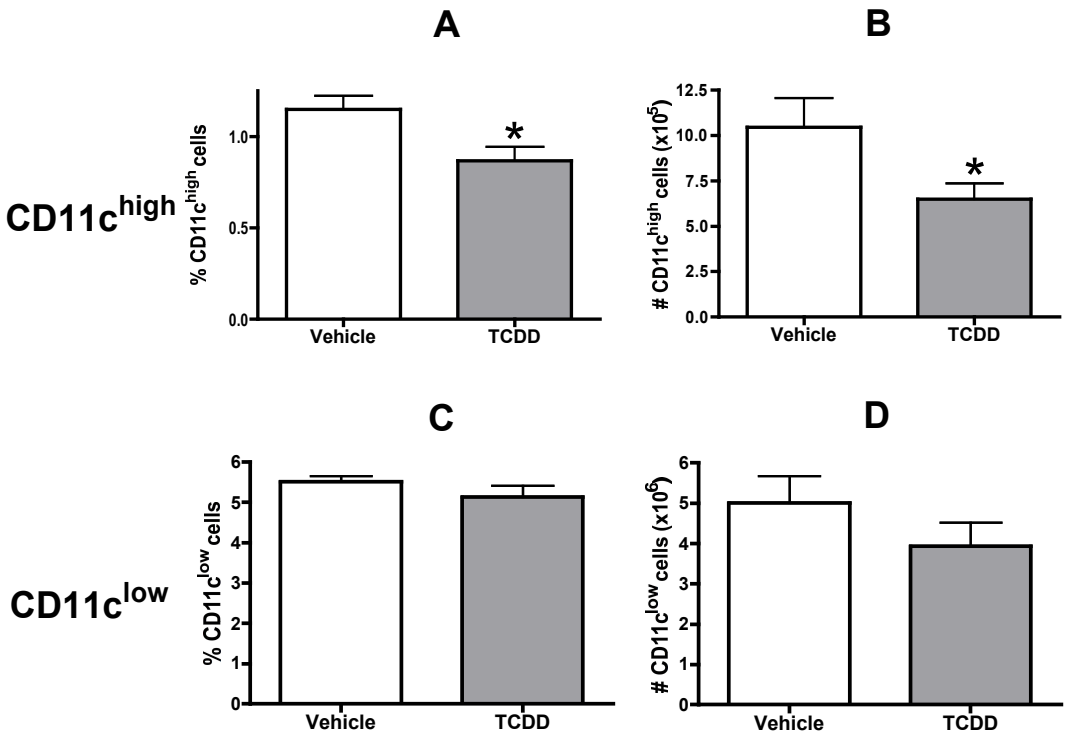

Figure 2-2. TCDD induces a loss of splenic CD11c^{high} but not CD11c^{low} DCs. C57Bl/6 mice were treated with vehicle or TCDD (15μg/kg). Spleens were harvested on day 7, processed and phenotyped by FACS analysis as described in *Materials and methods.* Bar graphs represent the percentage and numbers of splenic CD11c^{high} **(A, B)** and CD11c^{low} **(C, D)** DCs as determined by flow cytometry. Data represent means ± SEM from 6 mice per treatment group from 4 independent experiments. * indicates $p \leq 0.05$ for the comparision between vehicle- and TCDD-treated mice, respectively.

Therefore, several key activation markers were evaluated on both splenic CD11c populations. TCDD increased the expression of CD86, CD54 and the frequency of CD40 on $CD11c^{high}$ DCs. The frequency of both MHC II and CD11a on $CD11c^{high}$ DCs was downregulated by TCDD. $CD11c^{low}$ cells from TCDD-treated mice displayed a decrease in MHC II and CD11a frequency when compared to vehicle-treated controls. Although there was no effect on CD40, expression of both CD86 and CD54 on $CD11c^{low}$ DC increased following TCDD exposure (Table 2-1).

Because distinct DC subsets with unique functions have been recently characterized in the mouse spleen, we examined the effects of TCDD on these DC subsets. $CD11c^{high}$ DCs expressing the phenotypic markers $CD8\alpha^{+}DEC205^{+}$ or $CD8\alpha^{-}33D1^{+}$ occupy different anatomical positions in the spleen and mediate distinct effector functions (Dudziak *et al.*, 2007). TCDD caused a 40 percent reduction in the number of splenic $CD11c^{high}$ $CD8\alpha^{-}$ $33D1^{+}$ DCs on day 7 but not on day 3 or 5 (Table 2-2 and data not shown). This effect was dependent on the expression of the AhR as no loss of $CD11c^{high}CD8\alpha^{-}33D1^{+}$ DCs occurred in AhR knockout mice exposed to TCDD (Table 2-2). In contrast, the number of $CD11c^{high}$ $CD8\alpha^{+}$ $DEC205^{+}$ DCs subset was unaffected by TCDD. Consistent with the study by Vorderstrasse and coworkers, an upregulation of $CD8\alpha^{+}$ by 10 percent was observed on $CD11c^{high}$ DCs isolated from TCDD-treated mice (data not shown).

Additionally, splenic $CD11c^{low}$ cells can be segregated phenotypically into two subsets – plasmacytoid DCs (pDCs), which are $CD11c^{low}CD11b^{-}Ly6C^{+}B22$

TABLE 2-1

Effects of TCDD on the expression of key accessory molecules on splenic DCs[a]

		CD11c ^{high} DCs		CD11c ^{low} DCs	
		Vehicle	**TCDD**	**Vehicle**	**TCDD**
MHC II	Percentage	99 ± 0.2	**96 ± 0.6***	80 ± 0.5	**75 ± 0.7***
	MFI	5228 ± 231	5411 ± 301	1478 ± 77	1298 ± 105
CD86	Percentage	53 ± 3	56 ± 2	53 ± 1.6	54 ± 1.2
	MFI	1769 ± 64	**2174 ± 163***	1170 ± 9	**1379 ± 79***
CD40	Percentage	58 ± 3	**70 ± 4***	36 ± 1.3	33 ± 1.8
	MFI	546 ± 21	610 ± 46	870 ± 38	758 ± 54
CD11a	Percentage	96 ± 0.2	**82 ± 0.5***	92 ± 0.6	**86 ± 0.3***
	MFI	3912 ± 287	4505 ± 428	3906 ± 397	4305 ± 173
CD54	Percentage	95 ± 0.2	95 ± 0.3	89 ± 0.5	92 ± 0.6
	MFI	4450 ± 302	**5848 ± 540***	2518 ± 75	**3013 ± 165***

[a] C57Bl/6 mice were treated with TCDD (15μg/kg) or vehicle. On day 7, spleens were harvested and processed as described in the *Materials and Methods*. Expression of activation markers was determined by gating on the CD11c^{high} and CD11c^{low} DCs. Percent positive expression (%) and mean/median fluorescence intensity (MFI) was determined for each activation marker. Data represent the mean ± SEM of 5-6 mice/treatment and is representative of 2 independent experiments * indicates $p \leq 0.05$ for the comparision between vehicle- and TCDD-treated mice, respectively.

TABLE 2-2

Effects of TCDD on splenic DC subsets[a]

			Vehicle	TCDD
AhR $^{+/+}$	**CD11c^{high}**	CD8α^+DEC205$^+$DCs (x10^5)	3.2 ± 0.2	2.7 ± 0.7
		CD8α^- 33D1$^+$DCs (x10^5)	6.5 ± 0.6	3.7 ± 0.9*
AhR$^{-/-}$	**CD11c^{high}**	CD8α^+DEC205$^+$DCs (x10^5)	6.1 ± 0.6	6.9 ± 1
		CD8α^- 33D1$^+$DCs (x10^5)	5.5 ± 0.7	5.3 ± 0.5

[a] C57Bl/6 mice (AhR$^{+/+}$) and AhR knockout (AhR$^{-/-}$) mice were treated with vehicle or TCDD and 7 days later splenic DC subsets were prepared and analyzed as described in the *Materials and Methods*. Data represent the mean ± SEM from four to five mice/treatment. * indicates $p \leq 0.05$ for the comparision between vehicle-and TCDD-treated mice, respectively.

and Interferon-producing killer DCs (IKDCs), which express $CD11c^{low}CD11b^{-}$ $Ly6C^{-}B220^{+}$. In our studies neither percentages nor the numbers of splenic pDCs or IKDCs were affected following TCDD exposure of naïve mice (data not shown).

Effects of TCDD on the fate of splenic DCs in naive mice. Because TCDD selectively decreased $CD11c^{high}$ DCs in the spleen, we next investigated potential mechanisms responsible for this decline. We hypothesized that TCDD-induced decreases in the splenic $CD11c^{high}$ cells may be due to at least two outcomes: (a) increased DC death via apoptosis, or (b) increased DC emigration out of the spleen.

Fas-FasL interactions have been implicated in the TCDD-mediated death of various immune cells including BMDCs (Rhile *et al.*, 1996; Camacho *et al.*, 2001; Camacho *et al.*, 2002; Dearstyne and Kerkvliet, 2002; Ruby *et al.*, 2005). Therefore, we examined the potential role of Fas and FasL in the TCDD-induced loss of splenic $CD11c^{high}$ DCs. To evaluate this pathway, unimmunized C57Bl/6 $^{lpr/lpr}$ or C57Bl6 $^{gld/gld}$ mice were treated with vehicle or TCDD for 7 days. As shown in Figure 2-3, TCDD treatment decreased both the percentage and number of splenic $CD11c^{high}$ DCs when compared to vehicle-treated controls, in both the Fas–and FasL-deficient mice, respectively. In a separate experiment, apoptosis was directly evaluated in splenic DCs from wild-type C57Bl/6 mice on day 5 following TCDD exposure using two well-characterized markers of apoptosis, Annexin V and 7AAD. TCDD had no effect on the early or late

Figure 2-3

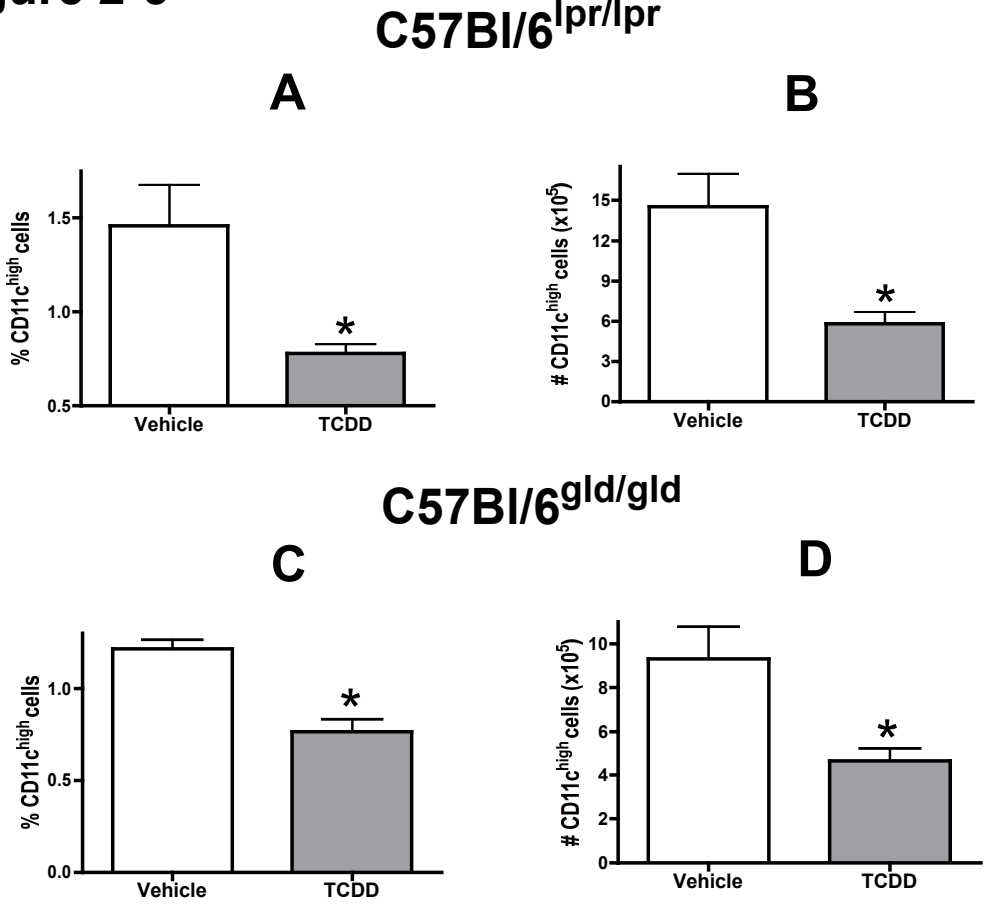

Figure 2-3. **The number and percentage of splenic CD11c^{high} DCs in lpr and gld animals are decreased following TCDD exposure.** Non-immunized C57Bl/6 $^{lpr/lpr}$ mice **(A, B)**, or C57Bl/6 $^{gld/gld}$ mice **(C, D)** were treated with vehicle or TCDD (15μg/kg) and the numbers of resident DCs in the spleen were determined on day 7. Data represent the means ± SEM from 5-6 mice per treatment group and are representative of two independent experiments for C57Bl/6$^{lpr/lpr}$ and one experiment for C57Bl/6$^{gld/gld}$ mice. * indicates $p \leq 0.05$ for the comparision between vehicle- and TCDD-treated mice, respectively.

apoptotic staining profiles of splenic CD11c^{high} cells when compared to controls (Table 2-3).

DCs reside in the periphery and upon encounter with antigen, undergo maturation. Maturation of DCs is characterized by phenotypic changes including the induction of the chemokine receptor CCR7 (Sallusto *et al.*, 1998; Forster *et al.*, 1999; Gunn, 2003; Scandella *et al.*, 2004; Randolph *et al.*, 2005; Sanchez-Sanchez *et al.*, 2006). Expression of this receptor allows DCs to home to T cell-rich areas in draining lymph nodes (Cyster, 1999; Scandella *et al.*, 2002; Scandella *et al.*, 2004). We hypothesized that a decrease in splenic CD11c^{high} DCs could be due to CCR7-mediated migration. To address this hypothesis, the expression of CCR7 was evaluated on splenic DCs to explore the possibility that increased migration might underlie the TCDD-induced decline of splenic CD11c^{high} cells. CCR7 expression was increased on splenic CD11c^{high} DCs in mice on day 5 following TCDD-treatment (Figure 2-4). CCR7 expression on CD11c^{low} DCs was unaffected by TCDD (data not shown).

DCs in the peripheral lymph nodes are affected by TCDD exposure. Because TCDD affects homeostasis of splenic DCs, we next examined if DCs that reside in peripheral lymph nodes are also affected by TCDD. We evaluated the effects of TCDD exposure on DCs in two sets of peripheral lymph nodes, the popliteal and brachial lymph nodes. In contrast to the spleen, the peripheral lymph nodes in mice contain a uniform CD11c^{+} DC population with no discernible CD11c^{high} and CD11c^{low} DCs (Figure 2-1). TCDD did not affect the frequency or

TABLE 2-3

Exposure to TCDD does not induce apoptosis[a]

	Vehicle	TCDD
% Early apoptosis	5.5 ± 0.3	5.3 ± 0.6
% Late apoptosis	5.6 ± 0.4	5.0 ± 0.2

[a] C57Bl/6 mice were treated with TCDD (15μg/kg) or vehicle for 5 days and spleens were harvested, processed and stained for expression of CD11c, Annexin V and 7AAD as described in *Materials and Methods.* Mature DCs were identified by gating on the $CD11c^{high}$ cells from vehicle- and TCDD- treated mice. Early apoptosis (Annexin V^+/ 7 AAD^-) and late apoptosis (Annexin V^+/$7AAD^+$) was evaluated. Data represent the mean ± SEM from 4-5 mice/ treatment.

Figure 2-4

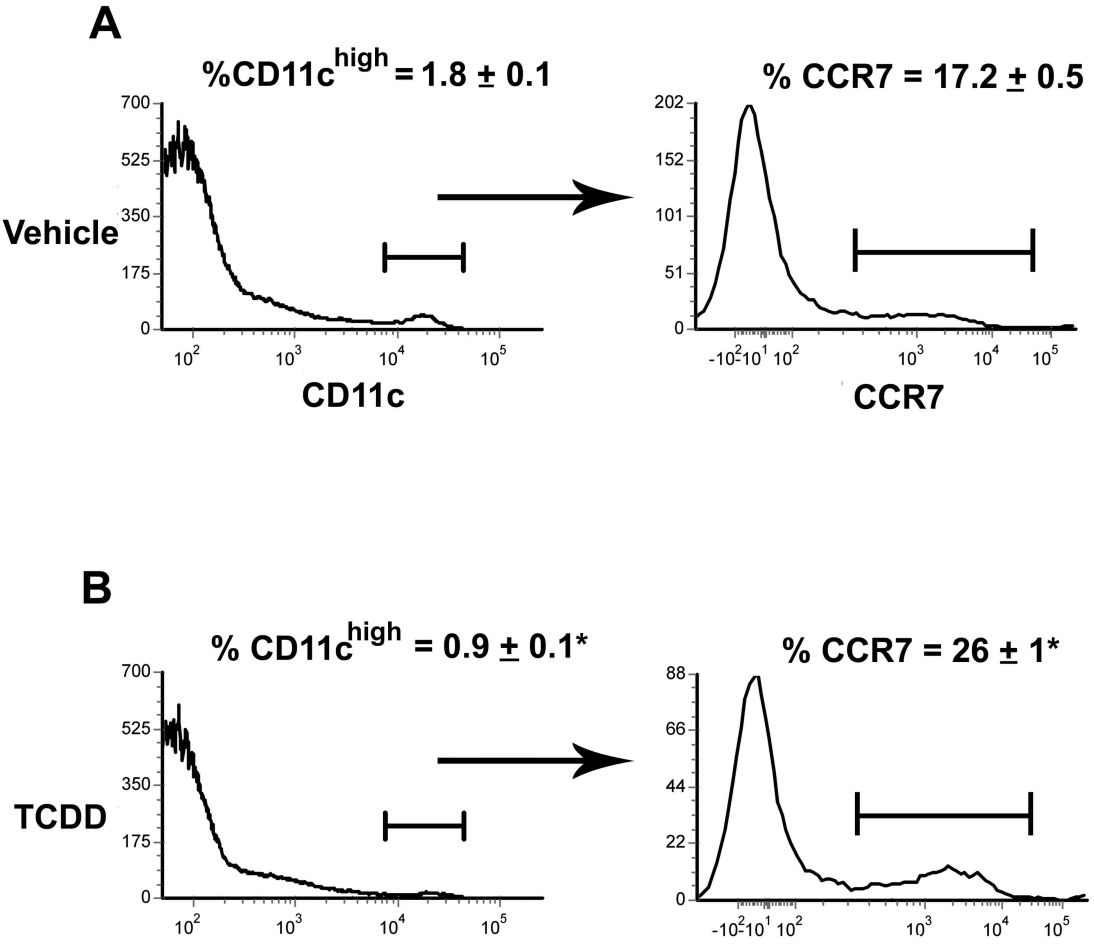

Figure 2-4. **TCDD increases CCR7 expression on splenic CD11c^{high} DCs.** C57Bl/6 mice were treated with vehicle or TCDD. On day 5, spleens were harvested and processed for flow cytometric analysis as described in the *Materials and Methods*. Representative histograms depict CCR7 expression gated on CD11c^{high} cells from Vehicle- **(A)** or TCDD-treated mice **(B)**. Values shown are mean ± SEM for 4-5 mice per treatment group and are representative of two independent experiments. * indicates $p \leq 0.05$ for the comparision between vehicle- and TCDD-treated mice, respectively.

the number of $CD11c^+$ cells in the popliteal and brachial lymph nodes on day 7 (Figure 2-5). As shown in Table 2-4, TCDD exposure enhanced expression of MHC II and CD40 but did not affect the expression of CD86 and CD11a or alter the percentage of $CD11c^+$ DCs that expressed these costimulatory molecules in the peripheral lymph nodes. Interestingly, on day 5 and 7 following treatment, $CD11c^+$ DCs in the popliteal and brachial lymph nodes of TCDD-treated mice expressed increased levels of CCR7 (Figure 2-6 and data not shown).

Figure 2-5

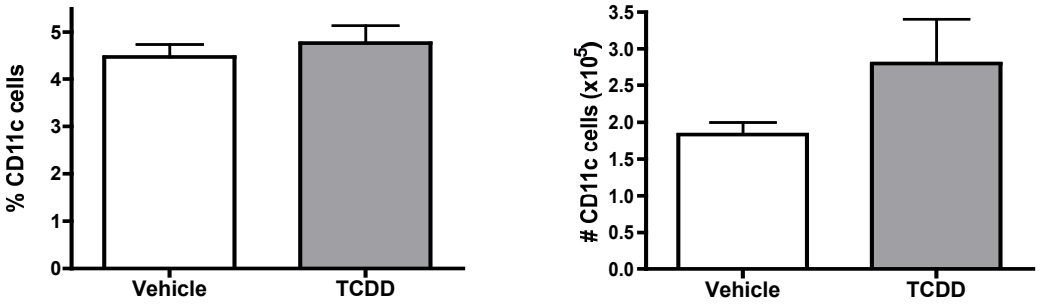

Figure 2-5. Effects of TCDD on DCs in the peripheral lymph nodes. The percentages and number of $CD11c^+$ DCs in the popliteal and brachial lymph nodes were determined from mice treated with TCDD (15μg/kg) or vehicle for 7 days. Lymph nodes were harvested and DCs analyzed by flow cytometry as previously described in the *Materials and Methods*. Data represent the mean ± SEM from 4-6 mice per treatment group. * indicates $p \leq 0.05$ for the comparision between vehicle- and TCDD-treated mice, respectively

TABLE 2-4

Effects of TCDD on the expression of accessory molecules on DCs from peripheral lymph nodes[a]

	$CD11c^+$ DCs	
	Vehicle	**TCDD**
MHC Class II MFI	1803 ± 13	**2828 ± 79***
CD86 MFI	3961 ± 271	4611 ± 386
CD40 MFI	814 ± 32	**1074 ± 18***
CD11a MFI	2846 ± 114	2706 ± 89

[a] C57Bl/6 mice were treated with TCDD or vehicle for 7 days and lymph nodes were harvested and processed for flow cytometric analysis. For each activation marker, results were generated by gating on the $CD11c^+$ DCs from vehicle- and TCDD-treated mice to determine the mean/median fluorescence intensity (MFI). Results are representative of two independent experiments and the data represent the mean ± SEM from 3 mice/treatment. * indicates $p \leq 0.05$ for the comparison between vehicle- and TCDD-treated mice, respectively.

Figure 2-6

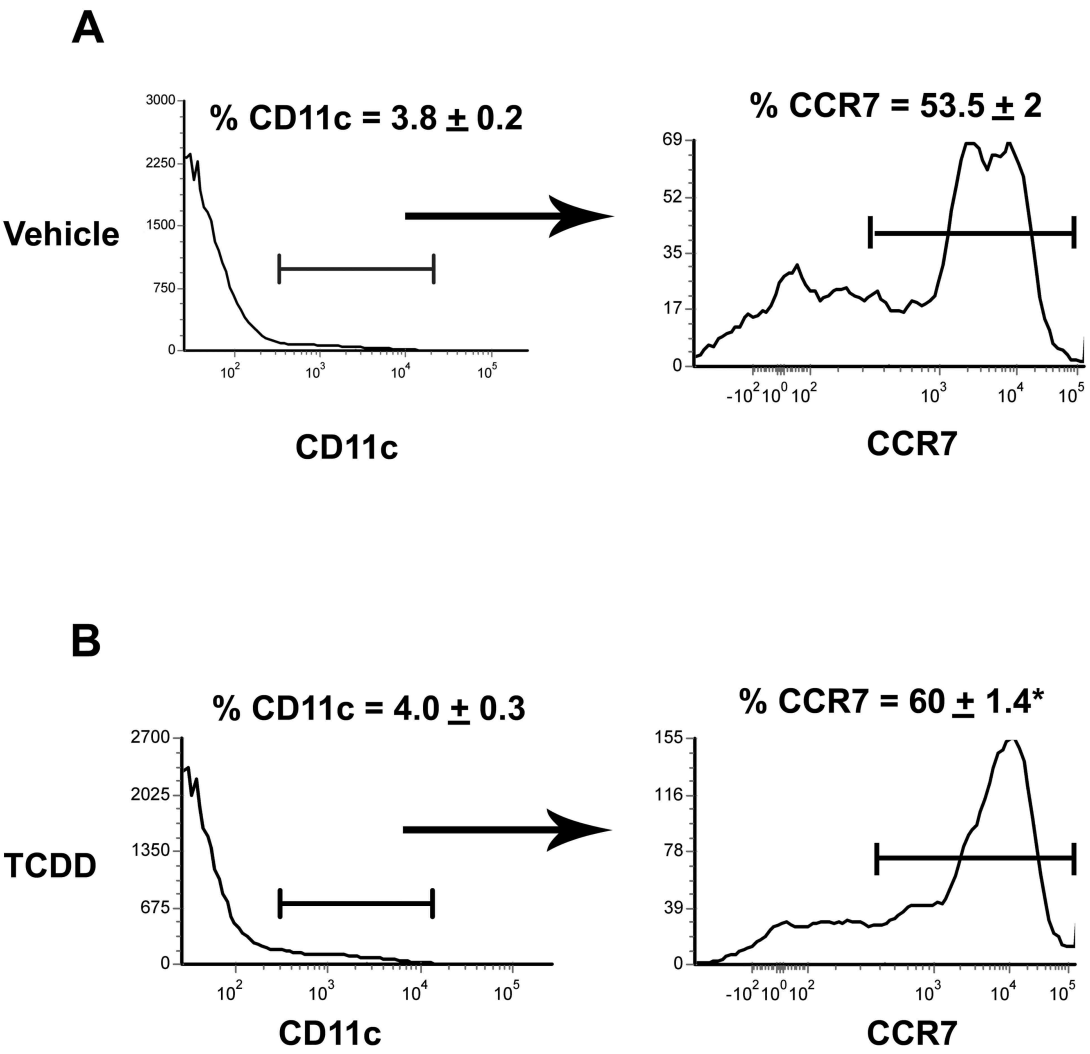

Figure 2-6. **Expression of CCR7 is increased on DCs from lymph nodes following TCDD exposure.** On day 5 following exposure to vehicle or 15 μg/kg TCDD, lymph nodes were harvested and analyzed by flow cytometric analysis as previously described. Representative histograms depict CCR7 expression on $CD11c^+$ cells in mice treated with vehicle **(A)** or TCDD **(B)**. Values represent the mean ± SEM of 5 mice per treatment group. * indicates $p \leq 0.05$ for the comparison between vehicle- and TCDD-treated mice for 2 independent experiments.

DISCUSSION

DCs play an important role in both the innate and adaptive arms of the immune system. They are professional antigen presenting cells, which regulate induction of T cell-mediated immunity by providing processed antigen to naïve T cells**.** The effects of TCDD exposure on distinct DC subsets in the spleen and the lymph nodes have not been previously examined. In our experiments we have evaluated the effects of TCDD on naïve splenic DCs- both $CD11c^{high}$ and $CD11c^{low}$ cells, in addition to naïve DCs in the peripheral lymph nodes. Both splenic DC subpopulations constitutively express AhR and therefore expected to be sensitive to TCDD.

Dendritic cells exist in various differentiation stages referred to as immature, mature and activated cells, which can be characterized phenotypically by their varying expression of the costimulatory molecules MHC II, CD86 and CD40. The seminal publication by Vorderstrasse et al., showed that oral administration of TCDD induced a loss of splenic $CD11c^{+}$ DCs in naïve mice (Vorderstrasse and Kerkvliet, 2001). Importantly, this study utilized low-density gradient separation in the isolation of DCs. This procedure selects for a distinct DC subpopulation from immune tissue and also affects DC activation. However, consistent with these findings, TCDD decreased the frequency and number of $CD11c^{+}$ DC. This decline was concomitant with modulation of MHC II, CD11a, CD40 and CD86 on the remaining $CD11c^{+}$ DCs in the spleen. Polarization of T cell differentiation into a Th1 or Th2 response can be significantly affected by the nature of the DC subset interacting with the T cell. A recent study conducted by

Dudziak and colleagues, identified two distinct CD11c^{high} splenic DC subsets in mice based on their expression of select phenotypic markers: CD8α^+ DEC205$^+$ DCs and CD8α^- 33D1$^-$ DCs (Dudziak *et al.*, 2007). These two distinct DC subsets differ in anatomical location and possess different effector functions (Vremec *et al.*, 1992; Dudziak *et al.*, 2007) The CD8α^+DEC205$^+$ DCs found in the splenic pulp specialize in cross presentation of exogenous Ags to CD8$^+$ T cells and have recently also been implicated in the generation and maintainance of splenic Tregs (Yamazaki *et al.*, 2008). Present in the mouse spleen, this splenic DC subset specializes in uptake of exogenous Ags and the activation of CD4$^+$ T cells via MHC II molecules (Dudziak *et al.*, 2007). Our results demonstrate the selective effects of TCDD in mediating a decline in the splenic CD11c^{high} CD8α^- 33D1$^+$ population, which are vital for the activation of CD4$^+$ Th1 helper cells. The selective loss of this CD4$^+$ T cell activating splenic DC subset, with the concomitant retention of the suppressive Treg-inducing CD8α^+DEC205$^+$ splenic CD11c population by TCDD may predispose naïve mice to be more susceptible to blood borne, extracellular pathogens and account for suppressed CD4 T cell reponses following TCDD exposure. However, further studies are necessary to specifically evaluate the functional capacity of the splenic DC subsets in response to extracellular antigens in TCDD-treated mice. Several investigators have linked TCDD-induced immunesuppression with induction of Tregs. AhR activation leads to generation of apadtive Tregs (Funatake *et al.*, 2005). Additionally, Vogel et al., have demonstrated increase in Treg marker Foxp3 in spleen of TCDD-treated mice (Vogel *et al.*, 2008). Our present findings provide

suggestive evidence that disruption of DC homeostasis following AhR activation may contribute to the generation of Tregs and subsequent immune suppression.

In contrast to the $CD11c^{high}$ cells, TCDD did not alter the frequency or the numbers of splenic $CD11c^{low}$ DCs. However, alteration of activation markers such as MHC class II in $CD11c^{low}$ DCs following exposure to TCDD suggests a modulation in the differentiation status of these cells. $CD11c^{low}$ DCs can be phenotypically and functionally characterized into at least two distinct populations, the IKDCs and pDCs. A tolerogenic function has been assigned to the pDC population in mice (Rutella *et al.*, 2006). Tolerogenic DCs can induce the generation and maintenance of antigen-specific unresponsiveness in central and peripheral immune tissues (Steinman *et al.*, 2003). It is thereby possible that the immunosuppressive effects of TCDD on T cell activation may be attributable to changes in the number and frequency of tolerogenic DCs. Contrary to our expectations, both the frequency and the number of pDCs were unaffected by TCDD. Collectively in the spleen, TCDD decreased the immunostimulatory subset of DCs but did not affect the tolerogenic DCs, essentially increasing their relative frequency after 7 days of exposure.

In sharp contrast to spleen, TCDD did not affect the number or frequency of DCs in the lymph nodes. It did however alter their expression of co-stimulatory markers such as MHC Class II and CD40. This modulation is reflective of alteration of the steady state differentiation status of DCs in lymph nodes upon TCDD exposure and establishes that TCDD, either directly or indirectly, has effects in the peripheral lymph nodes. Modulation of DC differentiation may alter

their functional abilty to interact with T cells and generate an activating immune response.

Exposure to TCDD has been reported to enhance cellular death in several leukocyte populations. Fas (CD95), a member of the TNF family is expressed on the surface of DCs as well as other immune cells. Upon binding to Fas ligand (CD95L), a series of downstream signaling cascades leads to the activation of terminal effector caspases and ultimately apoptosis (Adachi *et al.*, 1993; Nagata and Golstein, 1995; Peter and Krammer, 1998; Scaffidi *et al.*, 1998; Lenardo *et al.*, 1999; Dearstyne and Kerkvliet, 2002). Moreover, Fas-FasL interactions have been implicated in the TCDD–induced loss of various immune cells including T cells and DCs. (Rhile *et al.*, 1996; Kamath *et al.*, 1999; Camacho *et al.*, 2001; Dearstyne and Kerkvliet, 2002; Camacho *et al.*, 2005; Ruby *et al.*, 2005). We used Fas- deficient (lpr/lpr) and FasL-deficient (gld/gld) mice, defective in this extrinsic apoptotic pathway, to evaluate the possible involvement of Fas/FasL interactions in the decline of naïve splenic $CD11c^{high}$ DCs by TCDD. Splenic $CD11c^{high}$ DCs in both the gld and lpr mice declined in a manner similiar to that observed in the wild-type mice following TCDD exposure, suggesting that Fas-mediated apoptosis is not responsible for the loss of splenic $CD11c^{high}$ DCs in naïve mice. Collectively, these studies suggest that the TCDD-induced loss of naive splenic $CD11c^{high}$ DCs is independent of Fas-FasL interactions. Additionally, no evidence of TCDD-induced apoptosis was detected in the $CD11c^{high}$ DCs using Annexin V and 7-AAD on day 5 following TCDD exposure

of naïve mice. Taken together, our results demonstrate an overall lack of apoptosis in splenic CD11c^{high} DCs following exposure to TCDD.

Alternatively, the decline in splenic CD11c^{high} DCs upon TCDD exposure could be due to induced emigration. CCR7 is a chemokine receptor that plays a prominent role in the migration/trafficking of immune cells including DCs. This receptor specifically binds two ligands, CCL21 and CCL19, which are expressed constitutively by endothelial cells in the lymphatic vessels and high endothelial venules. Immature DCs, which comprise the majority of DCs in an unimmunized animal, also express this receptor and migrate to the lymph nodes (Sanchez-Sanchez *et al.*, 2006). However, in the absence of danger signals, these DCs contribute to peripheral tolerance against self-antigens (Scandella *et al.*, 2004; Sanchez-Sanchez *et al.*, 2006). Premature induction of migration, in the absence of danger signals may alter DC homeostasis in the periphery. Specifically for DCs, CCR7 expression is increased following activation. Interestingly, CCR7 expression was enhanced on the splenic CD11c^{high} DCs following TCDD exposure, suggesting the possibility that these cells were aberrantly being induced to emigrate out of the spleen. Also, CCR7 expression increased on DCs that resided in the peripheral lymph nodes in unimmunized mice treated with TCDD. The possibility exists that TCDD induces the release of proinflammatory cytokines by resident cells in the lymph nodes leading to upregulation of CCL21 on the lymphatic endothelium. Upregulation of a ligand for CCR7 could then induce chemotaxis of splenic DCs into the lymph nodes. However, no alteration in the frqeuency or the numbers of lymph node DCs was observed with TCDD. It

is possible that increased CCR7 expression on lymph node DCs could induce their emigration to the source of CCR7 ligands. The role of TCDD-induced increased CCR7 expression on lymph node DCs remains to be identified. However, additional experiments are necessary to establish if TCDD-induced migration of DCs is dependent on alterations in the expression of CCR7 or its ligands.

A seemingly plausible model emerges considering the progressive increase in CD11c expression, along with the modulation in the activation markers as DC differentiation proceeds (Figure 2-7). It is possible that TCDD selectively affects a subset of splenic $CD11c^{high}$ DCs increasing the CCR7 expression on these DCs, ultimately leading to their emigration out of the spleen. Because DCs acquire enhanced migratory abilities as they undergo differentiation, aberrant migration of splenic DCs may underlie the ability of TCDD to disrupt DC homeostasis in lymphoid organs. Alteration of DC differentiation and homoestasis by TCDD may generate dysfunctional DCs leading to immune suppression.

In conclusion, we have shown that TCDD affects DCs homeostasis by selectively decreasing a distinct subset ($CD11c^{high}$ $CD8\alpha^{-}$ $33D1^{+}$ DCs) in the splenic tissue of naïve mice. This loss of mature DCs was not attributed to Fas-mediated apoptosis. Instead, upregulation of CCR7 on the splenic $CD11c^{high}$ DCs provides suggestive evidence that migration of these DCs may be playing a role in TCDD-induced loss of splenic $CD11c^{high}$ DCs. In addition, our results

demonstrate increased activation of DCs in both the spleen and the lymph nodes following exposure of naïve mice to TCDD.

Figure 2-7

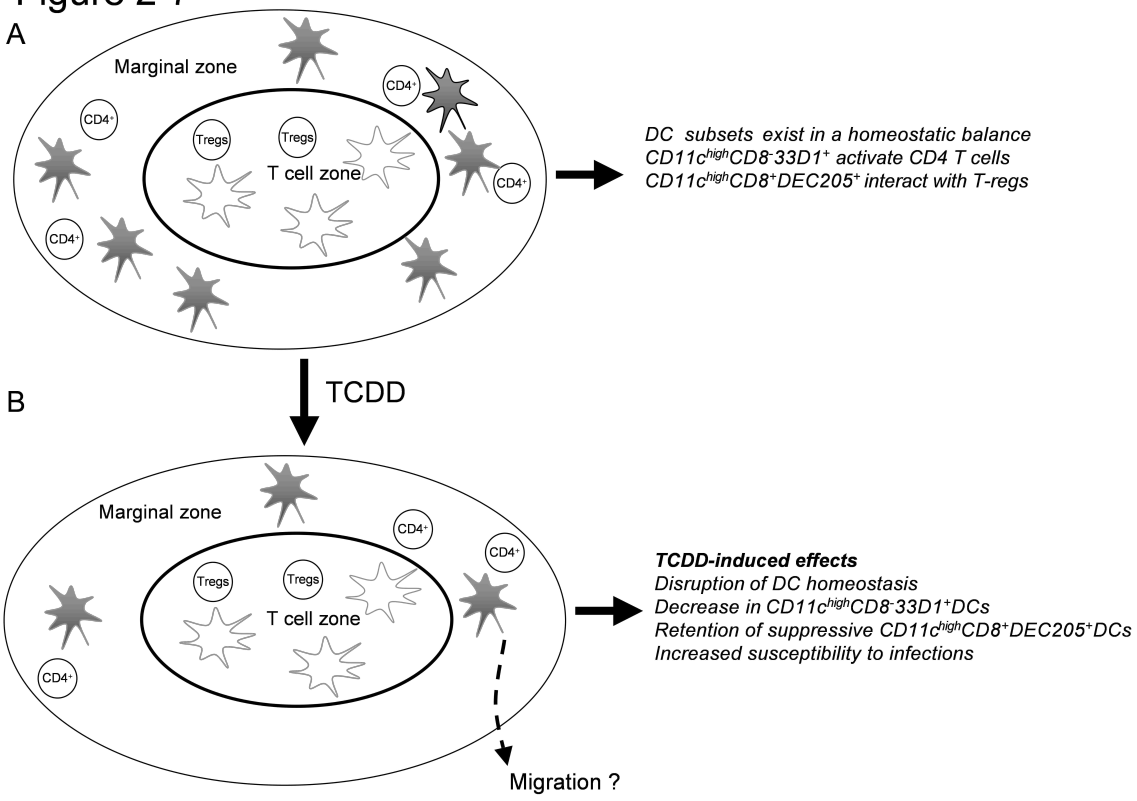

Figure 2-7. Schematic diagram of the effects of TCDD on splenic CD11c^{high} DCs.

A) In naïve condition, splenic CD11c^{high} DC subsets exist in a homeostatic balance. The CD11c^{high}CD8^{-}33D1^{+} DCs, represented in gray reside in the marginal zone and interact with CD4^{+} T cells. The CD11c^{high}CD8^{+}DEC205^{+} DCs residing in the splenic pulp area are specialized at inducing/maintaining Tregs. **B)** Following TCDD exposure the DC homeostasis balance is perturbed. TCDD induces the loss of CD11c^{high}CD8^{-}33D1^{+} DCs and retention of Treg-inducing CD11c^{high}CD8^{+}DEC205^{+} DCs.

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REFERENCES

Adachi, M., Watanabe-Fukunaga, R., and Nagata, S. (1993). Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc Natl Acad Sci U S A* **90**, 1756-1760.

Banchereau, J., Schuler-Thurner, B., Palucka, A. K., and Schuler, G. (2001). Dendritic cells as vectors for therapy. *Cell* **106**, 271-274.

Camacho, I. A., Hassuneh, M. R., Nagarkatti, M., and Nagarkatti, P. S. (2001). Enhanced activation-induced cell death as a mechanism of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity in peripheral T cells. *Toxicology* **165**, 51-63.

Camacho, I. A., Nagarkatti, M., and Nagarkatti, P. S. (2002). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induces Fas-dependent activation-induced cell death in superantigen-primed T cells. *Arch Toxicol* **76**, 570-580.

Camacho, I. A., Singh, N., Hegde, V. L., Nagarkatti, M., and Nagarkatti, P. S. (2005). Treatment of mice with 2,3,7,8-tetrachlorodibenzo-p-dioxin leads to aryl hydrocarbon receptor-dependent nuclear translocation of NF-kappaB and expression of Fas ligand in thymic stromal cells and consequent apoptosis in T cells. *J Immunol* **175**, 90-103.

Cyster, J. G. (1999). Chemokines and cell migration in secondary lymphoid organs. *Science (New York, N.Y* **286**, 2098-2102.

Dearstyne, E. A., and Kerkvliet, N. I. (2002). Mechanism of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced decrease in anti-CD3-activated CD4(+) T cells: the roles of apoptosis, Fas, and TNF. *Toxicology* **170**, 139-151.

Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M., and Nussenzweig, M. C. (2007). Differential antigen processing by dendritic cell subsets in vivo. *Science (New York, N.Y* **315**, 107-111.

Fernandez-Salguero, P. M., Hilbert, D. M., Rudikoff, S., Ward, J. M., and Gonzalez, F. J. (1996). Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity. *Toxicol Appl Pharmacol* **140**, 173-179.

Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* **99**, 23-33.

Funatake, C. J., Marshall, N. B., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2005). Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin generates a population of CD4+ CD25+ cells with characteristics of regulatory T cells. *J Immunol* **175**, 4184-4188.

Gunn, M. D. (2003). Chemokine mediated control of dendritic cell migration and function. *Semin Immunol* **15**, 271-276.

Gunn, M. D., Kyuwa, S., Tam, C., Kakiuchi, T., Matsuzawa, A., Williams, L. T., and Nakano, H. (1999). Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med* **189**, 451-460.

Kamath, A. B., Camacho, I., Nagarkatti, P. S., and Nagarkatti, M. (1999). Role of Fas-Fas ligand interactions in 2,3,7,8-tetrachlorodibenzo- p-dioxin (TCDD)-induced immunotoxicity: increased resistance of thymocytes from Fas-deficient (lpr) and Fas ligand-defective (gld) mice to TCDD-induced toxicity. *Toxicol Appl Pharmacol* **160**, 141-155.

Kerkvliet N., and G.R.Burleson. 1994. Immunotoxcity of TCDD and related halogenated hydrocarbons. In *Immunotoxicity and Immunopharmacology*. J.H.Dean, M.I. Luster, A.E.Munson and I.Kimber, eds. Raven Press, New York, p.97

Kerkvliet, N. I., Baecher-Steppan, L., Shepherd, D. M., Oughton, J. A., Vorderstrasse, B. A., and DeKrey, G. K. (1996). Inhibition of TC-1 cytokine production, effector cytotoxic T lymphocyte development and alloantibody production by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Immunol* **157**, 2310-2319.

Lenardo, M., Chan, K. M., Hornung, F., McFarland, H., Siegel, R., Wang, J., and Zheng, L. (1999). Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. *Annu Rev Immunol* **17**, 221-253.

Luster, M. I., Ackermann, M. F., Germolec, D. R., and Rosenthal, G. J. (1989). Perturbations of the immune system by xenobiotics. *Environ Health Perspect* **81**, 157-162.

Maraskovsky, E., Pulendran, B., Brasel, K., Teepe, M., Roux, E. R., Shortman, K., Lyman, S. D., and McKenna, H. J. (1997). Dramatic numerical increase of functionally mature dendritic cells in FLT3 ligand-treated mice. *Advances in experimental medicine and biology* **417**, 33-40.

MartIn-Fontecha, A., Sebastiani, S., Hopken, U. E., Uguccioni, M., Lipp, M., Lanzavecchia, A., and Sallusto, F. (2003). Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med* **198**, 615-621.

Mori, S., Nakano, H., Aritomi, K., Wang, C. R., Gunn, M. D., and Kakiuchi, T. (2001). Mice lacking expression of the chemokines CCL21-ser and CCL19 (plt mice) demonstrate delayed but enhanced T cell immune responses. *J Exp Med* **193**, 207-218.

Nagata, S., and Golstein, P. (1995). The Fas death factor. *Science (New York, N.Y* **267**, 1449-1456.

Perdew, G. H. (2008). Ah receptor binding to its cognate response element is required for dioxin-mediated toxicity. *Toxicol Sci* **106**, 301-303.

Peter, M. E., and Krammer, P. H. (1998). Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. *Curr Opin Immunol* **10**, 545-551.

Pulendran, B., Lingappa, J., Kennedy, M. K., Smith, J., Teepe, M., Rudensky, A., Maliszewski, C. R., and Maraskovsky, E. (1997). Developmental pathways of dendritic cells in vivo: distinct function, phenotype, and localization of

dendritic cell subsets in FLT3 ligand-treated mice. *J Immunol* **159**, 2222-2231.

Randolph, G. J., Sanchez-Schmitz, G., and Angeli, V. (2005). Factors and signals that govern the migration of dendritic cells via lymphatics: recent advances. *Springer Semin Immunopathol* **26**, 273-287.

Rhile, M. J., Nagarkatti, M., and Nagarkatti, P. S. (1996). Role of Fas apoptosis and MHC genes in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity of T cells. *Toxicology* **110**, 153-167.

Ruby, C. E., Funatake, C. J., and Kerkvliet, N. I. (2005). 2,3,7,8 Tetrachlorodibenzo-p-Dioxin (TCDD) Directly Enhances the Maturation and Apoptosis of Dendritic Cells In Vitro. *J Immunotoxicol* **1**, 159-166.

Rutella, S., Danese, S., and Leone, G. (2006). Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood* **108**, 1435-1440.

Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C. R., Qin, S., and Lanzavecchia, A. (1998). Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* **28**, 2760-2769.

Sanchez-Sanchez, N., Riol-Blanco, L., and Rodriguez-Fernandez, J. L. (2006). The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *J Immunol* **176**, 5153-5159.

Sato, K., and Fujita, S. (2007). Dendritic cells: nature and classification. *Allergol Int* **56**, 183-191.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *Embo J* **17**, 1675-1687.

Scandella, E., Men, Y., Gillessen, S., Forster, R., and Groettrup, M. (2002). Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood* **100**, 1354-1361.

Scandella, E., Men, Y., Legler, D. F., Gillessen, S., Prikler, L., Ludewig, B., and Groettrup, M. (2004). CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. *Blood* **103**, 1595-1601.

Shepherd, D. M., Steppan, L. B., Hedstrom, O. R., and Kerkvliet, N. I. (2001). Anti-CD40 Treatment of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-exposed C57Bl/6 mice induces activation of antigen presenting cells yet fails to overcome TCDD-induced suppression of allograft immunity. *Toxicol Appl Pharmacol* **170**, 10-22.

Steinman, R. M., Hawiger, D., and Nussenzweig, M. C. (2003). Tolerogenic dendritic cells. *Annu Rev Immunol* **21**, 685-711.

Vogel, C. F., Goth, S. R., Dong, B., Pessah, I. N., and Matsumura, F. (2008). Aryl hydrocarbon receptor signaling mediates expression of indoleamine 2,3-dioxygenase. *Biochemical and biophysical research communications* **375**, 331-335.

Vorderstrasse, B. A., Dearstyne, E. A., and Kerkvliet, N. I. (2003). Influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the antigen-presenting activity of dendritic cells. *Toxicol Sci* **72**, 103-112.

Vorderstrasse, B. A., and Kerkvliet, N. I. (2001). 2,3,7,8-Tetrachlorodibenzo-p-dioxin affects the number and function of murine splenic dendritic cells and their expression of accessory molecules. *Toxicol Appl Pharmacol* **171**, 117-125.

Vremec, D., Zorbas, M., Scollay, R., Saunders, D. J., Ardavin, C. F., Wu, L., and Shortman, K. (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med* **176**, 47-58.

Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adorini, L., Zimmermann, V. S., Davoust, J., and Ricciardi-Castagnoli, P. (1997a). Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J Exp Med* **185**, 317-328.

Winzler, C., Rovere, P., Zimmermann, V. S., Davoust, J., Rescigno, M., Citterio, S., and Ricciardi-Castagnoli, P. (1997b). Checkpoints and functional stages in DC maturation. *Advances in experimental medicine and biology* **417**, 59-64.

Yamazaki, S., Dudziak, D., Heidkamp, G. F., Fiorese, C., Bonito, A. J., Inaba, K., Nussenzweig, M. C., and Steinman, R. M. (2008). CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J Immunol* **181**, 6923-6933.

CHAPTER 3

Functional and phenotypic effects of AhR activation in murine, bone marrow-derived dendritic cells

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ABSTRACT

Aryl hydrocarbon receptor (AhR) activation by its prototypical ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces immune suppression. Dendritic cells (DCs) are key antigen presenting cells governing T cell activation and differentiation. However, the consequences of AhR activation in DCs are not fully defined. We hypothesize that AhR activation alters DC differentiation and generates dyfunctional DCs. To test this hypothesis, inflammatory BMDCs were generated in the presence of vehicle or TCDD. TCDD decreased CD11c expression but increased MHC class II, CD86 and CD25 expression on BMDCs. The AhR ligands 6-formylindolo[3,2-b]carbazole (FICZ) and 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid (ITE) elicited similar effects to TCDD on BMDCs. The effects were strictly AhR-dependent but not exclusively DRE-mediated. TCDD downregulated LPS- and CpG-induced NF-kB p65 levels and induced a trend towards upregulation of RelB levels in BMDCs. Induction of indoleamine 2,3–dioxygenase (IDO) and TGF-β3 has been implicated in the generation of Tregs following AhR activation. AhR activation by TCDD increased IDO1, IDO2 and TGF-β3 mRNA levels in BMDCs as compared to vehicle. Interestingly, despite the induction of a regulatory phenotype, TCDD-treated BMDCs failed to suppress antigen-specific T cell activation.

Keywords: Aryl hydrocarbon receptor (AhR); dendritic cells (DCs); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); NF-kB signaling; Indole amine-2,3-dioxygenase (IDO)

Abbreviations: AhR, Aryl hydrocarbon receptor; Ag, Antigen; BMDCs, Bone marrow-derived dendritic cells; DCs, dendritic cells; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TLR, Toll like receptors; FICZ, 6-formylindolo[3,2-b]carbazole; IDO, Indoleamine-2,3-dioxygenase, ITE, 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid.

INTRODUCTION

The Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor mediating the toxic effects of environmental pollutants such as TCDD. Following ligand binding, the AhR translocates into the nucleus, where in conjunction with its dimeric partner the Aryl hydrocarbon receptor nuclear translocater (ARNT) it binds to dioxin response elements (DREs) in target genes. AhR activation has been recognized as the prime mechanism by which environmental pollutants such as dioxins exert their adverse effects. The prototypical dioxin, 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD), is a high affinity, exogenous AhR ligand. Exposure to very low doses of dioxin produces immune suppression via activation of the AhR in many animal species, making them highly susceptible to infectious diseases and cancer (Kerkvliet, 2002). More recently, various tryptophan metabolites such as 6-formylindolo[3,2-b]carbazole (FICZ) and 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid (ITE) have been described as high-affinity natural AhR ligands (Oberg *et al.*, 2005; Henry *et al.*, 2006). Few studies have examined the effects of these natural AhR ligands on the immune system. Initial studies demonstrated that FICZ, a high affinity AhR agonist induced Th17 differentiation and inhibited Treg development by TGF-β (Quintana *et al.*, 2008). Contrasting their findings, Kimura et al demonstrated Treg induction by TGF-β that was enhanced when the AhR was activated by TCDD or FICZ (Kimura *et al.*, 2008). FICZ also increased the expression of CD86 on human U937 monocyte-derived DCs (Veldhoen *et al.*, 2008; Vogel *et al.*, 2008) Studies conducted by Henry et al., showed that similar to TCDD, ITE

alters thymocyte differentiation *in vitro* (Henry *et al.*, 2006). However, the effects of these natural AhR ligands on dendritic cells have not been previously described.

Dendritic cells (DCs) are key immune cells that bridge the gap between innate and adaptive immunity. Progressive differentiation of a DC is characterized by upregulation of MHC II and costimulatory molecules such as B7-2 (CD86) (Sato and Fujita, 2007). In addition to activating T cells, DCs constitute part of the first line of defense against invading pathogens and play a critical role in the generation of effective innate immunity to pathogenic challenge. The prime innate functions executed by DCs are their ability to recognize unique pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) such as toll like receptors (TLRs), ability to uptake antigens and secrete cytokines. Additionally, studies have shown the existence of distinct DC subsets *in vivo* (Shortman and Naik, 2007). These include the steady-state DCs and inflammatory DCs. Steady-state DCs are mostly lymphoid organ resident. The inflammatory DCs, on the other hand, constitute a novel DC population that are absent in the steady-state and appear only following an inflammatory response. These DCs, characterized by copious production of TNF-α and expression of inducible NO synthase (iNOS), are derived *in vitro* using GM-CSF/IL4 (Xu *et al.*, 2007).

Studies by Vorderstrasse and colleagues established DCs as a sensitive target of TCDD, demonstrating a loss of splenic DCs following AhR activation (Vorderstrasse and Kerkvliet, 2001). Additional studies have linked functional

alterations of DCs to defective T cell responses and immune suppression following AhR activation. For instance, studies conducted by Ruby et al., showed that TCDD-treatment of BMDCs enhanced TNF-α-induced maturation, and augmented CD95-mediated BMDC apoptosis (Ruby et al., 2005). Recently, AhR activation by TCDD was also demonstrated to induce down regulation of RelB mRNA levels in GM-CSF-derived BMDCs (Lee *et al.*, 2007). DCs play a central role in both innate and adaptive immunity, however the effects of AhR activation on DCs remain poorly understood. Thus, the aim of this study was to characterize the effects of AhR activation on the fate and function of inflammatory DCs. We hypothesize that AhR activation alters the differentiation of murine inflammatory DCs, an effect that may underlie the generation of TCDD-induced immune suppression. In this study, we explored the effects of AhR activation on DC differentiation, antigen-uptake, TLR responsiveness and production of Treg-inducing mediators. Mechanistically, we examined the role of DREs in the generation of AhR-induced effects on DCs. The data presented in this paper provides new information on the consequences associated with AhR activation in DCs.

MATERIALS AND METHOD

Animals: 6-8 week old C57Bl/6 ($AhR^{+/+}$) mice were purchased from Jackson Laboratory (Bar Harbor, ML). Breeding pairs of AhR knockout mice ($AhR^{-/-}$) were kindly provided by Dr. Paige Lawrence (URMC, Rochester, NY). $AhR^{dbd/dbd}$ and $AhR^{nls/nls}$ mutant mice were generously provided by Dr. Chris Bradfield and Dr. Ed Glover (University of Wisconsin-Madison) and subsequently maintained at UM. Animal experiments were approved by the UM IACUC and adhered to the current NIH guidelines for animal usage. Animals were provided rodent chow and tap water ad libitum.

Chemicals*:* TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) in DMSO was obtained from Cambridge Isotope laboratories Inc., (Woburn MA). 6-formylindolo[3,2-b]carbazole (FICZ) and 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid (ITE) were obtained from BIOMOL (Plymouth Meeting, PA) and Tocris (Ellisville, Missouri), respectively. DMSO was used as a vehicle for all AhR ligands. Unless specified, TCDD was used at a concentration of 10nM. Final concentration of the solvent in culture was below 0.1% DMSO and did not induce cytotoxicity.

Generation of BMDCs*:* Bone marrow-derived dendritic cells were prepared using methods as described by Inaba and coworkers (Inaba *et al.*, 1992). Briefly, bone marrow cells were flushed from the femurs of C57Bl/6 or AhR mutant mice using complete RPMI (cRPMI) media. Bone marrow cells were centrifuged at low speed and red blood cells were removed by gradient centrifugation using lympholyte-M (Cedarlane; Hornby, Canada). Bone marrow cells were washed

twice and plated at a density of $1x10^6$cells/ml in tissue culture flasks or 6 well plates. Cells were cultured in media with 30ng/ml GM-CSF and Vehicle or TCDD (10nM) for 3 days at 37° C and 5 % CO_2. On days 3 and 5, non-adherent cells were collected washed once and reseeded. Fresh media, GM-CSF (30ng/ml), vehicle or TCDD (10nM) was added to the culture flask. Non-adherent cells were harvested on day 7 and BMDCs were purified (≥ 85-90%) with CD11c-APC mAb and anti-APC beads (Miltenyi Biotec, Auburn, CA) using MACS separation columns as per the manufacturer's instructions.

Immunophenotypic analysis of BMDCs*:* BMDCs were stained for FACS analysis as previously described (Shepherd *et al.*, 2001). Briefly, rat, hamster or mouse IgG block was added to prepared leukocytes for 10 minutes on ice to prevent non-specific binding. Cells were stained for 10 minutes on ice cells with monoclonal antibodies (mAb) from BDPharmingen (San Diego, CA): CD11c (HL3), MHCII (2G9), CD86 (GL1), CD45.2 (104), CD62L (MEL14), CD44 (IM7) and Biolegend (San Diego, CA): CD25 (PC61). All mAbs used were optimally-titrated and isotype-matched mAbs were used as controls. Acquisition of 40,000-300,000 events was performed using a FACS Aria flow cytometer. (BD biosciences) and analyzed by BD FACS Diva software (Version 4.0).

Activation of BMDCs with TLR ligands and cytokine assays: Purified vehicle or TCDD-treated BMDCs were cultured in 6 well plates at a density of $1x10^6$ per well and stimulated with LPS at 1 μg/ml (Sigma) or CpG at 0.5μM (Invivogen, SA, USA) for 24 hrs. IL-6, TNF-α, IL-12p70 and IL-10 levels in the supernatants from TLR ligand-stimulated BMDCs were measured by enzyme linked immunosorbent

assay (ELISA). Samples were analyzed as per the manufacturer's instructions using cytokine-specific BD ELISA kits (BD Pharmingen)

NO measurement: NO levels in supernatants from TLR-activated BMDCs were assessed using the Griess assay. The assay was conducted as per the manufacturer's instructions (Promega, Madison, USA).

Antigen uptake: To measure phagocytic potential, BMDCs were incubated with acetylated LDL-FITC (Molecular probes, Oregon, USA) for 1.5 hours, FITC–ovalbumin (Molecular probes, Oregon, USA) for 12 hours or latex beads-FITC (Polysciences, Warrington, PA) for 6 hours. Antigen uptake was subsequently assessed by flow cytometry.

T cell activation: To assess the ability of BMDCs to activate $CD4^+$ T cell in an Ag-specific fashion, the OT II adoptive transfer model was employed. Briefly, $CD45.2^+$ BMDCs were generated in the presence of GM-CSF and TCDD (10nM) or vehicle. On day 7, BMDCs were purified and loaded with whole OVA. Vehicle- or TCDD-treated, antigen-laden BMDCs ($1x10^6$/footpad) were transferred into host CD45.1 mice that had previously received TCR transgenic OT II T cells intravenously ($2x10^6$/mouse) one day prior. Lymph nodes were harvested on day 4 post-immunization and donor DCs and OT II T cells analyzed by flow cytometry. Co-expression of CD45.2/CD11c or $CD4^+$/Thy1.1 was used to track donor DCs or OT II T cells, respectively. The activation status of donor OT II T cells was assessed phenotypically using the markers CD44 and CD62L.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR): Total RNA was isolated using Trizol reagent and quantified by

absorbance at 260nm. Single strand cDNA synthesis was carried using a RT-PCR kit (SAbiosciences) and prepared as per the manufacturer's instructions. Specific primers for IDO1, IDO2, TGF-β1, TGF-β2, TGF-β3, latent transforming growth factor beta binding protein 3 (Ltbp3), Aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2), Thrombospondin 1 (Thbs1), Plasminogen activator (Plat) and β-actin were obtained from SABiosciences. Quantitative detection of mRNA expression was performed on a Light cycler Instrument (Biorad IQ^{TM} 5, Multicolor Real Time PCR Detection Systems) using SYBR green (SAbiosciences) according to the manufacturer's instructions.

NF-kB activity: Purified BMDCs ($1.5x10^6$/well) were stimulated with LPS (1μg/ml) or CpG (0.5μM/ml) for 30 minutes in 6-well plates. Cells were harvested and nuclear protein extracts prepared using the Active Motif nuclear lysis kit (Active Motif, Carlsbad, CA). The protein content of the nuclear extract was measured using a BCA protein assay kit (Pierce, Rockford, IL). Nuclear protein (2.5 μg/ml per well) was then added to the Active Motif TransAM NF (Motif, Carlsbad, CA) and assayed according to the manufacturer's specifications.

Statistical analysis: Student's t test was used to compare means of vehicle-treated groups to TCDD–exposed groups. Data sets with multiple comparisions were evaluated by one-way analysis of variance (ANOVA) with Bonferroni's post test. For analysis, values of $p \leq 0.05$ were considered significant.

RESULTS

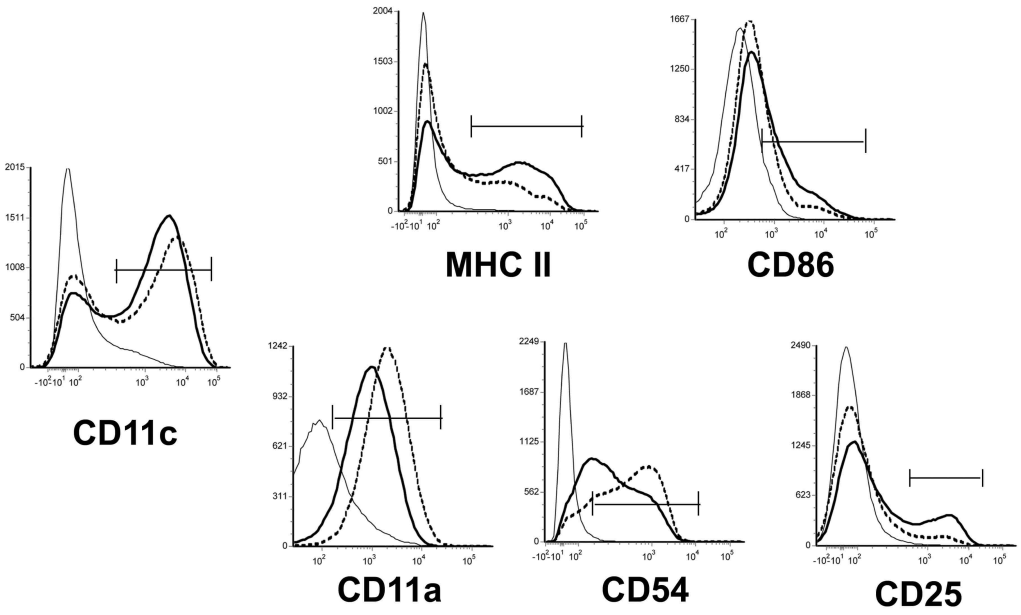
AhR activation alters BMDC growth and differentiation: We first examined the effects of TCDD on the growth of BMDCs. Bone marrow cells were induced to differentiate into DCs with GM-CSF in the presence of vehicle or TCDD (10nM) for 7 days. On day 7 both adherent and non-adherent fractions were harvested. TCDD increased the number of non-adherent cells by 50 percent, but had no effect on the adherent cells obtained on day 7 when compared to vehicle-treated controls (Table 3-1). Both vehicle- and TCDD-treated BMDCs were >95% viable (data not shown). Non-adherent cells were immunophenotyped using the murine DC marker, CD11c and costimulatory/differentiation markers MHC Class II, CD86, CD11a, CD54 and CD25. TCDD increased the frequency of $CD11c^{+}$ BMDCs but decreased the relative expression of this lineage marker when compared to vehicle-treated controls. Additionally, TCDD increased MHC II, CD86 and CD25 expression but decreased CD11a and CD54 expression on BMDCs (Figure 3-1). TCDD altered BMDC differentiation at very low concentrations. A concentration of 0.01nM TCDD upregulated the expression of CD11c, MHC Class II^{high} and $CD86^{high}$ expression on BMDCs when compared to vehicle-treated controls (Figure 3-2). In addition to TCDD, many natural (TCDD-like) ligands bind and activate the AhR. To examine the affects of non-TCDD chemicals on BMDC differentiation, cells were treated with two natural AhR ligands, FICZ and ITE. Similar to TCDD, FICZ and ITE decreased CD11c expression and increased levels of MHC Class II and CD86 on the BMDCs (Figure 3-3).

Table 3-1: Effects of TCDD on the number of murine bone marrow dendritic cells [a]

		Vehicle	TCDD
Number of cells ($x10^6$)	Non-adherent	16.3 ± 1.2	24.0 ± 4.6*
	Adherent cells	9.3 ± 0.7	7.1 ± 2.9

[a] BMDCs were grown in presence of vehicle or TCDD. Cells were harvested on day 7 and ennumerated as described in the *materials and methods* section. Viability of the cells was ≥ 95% as determined by trypan blue staining Data represent the mean ± SEM of 9 independent samples per treatment group. *$p<0.05$ indicates significant differences between vehicle and TCDD-treated groups.

Figure 3-1



		Vehicle	TCDD
CD11c	Percentage	56.7 ± 0.4	60.6 ± 0.3*
	MFI	7737 ± 160	5138 ± 54*
MHC II	Percentage	38.7 ± 0.8	60.4 ± 0.6*
	MFI	690.8 ± 8	1182 ± 24*
CD86	Percentage	15.7 ± 0.3*	27.2 ± 0.3*
	MFI	1905 ± 56	2278 ± 29*
CD11a	Percentage	81.2 ± 0.7	53.6 ± 0.7*
	MFI	3304 ± 53	2147 ± 20*
CD54	Percenatage	81.7 ± 0.4	67.0 ± 0.5*
	MFI	797 ± 24	654 ± 7.2*
CD25	Percentage	4.8 ± 0.2	18.6 ± 0.4*
	MFI	1775 ± 8	2204 ± 11*

Figure 3-1: TCDD alters the expression of differentiation markers on BMDCs: Bone marrow cells were induced to differentiate into immature DCs with GM-CSF in the presence of vehicle (represented by dotted line in the histograms) or 10 nM TCDD (thick line as indicated in the histograms). Isotype staining is shown in gray lines. Non-adherent cells were harvested on day 7 and stained with CD11c, MHC Class II, CD86, CD54, CD11a and CD25 and their expression determined on $CD11c^+$ BMDCs by FACS analysis. Data shown represent non-adherent cells obtained from 4 separate flasks per treatment group and is representative of 3 independent experiments. *$p<0.05$ indicates significant differences between vehicle-and TCDD-treated groups.

Figure 3-2

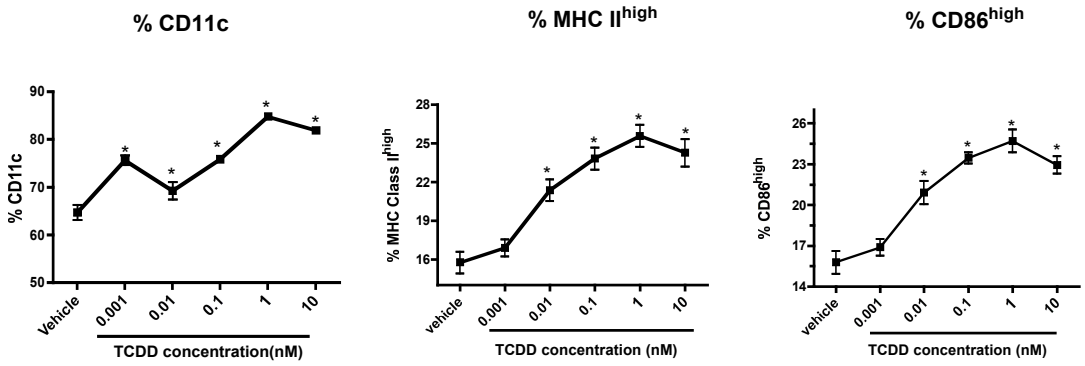


Figure 3-2: Low concentrations of TCDD alter BMDC differentiation: Bone marrow cells were generated in the presence of GM-CSF and vehicle or varying TCDD concentrations (0.001nM-10nM). Non-adherent cells were harvested on day 7 and stained for CD11c, MHC II and CD86. Expression of MHC II and CD86 was determined on the $CD11c^{+}$-gated population. Data represent mean ± SEM of 6 samples at each concentration. *$p < 0.05$ indicates significant differences between the vehicle- and TCDD-treated group.

Figure 3-3

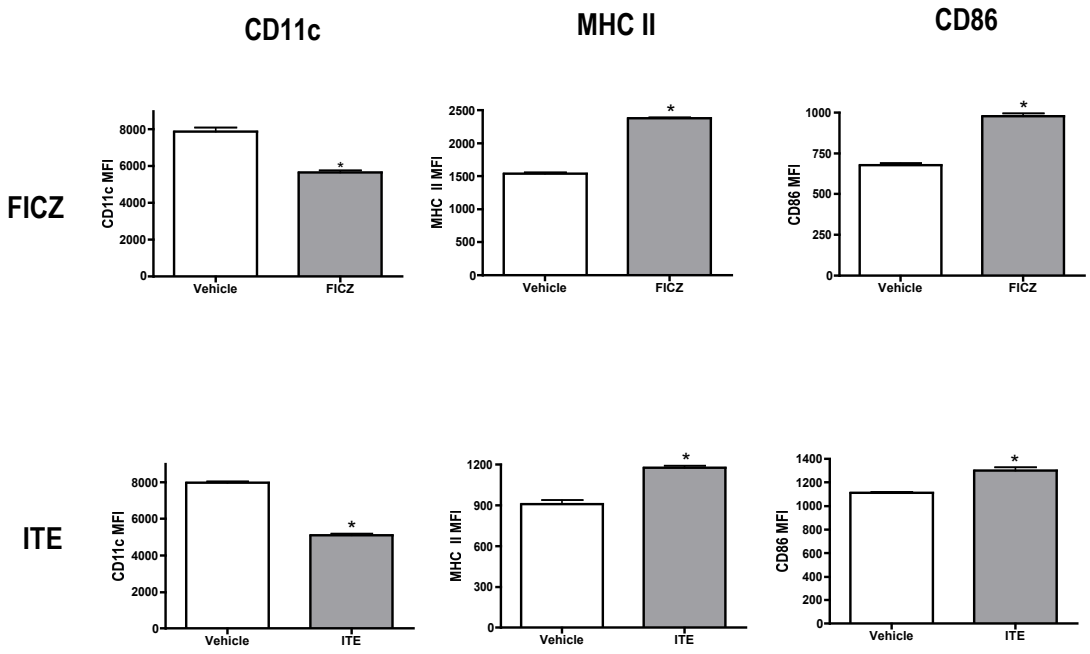


Figure 3-3: Natural AhR agonists alter BMDC differentiation: Bone marrow-derived dendritic cells were grown in presence of vehicle or the natural AhR agonists, FICZ and ITE. Non-adherent cells were harvested on day 7 and stained for CD11c, MHC II and CD86. Expression of MHC II and CD86 was determined on $CD11c^{+}$-gated population. *indicates significant differences between treated and respective vehicle control groups. Data represent mean ± SEM of 4 samples per treatment group for 2 independent experiments.

Effects of TCDD on BMDC differentiation are AhR-dependent but are not exclusively DRE-mediated: To define the role of the AhR in TCDD-induced alterations in the differentiation of BMDCs, we analyzed BMDCs from $AhR^{-/-}$, $AhR^{dbd/dbd}$ and $AhR^{nls/nls}$ mice. TCDD did not affect CD11c, MHC II and CD86 expression on $AhR^{-/-}$ BMDCs when compared to vehicle-treated controls (Figure 3-4). $AhR^{nls/nls}$ and $AhR^{dbd/dbd}$ mice express a mutant AhR that binds ligand but fails to translocate in the nucleus or bind DREs, respectively. BMDCs from $AhR^{dbd/dbd}$ mutant mice displayed decreased CD11c and increased MHC II and CD86 expression following TCDD treatment (Figure 3-4). On the other hand, BMDCs from $AhR^{nls/nls}$ mice displayed decreased CD11c and CD86, and a slight but significant increase in the expression of MHC II following TCDD treatment.

Effects of TCDD on TLR responsiveness and TLR ligand-induced production of inflammatory mediators: To evaluate the effects of TCDD on the innate responsiveness of BMDCs, cells were cultured with the TLR ligands, LPS (TLR4 agonist) and CpG (TLR9 agonist). Consistent with our previous results, TCDD increased MHC class II^{high} expression on unstimulated BMDCs when compared to the unstimulated vehicle controls (Figure 3-5). As expected, LPS- and CpG-treatment increased MHC Class II^{high} frequency of BMDCs when compared to the unstimulated controls. TCDD significantly increased the LPS- and CpG-induced frequency of BMDCs expressing high levels of MHC Class II^{high} compared to respective vehicle-treated controls. TLR4 and TLR9 mRNA

Figure 3-4

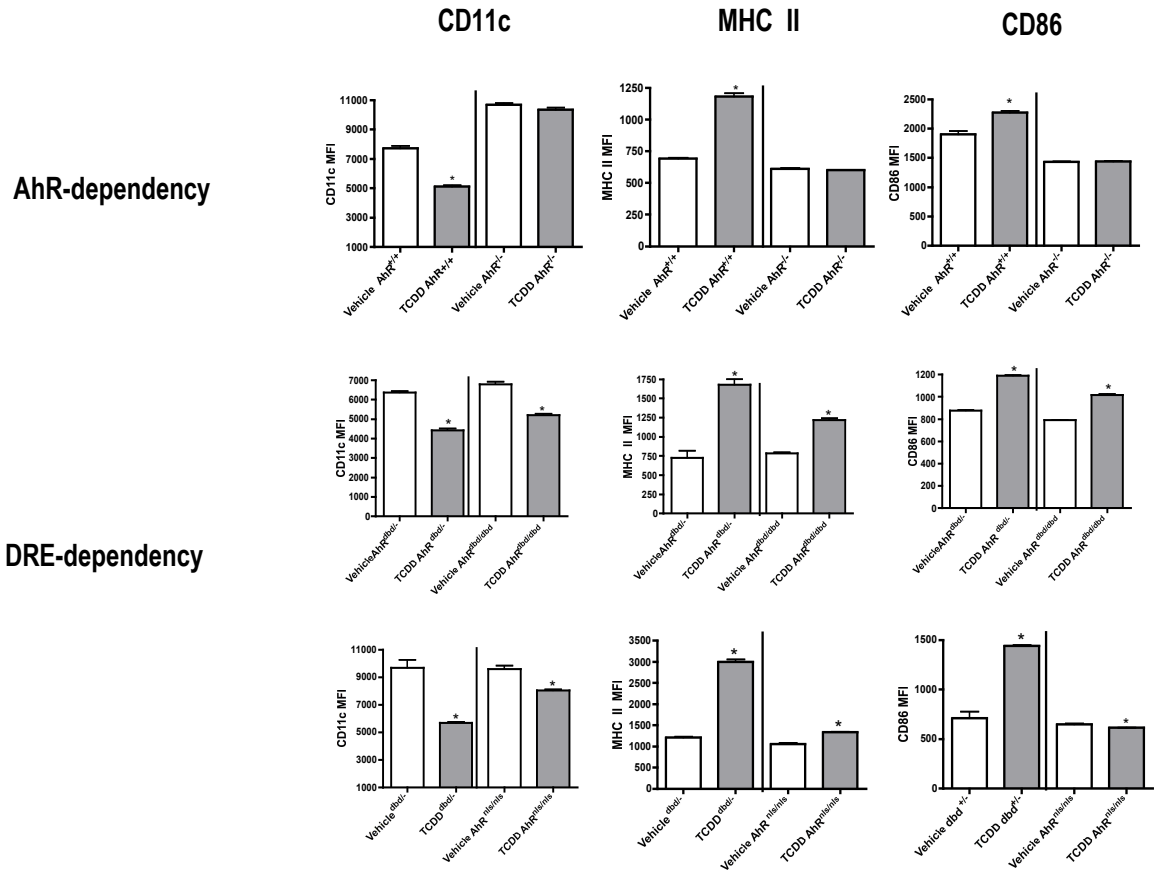


Figure 3-4. The effects of TCDD on BMDCs are AhR-dependent and partially DRE-dependent: BMDCs from $AhR^{+/+}$, $AhR^{dbd/-}$, $AhR^{dbd/dbd}$ and $AhR^{nls/nls}$ were treated with vehicle or TCDD. Non-adherent cells were harvested on day 7 and stained for CD11c, MHC II and CD86. Expression of MHC II and CD86 was determined on the CD11c gated population and analyzed by Facs aria. Data represent mean ± SEM of non-adherent cells obtained from 3-4 flasks per treatment goup. *$p<0.05$ indicates significant differences between respective vehicle- and TCDD-treated groups.

Figure 3-5

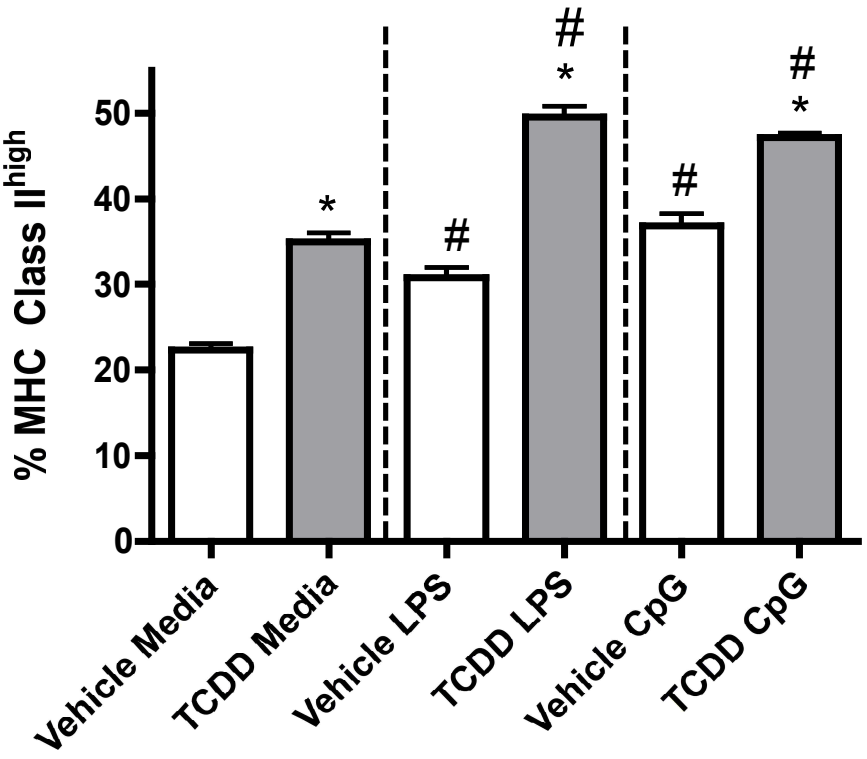


Figure 3-5. Effects of TCDD on LPS- and CpG–induced MHC Class II^{high} expression: BMDCs were grown in vehicle or TCDD and non-adherent cells were harvested on day 7. BMDCs were purified and treated with LPS or CpG for 24 hours. Cells were harvested after 24 hours and MHC class II expression was assessed. Data represent mean ± SEM of 9 samples per treatment group and is representative of 3 independent experiments. *$p<0.05$ indicates significant differences between the respective vehicle- and TCDD-treated groups. $^{\#}p<0.05$ indicates significant differences between TLR-stimulated and respective untreated groups.

expression was not affected by TCDD as measured by RT-PCR (data not shown). Additionally, AhR activation by TCDD increased both LPS- and CpG-induced IL-6 and TNF-α production but decreased NO production by BMDCs (Table 3-2). TCDD did not affect IL-10 production by the BMDCs when compared to the vehicle-treated controls. Although TCDD did not alter the LPS-induced IL-12p70 production, it decreased the CpG-induced IL-12p70 production by the BMDCs when compared to vehicle-treated BMDCs.

TCDD decreases LPS- and CpG-induced p65 but not RelB activity: NF-kB signaling in DCs is activated following TLR stimulation. Additionally, based on the potential of ligand-activated AhR to physically interact with NF-kB and alter immune function, we evaluated NF-kB signaling in BMDCs (Ruby *et al.*, 2002; Vogel *et al.*, 2007a). TCDD decreased p65, but not RelB activity in unstimulated BMDCs when compared to unstimulated vehicle controls. LPS- and CpG-treatment increased p65 but not RelB activity in BMDCs when compared to the unstimulated controls. TCDD significantly decreased LPS-and CpG-induced p65 activity in BMDCs when compared to respective vehicle-treated controls. TCDD generated a trend towards upregulation of LPS- and CpG-induced RelB levels in BMDCs when compared to the respective vehicle-treated controls (Figure 3-6).

Antigen uptake by BMDCs is modulated by TCDD: Dendritic cells take up antigens by phagocytosis and receptor-mediated endocytosis. To monitor the effects of TCDD on antigen uptake, BMDCs were evaluated for uptake of both soluble and particulate antigens. As determined by flow cytometry, TCDD

Figure 3-6

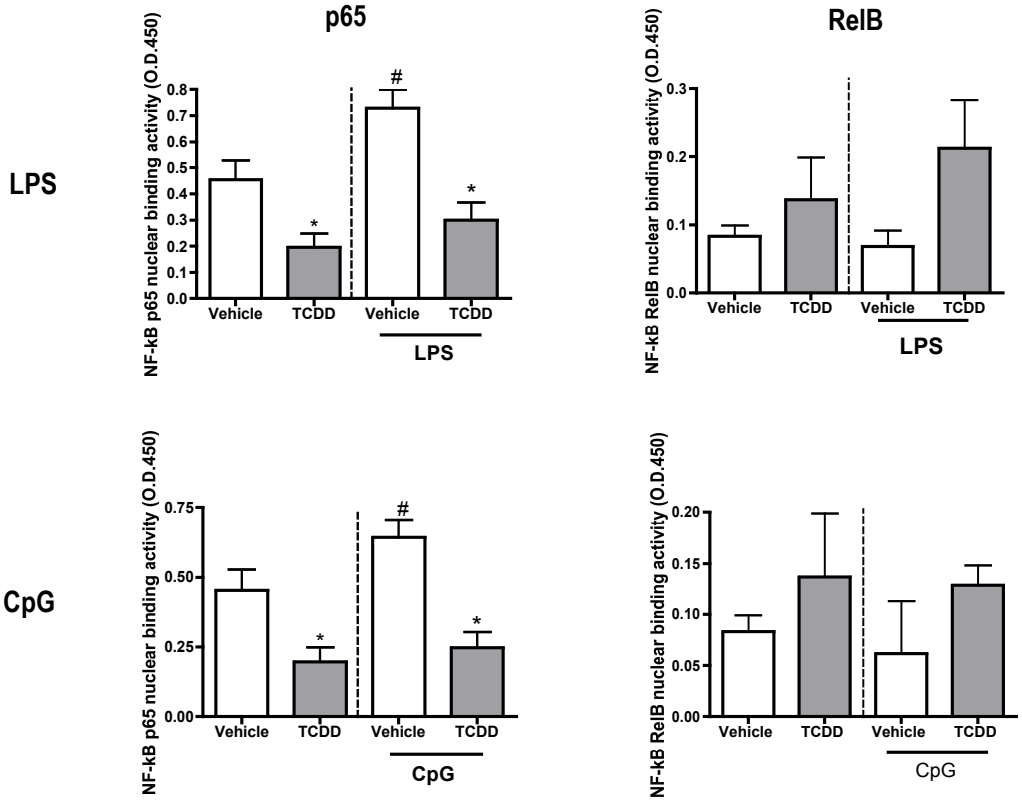


Figure 3-6. LPS-and CpG-induced p65 but not Rel B activity is reduced by TCDD: Vehicle- or TCDD-treated BMDCs were stimulated with LPS (1 μg/ml) or CpG (0.5μM) for 0.5 hours. Cells were harvested and nuclear proteins isolated. p65 and RelB activity was measured using the TransAM activity ELISA. * indicates significant differences between LPS- or CpG-stimulated groups compared to respective vehicle-treated control groups. Data represent mean ± SEM of 3 samples per treatment group.

Table 3-2: Effects of TCDD on cytokine production by BMDCs[a]

	TNF-α (pg/ml)	
	Vehicle	**TCDD**
Media	BD	BD
LPS	4286 ± 407	9565 ± 266*
CpG	5154 ± 207	8556 ± 399*

	IL-6 (pg/ml)	
	Vehicle	**TCDD**
Media	BD	BD
LPS	21960 ± 2000	33080 ± 3592*
CpG	22660 ± 1135	30590 ± 3029*

	IL-10 (pg/ml)	
	Vehicle	**TCDD**
Media	BD	BD
LPS	1310 ± 76	1538 ± 282
CpG	694± 64	719 ± 43

	IL-12p70 (pg/ml)	
	Vehicle	**TCDD**
Media	BD	BD
LPS	268 ± 36	271 ± 34
CpG	640 ± 28	581 ± 20*

	NO (μM)	
	Vehicle	**TCDD**
Media	0.4 ± 0.1	0.5 ± 0.1
LPS	20 ± 2.2	4 ± 0.3*
CpG	16.2 ± 1.5	3.2 ± 0.2*

[a]BMDCs were stimulated with LPS or CpG as described in *Materials and Methods.* Supernatants were collected and levels of TNF-α, IL-6, IL-10 and IL-12p70 determined by ELISA. Levels of NO were determined using the Griess reagent. Data represent mean ± SEM of 4-9 samples for TNF-α, IL-6, IL-10 and NO and is representative of 2 independent experiments. Data represent mean ± SEM of 12-15 samples pooled from 2 separate experiments for IL-12p70. * $p \leq 0.05$ indicates significant differences between LPS- and CpG–stimulated BMDCs as compared to respective vehicle-treated controls. BD represents below detection.

decreased BMDC uptake of the soluble antigens, ovalbumin and acetylated-LDL, by 25 and 10 percent, respectively (Figure 3-7). Conversely, TCDD increased the uptake of the particulate antigen, latex beads, by 40 percent when compared to the vehicle-treated controls.

AhR activation upregulates mRNA levels of suppressive T reg-inducing mediators. Potent T reg-inducing mediators such as IDO1, IDO2, Thbs1 and TGF-β3 have been implicated in the generation of immune suppression following AhR activation (Lawrence *et al.*, 2008; Marshall *et al.*, 2008; Vogel *et al.*, 2008). In this context, we examined the effects of AhR activation on the generation of suppressive mediators in BMDCs. TCDD-treated BMDCs upregulated IDO1, IDO2 and TGF-β3 levels by 18-, 24- and 11-fold, respectively (Table 3-3). TCDD had no effect on the expression of LTBP, Aldh, Thbs1 or Plat in the BMDCs. Following LPS treatment, TCDD-BMDCs displayed increased levels of IDO1, IDO2, TGF-β1, TGF-β2 and Thbs1 when compared to vehicle-treated BMDCs.

AhR-activated BMDCs do not inhibit antigen-specific T cell responses: We tested the ability of AhR-activated BMDCs to generate antigen-specific T cell activation using the modified OT II adoptive transfer model. Vehicle- or TCDD-treated donor BMDCs ($CD45.2^+$) were pulsed *in vitro* with whole ova to load these APCs with antigen. Ova-loaded DCs were then used to activate adoptively transferred, ova-specific OT II/Thy1.1 $CD4^+$ T cells that had been adoptively transferred into CD45.1 host mice. Peripheral lymph nodes were harvested on day 4 following BMDC immunizations. Donor DCs obtained from mice receiving

Figure 3-7

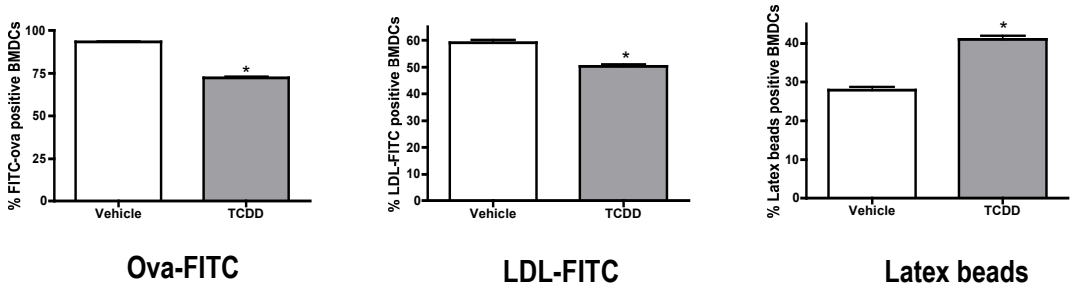


Figure 3-7. TCDD alters antigen uptake by BMDCs: Vehicle- or TCDD-treated BMDCs were purified and evaluated for the ability to take up FITC-conjugated whole ovalbumin (FITC–Ova), low-density lipoprotein (LDL-FITC) and latex beads was assessed. Cells were exposed to FITC-conjugated ovalbumin overnight, LDL-fitc for 1.5 hours and FITC-latex beads for 6 hours prior to harvesting. Data represent mean ± SEM of 9-12 samples per treatment group for 2 independent experiments. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated samples.

Table 3-3: AhR activation alters expression of suppressive mediators[a]

	Unactivated		*LPS-activated*	
	Fold change	***p value***	**Fold change**	***P value***
IDO1	**18***	*0.0001*	**22***	*≤0.001*
IDO2	**24***	*≤0.001*	**25***	*≤0.001*
TGFβ1	**0.4**	*0.1*	**1.8***	*0.02*
TGFβ2	**0.9**	*0.9*	**2.3***	*0.008*
TGFβ3	**11***	*0.001*	**0.01**	*0.4*
Ltbp3	**0.2**	*0.3*	**1.3***	*0.001*
Aldh1a2	**0.8**	*0.6*	**0.6**	*0.09*
Thbs1	**0.4**	*0.07*	**1.9***	*0.01*
Plat	**1.2**	*0.4*	**1.0**	*0.8*

[a]Gene expression in resting and LPS-activated BMDCs was evaluated by real-time RT-PCR. The results are expressed as TCDD-induced fold change after normalization with the housekeeping gene, β-actin. *N* = 3-4 per treatment group. * p≤ 0.05 indicates significant differences between vehicle- and TCDD-treated BMDCs.

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Figure 3-8

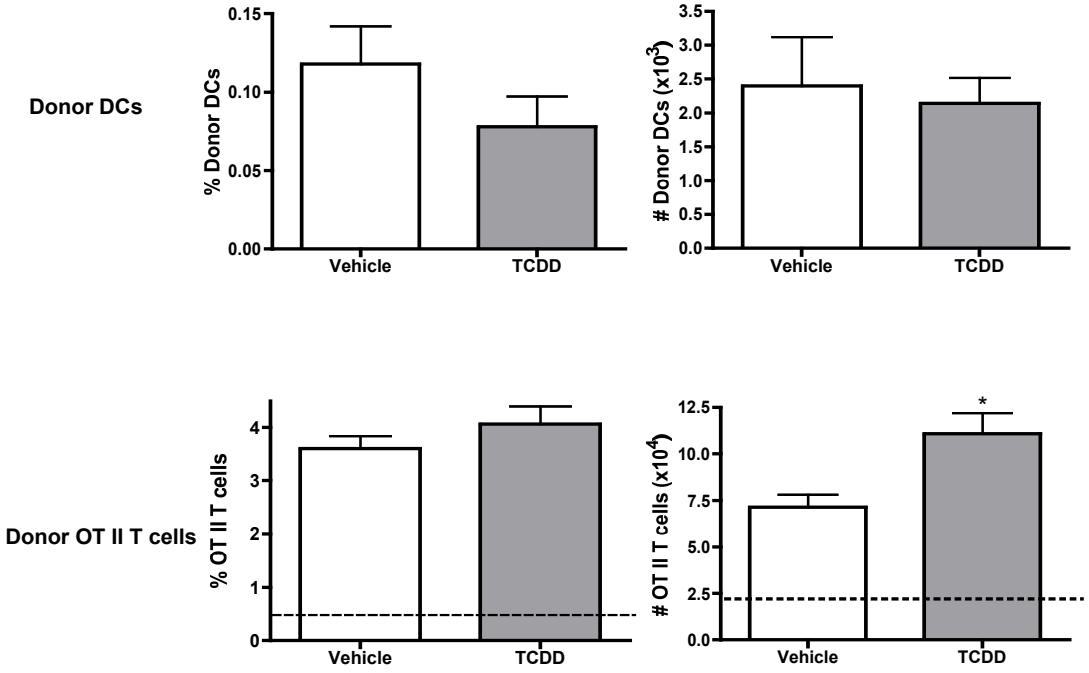

Figure 3-8. Ability of TCDD-treated BMDCs to activate antigen-specific T cells: $CD4^+$ T cells from OT II Thy1.1 were adoptively transferred into congenic CD45.1 host mice on day -1 relative to immunization. On day 0, whole ova-loaded BMDCs, that were either vehicle- or TCDD-treated were injected into adoptively transferred mice. On day 4 post-immunization, popliteal lymph nodes were harvested from host mice and analyzed by flow cytometry. Analysis was based on the differences in the phenotypic expression of cell surface molecule between donor DCs (CD45.2/CD11c) and donor T cells (CD4/Valpha2/Thy1.1) from host mice (CD45.1/Thy1.2). Data represent mean ± SEM of 4-5 animals per treatment group. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD–treated groups. Dashed line represents the status of donor T cells in the brachial lymph nodes.

TCDD-treated BMDCs, displayed reduced MHC Class II frequency when compared to vehicle-treated BMDCs. Ova-specific OT II T cells from the popliteal lymph nodes exhibited significant clonal expansion when compared to the OT-II T cells from non-draining BLNs (Figure 3-8 + data not shown). Host mice, immunized with TCDD-treated BMDCs displayed an increase in the number, but not the frequency of OT II T cells. Additionally, no differences were observed in the expression of the activation markers CD62L and CD44 on donor T cells from mice receiving TCDD-treated BMDCs when compared to donor T cells from mice receiving vehicle-treated BMDCs (Figure 3-8).

DISCUSSION

Dendritic cells are professional antigen presenting cells that play an important role in both the innate and adaptive arms of the immune systems. In this study, we have characterized the consequences of AhR activation on the innate and adaptive functions of BMDCs and examined the role of DRE-mediated events in the generation of these effects. We demonstrate that AhR activation alters BMDC differentiation and induces a regulatory phenotype, but surprisingly without altering the ability of the DCs to initiate Ag-specific activation of naïve, $CD4^+$ T cells.

Dendritic cells exist in multiple, functionally distinct stages of differentiation. Several investigators have linked TCDD-induced modulation of DC differentiation to the generation of defective T cell responses (Vorderstrasse and Kerkvliet, 2001; Lee *et al.*, 2007). In our study, TCDD decreased expression of CD11c but increased MHC II and CD86 levels on BMDCs. Engagement of B7 (CD86) molecules on a DC with CD28 or CTLA4 on the surface of the T cell can activate or impair T cell responses, respectively (Krummel and Allison, 1995; Lenschow *et al.*, 1996). Successful T cell activation involves not only interactions between the TCR and Ag-associated MHC II, but also costimulatory interactions such as CD28 and CD86. In this context, studies have linked TCDD-induced increases in CD86 expression on DCs with increased T cell activation in an MLR response (Lee *et al.*, 2007). However, AhR activation has also been shown to induce CTLA4 expression on T cells which can bind to CD86 on a DC with high

affinity and impair T cell responses (Funatake *et al.*, 2005). Thus, in the context of AhR activation, ligation of CD86 on DCs with CTLA4 on T cells could alternatively contribute towards the generation of immune suppression. Furthermore, consistent with previous studies documenting TCDD-induced modulation of the adhesion molecules CD11a and CD54, TCDD decreased the expression of both these molecules on BMDCs (Shepherd *et al.*, 2001; Vorderstrasse and Kerkvliet, 2001). The decreased expression of adhesion molecules on DCs could contribute to defective T cell activation and subsequent immune suppression. TCDD-induced immune suppression has also been linked to increased CD25 expression on T cells (Funatake *et al.*, 2005). More recently, CD25 was identified as a phenotypic marker for regulatory DCs (DCregs) (Driesen *et al.*, 2008) (von Bergwelt-Baildon et al., 2006). TCDD increased CD25 expression on BMDCs, suggesting the induction of a regulatory phenotype in the BMDCs. Taken together, TCDD-induced alteration of molecules involved in Ag-presentation, costimulation and adhesion could result in defective T cell activation contributing to immune suppression. Recently, ligand-specific activation of the AhR has been shown to generate Tregs or Th17 cells (Quintana *et al.*, 2008). However, the effects of ligand-specific activation in inflammatory DCs have not been previously described. Interestingly, the AhR ligands, FICZ and ITE generated phenotypical alterations in BMDCs similar to TCDD, demonstrating a lack of differential responsiveness of DCs to an array of AhR ligands.

While the AhR is essential for TCDD-induced immunotoxicity, no

information exists regarding the role of DREs in TCDD-induced alterations of DCs. We investigated the role of the AhR and DREs in TCDD-induced alteration in DC differentiation using BMDCs from AhR null, $AhR^{nls/nls}$ and $AhR^{dbd/dbd}$ mice. While the AhR null mice, lacking functional AhR receptor, enabled us to determine the role of the AhR in the generation of TCDD-induced alterations in DCs, involvement of DRE-mediated events was assessed using the $AhR^{nls/nls}$ and $AhR^{dbd/dbd}$ mice expressing a mutant AhR that binds ligand but fails to translocate into the nucleus or bind DREs, respectively. Our results show that the effects of AhR activation in BMDCs are strictly AhR-dependent but not exclusively DRE-mediated. TCDD-treated BMDCs from $AhR^{dbd/dbd}$ mice displayed increased MHC II and CD86 expression when compared to vehicle-treated $AhR^{dbd/dbd}$ BMDCs. However, TCDD-induced increases in MHC II and CD86 expression in AhR $^{dbd/dbd}$ BMDCs did not reach similar levels to those observed in TCDD-treated BMDCs from $AhR^{dbd/-}$ control mice. This suggested that a non-DRE mediated mechanism was contributing to the TCDD-induced differentiation of the DCs. Of the several non-DRE mediated mechanisms implicated in TCDD immunotoxicity, interactions of the activated AhR with NF-kB signaling components can induce defects in DCs following TCDD exposure (Ruby *et al.*, 2002; Vogel and Matsumura, 2008). Thus, one possible explanation for the partial effects of TCDD on BMDC differentiation as observed in DCs from $AhR^{dbd/dbd}$ and $AhR^{nls/nls}$ mice could be due to altered NF-kB signaling.

A primary function of DCs is the recognition of PAMPs via PRRs such as TLRs. Based on the observed TCDD-induced modulation of DC differentiation,

we assessed whether TCDD-exposed BMDCs were responsive to PAMPs via TLR activation. TCDD increased BMDC responsiveness to TLR stimulation via TLR4 and TLR9 without affecting the basal mRNA levels of these receptors. Following activation DCs secrete cytokines that orchestrate developing immune responses. In our study, TCDD increased LPS- and CpG-induced IL-6 and TNF-α production by BMDCs but decreased their production of nitric oxide. Multiple possibilities could account for these observed effects. NF-kB independent transcription factor, LPS-Induced TNF-α Factor (LITAF), is a transcription factor that has been implicated in the LPS-induced expression of TNF-α and other inflammatory cytokines (Sun *et al.*, 2004; Tang *et al.*, 2005). LITAF contains 7 DRE sequences and upregulation of LITAF in DCs could underlie our observed TCDD-induced increases in LPS-induced proinflammatory cytokine secretion (Sun *et al.*, 2004). Recent studies have, however, documented the ability of activated AhR to negatively regulate inflammatory responses in human monocyte-derived DCs and murine macrophages (Lawrence *et al.*, 2008; Kimura *et al.*, 2009). Discrepancies in these results could be attributed to differences in the AhR agonists used in the respective studies (VAGF347 versus TCDD) or in the cells that were examined (human monocyte DCs versus murine DCs or macrophages). In addition, activation of inflammatory DCs by LPS or CpG usually induces upregulation of nitric oxide synthase (NOS) and subsequently increased NO production. AhR activation by TCDD has been shown to increase pulmonary inducible NO synthase (iNOS) levels contributing to enhanced IFN-γ production in the lungs (Neff-LaFord *et al.*, 2007). Contrary to these reports, in

our study TCDD decreased NO production by both unstimulated and TLR-activated BMDCs. Additonal investigation is warranted to further understand the effect of TCDD on NO production by BMDCs.

A previous study examining the effects of TCDD on BMDCs reported decreased mRNA levels of IL-10 but not IL-12 following LPS-stimulation (Lee *et al.*, 2007). Contrary to this study, our data indicates that TCDD does not alter the production of IL-10 and decreases CpG-induced IL-12 production by BMDCs. Different DC preparations (unpurified versus purified DCs) used in these two studies could account for the observed differences. Under *in vivo* conditions, inflammatory DCs function to limit infection by their secretion of inflammatory cytokines. Induction of inflammation is a hallmark feature of TCDD-induced immunotoxicity and our results suggest a role for DCs in TCDD-induced inflammatory responses. However, how TCDD-induced alterations of inflammatory DCs impacts the development of acute inflammation remains to be determined.

NF-kB is a major signaling pathway involved in the differentiation of DCs and their responsiveness to TLR stimulation. Furthermore, several studies have linked physical interactions of activated AhR with NF-kB p65 and RelB, in the generation of TCDD-induced cellular dysfunction and immune suppression (Ruby *et al.*, 2002; Camacho *et al.*, 2005; Vogel *et al.*, 2007a; Vogel *et al.*, 2007b; Vogel and Matsumura, 2008). Because TCDD alters the differentiation of DCs and their responsiveness to TLRs, we investigated the effects of AhR activation on NF-kB

signaling in inflammatory BMDCs. TCDD suppressed basal p65 activity in unstimulated BMDCs as well as BMDCs activated by LPS and CpG. Furthermore, a trend towards increased RelB activity was also observed in TCDD-treated BMDCs. Binding of RelB/AhR to specific response elements (RelB/AhRE) in certain target genes has been linked to the regulation of cytokines and chemokines such as IL-8 and BLC following TCDD exposure (Vogel *et al.*, 2005; Vogel *et al.*, 2007b; Vogel and Matsumura, 2008). It would be of interest to determine if the enhanced production of IL-6 and TNF-α by TCDD-treated BMDCs is also regulated by increased interactions between activated AhR and RelB. Overall, our data suggest that TCDD-induced modulation of DC differentiation and their responsiveness to TLR stimulation may be mediated via altered NF-kB signaling.

Dendritic cells capture antigens via distinct mechanisms. While soluble antigen uptake primarily involves macropinocytic or receptor-mediated endocytic events, particulate antigens are internalized primarily via phagocytosis (Banchereau *et al.*, 2000). TCDD decreased the uptake of the soluble antigens ovalbumin and LDL but increased phagocytosis of latex beads by inflammatory DCs. These results suggest that TCDD differentially impacts mechanisms of antigen uptake. Studies have shown macrophage mannose receptor and scavenger receptor A to mediate the endocytosis of ova and acetylated LDL, respectively (Pearson *et al.*, 1993; Becker *et al.*, 2006; Burgdorf *et al.*, 2007). Therefore, based on our results, we hypothesize that decreased expression of these receptors in BMDCs following exposure to TCDD leads to decreased

uptake of soluble antigens. Conversely, TCDD increased the uptake of latex beads by BMDCs. So far, only one study has described effects of TCDD on the uptake of latex beads. In this *in vivo* study, uptake of latex beads by splenic DCs was unaffected following TCDD exposure (Vorderstrasse *et al.*, 2003). The observed discrepancy in our results could be attributed to distinct antigen uptake mechanisms in the two functionally distinct DC populations (steady-state versus inflammatory), which could be differentially affected by TCDD.

Because our data demonstrated that TCDD altered antigen uptake, we assessed the effects of TCDD on the T cell stimulatory capacity of inflammatory BMDCs. Surprisingly, TCDD-treated BMDCs generated normal antigen-specific T cell responses *in vivo*. These results were unexpected, as TCDD not only induced a regulatory phenotype in BMDCs, but also increased their expression of several regulatory mediators including IDO1, IDO2 and TGF-β3. Several possibilities could account for the lack of T cell suppression by TCDD-treated BMDCs. Though our results demonstrated that TCDD alters antigen uptake of ovalbumin by BMDCs, it is possible that the observed decreases in antigen uptake do not reach a physiological threshold below which T cell activation is affected. Additionally, TCDD may increase the expression, but not the function or activity of the suppressive mediators IDO1, IDO2 and TGF-β3. In this case, we would not expect TCDD-treated BMDCs to contribute to the suppression of T cell activation. Recent studies have linked AhR activation in DCs with suppression of T cell responses (Hauben *et al.*, 2008). In these studies, AhR-activated DCs suppressed T cell responses only following LPS activation. Immature BMDCs did

not alter T cell responses. Thus, we also evaluated the expression of the suppressive mediators in BMDCs following LPS maturation. TCDD-treated BMDCs stimulated with LPS displayed increased expression of IDO1, IDO2, TGF-β1, TGF-β2 and Thbs1. However, future experiments are needed to determine whether TCDD-treated BMDCs stimulated with LPS can affect antigen-specific T cell responses.

Collectively, we show that AhR activation alters inflammatory DC differentiation, generating DCs that are hyperresponsive to TLR stimulation and exhibit a regulatory phenotype *in vitro*. Surprisingly, these BMDCs fail to alter antigen-specific T cell activation. However, further studies are needed to examine the potential for AhR-activated DCs to contribute to the generation of regulatory T cells and the suppression of T cell-mediated immune responses.

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REFERENCES

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., and Palucka, K. (2000). Immunobiology of dendritic cells. *Annu Rev Immunol* **18**, 767-811.

Becker, M., Cotena, A., Gordon, S., and Platt, N. (2006). Expression of the class A macrophage scavenger receptor on specific subpopulations of murine dendritic cells limits their endotoxin response. *Eur J Immunol* **36**, 950-960.

Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P. A., and Kurts, C. (2007). Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* **316**, 612-616.

Camacho, I. A., Singh, N., Hegde, V. L., Nagarkatti, M., and Nagarkatti, P. S. (2005). Treatment of mice with 2,3,7,8-tetrachlorodibenzo-p-dioxin leads to aryl hydrocarbon receptor-dependent nuclear translocation of NF-kappaB and expression of Fas ligand in thymic stromal cells and consequent apoptosis in T cells. *J Immunol* **175**, 90-103.

Driesen, J., Popov, A., and Schultze, J. L. (2008). CD25 as an immune regulatory molecule expressed on myeloid dendritic cells. *Immunobiology* **213**, 849-858.

Funatake, C. J., Marshall, N. B., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2005). Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin generates a population of CD4+ CD25+ cells with characteristics of regulatory T cells. *J Immunol* **175**, 4184-4188.

Hauben, E., Gregori, S., Draghici, E., Migliavacca, B., Olivieri, S., Woisetschlager, M., and Roncarolo, M. G. (2008). Activation of the aryl hydrocarbon receptor promotes allograft-specific tolerance through direct and dendritic cell-mediated effects on regulatory T cells. *Blood* **112**, 1214-1222.

Henry, E. C., Bemis, J. C., Henry, O., Kende, A. S., and Gasiewicz, T. A. (2006). A potential endogenous ligand for the aryl hydrocarbon receptor has potent agonist activity in vitro and in vivo. *Arch Biochem Biophys* **450**, 67-77.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* **176**, 1693-1702.

Kerkvliet, N. I. (2002). Recent advances in understanding the mechanisms of TCDD immunotoxicity. *Int Immunopharmacol* **2**, 277-291.

Kimura, A., Naka, T., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., Fujii-Kuriyama, Y., and Kishimoto, T. (2009). Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *J Exp Med*.

Kimura, A., Naka, T., Nohara, K., Fujii-Kuriyama, Y., and Kishimoto, T. (2008). Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc Natl Acad Sci U S A* **105**, 9721-9726.

Krummel, M. F., and Allison, J. P. (1995). CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* **182**, 459-465.

Lawrence, B. P., Denison, M. S., Novak, H., Vorderstrasse, B. A., Harrer, N., Neruda, W., Reichel, C., and Woisetschlager, M. (2008). Activation of the aryl hydrocarbon receptor is essential for mediating the anti-inflammatory effects of a novel low-molecular-weight compound. *Blood* **112**, 1158-1165.

Lee, J. A., Hwang, J. A., Sung, H. N., Jeon, C. H., Gill, B. C., Youn, H. J., and Park, J. H. (2007). 2,3,7,8-Tetrachlorodibenzo-p-dioxin modulates functional differentiation of mouse bone marrow-derived dendritic cells Downregulation of RelB by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Lett* **173**, 31-40.

Lenschow, D. J., Walunas, T. L., and Bluestone, J. A. (1996). CD28/B7 system of T cell costimulation. *Annu Rev Immunol* **14**, 233-258.

Marshall, N. B., Vorachek, W. R., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2008). Functional characterization and gene expression analysis of CD4+ CD25+ regulatory T cells generated in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Immunol* **181**, 2382-2391.

Neff-LaFord, H., Teske, S., Bushnell, T. P., and Lawrence, B. P. (2007). Aryl hydrocarbon receptor activation during influenza virus infection unveils a novel pathway of IFN-gamma production by phagocytic cells. *J Immunol* **179**, 247-255.

Oberg, M., Bergander, L., Hakansson, H., Rannug, U., and Rannug, A. (2005). Identification of the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole, in cell culture medium, as a factor that controls the background aryl hydrocarbon receptor activity. *Toxicol Sci* **85**, 935-943.

Pearson, A. M., Rich, A., and Krieger, M. (1993). Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. *J Biol Chem* **268**, 3546-3554.

Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., Caccamo, M., Oukka, M., and Weiner, H. L. (2008). Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* **453**, 65-71.

Ruby, C. E., Leid, M., and Kerkvliet, N. I. (2002). 2,3,7,8-Tetrachlorodibenzo-p-dioxin suppresses tumor necrosis factor-alpha and anti-CD40-induced activation of NF-kappaB/Rel in dendritic cells: p50 homodimer activation is not affected. *Mol Pharmacol* **62**, 722-728.

Ruby, C. E., Funatake, C. J., and Kerkvliet, N. I. (2005). 2,3,7,8 Tetrachlorodibenzo-p-Dioxin (TCDD) Directly Enhances the Maturation and Apoptosis of Dendritic Cells In Vitro. *J Immunotoxicol* **1**, 159-166.

Sato, K., and Fujita, S. (2007). Dendritic cells: nature and classification. *Allergol Int* **56**, 183-191.

Shepherd, D. M., Steppan, L. B., Hedstrom, O. R., and Kerkvliet, N. I. (2001). Anti-CD40 Treatment of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-

exposed C57Bl/6 mice induces activation of antigen presenting cells yet fails to overcome TCDD-induced suppression of allograft immunity. *Toxicol Appl Pharmacol* **170**, 10-22.

Shortman, K., and Naik, S. H. (2007). Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* **7**, 19-30.

Sun, Y. V., Boverhof, D. R., Burgoon, L. D., Fielden, M. R., and Zacharewski, T. R. (2004). Comparative analysis of dioxin response elements in human, mouse and rat genomic sequences. *Nucleic Acids Res* **32**, 4512-4523.

Tang, X., Marciano, D. L., Leeman, S. E., and Amar, S. (2005). LPS induces the interaction of a transcription factor, LPS-induced TNF-alpha factor, and STAT6(B) with effects on multiple cytokines. *Proc Natl Acad Sci U S A* **102**, 5132-5137.

Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J. C., and Stockinger, B. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* **453**, 106-109.

Vogel, C. F., Goth, S. R., Dong, B., Pessah, I. N., and Matsumura, F. (2008). Aryl hydrocarbon receptor signaling mediates expression of indoleamine 2,3-dioxygenase. *Biochem Biophys Res Commun* **375**, 331-335.

Vogel, C. F., and Matsumura, F. (2008). A new cross-talk between the aryl hydrocarbon receptor and RelB, a member of the NF-kappaB family. *Biochem Pharmacol*.

Vogel, C. F., Sciullo, E., Li, W., Wong, P., Lazennec, G., and Matsumura, F. (2007a). RelB, a new partner of aryl hydrocarbon receptor-mediated transcription. *Mol Endocrinol* **21**, 2941-2955.

Vogel, C. F., Sciullo, E., and Matsumura, F. (2007b). Involvement of RelB in aryl hydrocarbon receptor-mediated induction of chemokines. *Biochem Biophys Res Commun* **363**, 722-726.

Vogel, C. F., Sciullo, E., Wong, P., Kuzmicky, P., Kado, N., and Matsumura, F. (2005). Induction of proinflammatory cytokines and C-reactive protein in human macrophage cell line U937 exposed to air pollution particulates. *Environ Health Perspect* **113**, 1536-1541.

von Bergwelt-Baildon, M. S., Popov, A., Saric, T., Chemnitz, J., Classen, S., Stoffel, M. S., Fiore, F., Roth, U., Beyer, M., Debey, S., Wickenhauser, C., Hanisch, F. G., and Schultze, J. L. (2006). CD25 and indoleamine 2,3-dioxygenase are up-regulated by prostaglandin E2 and expressed by tumor-associated dendritic cells in vivo: additional mechanisms of T-cell inhibition. *Blood* **108**, 228-237.

Vorderstrasse, B. A., Dearstyne, E. A., and Kerkvliet, N. I. (2003). Influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the antigen-presenting activity of dendritic cells. *Toxicol Sci* **72**, 103-112.

Vorderstrasse, B. A., and Kerkvliet, N. I. (2001). 2,3,7,8-Tetrachlorodibenzo-p-dioxin affects the number and function of murine splenic dendritic cells and their expression of accessory molecules. *Toxicol Appl Pharmacol* **171**, 117-125.

Xu, Y., Zhan, Y., Lew, A. M., Naik, S. H., and Kershaw, M. H. (2007). Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol* **179**, 7577-7584.

CHAPTER 4

AhR-activation in dendritic cells and the generation of regulatory T cells

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ABSTRACT

Although Aryl hydrocarbon receptor (AhR) activation by its prototypical ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces regulatory T cell (Tregs), molecular mechanisms underlying immunosuppression are not well understood. Dendritic cells (DCs) are key antigen presenting cells governing T cell activation and differentiation. DCs are sensitive to TCDD and AhR activation induces a regulatory phenotype in DCs. We hypothesize that AhR-activated dendritic cells (DCs) induce the generation of adaptive Tregs ultimately contributing to immune suppression. To test this hypothesis, purified bone marrow-derived DCs (BMDCs), generated in the presence of vehicle or TCDD were assessed for induction of adaptive Tregs *in vitro*. Additionally, suppression of T cell activation *in vivo* by LPS-stimulated, vehicle- and TCDD-treated BMDCs was assessed. TCDD-treated BMDCs did not induce the generation of adaptive T regs *in vitro* or suppress T cell activation *in vivo*. These results suggest that AhR-activated regulatory DCs do not induce the generation of adaptive Tregs and that AhR-induced Treg development may be a direct effect of AhR activation on T cells. Alternatively, AhR-activated DCs may contribute to the expansion of natural Tregs, a possibility that remains to be tested.

Keywords: Aryl hydrocarbon receptor (AhR); dendritic cells (DCs); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); Indole amine-2,3-dioxygenase (IDO) ; FICZ, 6-formylindolo[3,2-b]carbazole.

Abbreviations: AhR, Aryl hydrocarbon receptor; Ag, Antigen; BMDCs, Bone marrow-derived dendritic cells; DCs, dendritic cells; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin TCDD; IDO, Indoleamine-2,3-dioxygenase.

INTRODUCTION

TCDD is an environmental pollutant that induces profound immunosuppression via activation of the Aryl hydrocarbon receptor (AhR)(Ema *et al.*, 1994b; Okey *et al.*, 1994). Following ligand binding, the AhR translocates into the nucleus, where in conjunction with its dimeric partner the Aryl hydrocarbon receptor nuclear translocater (ARNT) it binds to the dioxin response elements (DREs) in target genes. Several studies have documented TCDD exposure to decrease $CD4^+$ T cell function and induce the generation of regulatory T cells (Tregs), which function to maintain tolerance (Kerkvliet *et al.*, 2002; Quintana *et al.*, 2008). Two distinct types of regulatory T cells have been described: natural Tregs that arise in the thymus, and inducible/adaptive Tregs, that form from $CD4^+CD25^-Foxp3^-$ precursors in the periphery (Shevach, 2006; Sakaguchi *et al.*, 2008; Yamazaki and Steinman, 2009).

Several investigators have described the induction of Tregs following TCDD exposure (Funatake *et al.*, 2005; Marshall *et al.*, 2008; Quintana *et al.*, 2008). Additionally, the increase in Tregs following AhR activation has been associated with an increased production of Treg mediators including TGF-β3 and indoleamine-2, 3-dioxygenases (IDO) (Marshall *et al.*, 2008; Vogel *et al.*, 2008). Despite these reports, little is known about the mechanisms underlying generation of Tregs following TCDD exposure.

Dendritic cells (DCs) are central antigen presenting cells that express the AhR constitutively. We have previously demonstrated that TCDD induces a regulatory phenotype in BMDCs that is characterized by an increased expression

of IDO1, IDO2 and TGF-β3. Thus, the induction of regulatory DCs following AhR activation may underlie generation of Tregs and subsequent immune suppression. We hypothesize that AhR-activated DCs promote the generation of adaptive Tregs. In this study, we evaluated the role of DREs in the TCDD-induced expression of suppressive regulatory mediators in BMDCs and examined the ability of TCDD-exposed APCs to induce Tregs.

MATERIALS AND METHODS

Animals: 6-8 week old C57Bl/6 ($AhR^{+/+}$) mice were purchased from Jackson Laboratory (Bar Harbor, ML). Breeding pairs of AhR knockout mice ($AhR^{-/-}$) were kindly provided by Dr. Paige Lawrence (URMC, Rochester, NY). $AhR^{nls/nls}$ and $AhR^{dbd/dbd}$ mice were initially acquired from Dr. Chris Bradfield and Dr. Ed Glover (University of Wisconsin-Madison) and subsequently bred and maintained at UM. $AhR^{nls/nls}$ and $AhR^{dbd/dbd}$ mice bear a mutant AhR that binds the ligand, but is defective in nuclear translocation and binding the DREs, respectively. Animal experiments were approved by the UM IACUC and adhered to the current NIH guidelines for animal usage. Animals were provided rodent chow and tap water ad libitum.

Chemicals*:* TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) in DMSO was obtained from Cambridge Isotope laboratories Inc., (Woburn MA). 6-formylindolo[3,2-b]carbazole (FICZ) was obtained from BIOMOL. DMSO was used as a vehicle for all AhR ligands. TCDD and FICZ were used at a concentration of 10nM. Final concentration of the vehicle in culture was below 0.1% DMSO and did not induce cytotoxicity. rIL-2 was obtained from R and D systems (Minneapolis, MN) and TGF-β1 was obtained from ebiosciences (San Diego, CA).

Generation of BMDCs*:* Bone marrow-derived dendritic cells were prepared using methods from Inaba et al. (1992). Briefly, the bone marrow cells were flushed using complete RPMI (cRPMI) media from the bone shafts of C57Bl/6 or AhR mutant mice. Bone marrow cells were centrifuged at low speed and red blood cells were removed by gradient centrifugation using lympholyte-M

(Cedarlane, Hornby, Canada). Bone marrow cells were washed twice and plated at a density of $1x10^6$cells/ml in tissue culture flasks. Cells were cultured in media with GM-CSF (30ng/ml) and Vehicle or TCDD (10nM) for 7 days at 37° C and 5 % CO_2. On days 3 and 5, non-adherent cells were collected washed once and reseeded back into culture flask. Fresh media, GM-CSF (30ng/ml), vehicle or TCDD (10nM) was added to the culture flask and ultimately non-adherent cells harvested on day 7. BMDCs were purified (≥85-90%) using CD11c-APC antibody and anti-APC beads (Miltenyi Biotec, Auburn, CA) or CD11c beads (Miltenyi Biotec, Auburn, CA). Following purification, BMDCs were loaded with ova-peptide (50μg/ml cPRMI) and harvested after 2 hours. For some experiments BMDCs were simultaneously treated with LPS (1μg/ml) and ova-peptide (50μg/ml cPRMI) and harvested after 12 hours.

T cell purification: Splenocytes were isolated from OT II mice and prepared as previously described (Shepherd *et al.*, 2001). $CD4^+$ cells were isolated using the CD4 T cell isolation kit as per the manufacturer's instructions (Miltenyi, CA). Cell purity as determined by flow cytometry was ≥ 90%. Purified $CD4^+$ cells were labeled with anti-CD25 PE and MACS anti-PE beads and positively selected using LS columns (MACS separation columns). The purity of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells obtained was 75% and 85%, respectively.

Immunophenotypic analysis of BMDCs and T cells*:* Harvested immune cells were stained for FACS analysis as previously described (Shepherd *et al.*, 2001). Briefly, rat, hamster or mouse IgG block was added to prepared cells for 10 minutes on ice to prevent non-specific binding. Cells were stained for 10 minutes

on ice cells with monoclonal antibodies (mAb) from BDPharmingen (San Diego, CA): CD11c (HL3), MHCII (2G9), CD86 (GL1), CD62L (MEL14), CD44 (IM7) and Biolegend (San Diego, CA): CD25 (PC61). All mAbs used were optimally titrated and appropriately-labeled, isotype-matched mAbs were used as controls. Acquisition of 40,000-50,000 events was performed using a Facs Aria flow cytometer. (BD biosciences) and analyzed by BD FACS Diva software (Version 4.0).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR): Total RNA was isolated using Trizol reagent and quantified by absorbance at 260nm. Single strand cDNA synthesis was performed using a RT-PCR kit (SAbiosciences) and prepared as per the manufacturer's instructions. Specific primers for IDO1, IDO2, TGFβ-1, TGF-β2, TGF-β3, latent transforming growth factor beta binding protein 3 (Ltbp3), Aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2), Thrombospondin 1 (Thbs1), and β-actin were obtained from SABiosciences. Quantitative detection of mRNA expression was performed on a Light cycler Instrument (Biorad IQ^{TM} 5, Multicolor Real Time PCR Detection Systems) using SYBR green (SAbiosciences) according to the manufacturer's instructions.

In Vitro **Suppression Assay**: Purified $CD4^+CD25^+$ cells were suspended at a final concentration of $1x10^6$ cells/ml in complete RPMI (cRPMI) media and cultured in 6 well plates coated with plate bound anti-CD3 (2μg/ml) and supplemented with rIL-2 (250U/ml) and TGF-β1 (2ng/ml). $CD4^+CD25^-$ cells were co-cultured with DCs in a suppression assay as described below. Purified,

vehicle- or TCDD-treated BMDCs ($1x10^6$/ml) were loaded with ova-peptide ($OVA_{323-339}$) for 2 hrs in 6 well plates. Ova-peptide loaded DCs were subsequently co-cultured with purified, $CD4^+CD25^-$ OT II T cells at a DC to T cell ratio of 1:4, at a final concentration of 1 $x10^6$/ml in 6 well plates. After 4 days in culture, cells were harvested and stained for CD4, CD25 and Foxp3.

T cell activation: To assess the ability of LPS-activated BMDCs to activate $CD4^+$ T cell in an antigen-specific fashion, the OT II adoptive transfer model was employed. Briefly, $CD45.2^+$ inflammatory BMDCs were generated in the presence of vehicle or TCDD (10nM), purified and subsequently treated with LPS (1μg/ml) and ova-peptide (50μg/ml) for 12 hrs. LPS-activated, ova-peptide loaded BMDCs were injected ($1x10^6$/footpad) into host $CD45.1^+$ congenic mice that had previously received one day prior OT II T cells ($2x10^6$/mouse) intravenously. Popliteal Lymph nodes were harvested on day 5 post-immunization, processed and donor DCs and OT II T cells identified by flow cytometry. The expression of $CD45.2^+/CD11c^+$ or $CD4^+/Thy1.1^+$ cells was employed to track donor DCs or OT II T cells, respectively. The activation status of antigen-specific donor OT II T cells was assessed immunophenotypically using the markers CD44 and CD62L.

Statistical analysis: Student's t test was used to compare the means of vehicle-treated groups to TCDD–exposed groups. For analysis, values of $p \leq 0.05$ were considered significant.

RESULTS

TCDD-induced expression of IDO1 and IDO2 and TGFβ-3 in BMDCs is AhR-dependent. We have previously shown that AhR activation by TCDD increases the expression of IDO1, IDO2 and TGFβ-3 in inflammatory BMDCs. To determine the role of the AhR in the induction of these mediators, we assessed their expression in vehicle- or TCDD-treated BMDCs from AhR null and wildtype mice. As shown in table 4-1, TCDD increased the expression of IDO1, IDO2 and TGFβ3 in AhR wild type mice. On, the other hand, TCDD did not alter gene expression in AhR KO BMDCs, suggesting that the induction of these mediators following TCDD exposure was AhR-dependent (Table 4-1).

TCDD-induced expression of IDO1 and IDO2 but not TGF-β3 in BMDCs is DRE-dependent: To determine the role of the DRE-mediated events in the induction of these mediators, we assessed their expression in BMDCs from $AhR^{dbd/dbd}$ and $AhR^{nls/nls}$ mice. TCDD-treated BMDCs from $AhR^{dbd/dbd}$ increased TGFβ-2 and TGFβ-3 mRNA expression by 1.6- and 2.9- fold, respectively (Table 4-2A). Additionally, TCDD increased the expression of TGF-β2, TGF-β3, Aldh1a and Thbs in BMDCs from $AhR^{nls/nls}$ mice by 2.3-, 6.2-, 2.6- and 1.6-fold, respectively (Table 4-2B).

FICZ, a natural AhR ligand, increases expression of Treg-inducing mediators. More recently, tryptophan metabolites such as 6-formylindolo[3,2-b]carbazole (FICZ) have been described as high-affinity natural AhR ligands (Oberg *et al.*, 2005; Henry *et al.*, 2006). Our previous studies have demonstrated that FICZ generates phenotypical alterations in inflammatory BMDCs, similar to

Table 4-1: The AhR is required for TCDD-induced changes in the expression of regulatory mediators[a]

	TCDD-induced fold change in AhR wildtype BMDCs	p value	**TCDD-induced fold change in AhR$^{-/-}$ BMDCs**	p value
IDO1	**18***	*0.0001*	**0.6**	*0.4*
IDO2	**24***	*≤0.001*	**1.3**	*0.2*
TGFβ1	**0.4**	*0.1*	**0.9**	*0.8*
TGFβ2	**0.9**	*0.9*	**0.9**	*0.8*
TGFβ3	**11***	*0.001*	**0.5**	*0.1*
Ltbp3	**0.2**	*0.3*	**1.0**	*0.9*
Aldh1a2	**0.8**	*0.6*	**1.0**	*0.6*
Thbs1	**0.4**	*0.07*	**1.1**	*0.1*

[a]Gene expression was evaluated in AhR wild-type and AhR null BMDCs by qRT-PCR. The results are expressed as TCDD-induced fold change when compared to the vehicle-treated controls after normalization with the housekeeping gene, β actin. The experiment was conducted with $N = 3$ per treatment group. *$p \leq 0.05$ was considered significant for all analysis conducted between vehicle-treated and TCDD-treated BMDCs.

Table 4-2: Role of DRE-mediated events in the TCDD-induced gene expression of regulatory mediators in BMDCs

A.

	TCDD-induced fold change in $AhR^{dbd/-}$ BMDCs	p value	TCDD-induced fold change in $AhR^{dbd/dbd}$ BMDCs	p value
IDO 1	4.5*	*0.003*	-1.5*	*0.03*
IDO 2	7.6*	*0.0004*	0.8	*0.6*
TGFβ1	0.8	*0.3*	0.8	*0.2*
TGFβ2	1.7	*0.2*	1.6*	*0.04*
TGFβ3	15*	*≤0.001*	2.9*	*0.008*
Ltbp3	-1.8*	*0.02*	-2.1*	*0.005*
Aldh1a	1.4	*0.2*	1.5	*0.07*
Thbs1	0.8	*0.5*	0.1	*0.1*

B.

	TCDD-induced fold change in $AhR^{dbd/-}$ BMDCs	p value	TCDD-induced fold change in $AhR^{nls/nls}$ BMDCs	p value
IDO 1	8.5*	*0.005*	2.5	*0.1*
IDO 2	1.9*	*0.04*	1.2	*0.4*
TGFβ1	0.8	*0.2*	0.8	*0.2*
TGFβ2	1.7	*0.2*	2.3*	*0.004*
TGFβ3	8.8*	*0.009*	6.2*	*0.001*
Ltbp3	0.7	*0.14*	0.9	*0.7*
Aldh1a	1.5*	*0.004*	2.6*	*0.001*
Thbs1	1.2	*0.33*	1.6*	*0.01*

[a] Gene expression was evaluated from **A)** $AhR^{dbd/-}$ and $AhR^{dbd/dbd}$ **B)** $AhR^{dbd/-}$and $AhR^{nls/nls}$ BMDCs by qRT-PCR. The results are expressed as TCDD-induced fold change after normalization with the housekeeping gene, β actin. The experiment was conducted with $N = 3$ per treatment group. *$p \leq 0.05$ was considered significant for all analysis conducted between vehicle-treated and TCDD-treated BMDCs.

TCDD. These results suggest that unlike T cells AhR activation in DCs by exogenous or natural AhR ligands is not ligand-specific. To further assess the effects AhR activation by a natural ligand, we examined the induction of suppressive mediators in BMDCs following FICZ treatment. As shown in table 4-3, BMDCs displayed an increase in expression of IDO1, IDO2, TGF-β2, TGF-β3 and Aldh1a following exposure to FICZ when compared to vehicle-treated cells (Table 4-3).

Effects of TCDD-treated BMDCs on the generation of $CD4^+CD25^+$ $Foxp3^+$T cells in vitro: Regulatory T cells can be segregated into two distinct lineages referred to as natural T regs and adaptive Tregs. Several studies have shown have linked TCDD-induced immune suppression with the induction of Tregs. TCDD induced not only a regulatory phenotype in BMDCs, but also increased their expression of several suppressive mediators. We therefore, assessed the ability of TCDD-treated BMDCs to induce the conversion of $CD4^+CD25^-$ $CD4^+$ T cells into $CD4^+CD25^+$ $Foxp3^+$ T cells. Compared to vehicle-treated BMDCs, TCDD-treated BMDCs decreased the frequency and the number of $CD4^+CD25^+$ $Foxp3^+$ T cells (Figure 4-1).

LPS-activated, TCDD-treated BMDCs do not suppress antigen-specific T cell activation in vivo: We previously demonstrated that TCDD-treated BMDCs, displaying high expression of suppressive mediators fail to suppress antigen-specific T cell activation in an OT II adoptive transfer model. Recent studies have

Table 4-3: AhR activation by natural ligand FICZ alters gene expression[a]

	FICZ-induced fold change in AhR wildtype BMDCs	p value
IDO 1	7.4*	*0.003*
IDO 2	14*	*0.001*
TGFβ1	0.5	*0.11*
TGFβ2	2.2*	*0.006*
TGFβ3	9.3*	*0.001*
Ltbp3	0.8	*0.12*
Aldh1a2	1.2*	*0.02*
Thbs	0.7	*0.5*

[a]Gene expression was evaluated from BMDCs by qRT-PCR. The results are expressed as FICZ-induced fold change after normalization with the housekeeping gene, β actin. The experiment was conducted with $N = 3$ per treatment group. * $p \leq 0.05$ indicates significant differences between vehicle-treated and FICZ-treated BMDCs.

linked AhR activation in DCs with suppression of T cell responses (Hauben *et al.*, 2008). These studies demonstrated AhR-activated DCs to suppress T cell responses only following LPS stimulation. Immature BMDCs did not alter T cell responses. Thus, we also evaluated the expression of the suppressive mediators in BMDCs following 8 hours LPS maturation. TCDD-treated BMDCs stimulated with LPS displayed increased levels of IDO1, IDO2 and TGF-β3 as compared to LPS-stimulated, vehicle-treated BMDCs (Table 4-4). We next tested the ability of LPS-activated, TCDD-treated BMDCs to suppress antigen-specific T cell activation *in vivo* using a modified OT II adoptive transfer model. LPS-activated, vehicle- or TCDD-treated BMDCs were pulsed *in vitro* with ova-peptide to generate ova-loaded DCs capable of activating adoptively transferred, ova-specific OT II $CD4^+$ T cells *in vivo*. Peripheral lymph nodes were harvested on day 5 following BMDC immunizations. No effects on the number, frequency or activation profile of donor DCs were observed in mice receiving LPS-activated, TCDD-treated BMDCs as compared to LPS-activated, vehicle-treated BMDCs (Figure 4-2). Ova-specific OT II T cells from the popliteal lymph nodes exhibited significant clonal expansion when compared to the OT II T cells from non-draining BLNs, indicating antigen-specific activation. However, no differences in the frequency, number or the activation profile of the OT II T cells were detected in mice immunized with antigen-laden, LPS-stimulated donor BMDCs treated with vehicle or TCDD.

Table 4-4

Effects of 8 hours LPS stimulation on TCDD-induced regulatory gene expression in BMDCs.

	TCDD-induced fold change following 8 hrs LPS stimulation	p value
IDO 1	15*	*0.0004*
IDO 2	14*	*0.002*
TGFβ1	1.7	*0.05*
TGFβ2	-2.3	*0.01*
TGFβ3	5.7*	*0.003*
Aldh1a	-2.6*	*0.01*
Thbs1	1.0	*0.5*

[a]Gene expression was evaluated by qRT-PCR in vehicle- and TCDD-treated BMDCs stimulated with LPS for 8 hours. The results are expressed as TCDD-induced fold change when compared to vehicle-treated controls after normalization to the housekeeping gene, β actin. The experiment was conducted with $N = 2$ per treatment group. *$p \leq 0.05$ was considered significant for all analysis conducted between vehicle-treated and TCDD-treated BMDCs.

Figure 4-1

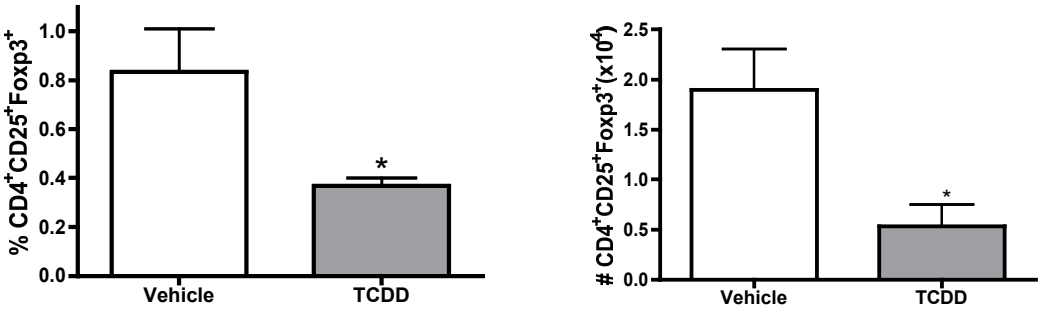
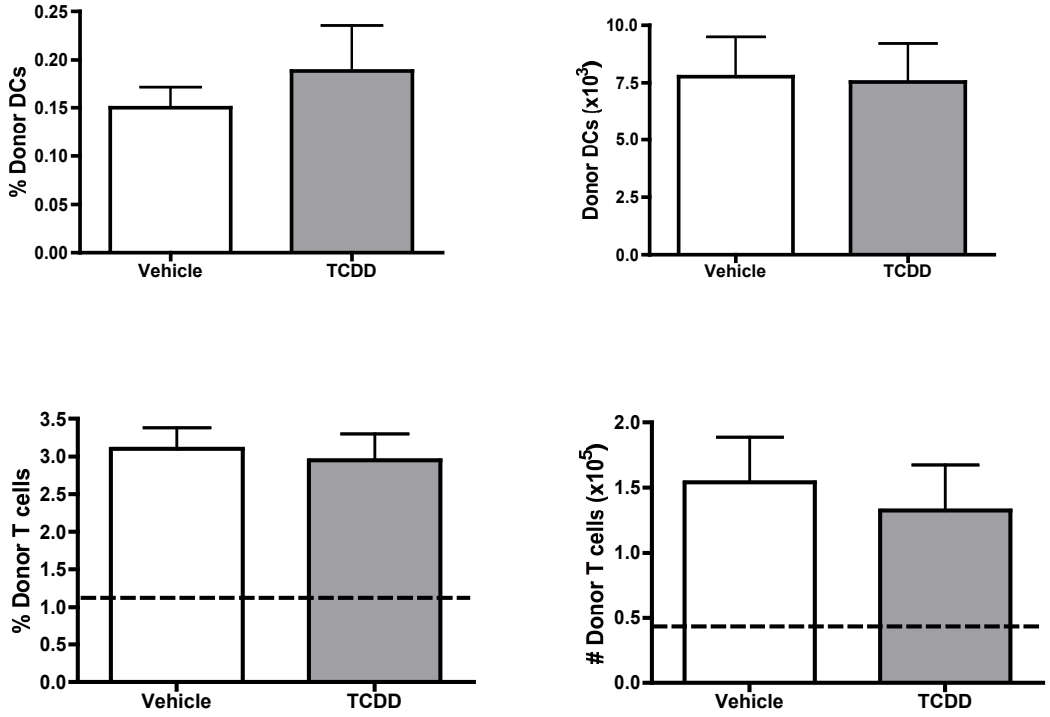


Figure 4-1. Effects of TCDD-treated BMDCs on the generation of Foxp3$^+$ Tregs from conventional CD4$^+$ T cells.

Ova-peptide loaded, vehicle or TCDD BMDCs were co-cultured with purified $CD4^+CD25^-$ OT II T cells in 6 well plates at a 1:4 DC to T cell ratio at a final concentration of 1 x10^6 total cells per well. After 4 days in culture, cells were harvested and the frequency and number of $CD4^+CD25^+Foxp3^+$ T cells determined. Data represent mean ± SEM of n=3 per treatment group. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD–treated groups

Figure 4-2



		Vehicle	TCDD
DC activation markers	% CD86	35 ± 3.6	38 ± 10.3
	% MHC II	62 ± 3.7	55 ± 3.2
T cell activation markers	CD62 L MFI	284 ± 18	302 ± 36
	CD44 MFI	2150± 119	1884 ± 87.4

Figure 4-2. Effects of LPS-matured, TCDD-treated, ova-peptide loaded BMDCs on antigen-specific T cell proliferation *in vivo*: $CD4^+$ T cells from OT II Thy1.1 mice were adoptively transferred into CD45.1 host mice, 24 hours before immunization with LPS-stimulated, ova-peptide loaded BMDCs, that were either vehicle- or TCDD-treated. On day 5 post-immunization, popliteal lymph nodes were harvested and the donor DCs and donor T cells analyzed by flow cytometry as described in *materials and methods*. Data represent mean ± SEM of 4-5 animals per treatment group. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD–treated groups.

DISCUSSION

We have previously reported that AhR activation by TCDD induces a regulatory phenotype in DCs characterized by an increased expression of IDO1, IDO2 and TGF-β3 mRNA levels. However, these studies did not address the effects of regulatory DCs on T cell differentiation, specifically the generation of regulatory T cells. In this study, we have assessed the role of the AhR and DRE-mediated events in the induction of regulatory mediators in DCs and evaluated the potential of AhR-activated BMDCs to induce adaptive Tregs.

TCDD-induced toxicity is associated with activation of both DRE and non–DRE-mediated AhR signaling pathways. We have previously demonstrated that TCDD modulates BMDC differentiation via mechanisms that are strictly AhR-dependent but not exclusively DRE-mediated (unpublished data). Consistent with these studies, TCDD-induced expression of regulatory mediators in BMDCs was found to be AhR-dependent. The potential involvement of DREs in the expression of these mediators was further assessed using BMDCs from $AhR^{dbd/dbd}$ and $AhR^{nls/nls}$ mice harboring a mutant AhR that binds ligand but fails to translocate to the nucleus or bind to DREs, respectively. TCDD did not significantly increases the expression of IDO1 or IDO2 in $AhR^{dbd/dbd}$ or $AhR^{nls/nls}$ BMDCs, suggesting that their induction by TCDD required binding of the activated AhR to the DREs. Conversely, TCDD increased expression of both TGF-β2 and TGF-β3 in $AhR^{dbd/dbd}$ and $AhR^{nls/nls}$ BMDCs. Interestingly, increases in TGF-β3 expression by TCDD in both $AhR^{dbd/dbd}$ and $AhR^{nls/nls}$ BMDCs were less compared to TCDD-induced increases in $AhR^{dbd/-}$ control BMDCs suggesting that TCDD-induced expression of TGF-β2 and TGF-β3 expression in

inflammatory BMDCs may be partially regulated by DRE-mediated events. Other genes that were slightly but significantly upregulated in $AhR^{nls/nls}$ BMDCs following TCDD treatment included Thbs1 and Aldh1a. Aldh1a, an enzyme involved in the production of retinoic acid is implicated in the production of Tregs (Elias *et al.*, 2008). A slight but significant increase in Aldh1a expression in $AhR^{nls/nls}$ BMDCs following TCDD treatment suggests the involvement of cytoplasmic, non-DRE mediated signaling events in the induction of regulatory mediators following AhR activation.

Recently studies have identified natural ligands for the AhR such as the tryptophan photoproduct, FICZ. Initial studies demonstrated that FICZ, a high affinity AhR agonist, can induce Th17 differentiation and inhibit Treg (Quintana *et al.*, 2008). In contrast, Kimura et al., reported Treg induction by TGF-β that was enhanced when the AhR was activated by either TCDD or FICZ (Kimura *et al.*, 2008). FICZ has been shown to increase the expression of CD86 on human U937 monocyte-derived DCs (Veldhoen *et al.*, 2008; Vogel *et al.*, 2008). So far few studies have described the effects of natural AhR ligands on DCs. We have previously demonstrated that FICZ can generate phenotypical alterations in BMDCs, similar to TCDD in BMDCs, demonstrating a lack of AhR ligand specificity in DCs. These results raised the possibility that similar to TCDD, AhR activation by FICZ could also induce the generation of regulatory DCs. FICZ increased the expression of IDO1, IDO2, TGF-β2 and TGF-β3 and Aldh1a. Although, we have not validated the activity of these suppressive mediators, these results suggest that AhR-activation by FICZ could contribute to Treg

development. However, further studies are needed to examine the effects of FICZ-treated DCs on the polarization of T cells into Th17 or Tregs cells.

Several investigators have linked the generation of immune suppression following TCDD exposure to the induction of Tregs (Marshall *et al.*, 2008; Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). In a graft versus host model, Marshall and colleagues demonstrated the suppressive function of TCDD-$CD4^+$ T cells to be independent of Foxp3 expression, suggesting that ligand-activated AhR functions as an alternative to Foxp3 in naïve T cells (Marshall *et al.*, 2008). Conversely, TCDD-induced suppression of EAE and diabetes in NOD mice has been linked to increased Foxp3 expression (Kerkvliet, 2009). Despite these studies, the mechanisms underlying the generation of AhR-induced Tregs remain poorly understood.

Recent studies have suggested that DCs can induce $Foxp3^+$ Tregs from $Foxp3^-$ precursors in the presence of exogenous TGF-β (Sakaguchi *et al.*, 2008). TCDD-treated BMDCs showed increased expression of TGF-β3, IDO1 and IDO2, raising the possibility that interactions of AhR-activated DCs with $Foxp3^-$ cells could promote the generation of adaptive/inducible Tregs. Contrary to our expectations, $Foxp3^+$ cells were not induced by TCDD-treated BMDCs. Additionally, TCDD-treated BMDCs stimulated with LPS failed to suppress antigen-specific T cell activation *in vivo*. These results were surprising, as LPS-matured, TCDD-treated BMDCs displayed increased mRNA levels of IDO1, IDO2 and TGF-β3, indicative of a regulatory phenotype. Several possibilities could

account for these observed effects. It is possible that TCDD does not increase the function or activity of these mediators. In the absence of functionally active DC-derived regulatory mediators, TCDD-treated BMDCs would not be expected to induce $Foxp3^+$ T cells. Several investigators have identified novel populations of adaptive Tregs including $CD4^+CD25^+$Tregs, $CD4^+CD25^-Foxp3^-$ Tregs, IL-10-induced Tregs, TGFβ-induced Tregs and $CD8^+CD28^-Foxp3^+$ T cells (Weiner, 2001; Barrat *et al.*, 2002; Zheng *et al.*, 2002; Apostolou and von Boehmer, 2004; Chen *et al.*, 2004; Kretschmer *et al.*, 2005; Battaglia *et al.*, 2006; Roncarolo *et al.*, 2006; Hansen *et al.*, 2007). The possibility that TCDD-treated BMDCs induce the generation of one or more of these distinct Treg populations remains to be tested. Studies described herein have exclusively assessed the effects of TCDD-treated DCs on the induction of adaptive Tregs. It remains to be tested whether TCDD-treated DCs affect the maintenance or the expansion of natural Tregs.

In summary, we have demonstrated that although AhR activation induces a regulatory phenotype in DCs, it does not contribute to induction of Treg. Our results suggest that the generation of Tregs following AhR activation may not be entirely attributable to DCs and that AhR activation of T cells may be essential for the induction of regulatory T cells.

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REFERENCES

Apostolou, I., and von Boehmer, H. (2004). In vivo instruction of suppressor commitment in naive T cells. *The Journal of experimental medicine* **199**, 1401-1408.

Barrat, F. J., Cua, D. J., Boonstra, A., Richards, D. F., Crain, C., Savelkoul, H. F., de Waal-Malefyt, R., Coffman, R. L., Hawrylowicz, C. M., and O'Garra, A. (2002). In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *The Journal of experimental medicine* **195**, 603-616.

Battaglia, M., Gregori, S., Bacchetta, R., and Roncarolo, M. G. (2006). Tr1 cells: from discovery to their clinical application. *Seminars in immunology* **18**, 120-127.

Chen, T. C., Cobbold, S. P., Fairchild, P. J., and Waldmann, H. (2004). Generation of anergic and regulatory T cells following prolonged exposure to a harmless antigen. *J Immunol* **172**, 5900-5907.

Elias, K. M., Laurence, A., Davidson, T. S., Stephens, G., Kanno, Y., Shevach, E. M., and O'Shea, J. J. (2008). Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* **111**, 1013-1020.

Ema, M., Ohe, N., Suzuki, M., Mimura, J., Sogawa, K., Ikawa, S., and Fujii-Kuriyama, Y. (1994). Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors. *The Journal of biological chemistry* **269**, 27337-27343.

Funatake, C. J., Marshall, N. B., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2005). Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin generates a population of CD4+ CD25+ cells with characteristics of regulatory T cells. *J Immunol* **175**, 4184-4188.

Hansen, W., Westendorf, A. M., Reinwald, S., Bruder, D., Deppenmeier, S., Groebe, L., Probst-Kepper, M., Gruber, A. D., Geffers, R., and Buer, J. (2007). Chronic antigen stimulation in vivo induces a distinct population of antigen-specific Foxp3 CD25 regulatory T cells. *J Immunol* **179**, 8059-8068.

Hauben, E., Gregori, S., Draghici, E., Migliavacca, B., Olivieri, S., Woisetschlager, M., and Roncarolo, M. G. (2008). Activation of the aryl hydrocarbon receptor promotes allograft-specific tolerance through direct and dendritic cell-mediated effects on regulatory T cells. *Blood* **112**, 1214-1222.

Henry, E. C., Bemis, J. C., Henry, O., Kende, A. S., and Gasiewicz, T. A. (2006). A potential endogenous ligand for the aryl hydrocarbon receptor has potent agonist activity in vitro and in vivo. *Archives of biochemistry and biophysics* **450**, 67-77.

Kerkvliet, N. I. (2009). AHR-mediated immunomodulation: the role of altered gene transcription. *Biochemical pharmacology* **77**, 746-760.

Kerkvliet, N. I., Shepherd, D. M., and Baecher-Steppan, L. (2002). T lymphocytes are direct, aryl hydrocarbon receptor (AhR)-dependent targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): AhR expression in both CD4+ and CD8+ T cells is necessary for full suppression of a cytotoxic T lymphocyte response by TCDD. *Toxicology and applied pharmacology* **185**, 146-152.

Kimura, A., Naka, T., Nohara, K., Fujii-Kuriyama, Y., and Kishimoto, T. (2008). Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 9721-9726.

Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M. C., and von Boehmer, H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. *Nature immunology* **6**, 1219-1227.

Marshall, N. B., Vorachek, W. R., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2008). Functional characterization and gene expression analysis of CD4+ CD25+ regulatory T cells generated in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Immunol* **181**, 2382-2391.

Oberg, M., Bergander, L., Hakansson, H., Rannug, U., and Rannug, A. (2005). Identification of the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole, in cell culture medium, as a factor that controls the background aryl hydrocarbon receptor activity. *Toxicol Sci* **85**, 935-943.

Okey, A. B., Riddick, D. S., and Harper, P. A. (1994). The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. *Toxicology letters* **70**, 1-22.

Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., Caccamo, M., Oukka, M., and Weiner, H. L. (2008). Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* **453**, 65-71.

Roncarolo, M. G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., and Levings, M. K. (2006). Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological reviews* **212**, 28-50.

Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008). Regulatory T cells and immune tolerance. *Cell* **133**, 775-787.

Shepherd, D. M., Steppan, L. B., Hedstrom, O. R., and Kerkvliet, N. I. (2001). Anti-CD40 Treatment of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-exposed C57Bl/6 mice induces activation of antigen presenting cells yet fails to overcome TCDD-induced suppression of allograft immunity. *Toxicology and applied pharmacology* **170**, 10-22.

Shevach, E. M. (2006). From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* **25**, 195-201.

Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J. C., and Stockinger, B. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* **453**, 106-109.

Vogel, C. F., Goth, S. R., Dong, B., Pessah, I. N., and Matsumura, F. (2008). Aryl hydrocarbon receptor signaling mediates expression of indoleamine 2,3-dioxygenase. *Biochemical and biophysical research communications* **375**, 331-335.

Weiner, H. L. (2001). Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunological reviews* **182**, 207-214.

Yamazaki, S., and Steinman, R. M. (2009). Dendritic cells as controllers of antigen-specific Foxp3+ regulatory T cells. *J Dermatol Sci* **54**, 69-75.

Zheng, S. G., Gray, J. D., Ohtsuka, K., Yamagiwa, S., and Horwitz, D. A. (2002). Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors. *J Immunol* **169**, 4183-4189.

CHAPTER 5

CONCLUSIONS

AhR activation by TCDD profoundly suppresses cell-mediated and humoral immune responses. However, the specific mechanisms and molecular targets underlying these immunotoxic effects remain undefined. Studies have shown that expression of the AhR is inducible in lymphocytes and the debate whether lymphocytes are direct or indirect targets of AhR activation continues. In this context, it has been hypothesized that dendritic cells (DCs), that express AhR constitutively, may be directly affected by TCDD and induce suppression of T-cell mediated immune responses. To address this hypothesis Vorderstrasse and colleagues, initially characterized the effects of AhR activation in DCs. Their study demonstrated a decrease in splenic DCs concomitant with modulation of maturation markers (Vorderstrasse and Kerkvliet, 2001). Although TCDD decreased splenic DC numbers, no effect on the *in vivo* or *in vitro* antigen processing ability of these DCs was observed. Later studies by Ruby et al., documented TCDD to suppress NF-kB/Rel activation (Ruby *et al.*, 2002). Despite these studies establishing DCs sensitivity to AhR activation, the consequences of AhR activation on the innate and adaptive function of DCs remained undefined. To address this deficiency, we investigated the consequences of AhR activation on the fate and function of DCs. We postulated that AhR activation generates defects in DCs, contributing to aberrant innate and adaptive immune responses, culminating in TCDD-induced immune suppression.

In our study we first examined the effects of AhR activation in steady-state DCs in naïve mice. TCDD altered DC homeostasis by selectively decreasing the $CD11c^{high}CD8\alpha^{-}33D1^{+}$ splenic DCs subset specialized at activating $CD4^{+}$ T cells but did not affect the regulatory $CD11c^{high}CD8\alpha^{+}DEC205^{+}$ splenic DCs. Thus, decreases in the $CD4^{+}$ T cell activating DCs by TCDD may significantly increase susceptibility to infectious agents, especially pathogens that are considered blood-borne. Corroborating these *in vivo* findings, recent data from our laboratory has shown TCDD to decrease LPS- and CpG-induced IL-6 and TNF- α production by Flt3L-derived, steady-state DCs *in vitro (*unpublished data). Following pathogenic insult, TCDD-induced decreases in proinflammatory cytokine production by DCs may lead to defective pathogenic clearance. Thus, disruption of DC homeostasis as well as modulation of DC differentiation following AhR activation would prevent DCs from effectively activating $CD4^{+}$ T cells, resulting in defective $CD4^{+}$ T cell responses and immune suppression.

Recently, several reports have linked TCDD-induced immune suppression with induction of Tregs (Funatake *et al.*, 2005; Marshall *et al.*, 2008). However, little information exists on the mechanisms underlying induction of Tregs and subsequent immune suppression following TCDD exposure. Dendritic cells contribute to the generation of Tregs via multiple mechanisms. One such mechanism is the production of Treg-inducing mediators such as IDO and TGF-β. Studies from our laboratory have shown an increased production of regulatory

mediators including both IDO and TGF-β3 by steady-state DCs treated with TCDD (unpublished data). Therefore, induction of Tregs and subsequent immune suppression following TCDD exposure could result not only from a selective retention of Treg-inducing $CD11c^{high}CD8\alpha^{+}DEC205^{+}$ DCs in the periphery, but also from an increased production of suppressive mediators by steady-state DCs. Taken together, in naive mice, AhR activation disrupts DC homeostasis, inducing a loss of DCs that contribute to $CD4^{+}$ T cell activation. The selective loss of this subset, concomitant with the retention of Treg-inducing DCs may increase susceptibility to infection and contribute to defective adaptive immune responses following TCDD exposure.

Dendritic cells exist in multiple, functionally distinct stages of differentiation both *in vitro* and *in vivo*. These include but are not limited to the steady-state DCs and inflammatory DCs. As previously described, TCDD altered steady-state DC homeostasis and differentiation. Inflammatory DCs, on the other hand, constitute a novel DC population that are absent in the steady-state and appear only following an inflammatory insult. Because we observed disruption of steady-state DC homeostasis, we assessed the direct effects of AhR activation in inflammatory DCs and the contribution of AhR-activated DCs in the generation of inflammatory responses. TCDD induced a regulatory phenotype in inflammatory DCs characterized by an increased expression of the suppressive mediators IDO1, IDO2 and TGF-β3. The induction of a regulatory phenotype in inflammatory DCs raised the possibility that interactions of these DCs with T cells

may generate regulatory T cells or suppress T cell responses directly. Surprisingly, despite the induction of regulatory mediators, AhR-activated DCs did not suppress T cell activation, raising the possibility that AhR activation in both T cells and DCs may be essential for Treg induction or suppression of T cell activation. This possibility is consistent with studies from our laboratory describing the effects of AhR activation in DCs during the generation of antigen-specific T cell responses. In our study, host mice treated with TCDD displayed increased deletion of donor, antigen-laden DCs contributing to decreased expansion of antigen-specific T cells. Though results from these experiments demonstrated the requirement for the AhR in DCs to be essential for the suppression of antigen-specific T cell responses, they did not exclude the possibility that antigen-specific $CD4^+$ T cells may be direct targets of AhR activation resulting in suppression of T cell activation.

Recently, several non-DRE-mediated or non-canonical AhR signaling mechanisms underlying toxic manifestations of TCDD have been identified. Of these, interactions of activated AhR with NF-kB signaling components were shown to induce defects in DCs following TCDD exposure (Ruby *et al.*, 2002; Vogel *et al.*, 2008; Vogel and Matsumura, 2008). Consistent with these findings, in our study, TCDD-induced modulation of inflammatory BMDCs and TLR responsiveness was only partially dependent on DRE-mediated events and led to altered NF-kB signaling. In addition to NF-kB signaling, an activated AhR interacts with other transcription factors such as retinoblastoma and E2F1. Assessing the potential interactions of the AhR with these transcription factors

and others may provide further insight into mechanisms by which AhR activation modulates DC function.

Traditionally referred to as an orphan receptor, recent studies have identified candidate natural ligands for the AhR ranging from dietary components such as flavones to bilirubin. In our study, natural AhR ligands, 6-formylindolo[3,2-b]carbazole (FICZ) and 2-(1H-Indol-3-ylcarbonyl)-4-thiazolecarboxylic acid (ITE) altered DC differentiation suggesting that endogenous or natural AhR ligands can also induce immunomodulation. Additionally, recent studies have revealed physiological functions of the AhR. In one of these studies, the AhR was shown to negatively regulate LPS-induced inflammatory responses in macrophages, demonstrating that the physiologic function of the receptor extends beyond mediating the toxicities of exogenous environmental pollutants (Kimura *et al.,* 2009). Characterizing the effects of natural AhR ligands and their specific mechanisms in immune cells may provide insights into the physiological functions of the AhR receptor in the immune system and help devise strategies by which AhR activation can be therapeutically exploited.

Taken together, our research describes the effects of AhR activation in steady-state and inflammatory DCs and identifies mechanisms that may underlie TCDD-induced immune suppression. Our results show that AhR activation induces defects in DCs that could contribute to the generation of defective $CD4^+$ T cell responses and subsequent immune suppression. In addition to DCs and

$CD4^+$ T cells, many different types of immune cells express the AhR. Future studies examining the consequences of interactions between DCs and immune cells such as the Natural Killer cells, B cells and $CD8^+$T cells, in the context of AhR activation, may unravel additional mechanisms underlying TCDD-induced immune suppression and help understand the normal physiological function of the AhR.

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REFERENCES

Funatake, C. J., Marshall, N. B., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2005). Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin generates a population of CD4+ CD25+ cells with characteristics of regulatory T cells. *J Immunol* **175**, 4184-4188.

Kimura, A., Naka, T., Nakahama, T., Chinen, I., Masuda, K., Nohara, K., Fujii-Kuriyama, Y., and Kishimoto, T. (2009). Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *The Journal of experimental medicine* **206**, 2027-2035.

Marshall, N. B., Vorachek, W. R., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. (2008). Functional characterization and gene expression analysis of CD4+ CD25+ regulatory T cells generated in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Immunol* **181**, 2382-2391.

Ruby, C. E., Leid, M., and Kerkvliet, N. I. (2002). 2,3,7,8-Tetrachlorodibenzo-p-dioxin suppresses tumor necrosis factor-alpha and anti-CD40-induced activation of NF-kappaB/Rel in dendritic cells: p50 homodimer activation is not affected. *Molecular pharmacology* **62**, 722-728.

Vogel, C. F., Goth, S. R., Dong, B., Pessah, I. N., and Matsumura, F. (2008). Aryl hydrocarbon receptor signaling mediates expression of indoleamine 2,3-dioxygenase. *Biochemical and biophysical research communications* **375**, 331-335.

Vogel, C. F., and Matsumura, F. (2008). A new cross-talk between the aryl hydrocarbon receptor and RelB, a member of the NF-kappaB family. *Biochemical pharmacology*.

Vorderstrasse, B. A., and Kerkvliet, N. I. (2001). 2,3,7,8-Tetrachlorodibenzo-p-dioxin affects the number and function of murine splenic dendritic cells and their expression of accessory molecules. *Toxicology and applied pharmacology* **171**, 117-125.