2012

CONSEQUENCES OF ARYL HYDROCARBON RECEPTOR ACTIVATION IN STEADY-STATE AND INFLAMMATORY DENDRITIC CELLS

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Dissertation

Presented in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in Toxicology

The University of Montana
Missoula, MT

May 2012

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a steroid-like transcription factor that mediates the toxicity of various environmental pollutants, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and TCDD-like compounds. Exposure to environmentally relevant levels of TCDD and TCDD congeners has been shown to cause immune suppression, yet the mechanisms underlying TCDD-induced immune suppression remain poorly understood. Dendritic cells (DCs) are professional antigen presenting cells (APCs) that play an integral role in both innate and adaptive immune responses. DCs constitutively express AhR and represent a highly sensitive immune cell population to AhR activation by environmental pollutants. In a murine inflammatory bone marrow-derived DC (BMDC) model, AhR activation has been shown to alter surface molecule expression and induce immnuoregulatory genes. To further these studies, we examined AhR activation in murine steady-state BMDCs, DCs that *in vitro* closely resemble DCs found in peripheral immune tissues *in vivo*. We found that similar to inflammatory BMDCs, TCDD-induced AhR activation disrupts steady-state BMDC differentiation, responsiveness to innate danger signals, and induces immunoregulatory gene expression. AhR activation has been implicated in the polarization of regulatory T cells (Tregs), which are capable of inhibiting immune responses and may contribute to the observed immune suppression following exposure to TCDD. Because both inflammatory and steady-state BMDCs upregulated immunoregulatory gene expression in response to TCDD, their potential to suppress $CD4^+$ T cells responses was examined in antigen-specific DC:$CD4^+$ T cell co-cultures *in vitro*. Both inflammatory and steady-state AhR activated BMDCs altered antigen-specific $CD4^+$ T cell responses and induced $Foxp3^+$ Tregs when compared to vehicle-treated BMDCs. These *in vitro* studies were followed by a series of *in vivo* studies, in which we demonstrated that TCDD-exposed mice immunized with antigen-laden BMDCs, or antigen with adjuvant generated an increased frequency of $Foxp3^+$ Tregs and subsequently dampened antigen-specific $CD4^+$ effector T cell responses. Furthermore, DCs from TCDD-treated mice displayed increased expression of immunoregulatory genes, suggesting that they may be contributing to deficient $CD4^+$ effector T cells responses and the generation of Tregs as observed in our *in vitro* studies. Overall, these studies significantly advance our understanding of how AhR activation by TCDD and TCDD-like chemicals induces immune suppression.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my parents for their never-ending support and encouragement. They instilled in me a positive outlook in life and for that I am truly grateful. I would also like to thank my brother and sister for all of their support and help throughout graduate school and life.

I owe my deepest gratitude to my mentor Dr. David M. Shepherd for all of his advice, encouragement and assistance. He has fully supported my efforts as a graduate student and significantly contributed to my development as an independent scientist. For his efforts' I am truly thankful. I would also like to thank my committee members for supporting me through the graduate school process and aiding in my scientific development.

I would like also to thank all of the people who have helped me in one way or another throughout my doctoral studies. Jasihree Bankoti and Andrea Miller for teaching me lab techniques and helping with experiments and for all of their kind words of encouragement. Dr. Celine Beamer for her vast wealth of knowledge and technical assistance. Pam Shaw for teaching me flow cytometry, sorting cells and troubleshooting, I couldn't have done it without her. Britten Postma for her help with the mice. Alison Kearns for all of her wonderful support both in lab and in life. Lastly, all the graduate students and friends that have helped me out in more ways than one. This was all made possible with a little help from my friends.

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CHAPTER 1

INTRODUCTION

This dissertation describes the effects of aryl hydrocarbon receptor (AhR) activation in two distinct dendritic cell populations. The introduction will provide: 1) an overview of the immune system, 2) an in depth review of dendritic cell biology and T cell activation, and 3) describe the immune toxicity of environmental toxicants that activate the AhR.

The Immune System

The immune system is designed to protect against foreign pathogens and provide tolerance to self. Through the process of evolution the immune system has formed two distinct branches, the innate and adaptive immune systems. These two systems often work in concert to recognize, alert and remove invading microorganisms to provide full protection to the host. The innate immune system provides the first line of defense, while the adaptive immune system provides additional defense and memory.

Innate Immunity

The innate immune system, being the first line of defense is designed to 1) provide a barrier, 2) recognize a wide range of foreign organisms or xenobiotics and 3) effectively remove foreign organisms or xenobiotics with minimal damage to the host. The skin and mucosal surfaces make up the general barriers of the innate immune system, acting as shields to keep pathogens from gaining entry into the body. If the barrier function is compromised, various innate immune cells have the ability to broadly

recognize pathogens, mount a rapid response against the pathogen and remove it from the body.

The leukocytes that constitute the innate immune system are neutrophils, basophils and mast cells, eosinphils, natural killer cells, macrophages and dendritic cells, with secondary support from cells that make up epithelial and mucosal layers (Figure 1.1). The innate immune system also contains specific innate lymphocyte populations such as B1 B cells, γδ T cells and natural killer T cells. Neutrophils or polymorphonuclear cells (PMNs) are professional phagocytes that engulf microorganisms and damaged cells. Neutrophils are short-lived and make up ~50% of the circulating leukocyte population. They are recruited from circulation to damaged sites by chemokines and various microbial products. Neutrophils have the ability to destroy pathogens through phagocytosis, breakdown via intracellular endosomal/lysosomal pathways and the production of antimicrobial proteins. Neutrophils also secrete reactive oxygen species during oxidative bursts to destroy extracellular pathogens. Basophils and mast cells are circulating and tissue resident non-phagocytic leukocytes, respectively, that aid in the defense against parasites through the release of cytoplasmic granules containing compounds such as proteases, major basic protein and cytokines. Basophils and mast cells make up ~1% of the leukocyte population and are also effector cells in allergic responses, secreting histamine and other pro-inflammatory compounds following IgE-mediated Fc receptor activation. Eosinophils are circulating weakly phagocytic cells that release cytotoxic granules to defend against extracellular parasites, primarily helminths. Eosinophils are mainly tissue resident leukocytes and make up ~3% of the total immune cell population. Natural killer (NK) cells are derived from lymphoid progenitor cells, but

they contribute to innate immune responses. NK cells recognize virally infected cells and tumor cells and induce their apoptosis through the release of lytic granules. In addition to neutrophils, macrophages help ingest and clear microorganisms, as well as clear apoptotic cells to help control cellular turnover and support self-tolerance. Derived from monocytes in the blood, macrophages are rapidly recruited to sites of inflammation to provide additional help in clearing infected or dying cells and aid in the inflammatory response through the production and secretion of pro-inflammatory cytokines such as IL-6 and TNFα and various chemokines. Within tissues, macrophages take on specialized roles. For example, macrophages present in the liver, known as Kupffer cells, and in the spleen primarily scavenge senescent cells, whereas alveolar macrophages present in the lung phagocytose inhaled particles and regulate inflammation. Within the innate immune system, similar to macrophages, dendritic cells also help in the recognition and removal of pathogens. Pathogen recognition in the innate immune system is achieved through a highly evolved receptor network.

Innate immune cells use a class of receptors known as pathogen recognition receptors (PRRs) to identify foreign organisms. PRRs recognize pathogen-associated molecular patterns (PAMPs) that are widely expressed on different microorganisms. PRRs are primarily expressed in neutrophils, macrophages, natural killer cells and dendritic cells as well as B lymphocytes (Janeway and Medzhitov, 2002; Janssens and Beyaert, 2003). The power of PRRs and the innate immune system comes from its ability to recognize a wide variety of PAMPs. This recognition is accomplished through the expression of different PRRs including: scavenger receptors that recognize low-density lipoproteins; mannose receptors that bind mannose-containing carbohydrates found on

various fungal and protozoan pathogens; C-type lectin receptors that recognize foreign glucan structures; and Toll-like receptors (TLRs) and nucleotide oligomerization domain receptors (NODs), which recognize both extracellular and intracellular PAMPs.

Figure 1.1

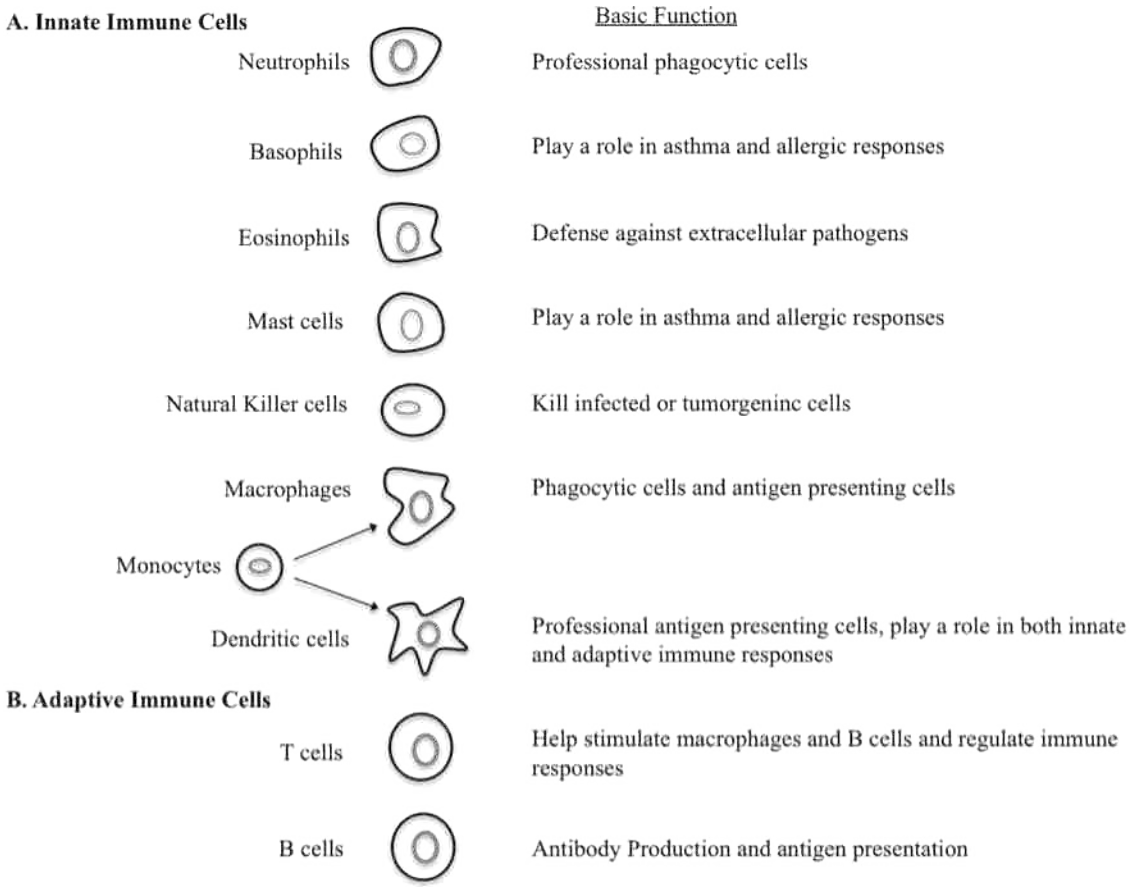

Figure 1.1. Cells of the innate and adaptive immune systems and their basic function.

There are 13 TLRs described in mice and 10 in humans (Janssens and Beyaert, 2003). TLRs can be broken into two groups based on location and substance recognized: extracellular membrane-bound and intracellular TLRs (Figure 1.2). Membrane-bound TLRs include TLR 1, 2, 4, 5 and 6 and function to recognize bacterial and fungal cell wall components and motor proteins such as flagellin. TLRs 3, 7 and 9 are intracellular PRRs contained in endosomes, binding mainly viral nucleic acids. Specifically, TLR2 recognizes lipoteichoic acid and peptiglycans on gram-positive bacteria and is constitutively expressed on macrophages, dendritic cells and B lymphocytes. TLR2 also forms heterodimeric complexes with TLR1 and TLR6, effectively increasing the number of foreign ligands detected by TLR2. TLR4 is responsible for detecting lipopolysassricde (LPS), a constituent of gram-negative bacterial cell walls, and is expressed on macrophages, dendritic cells, neutrophils, mast cells, as well as B lymphocytes and various non-immune cells. Flagellin, a bacterial protein used in propulsion, is detected by TLR5, which is expressed on macrophages and dendritic cells. The intracellular TLRs 3, 7 and 9 recognize viral double-stranded DNA (dsDNA), viral ssRNA and unmethylated cytosine-phosphate-guanine (CpG) viral DNA motifs, respectively, and are expressed in macrophages, dendritic cells and B lymphocytes at varying levels (Medzhitov, 2001; Janeway and Medzhitov, 2002). TLR11, 12 and 13 in mice are less characterized, but are thought to play a role in the detection of pathogenic organisms of the urinary tract (Medzhitov, 2001; Janssens and Beyaert, 2003).

The NOD receptor family consists of over 20 intracellular NOD receptors. NOD1 and NOD2 are the best characterized and considered prototypical NOD receptors. NOD1 and NOD2 receptors recognize peptidoglycans expressed on both gram-positive and

gram-negative bacteria. In addition to membrane-bound and intracellular PRRs, the innate immune system also employs secreted PRRs to detect foreign organisms and elicit immune responses.

Similar to membrane-bound and intracellular PRRs, secreted PRRs recognize PAMPs to help activate the complement system and to opsonize pathogens for phagocytosis. Secreted PRRs, also known as acute-phase proteins, are integral to acute-phase responses induced by early systemic inflammation in response to infection (Janeway, 2008). Acute-phase proteins are classified into four groups: collectins, pentraxins, lipid transferases and peptidoglycan recognition proteins (PGRPs). Each group consists of a large number of proteins with the more common acute-phase proteins being C-reactive protein, serum amyloid A and serum amyloid P, mannose-binding lectin, fibrinogen, and the surfactants SP-A and SP-D. These acute-phase proteins orchestrate the activation of the complement system and overall aid in pathogen recognition and destruction. In addition, many secreted PRRs have bactericidal properties to help destroy or weaken bacteria (Janeway and Medzhitov, 2002; Janeway, 2008; Murphy *et al.*, 2008).

Overall, the innate immune system employs highly efficient mechanisms to detect, destroy and remove injurious foreign organisms. As the first line of defense, the innate immune system effectively clears the majority of harmful microorganisms. While the innate immune system provides rapid host protection, it lacks two important characteristics, specificity and memory. Fortunately, these attributes are what define the adaptive immune system.

Figure 1.2

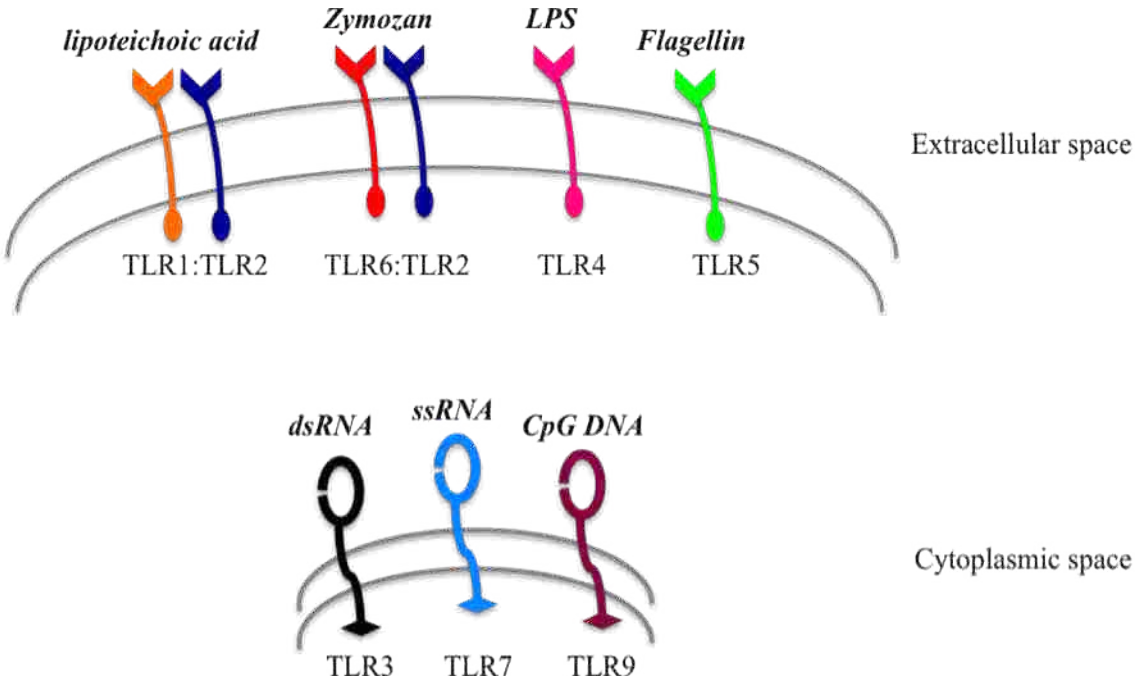

Figure 1.2. Toll-like receptors and their common ligands.

Adaptive Immunity

To provide more comprehensive host recognition of both foreign and self-antigens, mammals as well as various non-mammal species have developed a second line of defense known as the adaptive immune system. This second branch consists of specialized immune cells that interact with one another and can communicate with the innate immune system. Initial adaptive immune responses are slower than those of the innate immune system, taking from 5-7 days to initiate a response and 7-14+ days to clear a pathogen. However, during the course of the immune response, memory cells develop and upon secondary exposure the adaptive immune response is rapid, taking only a few days to mount a robust full response. The leukocytes involved in adaptive immune responses are T cells and B cells and macrophages and dendritic cells (Figure 1.1).

T and B lymphocytes are derived from common lymphoid progenitors and are unique in that they are the only cells in the body that express antigen receptors designed to recognize only a single specific antigen and are capable of clonal expansion following recognition and activation. T lymphocytes develop in the thymus from $CD8^-$ $CD4^-$ precursor cells into committed $CD4^+$ T cells or $CD8^+$ T cells following a complex process of positive and negative selection. The selection process is designed to produce individual T cells that recognize only antigens processed and presented on major histocompatibility complex (MHC) molecules by antigen presenting cells (APCs) and to limit the generation of autoreactive T cells that respond to self-antigens. B lymphocytes develop in the medulla ossium rubra of the bone marrow and, similar to T cell development, the generation of self-reactive B cells is minimized before exodus from the bone marrow. In contrast to T cells, which only recognize processed antigens on MHC

molecules presented primarily by APCs, B cells recognize antigen in its natural unprocessed form. In the periphery, T cells and B cells in circulation and in secondary and tertiary lymphoid tissues coordinate effective humoral and cell-mediated immune responses to a diverse range of pathogens.

B cells are responsible for the production of antibodies, which are the defining quality of humoral immunity. B cells express surface immunoglobulin B cell receptors (BCR) that recognize antigens in their natural form, whether soluble or bound to receptors on other immune cells. Activation through the BCR occurs in response to thymus-independent antigens (TI) or thymus-dependent antigens (TD). B cell activation through TI antigens is mediated through BCR signaling and TLR-antigen recognition or extensive BCR cross-linking, whereas TD activation requires BCR signaling and T helper cell interactions that deliver a second requisite activation signal. There are three main classes of B cells: B1, marginal zone and follicular B cells. B1 and marginal zone B cells are primarily responsible for antibody production in response to TI antigens, while follicular B cells are primarily involved in TD antigen responses. Activated B cells in the spleen or lymph nodes clonally expand following BCR antigen recognition and cognate antigen T cell help. T cells move into primary B cell follicles and help form germinal centers where B cells go through a process of class-switching and affinity maturation, ultimately forming plasma cells or memory B cells. The majority of the plasma cells formed in the germinal center travel to the bone marrow where they produce and secrete high-affinity antigen-specific antibodies.

Initial antibody responses are characterized by IgM production, which requires no class switching. However, IgG is the most abundant antibody found in the plasma,

secreted following B cell activation and subsequent class switching. IgA and IgE are also produced following class switching, in response to different antigens and specific signals from T cells. IgD is not secreted but rather a surface-bound antibody. Antibodies perform three main functions to help clear pathogens. First, they can bind and neutralize bacteria and toxins in the blood, effectively blocking their entry into cells. Second, antibodies can opsonize pathogens targeting and making them suitable for phagocytosis and destruction. Third, antibodies can activate the complement pathway. Overall, B cell antibody production is integral to adaptive humoral immune responses and often complements cell-mediated immune responses.

$CD8^+$ T cells are responsible for the majority of cell-mediated responses designed to detect and destroy host cells infected with an intracellular pathogen. Upon recognizing their cognate antigen, primarily viral and intracellular bacterial antigens, presented in the context of MHC class I, $CD8^+$ T cells become activated and develop into $CD8^+$ effector cells also known as cytotoxic T lymphocytes (CTLs). In turn, CTLs target infected cells expressing viral components on MHC class I and initiate cell death through the release of cytotoxic granules containing perforin and granzymes and through apoptotic signaling interactions between Fas ligand expressed on the CTL and Fas expressed on the target cell. While $CD8^+$ effector cells are highly efficient and specific cytotoxic T cells, they often require help from $CD4^+$ T cells to become fully activated or for the generation of $CD8^+$ memory T cells.

In contrast to $CD8^+$ T cells, $CD4^+$ T cells have the ability to differentiate into discrete $CD4^+$ effector or T helper (Th) cells. The role of $CD4^+$ T cells following activation is not to lyse infected cells, but rather to help other immune cells mount

specific immune responses. Naïve $CD4^+$ T cells that recognize antigen presented via MHC class II can develop into either Th1, Th2, Th17 or regulatory T cells (Tregs) (Figure 1.3). In addition to these classical Th cells, in humans Th22 cells have recently been described that characteristically produce IL-22 (Wolk *et al.*, 2010). In mice the current state of affairs is that a subset of Th17 cells produce IL-22, rather than a distinct Th22 cell (Wolk *et al.*, 2010). $CD4^+$ T cell polarization is dependent on the type of antigen and the prevailing cytokine environment (Figure 1.3). Once differentiated, Th1 cells help alert and activate macrophages to the presence of intracellular bacteria, effectively enabling macrophages to destroy infected cells. In response to intracellular pathogens, Th1 cells secrete IFNγ and IL-2 to help stimulate macrophages and $CD8^+$ effector cells. During Th1-mediated immune responses B cells following interactions with Th1 cells produce IgG1 and IgG3 antibodies in humans and IgG2a and IgG2b in mice to aid in the detection and clearance of infected cells. Th2 cells help elicit responses to extracellular parasites through the production of IL-4, IL-5, IL-10, IL-13 and IL-25, which recruit and activate mast cells, basophils and eosinophils. Th2 cells instruct B cells to produce IgE, which allows eosinophils to target and destroy extracellular parasites and activates mast cells and basiophils. Recently discovered and characterized, Th17 cells function in the defense against extracellular bacteria and fungi by producing the characteristic cytokine IL-17 as well as IL-21, IL-22 and IL-23. IL-17 helps recruit neutrophils to sites of inflammation and bacterial burden. Th1 and Th17 cells also contribute to the pathology of various autoimmune diseases, allergy and asthma. Regulatory T cells help coordinate appropriate responses and restrain adverse pathologic responses throughout the course of an immune response.

Figure 1.3

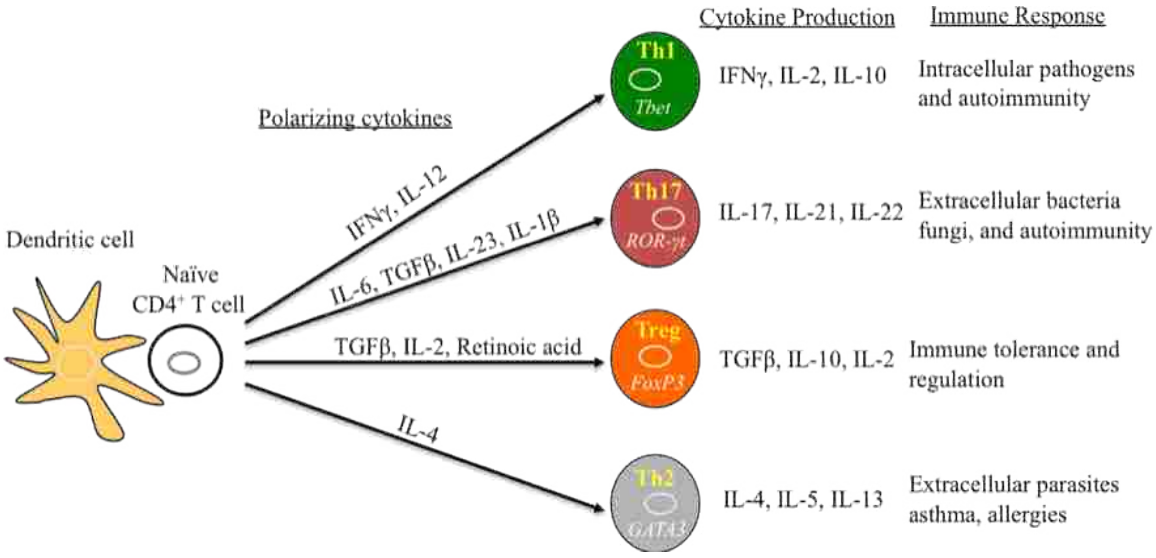

Figure 1.3. $CD4^+$ effector T cell polarization.

Regulatory T cells are separated into two categories, induced Tregs (iTregs) and natural Tregs (nTregs). The later are formed in the thymus during T cell development and play a key role in preventing autoimmune disease by maintaining peripheral T cell tolerance. $CD4^+$ nTregs have a diverse T cell receptor (TCR) repertoire and are defined by the lineage specific transcription factor Forkhead box P3 (Foxp3) and various surface molecules, including CD25, cytotoxic T-lymphocyte antigen 4 (CTLA-4), Helios and glucocorticoid-induced tumor necrosis factor receptor (GITR). Induced Tregs, also known as adaptive Tregs, develop from naïve $CD4^+$ T cells in the periphery following interactions with tolerogenic DCs. Classical iTregs, once formed, express Foxp3 and high levels of CD25, CTLA-4 and GITR. However, there are well-defined iTreg subsets that do not express Foxp3, such as regulatory type 1 (Tr1) and Th3 Tregs (Table 1.1) (Li and Boussiotis, 2011). Tr1 cells are characterized by their production of IL-10, while Th3 Tregs produce significant amounts of TGFβ.

Tregs help control ongoing immune responses and reactions to self-antigens through the production of inhibitory cytokines, metabolic disruption of effector responses, cytolysis and APC modulation (Vignali *et al.*, 2008). IL-10, IL-35 and TGFβ produced by Tregs act as anti-inflammatory and inhibitory cytokines that dampen effector immune cell responses. TGFβ is also essential in instructing naïve T cells to become iTregs. High levels of CD25, the high-affinity IL-2 receptor (IL-2Rα), expressed on Tregs allows them to efficiently sequester IL-2, which is required by effector T cells for proper proliferation and expansion. In addition to sequestering IL-2, Tregs express the ectoenzymes CD39 and CD73 that generate extracellular adenosine. Adenosine binds $A_{2A}R$ receptors on effector T cells and inhibits effector T cell proliferation and induces

TGFβ expression (Ernst *et al.*, 2010). Metabolic disruption also occurs through the transfer of cAMP from Tregs to effector cells, effectively inhibiting effector T cell cytokine response and proliferation (Bopp *et al.*, 2007; Ernst *et al.*, 2010). Furthermore, CTLA4 and LAG3 expressed on Tregs inhibit DC function through interactions with CD80/86 and MHC class II molecules expressed on DCs, respectively (Vignali *et al.*, 2008).

Overall, the adaptive immune system with both cell-mediated and humoral immune responses offers protection against a wide variety of pathogens. With the rapid responses of the innate immune system and the specificity and memory of the adaptive immune system, mammals are well suited to fend off harmful microorganisms. Moreover these systems effectively control ongoing responses and provide tolerance to self-proteins and detect tumorogenic cells. All of this is accomplished through coordinated communication between the innate and adaptive arms of the immune system. Dendritic cells are the only leukocytes to play a fundamental role in both innate and adaptive immune responses and in doing so link the two systems together.

Table 1.1

$CD4^+$ Regulatory T cells (Tregs)	Typical Phenotype	Development	Suppressor Function
Natural Tregs (nTreg)	$CD4^+$ $CD25^{high}$ $FoxP3^{high}$ $GITR^+$ $CTLA\text{-}4^+$	Thymus	Cell-cell inhibition through CTLA-4, GITR. Also produce IL-10 and TGFβ
Classical Induced Treg (iTreg)	$CD4^+$ $CD25^{high}$ $FoxP3^{high}$ $GITR^+$ $CTLA\text{-}4^+$	Periphery	Cell-cell inhibition through CTLA-4, GITR. Also produce IL-10 and TGFβ
Type 1 Induced Treg (Tr1)	$CD4^+$ $CD25^-$ $FoxP3^-$	Periphery	Produce copious amounts of anti-inflammatory IL-10
T helper 3 Induced Treg (Th3)	$CD4^+$ $CD25^-$ $FoxP3^-$	Periphery	Secrete large amounts of TGFβ

Table 1.1. Regulatory T cell classification and function.

Dendritic Cells

Dendritic cells are integral in mounting proper immune responses and regulating autoimmune reactions to maintain self-tolerance. They are important cells in both innate and adaptive immune responses and provide distinct functions to each system.

Dendritic Cell Lineage and Subsets

Dendritic cells, referred to as DCs hereafter, are derived from both myeloid and lymphoid progenitor cells (Banchereau *et al.*, 2000). The vast majority of DCs are of myeloid origin, while DCs derived from lymphoid precursors represent a small fraction of the total DC population (Yang *et al.*, 2005; Shortman and Naik, 2007a; Naik, 2008a). DCs are highly specialized immune cells and are commonly classified into distinct subsets depending on their phenotype, function and location. While there is ongoing discussion and research regarding DC classification, DCs are commonly classified into three categories: plasmacytoid DCs, conventional or classical DCs and inflammatory DCs (Figure 1.4). There is an additional class of DCs that develop from pre-DCs and circulating monocytes, depending on the tissue and inflammatory milieu. Both pre-DCs and monocytes have a unique surface molecule phenotype before their recruitment to DC status. However, pre-DCs or monocytes upon maturation typically develop into inflammatory DCs. In mice, all DC classes express the DC lineage marker CD11c and MHC class II, to varying degrees (Table 1.2). Each DC class is further identified through the expression of specific surface molecules (Table 1.2). Each class of DCs also has a specialized function, providing unique support to both innate and adaptive immune responses.

Table 1.2

Dendritic Cells	Typical Phenotype	Origin	Function/ Tolerogenic Status (-/+)
Plasmacytoid DCs	$CD11c^{low}/CD11c^{intermdiate}$, MHC class II^{low}, $B220^{+}$, $Ly6C/Gr1^{+}$, $CD11b^{-}$	CMP/CLP	Produce IFNα/β in response to viral detection/ ++
Conventional DCs			
Dermal/Langerhan DCs	$CD11c^{High}$, $CD11b^{+}$ $DEC205^{+}$ $CD103^{-}$	Unknown	Classical migratory APCs Mediate contact hypersensitivity reactions/ -
	$CD11c^{High}$, $DEC205^{+}$, $Langerin^{+}$ $CD103^{+}$	CMP	
Interstitial DCs	$CD8^{+}$, $CD4^{-}$ $CD11c^{High}$, MHC class II^{+}, $DEC205^{+}$, $33D1^{-}$, $CD11b^{-}$	CMP	Cross-presentation induces Th1 responses/ ++
	$CD8^{-}$, $CD4^{+}$, $CD11c^{High}$, MHC class II^{+}, $DEC205^{-}$, $33D1^{+}$, $CD11b^{+}$	CMP	Initiate Th2 responses/-
	$CD8^{-}$, $CD4^{-}$, $CD11c^{High}$, MHC class II^{+}, $DEC205^{-}$ and $33D1^{+}$	CMP	Unknown/ -/+
Inflammatory DCs	$CD11c^{Intermediate}$, $CD8^{-}$ $CD4^{-}$ $CD11b^{+}$, $DEC205^{+}$	MDP/ Monocytes	Help initiate and aid in inflammatory responses produce TNFα, IL-6 and NO/ -

Table 1.2. Dendritic cell subsets, phenotype, and basic function.

Plasmacytoid DCs (pDCs) characteristically produce type-I interferons in response to viral infections and are derived from both lymphoid and myeloid progenitor cells (Shortman and Naik, 2007a; Geissmann *et al.*, 2010). Immature pDCs are found in the bone marrow, circulation and in peripheral organs. In the spleen and lymph nodes, pDCs reside in the marginal zone and T cell rich microenvironments, respectively. Compared to conventional or inflammatory DCs, pDCs are long-lived and constitutively express high levels of TLR7 to recognize viral RNA. Viral recognition causes pDC activation; in turn, activated pDCs produce copious amounts of IFNα/β and IL-10. While essential in viral detection, pDCs in the steady state are tolerogenic, presenting largely non-viral antigens to T cells in a fashion that induces regulatory T cells and/or dampens T cell activation (Yang *et al.*, 2005; Shortman and Naik, 2007a). Their tolerogenic function is in part due to their inherent production of TGFβ and IL-10 and their constitutive expression of indoleamine 2,3 dioxygenase (IDO), a known immune regulatory enzyme (Mellor and Munn, 2004). The pDC lineage in mice is identified as $CD11c^{low}$/$CD11c^{intermdiate}$, MHC class II^{low}, $B220^{+}$, $Ly6C/Gr1^{+}$, $CD11b^{-}$ (Table 1.2) (Geissmann *et al.*, 2010). In mice, pDCs are also identified by their surface expression of mouse pDC antigen 1 (mPDCA-1). Overall, pDCs offer innate protection against viral pathogens and provide tolerogenic signals to adaptive immune responses. However, pDCs are poor antigen presenting cell as compared to conventional DCs.

Conventional or classical DCs (cDCs) are myeloid-derived DCs that upon further differentiation make up the majority of circulating/migratory and specialized tissue-resident DCs. Conventional DCs are further separated into two categories, migratory DCs and interstitial DCs (Shortman and Naik, 2007a; Naik, 2008a; Geissmann *et al.*, 2010).

Migratory DCs are the classical DC population, in that they survey peripheral tissues and upon recognition of foreign antigen, migrate to draining lymph nodes and present antigen to T cells, effectively eliciting an antigen-specific immune response. Examples of migratory cDCs include dermal DCs and Langerhans cells. Dermal DCs are present in the dermis of the skin and express $CD11c^{High}$, CD11b and DEC205 on their surface (Table 1.2). Langerhans cells are abundant in the skin and express $CD11c^{High}$, DEC205 and langerin (Table 1.2). Dermal and Langerhans cells are often the first DCs to encounter antigens following a compromise in the skin's barrier function. They provide inflammatory signals to help modulate innate immune responses as well as initiate adaptive immune responses by rapidly migrating to skin draining lymph nodes to present foreign antigens to both $CD8^+$ and $CD4^+$ T cells.

In contrast to migratory DCs, interstitial DCs, also known as lymphoid-resident DCs, are abundant in all lymphoid tissues. As their name implies they rarely travel out of their resident lymphoid tissue. Interstitial DCs function by sampling antigens passing through the lymphatics and subsequently provide immunogenic and/or tolerogenic signals to T cells. Interstitial DCs are further classified into $CD8^+$, $CD8^-$ and $CD8^-$ $CD4^-$ DCs (Figure 1.4). $CD8^+$ cDCs are $CD11c^{High}$, MHC class II^+, $DEC205^+$, $33D1^-$, $CD11b^-$ and $CD4^+$ expressing, while $CD8^-$ express $CD11c^{High}$, MHC class II^+, $DEC205^-$, $33D1^+$, $CD11b^+$ and $CD4^+$ (Table 1.2). Double negative $CD8^-$ $CD4^-$ DCs express $CD11c^{High}$, MHC class II^+, $DEC205^-$ and $33D1^+$ (Table 1.2) (Wu and Liu, 2007; Pulendran *et al.*, 2008). All three interstitial DC subsets, $CD8^+$, $CD8^-$ and $CD8^-$ $CD4^-$ express the co-stimulatory molecules CD40, OX40 ligand, CD80 and CD86 and the cell adhesion molecules CD54 and CD11a (Lipscomb and Masten, 2002; Geissmann *et al.*, 2010).

$CD8^+$ DCs are found predominately in T cell rich areas of the spleen, lymph nodes and Peyer's patches of the small intestine. $CD8^-$ DCs reside in the marginal zones of the spleen and in subcapsular regions of lymph nodes. $CD8^-$ $CD4^-$ DCs are mostly concentrated in B cell follicles and T cell rich areas in the spleen and lymph nodes (Lipscomb and Masten, 2002).

Similar to migratory DCs, interstitial DCs, upon antigen recognition and activation present antigens to $CD4^+$ T cells, evoke antigen-specific immune responses. In the spleen, conventional $CD8^+$ DCs are known to induce Th1 responses, while $CD8^-$ DCs generate Th2 effector cells following antigen-specific interactions with naïve Th cells (Pulendran *et al.*, 2008; Yamazaki *et al.*, 2008). $CD8^+$ DCs also specialize in cross presentation, i.e. the internalization of extracellular antigens intended for presentation on MHC class II are instead process and presented on MHC class I to $CD8^+$ T cells. In addition, $CD8^+$ DCs expressing $DEC205^+$ are known inducers of regulatory T cells in the spleen. In contrast, $CD8^-$ DCs expressing $33D1^+$ are ineffective at inducing regulatory T cells (Naik, 2008a; Yamazaki *et al.*, 2008; Geissmann *et al.*, 2010).

Inflammatory DCs are a separate class of DCs that provide inflammatory signals in response to various bacteria, viruses and parasites. A key distinction between inflammatory DCs and conventional DCs is that inflammatory DCs are not present in the steady state. Inflammatory DCs arise and are present during periods of increased inflammation resulting from infection or autoimmune disease (Shortman and Naik, 2007a; Naik, 2008a; Simones *et al.*, 2010). Inflammatory DCs develop from myeloid pre-DCs and monocytes in the blood during inflammation and are recruited to sites of inflammation via chemokine receptor CCR2 signaling (Jia *et al.*, 2008; Bosschaerts *et al.*,

2010). Once developed, inflammatory DCs are identified as $CD11c^{Intermediate}$ $CD8^{-}$ $CD4^{-}$ $CD11b^{+}$ and $DEC205^{+}$ (Table 1.2). During periods of increased inflammation they are found in the spleen, lymph nodes and at the site(s) of inflammation. Inflammatory DCs produce significant amounts of TNFα and nitric oxide (NO) generated by inducible nitric oxide synthase (iNOS) (Naik, 2008a; Simones *et al.*, 2010). Consequently, inflammatory DCs are also known as TNFα/iNOS producing DCs (TIP-DCs) (Figure 1.4). *In vivo,* inflammatory DCs are weak antigen presenting cells as compared to cDCs and they are not required for the induction of T helper cells. Instead inflammatory DCs provide inflammatory signals such as TNFα, IL-6 and NO to help in the clearance of microorganisms through phagocytosis. Inflammatory DCs are generated in response to a variety of bacterial infections such as *Listeria monocytogenes, Brucella menitensis* and *Salmonella typhimurium* (Naik, 2008a; Simones *et al.*, 2010). They are also present during response to parasitic *Leishmania major,* and lymphocytic choriomeningitis and influenza viruses (Naik, 2008a). Due to the inherent inflammatory nature of many autoimmune diseases, inflammatory DCs also often contribute to the increased inflammation and overall disease pathology of many autoimmune diseases including type-1 diabetes and psoriasis (Naik, 2008a).

While DCs are commonly segregated into plasmacytoid, conventional and inflammatory DC classes, there are a number of specialized DCs present in different organs such as the lung, liver and intestinal tract. These specialized DCs are most likely conventional DCs. However, there is ongoing research to completely define the origin, phenotype and unique function of many of these organ-specific specialized DCs (Pulendran *et al.*, 2008).

Figure 1.4

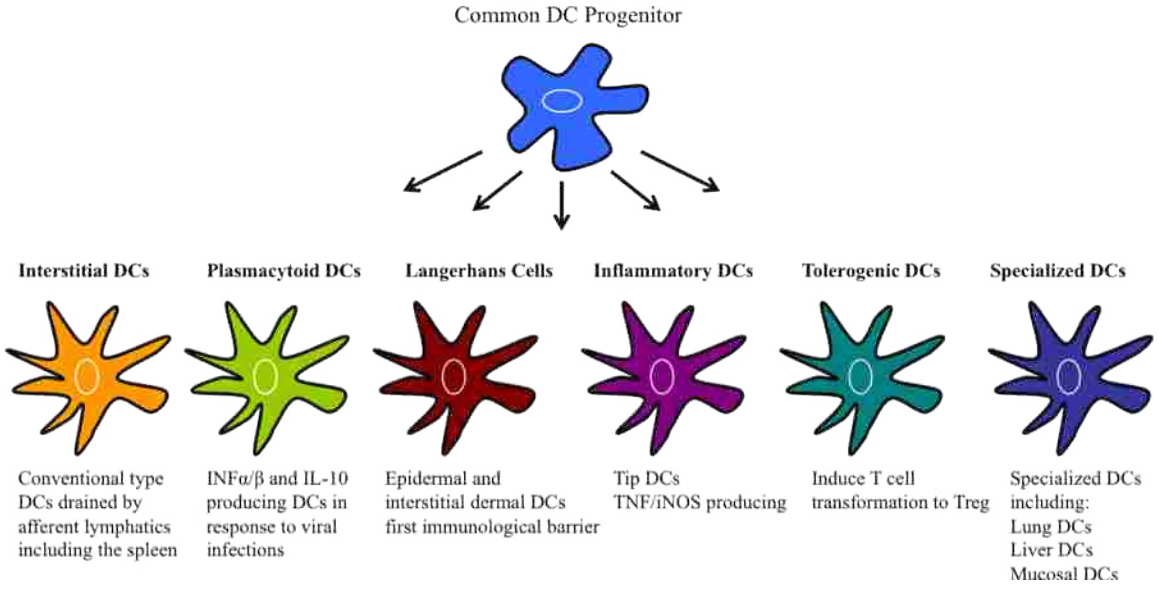

Figure 1.4. Dendritic cell subsets.

In vitro DC Model Systems

Due to the low frequency of DCs in humans and mice, *in vitro* model systems have been developed to greatly expand the number of DCs to utilize in scientific investigations. The most common method for deriving DCs from humans is to isolate $CD14^+$ peripheral blood mononuclear cells (PBMCs) or $CD34^+$ hematopoietic progenitor cells from the blood and drive them to become immature DCs *ex vivo* with granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 (Thomas *et al.*, 1993). In mice, progenitor cells are typically isolated from the bone marrow and induced to become immature DCs using GM-CSF with or without IL-4 (Inaba *et al.*, 1992). *In vitro* GM-CSF-derived DCs are well characterized and most closely resemble inflammatory DCs found *in vivo* during periods of inflammation (Inaba *et al.*, 1992; Naik *et al.*, 2007; Xu *et al.*, 2007). In contrast to the inflammatory DC model system, FMS-like tyrosine kinase receptor-3 ligand (Flt3 ligand) is used to derive DCs from bone marrow progenitor cells that emulate *in vivo* steady state DCs (Brasel *et al.*, 2000). The Flt3L model generates DCs similar to both plasmacytoid DCs and conventional DCs in mice (Xu *et al.*, 2007). Both inflammatory and steady state model systems have been used extensively to study the various facets of DCs.

Dendritic Cell Activation and Maturation

Before encountering antigen or danger signals, DCs usually persist in an immature state of differentiation. Immature DCs are highly mobile and survey the periphery or local microenvironment for antigens and PAMPs. Immature DCs are efficient at internalizing and processing antigens, but are poor T cell stimulators. Upon antigen recognition and uptake and/or PAMP recognition, DCs migrate to draining lymph

nodes or sites of inflammation. DC migration is mediated by an increased expression of chemokine receptors, primarily CCR7 (Banchereau *et al.*, 2000). Maturing DCs lose their ability to internalize and process antigens and in route to their final destination increase expression of MHC class I and MHC class II molecules, the co-stimulatory molecules CD80 and CD86, CD40 and OX40 ligand, and the adhesion proteins CD54 and CD11a as well as other surface molecules. During maturation, DCs increase gene expression and upregulate intracellular cytokines. Gene expression and cytokine profiles of maturing DCs are largely dependent on the type of DC and the maturation signal encounterd (Tureci *et al.*, 2003; Zhong *et al.*, 2009). Once mature, DCs are less mobile than their immature counterparts and are fully capable of activating T cells.

DC-Mediated CD4 T Cell Activation and Polarization

$CD4^+$ T cell activation and polarization into effector cells is accomplished through a series of steps that requires specific instruction from antigen presenting cells, hereafter referred to as APCs. Complete $CD4^+$ T cell activation is dependent on three signals (Figure 1.5).

First, a naïve $CD4^+$ T cell must recognize its cognate antigen via interactions between the T cell receptor (TCR) on the T cell surface and antigen properly presented on MHC class II by the APC. TCR engagement with cognate antigen presented on MHC class II and the cross-linking of CD4 induces a series of intracellular signaling events beginning with the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by the Src kinases Lck and Fyn. In turn, ITAM phosphorylation signals ZAP-70 to bind phosphorylated ITAM tyrosine residues and become activated by Lck-mediated phoshorylation. ZAP-70 then phosporylates LAT and SLP-76, which binds and activates

phospholipase C-γ (PLC-γ) resulting in a host of downstream signaling events ultimately activating NF-κB, NFAT and AP-1 transcription factors and inducing gene expression (Janeway, 2008).

Second, the $CD4^+$ T cell must receive co-stimulation through interactions between CD28 on the T cell and CD80/CD86 on the APC. CD28:CD80/CD86 interactions induce a number of intracellular signaling pathways including the phosphatidyl inositol-3 kinase (PI3K)/AKT pathway to help increase TCR signaling. Alternatively, CD80/CD86 can interact with CTLA4 expressed on the T cell, which induces inhibitory pathways and dampens T cell activation. If a T cell receives only signal 1, TCR activation, without signal 2 it becomes anergic, i.e. unresponsive and will be unable to fully activate and differentiate. If a T cell receives signal 1 and signal 2, TCR activation and co-stimulation, it will properly activate and begin the process of differentiation and clonal expansion. However, for full differentiation into effector status a third signal is required.

The third signal required for full differentiation comes from both the activating T cell and the APC. For example, IL-2 is required for proper clonal expansion and T cell activation. Primarily T cells produce IL-2 and depending on the type of $CD4^+$ effector response IL-2 is used in an autocrine fashion. The APC provides polarizing cytokines such as IL-1β, IL-2, IL-6, IL-10, IL-12, IL-23 and TGFβ, all of which affect T cell polarization in a specific manner (Figure 1.3). Following polarization, $CD4^+$ effector T cells secrete cytokines such as IFNγ, IL-4, IL-17 and IL-23 and TGFβ to maintain their Th1, Th2, Th17 or Treg status, respectively (Figure 1.3). DCs are the primary APCs responsible for inducing the majority of T cell-mediated immune responses. DCs also

play an important role in T cell tolerance and immune regulation through the induction and expansion of regulatory T cells.

Figure 1.5

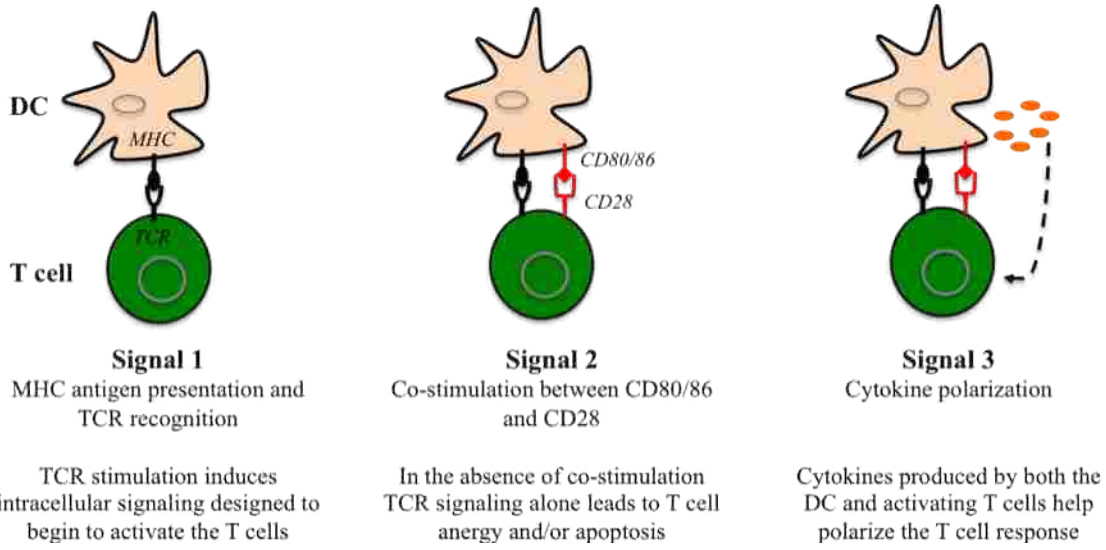

Figure 1.5. Signals required for proper T cell activation and polarization.

Immune Tolerance

Tolerance is the ability of the immune system to distinguish between self and non-self antigens, such that an immune response against self-proteins and tissues should not occur. Complete tolerance is established by both central tolerance and peripheral tolerance. Central tolerance is strictly maintained during lymphocyte development and is designed to remove T cells and B cells that react to self-antigens during their development in the thymus and bone marrow, respectively. Peripheral tolerance is maintained following lymphocyte development and migration into the periphery, where a handful of T cells that recognize self-antigens may have escaped deletion during central tolerance. Moreover, regulating and ending effective immune responses are key components of peripheral tolerance. Peripheral tolerance mechanisms include T cell apoptosis, T cell anergy and the induction of regulatory T cells.

T cell apoptosis and T cell anergy are commonly induced when $CD4^+$ T cells interact with immature DCs or via transient or unsuccessful DC:T cell interactions. Immature DCs lack the ability to fully stimulate a T cell through co-stimulation, the second signal required for full T cell activation. The deficiency in co-stimulatory signals coupled with TCR signaling causes T cells to become apoptotic or anergic (Shortman and Naik, 2007a). Mature DCs can also induce T cell apoptosis or anergy through specific interactions and signaling through CD80/CD86 and programmed cell death 1 (PD-1) expressed on the DC and CTLA-4 and PD-Ligand 1 or PD-Ligand 2 (PD-L1, PD-L2) expressed on T cells (Carter and Carreno, 2003; Fife and Bluestone, 2008). These same signals can also lead to the generation of active tolerance through the induction of regulatory T cells (Fife and Bluestone, 2008; Maldonado and von Andrian, 2010).

Tolerogenic DCs

The generation of regulatory T cells, whether classical iTregs, Tr1 and Th3 Tregs or the expansion of nTregs is largely dependent on interactions with tolerogenic DCs. Tolerogenic DCs inhibit the generation of $CD4^+$ effector responses and can directly induce regulatory T cells. Due to the heterogeneity of DC populations, tolerogenic DCs are present in all DC subpopulations, with varying degrees of tolerogenic capacity. For example, plasmacytoid DCs, $CD103^+$ DCs in the gut and $CD8^+$ $DEC205^+$ DCs in the spleen are highly tolerogenic (Pulendran *et al.*, 2008; Yamazaki *et al.*, 2008; Maldonado and von Andrian, 2010). $CD8^-$ $33D1^+$ DCs in the spleen, $CD103^+$ DCs in the skin and $CD11b^+$ dermal DCs and Langerhans cells are relatively non-tolerogenic, but rather highly stimulatory (Pulendran *et al.*, 2008; Maldonado and von Andrian, 2010). Regardless of the DC subtype, the tolerogenic or regulatory capacity of a DC is defined by the expression of specific inhibitory cytokines, enzymes and surface molecules.

The most common molecules produced by tolerogenic DCs are IL-10, TGFβ, retinoic acid, and IDO1/IDO2. IL-10 is an anti-inflammatory cytokine that dampens ongoing T cell responses and helps polarize iTregs. TGFβ is essential to both nTreg and iTreg development and survival in the periphery. TGFβ modulates T cell differentiation, inhibits T cell proliferation, induces Treg generation and dampens the stimulatory capacity of APCs (Li *et al.*, 2006; Yoshimura *et al.*, 2010). Moreover, mice that lack TGFβ1 suffer from serve multiorgan autoimmune disease and die shortly after birth, highlighting the importance of TGFβ in immune tolerance (Kulkarni *et al.*, 1993). Retinoic acid in the presence of TGFβ contributes towards the generation of regulatory T cells rather than Th17 cells, cells that also require TGFβ for their differentiation (Weiner

et al., 2011). IDO1 and IDO2 are involved in tryptophan catabolism, which has a two-fold effect on effector T cells. First, tryptophan is required for proper effector T cell proliferation. Thus, decreasing levels of tryptophan help limit effector T cell expansion and activation. Second IDO-mediated tryptophan catabolism generates kynurenine. Kynurenine can instruct naïve T cells to become regulatory T cells (Mezrich *et al.*, 2010). DCs express and utilize these tolerogenic mediators in various fashions. For instance, $CD103^+$ DCs in the gut constitutively express aldehyde dehydroxygenase (Aldh1a1 and Aldh1a2), which converts vitamin A present at high levels in the gut to retinoic acid (Coombes *et al.*, 2007; Del Rio *et al.*, 2010). Plasmacytoid DCs express higher levels of IDO1 and IDO2 than do conventional DCs, contributing to their enhanced capacity to induce Tregs in the periphery (Mellor and Munn, 2004; Yang *et al.*, 2005). Plasmacytoid DCs also produce TGFβ to aid in their tolerogenic responses. $CD8^+$ $DEC205^+$ DCs in the spleen produce copious amounts of TGFβ, effectively inducing iTregs, while maintaining nTreg populations (Naik, 2008a; Yamazaki *et al.*, 2008; Shortman and Heath, 2010). Although they have different phenotypes and tolerogenic capacities, tolerogenic DCs can be found in almost all tissues and organs and are present in the steady state and throughout the course of various immune responses and disease states. Over the past twenty years, there has been a concerted effort to identify or generate compounds that induce tolerogenic DCs in order to intentionally regulate immune responses and treat autoimmune diseases.

Various biological and pharmacological agents, natural compounds and environmental pollutants are capable of generating tolerogenic DCs that can subsequently induce regulatory T cells. The two most common biological agents used to generate

tolerogenic DCs are TGFβ and IL-10. In general, both TGFβ and IL-10 are anti-inflammatory cytokines. In DCs, active TGFβ induces both SMAD-dependent and SMAD-independent pathways, which control the expression of regulatory genes including IL-10, IDO and TGFβ (Fainaru *et al.*, 2007). TGFβ can also inhibit NF-κB signaling, causing DCs to remain in an immature state and function as tolerogenic DCs (Fainaru *et al.*, 2007). Similar to TGFβ, IL-10 signaling via the IL-10 receptor and JAK/Stat3 helps maintain an immature DC phenotype (Murray, 2006). Furthermore, IL-10 signaling induces suppressor of cytokine signaling 3 (SOCS3), which negatively regulates the production of inflammatory IL-6 in DCs (Murray, 2006). In addition to TGFβ and IL-10, anti-inflammatory glucocorticoids such as dexamethasone are used to generate tolerogenic DCs. As a pharmacological agent, dexamethasone binds the glucocorticoid receptor (GCR). In DCs, GCR activation upregulates the expression of genes that dampen inflammatory signals by inhibiting canonical NF-κB signaling and inducing the expression of anti-inflammatory mediators including IL-10, TGFβ, IDO and GITR ligand (Leung and Bloom, 2003; Iruretagoyena *et al.*, 2006). Collectively, dexamethasone and other glucocorticoids inhibit DC maturation and increase levels of anti-inflammatory molecules, effectively generating tolerogenic DCs capable of inducing regulatory T cells and dampening autoimmune diseases. In addition to glucocorticorids, other pharmacological and natural compounds such as aspirin, 1,25-dihydroxyvitamin D3 (the reactive metabolite of vitamin D3), resveratrol and curcumin can also induce toloerogenic DCs (Maldonado and von Andrian, 2010). Moreover, antibodies against CD200R on DCs can generate tolerogenic DCs, which are capable of inducing regulatory T cells (Maldonado and von Andrian, 2010). Many of these compounds work to increase

the expression of anti-inflammatory/regulatory mediators in DCs and inhibit DC maturation. More recently, chemicals known to activate the aryl hydrocarbon receptor have been reported to induce regulatory T cells and tolerogenic DCs.

Aryl Hydrocarbon Receptor Biology, Toxicity and Immune Modulation

The aryl hydrocarbon receptor (AhR) is an evolutionarily conserved, steroid-like orphan receptor. Depending on the species, the *AhR* gene encodes a 95-110kDa protein. The AhR is part of the basic helix-loop-helix-Per-ARNT-Sim (PAS)-containing transcription factor family and plays a role in many physiological functions including circadian rhythm, cell cycle, fetal development, tumorogenesis, lipid metabolism, cardiovascular function and immune responses (Nguyen and Bradfield, 2008; Denison *et al.*, 2011; Stejskalova *et al.*, 2011; Zhang, 2011).

AhR Signaling

AhR signaling is ligand-activated and includes both canonical and non-canonical pathways. The canonical AhR signaling pathway is well defined (Figure 1.6). The AhR is held quiescent in the cytoplasm by heat shock protein 90 (HSP90), HBV X-associated protein 2 (XAP2) and p23 chaperone proteins. Upon ligand binding, the AhR sheds its chaperone proteins exposing an N-terminal nuclear localization sequence (NLS). The NLS facilitates nuclear entry and the ligand:AhR complex moves into the nucleus bound to importin-β. Inside the nucleus, the ligand:AhR complex heterodimerizes with AhR nuclear translocator (ARNT). The ligand:AhR:ARNT complex then binds dioxin response elements (DREs) consisting of a core GCGTG sequence in the DNA. Upon DRE binding the AhR:ARNT complex recruits transcriptional co-activators and/or co-

repressors and modulates target gene expression. Prototypical AhR target genes include the phase I drug-metabolizing enzymes cytochrome P450 CYP1A1, CYP1A2 and CYP1B1 and phase II enzymes NQO1, GSTA2, UGT1A1 and UGT1A6 all of which contain multiple DREs in their promotor/enhacer regions (Denison *et al.*, 2002; Kohle and Bock, 2007). AhR signaling is controlled through a self-initiating feedback loop, where ligand:AhR:ARNT complexes induce AhR repressor (AhRR) expression. AhRR negatively controls AhR signal transduction by competing for ARNT and AhRR:ARNT in turn bind DREs, effectively blocking AhR

ARNT binding and gene transcription (Nguyen and Bradfield, 2008). Following gene transcription, the ligand:AhR:ARNT complex is exported into the cytosol, mediated by the AhR nuclear export signal (NES) and CRM1 export protein, and ubiquitin-tagged for proteosomal degradation (Nguyen and Bradfield, 2008).

Figure 1.6

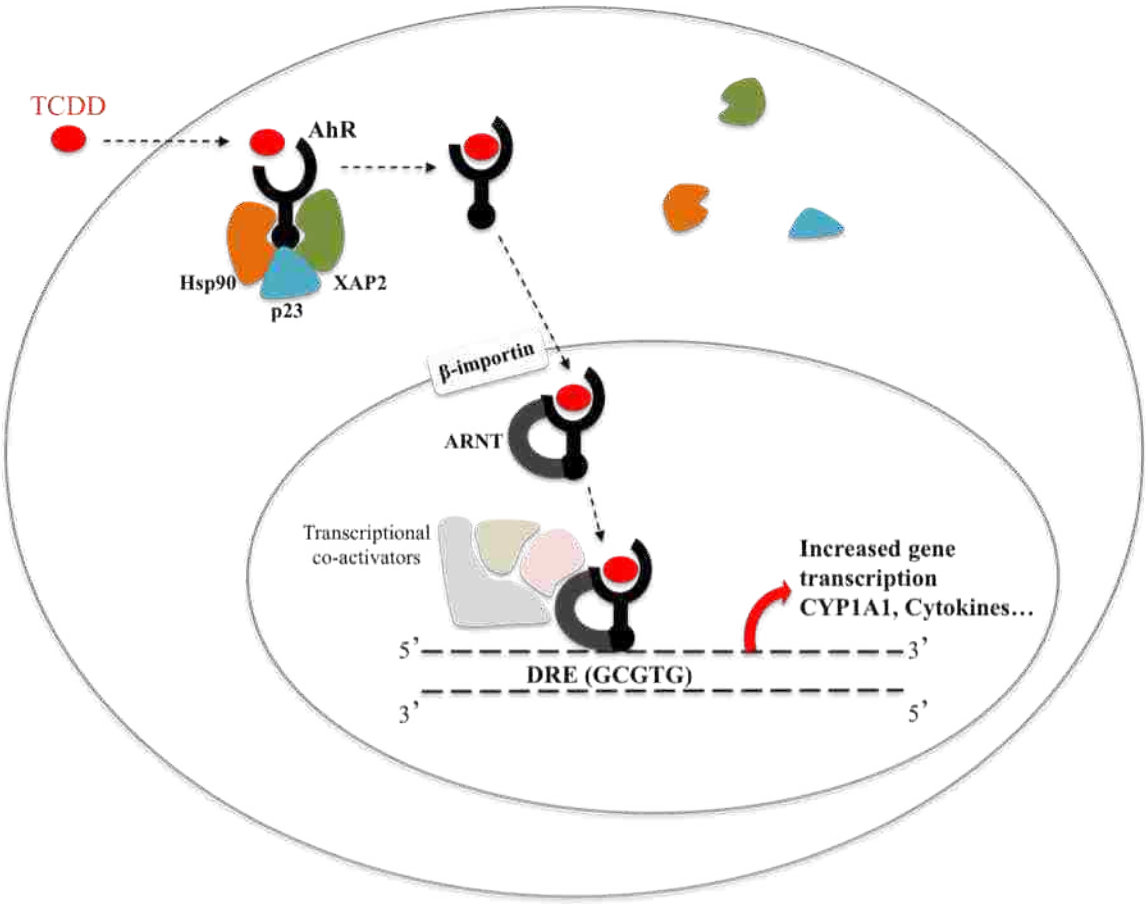

Figure 1.6. Canonical AhR signaling pathway.

Non-canonical AhR pathways are less well understood and involve interactions with multiple intracellular signaling pathways that control cell cycle, inflammation and hormone signaling (Vogel and Matsumura, 2009; Swedenborg and Pongratz, 2010; Denison *et al.*, 2011). Canonical DRE-depdendent AhR signaling can drive the expression of $p27^{kip1}$, an inhibitory cell cycle-dependent kinase (CDK) that contributes to retinoblastoma protein (Rb) inactivation and cell cycle arrest (Pang *et al.*, 2008). In a non-canonical fashion, ligand-activated AhR can directly bind Rb and disrupt Rb phosphorylation and subsequent activation required for cell cycle progression (Ma *et al.*, 2009).

Ligand-activated AhR can also physically interact with NF-κB family members and Signal Transducer and Activator of Transcription (STAT) proteins to control immune and inflammatory gene expression. Early work done by Ruby and co-workers demonstrated that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure decreases NF-κB/RelA binding and NF-κB/RelA entry into the nucleus in DC2.4 cells following TNFα or anti-CD40 activation (Ruby *et al.*, 2002). These studies also demonstrated that transfected AhR physically associated with RelA in DC2.4 cells in response to TNFα stimulation (Ruby *et al.*, 2002). Recently, Vogel and colleagues using the human macrophage cell line U937 were able to demonstrate that TCDD induces IL-8 through an AhR-dependent mechanism that involved direct interactions between AhR and RelB (Vogel *et al.*, 2007a). In these studies ligand activated-AhR was shown to physically interact with RelB, forming AhR/RelB complexes that bound DRE/NF-κB like response elements in the promoter region of the IL-8 gene (Vogel *et al.*, 2007a). In addition to AhR/NF-κB crosstalk, the AhR has been shown to interact with various STAT proteins.

In peritoneal macrophages the AhR was shown to interact with STAT1 and p50 following LPS stimulation (Kimura *et al.*, 2009). The AhR/STAT1/p50 complexes were shown to reduce LPS-induced IL-6 production by binding specific sites upstream of the IL-6 transcriptional start site and inhibiting promoter activity (Kimura *et al.*, 2009). Furthermore, under Th17 polarizing conditions Kimura and colleagues demonstrated that the AhR contributed to the induction of Th17 cells through direct physical interactions with STAT1 and STAT5 (Kimura *et al.*, 2008). AhR:STAT4 interactions have also been observed during acute graft vs. host responses in $CD4^+$ T cells following TCDD exposure (Marshall *et al.*, 2008).

Non-canonical AhR/estrogen receptor (ER) interactions and signaling have also been described. The majority of AhR:ER interactions are anti-estrogenic (Ohtake *et al.*, 2007; Swedenborg and Pongratz, 2010; Denison *et al.*, 2011) Since the AhR is a steroid-like receptor, it competes for transcriptional co-activators used by ERα and ERβ and can dampen ER-mediated gene transcription (Denison *et al.*, 2011). In a similar fashion, AhR competes for ARNT, which is also used by ERα and ERβ to enhance ER target gene transcription (Ruegg *et al.*, 2008). Ohtake and colleagues also demonstrated that ligand-activated AhR can directly interact with ERα/ERα subunits, which causes ER polyubiquitination and subsequent proteosomal degradation (Ohtake *et al.*, 2003). The ubiquitination and proteosomal degradation is due to the ability of ligand-activated AhR to recruit E3 ubiquitin ligase complexes (Ohtake *et al.*, 2003; Ohtake *et al.*, 2007). In turn, the E3 ligase complex recruits E2 which ubiquitinates ERα, effectively inducing ERα degradation through the 26S proteosomal pathway. ER degradation is not observed in AhR null mice, suggesting that the AhR is required for ER degradation following

exposure to AhR ligands (Mimura and Fujii-Kuriyama, 2003; Ohtake *et al.*, 2009). In contrast to the anti-estrogenic effects of ligand-activated AhR, AhR:ER interactions have also been shown to increase ER target gene transcription (Denison *et al.*, 2011). It is thought that ligand-activated AhR:ARNT complexes bind ERα/ERα bound to estrogen response elements (EREs), which then recruits transcriptional co-activators to enhance ER target gene expression (Ohtake *et al.*, 2009; Swedenborg and Pongratz, 2010). In a similar but reverse fashion, ERα/ERα can bind ligand:AhR:ARNT complexes bound to DREs and help recruit transcriptional regulators to control DRE-mediated gene transcription (Ohtake *et al.*, 2009; Denison *et al.*, 2011).

In addition to the above-mentioned non-canonical signaling pathways, the AhR has been described to interact with mitogen-activated and calcium/calmodulin-dependent protein kinases and Nrf2 and TGFβ signaling proteins (Gomez-Duran *et al.*, 2009; Denison *et al.*, 2011). However, AhR crosstalk with various intracellular signaling pathways tends to be cell-type and tissue-dependent. Moreover, there are AhR ligand-specific effects associated with both canonical and non-canonical AhR signaling.

AhR Ligands

Halogenated aromatic hydrocarbons (HAHs) were the first chemicals shown to bind and activate the AhR (Harris *et al.*, 1973). Within the family of HAHs, TCDD binds the AhR with the highest affinity. TCDD remains in the environment for extended periods of time and is broken down slowly through UV degradation. Moreover, TCDD is is poorly metabolized and can bioaccumulate in humans and animals, with a half-life in of approximately 7-10 years in humans and 7-10 days in mice. For these reasons, TCDD is considered the prototypical AhR ligand. Many TCDD-congeners, such as 2,3,7,8-

tetrachlorodibenozofuran and 2,3,6,7-tetrachloronapthalene are capable of binding and activating the AhR, albeit with lower affinity than TCDD (Figure 1.7). Various polycyclic aromatic hydrocarbons (PAHs) can also bind and activate the AhR. 3-methylcholanthrene, benzo[a]pyrene (BaP) and 7,12-dimethylbenzanthracene (DMBA) are common PAH AhR ligands. BaP and DMBA are unique PAHs in that their metabolic activation into carcinogenic compounds is mediated by AhR-activated CYP1A1 expression. Furthermore, various polychlorinated biphenyls (PCBs) such as 3,3',4,4',5-pentachlorobiphenyl (PCB 126) can bind and activate the AhR. In comparison to TCDD, the PAHs and PCBs listed above are approximately 10-1000 times less potent that TCDD at inducing AhR activation (Nguyen and Bradfield, 2008). These anthropogenic compounds are considered exogenous AhR ligands.

Figure 1.7

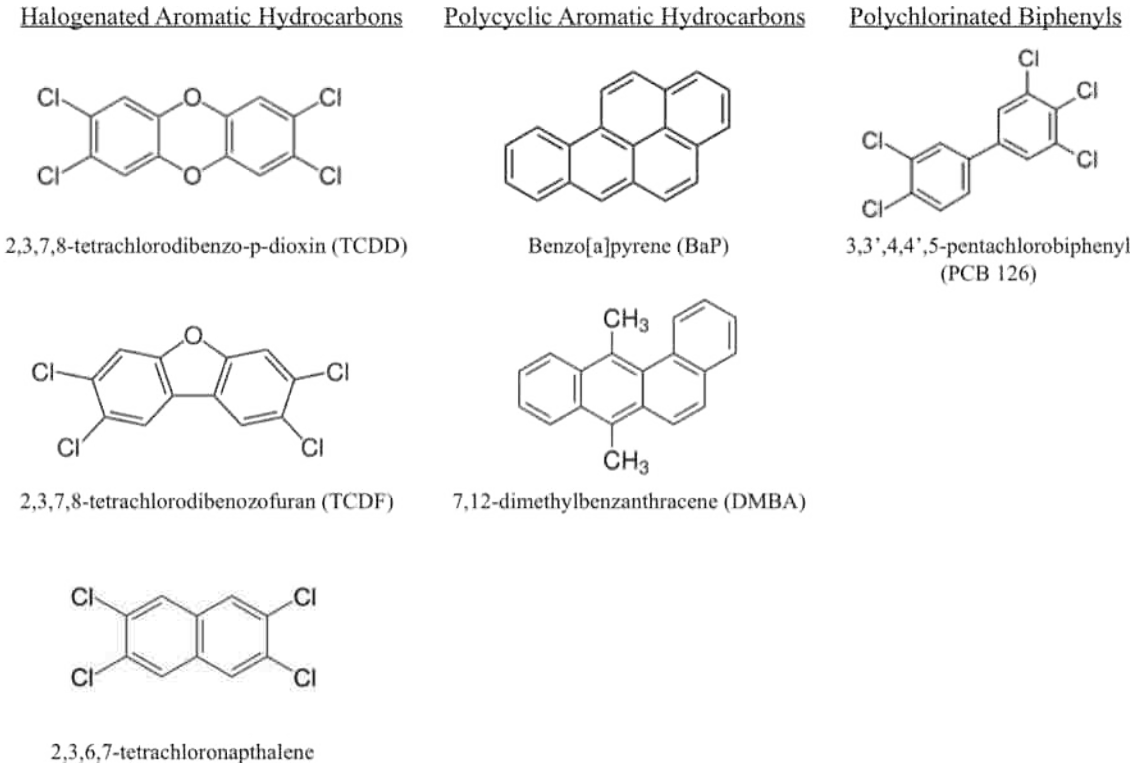

Figure 1.7. Environmental pollutants and AhR ligands.

Recently, many endogenous and natural AhR ligands have also been described. Some of the more common endogenous ligands include indirubin, bilirubin, and the arachidonic acid metabolites lipoxin A4 and prostaglandin G2 (Nguyen and Bradfield, 2008; Stejskalova *et al.*, 2011). The catabolism of tryptophan by IDO produces kynurenine that is capable of activating the AhR (Nguyen and Bradfield, 2008; Mezrich *et al.*, 2010). In addition, the tryptophan UV- and visible light-catabolite, 6-formylindolo[3,2-b]carbazole (FICZ), has an affinity for the AhR close to that of TCDD (Oberg *et al.*, 2005; Wincent *et al.*, 2009). 2-(1'H-Indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) also has a high AhR affinity, but it is not clear whether ITE is an endogenous AhR ligand or an artifact produced during isolation from biological tissues (Henry *et al.*, 2006; Nguyen and Bradfield, 2008). AhR ligands are also found in natural dietary sources. Known natural AhR ligands include compounds found in cruciferous vegetables such as indole-3-carbinol (I3C) and 3,3'-diindolymethane (DIM) and various flavones such as quercetin and genistein that are found in a variety of fruits and vegetables (Nguyen and Bradfield, 2008; Kerkvliet, 2009). Furthermore, various pharmacological compounds including: leflunomide, omeprazole, lansoprazole, primaquine and VAF347 have been shown to bind and activate the AhR (Hauben *et al.*, 2008; Kerkvliet, 2009; Stejskalova *et al.*, 2011).

A number of AhR antagonists have also been described. Within the flavone compound group, α-naphthoflavone is a known AhR antagonist, while β-naphthoflavone is an AhR agonist (Stejskalova *et al.*, 2011). Resveratrol, a polyphenolic antioxidant compound found primarily in grapes, is a well known AhR antagonist (Stejskalova *et al.*, 2011). New synthetic AhR antagonists have also been developed such as CH223191,

GNF351 and SR1 (Denison *et al.*, 2011). Many of the well-known and newly synthesized AhR antagonists work as ligand-dependent antagonists. Ligand-dependent antagonists block specific classes of AhR ligands while permitting other classes to freely bind and activate the AhR. For example, α-naphthoflavone and CH223191 effectively inhibit TCDD and other HAHs from binding and activating the AhR, while allowing different flavonoids, indirubin and various PAHs to bind and activate the AhR (Zhao *et al.*, 2010; Smith *et al.*, 2011). In contrast, GNF351 is a complete AhR antagonist, in that it effectively inhibits all AhR binding and activation no matter the class of AhR ligand (Smith *et al.*, 2011). StemRegenin 1, known as SR1, was shown to greatly enhance the proliferation and engraftment of human $CD34^+$ hematopoietic stem cells by antagonizing the AhR (Boitano *et al.*, 2010). SR1 effectively inhibited TCDD-mediated CYP1B1 gene expression and was shown to directly interact with the AhR in competitive binding assays with indirubin, suggesting that SR1 may be a complete AhR antagonist (Boitano *et al.*, 2010).

Another class of AhR ligands has been recently described as selective AhR modulators (SAhRMs). SAhRMs bind and activate the AhR in a pathway specific fashion. For example, the known AhR agonists DIM and 6-methyl-1,3,8-trichlorordibenzofuran (MCDF) were shown to work as SAhRMs by activating the AhR and exclusively inducing anti-estrogenic effects without inducing canonical AhR signaling and CYP1A1 expression (Denison *et al.*, 2011). Moreover, binding and activation of the AhR with the SAhRMs 3',4'-dimethoxy-α-naphthoflavone (DMF), SGA360 or WAY-169916 causes suppression of acute phase proteins in an AhR-dependent, but AhR:ARNT:DRE-independent fashion (Murray *et al.*, 2010). The exact

mechanisms of action of SAhRMs are currently unknown. Moreover, there exist interspecies differences in the binding affinity and relative involvement of non-canonical and canonical AhR pathways with various AhR agonist, antagonists and SAhRMs.

AhR polymorphisms and AhR$^{-/-}$ and Mutant Mice

In mice there exists a ~10-fold difference in TCDD sensitivity between different strains (Ema *et al.*, 1994; Okey *et al.*, 2005). This difference occurs between mouse strains on a low affinity background such as DBA2 mice and high affinity backgrounds such as C57BL/6 mice (Ema *et al.*, 1994). DBA2 mice express an allelic variation termed AhR^{d}. The AhR^{d} low affinity receptor has two single nucleotide polymorphisms (SNPs), one at codon 375, which causes a valine to alanine variant and a second in the stop codon, which lengthens the C-terminal end of the AhR protein as compared to the C57BL/6 AhR^{b} encoded protein (Ema *et al.*, 1994). The high affinity background in CD57B/6 (AhR^{b-1}) and Balb/c (AhR^{b-2}) mice is the dominant responsive allele. Interestingly, sequencing studies have shown that most humans carry an equivalent low affinity AhR^{d} with an equivalent valine to alanine SNP in codon 381 (Okey *et al.*, 2005). The AhR is highly conserved evolutionarily and mammalian species as diverse as rats, guinea pigs, hamsters and humans all harbor AhR polymorphisms similar to DBA2 mice (Garte and Sogawa, 1999; Harper *et al.*, 2002; Okey *et al.*, 2005).

AhR knockout mice (AhR$^{-/-}$) have been integral in defining the AhR signaling pathways and the toxicity of various AhR ligands. AhR$^{-/-}$ mice were developed in 1996 in two separate laboratories (Fernandez-Salguero *et al.*, 1996; Schmidt *et al.*, 1996). Since the development of AhR$^{-/-}$, additional AhR mutant mice including AhR$^{dbd/dbd}$ and AhR$^{nls/nls}$ mice have been generated to gain a better understanding of AhR signaling and

ligand-specific affects (Bunger *et al.*, 2003; Bunger *et al.*, 2008). $AhR^{dbd/dbd}$ mice have a mutation in the DNA binding domain (DBD) sequence of the AhR, disrupting AhR binding to DREs within target genes (Bunger *et al.*, 2008). These mice have been essential in describing DRE-independent signaling events. $AhR^{nls/nls}$ mice carry a mutation in the nuclear localization sequence (NLS) of the AhR; hence the AhR doesn't traffic into the nucleus (Bunger *et al.*, 2003). $AhR^{nls/nls}$ mice have been used to define non-canonical signaling events that occur in the cytoplasm, such as the increase in Ca flux following TCDD exposure, and to better understand the requirement for AhR/nuclear protein interactions. Recently, conditional $AhR^{-/-}$ mice have been developed, in which the the AhR allele is floxed by loxP sites and upon crossing with Cre expressing mice the AhR can be eliminated in specific tissues or cell types (Walisser *et al.*, 2005).

AhR Associated Toxicity in Humans

TCDD is the most widely studied AhR ligand and over the last half century a large amount of data has been collected regarding its toxicity in humans. Humans are usually exposed to low levels of TCDD and other AhR ligands mainly through the food chain, especially from through foods rich in fats (Sweeney and Mocarelli, 2000; Baccarelli *et al.*, 2002; White and Birnbaum, 2009). It is important to note that TCDD has a long half-life in humans (~7-10 years) and can accumulate in adipose tissue. Thus, daily exposures can cause chronic long-term health affects. However, the majority of human data regarding TCDD exposure comes from retrospective studies following acute industrial accidental exposures. The first recorded exposure to TCDD was in 1949 following a trichlorophenol reactor explosion in Nitro, West Virginia. Between 1962 and

1970 the American military used a TCDD-contaminated herbicide known as Agent Orange. Consequently, service men were exposed to high levels of TCDD throughout the course of the Vietnam War. In 1971 to keep dust levels in check, roads in Times Beach, Missouri were sprayed with TCDD-contaminated oil and people and animals within the surrounding area came into direct contact with TCDD. Workers as well as townspeople were exposed to high levels of TCDD following an explosion at a chemical plant in Seveso, Italy in 1976. More recently in 2008, pork products from Ireland were recalled worldwide due to a TCDD contamination from the animal feed. In addition, levels of TCDD can be higher in farmed raised fish, particularly salmon, when compared to wild caught fish (Bethune *et al.*, 2006). Assessing populations from these exposures as well as many others, TCDD exposure has been shown to cause chloracne, a skin condition characterized by excessive blackheads and fluid filled cysts, cardiovascular disease, cancer, diabetes and reproductive and immune system aberrations (Sweeney and Mocarelli, 2000; White and Birnbaum, 2009). The best-documented and studied endpoint in humans exposed to TCDD is cancer. Retrospective cancer studies have been conducted in nearly all TCDD exposed cohorts and collectively the data shows a significant increase in all-cancer risk following TCDD exposure (White and Birnbaum, 2009; Boffetta *et al.*, 2011). Subsequently, the International Agency for Research (IARC) has classified TCDD as a known human carcinogen (NTP, 2006).

AhR Activation and Immune Modulation

Similar to the effects seen in humans, TCDD-induced AhR activation in animals has been shown to cause various cancers, liver injury, cardiovascular inflammation and damage, adverse developmental and reproductive effects, skin abnormalities and immune

alterations. With the use of various animal models, many of the mechanisms underlying the toxic effects of TCDD-induced AhR activation have been firmly established. While there is a considerable amount of research, both past and present, regarding TCDD-induced immune alterations, it is still not fully understood how AhR activation alters immune responses. Perhaps more importantly, the doses of TCDD and TCDD-congeners required to initiate cancer and organ-specific toxicities are well above day-to-day, physiological relevant, exposure levels. In contrast, the immune system has been shown to be highly sensitive to low dose TCDD exposures as well as other AhR ligands (Kerkvliet, 2002; Kerkvliet, 2012).

Over the last 35 years, TCDD and TCDD-congeners as well as prominent PAHs, such as BaP, have been shown to disrupt both innate and adaptive immune responses in various animal models. Early work done by Vos and Thigpen demonstrated that mice exposed to TCDD had an increased susceptibility to bacterial and viral infections (Harris *et al.*, 1973; Thigpen *et al.*, 1975). For example, influenza infections are exacerbated in mice exposed to TCDD, leading to an increased rate of mortality in mice exposed to TCDD (Burleson *et al.*, 1996; Vorderstrasse *et al.*, 2003). In fact, influenza host resistance is one of the most sensitive readouts of TCDD-induced immune suppression, with effects seen with doses as low as 10ng/kg TCDD (House *et al.*, 1990; Burleson *et al.*, 1996; Warren *et al.*, 2000). In response to influenza A, TCDD-exposed mice display lower IgM, IgG1, IgG2a and IgG2b levels in the plasma, decreased percent and number of $CD4^+$ and $CD8^+$ viral-specific lymphocytes in lung draining lymph nodes and dampened lung DC function (House *et al.*, 1990; Burleson *et al.*, 1996; Warren *et al.*, 2000; Jin *et al.*, 2010). Similar to decreased immune responses to influenza, TCDD has

been shown to quell immune responses in a P815 tumor graft vs. host model (Kerkvliet *et al.*, 1996). Following transfer of allogeneic P815 mastocytoma cells into C57BL/6 mice, TCDD-exposed mice displayed decreased IgM and IgG2a P815-specifc antibody levels and dampened $CD4^+$ Th1-mediated and $CD8^+$ CTL-mediated responses (Kerkvliet *et al.*, 1996). Furthermore, *ex vivo* spleoncytes from P815-injected mice produced significantly less IL-2 and IFNγ in response to P815 cells (Kerkvliet *et al.*, 1996). Interestingly, the IL-2 gene in mice contains functional DREs in its promoter region and increased IL-2 levels in response to TCDD exposure have been observed in different experimental systems (Kerkvliet, 2002; Kerkvliet, 2009). Taken together, data from host resistance and graft vs. host models as well as other models have shown that TCDD exposure significantly suppresses adaptive immune responses through direct effects on T cell, B cells and DCs. More recently, exposure to low doses of TCDD as well as other AhR ligands has been shown to induce the generation of functional regulatory T cells that are capable of suppressing ongoing immune responses (Funatake *et al.*, 2005b; Marshall *et al.*, 2008; Quintana *et al.*, 2008b; Quintana *et al.*, 2010).

Investigating the P815 graft vs. host further, Kerkvliet and colleagues demonstrated that TCDD exposure induced $CD4^+$ $CD25^+$ $CD62L^{low}$ T cells that suppressed the proliferation of naïve $CD4^+$ $CD25^-$ T cells *in vitro*, indicating that these TCDD-induced $CD4^+$ $CD25^+$ $CD62L^{low}$ T cells were regulatory in function (Funatake *et al.*, 2005b; Marshall *et al.*, 2008). This was a novel finding, in that AhR activation following TCDD exposure leads to the generation of Tregs, which may be responsible for dampening the acute graft vs. host inflammatory response. Seminal studies by Weiner and co-workers showed that TCDD exposure generated $Foxp3^+$ Tregs in an autoimmune

mouse model of multiple sclerosis (Quintana *et al.*, 2008b). Mice treated with TCDD and immunized with myelin oligodendrocyte glycoprotein (MOG) with complete Freund's adjuvant (CFA) to induce experimental autoimmune encephalomyelitis (EAE) displayed significant increases in $CD4^+$ $Foxp3^+$ Tregs when compared to vehicle-treated mice (Quintana *et al.*, 2008b). Using $Foxp3^{GFP}$ reporter mice it was demonstrated that a portion of naïve $CD4^+$ T cells converted to $CD4^+$ $Foxp3^+$ Tregs following TCDD exposure (Quintana *et al.*, 2008b). However, FICZ, the UV tryptophan catabolite shown to bind the AhR with similar affinity as TCDD, promoted the generation of Th17 cells *in vitro* instead of $Foxp3^+$ Tregs (Quintana *et al.*, 2008b). These studies demonstrated that TCDD-induced AhR activation leads to the generation of suppressive $CD4^+$ $Foxp3^+$ Tregs. In contrast, FICZ-induced AhR activation may promote the generation of inflammatory Th17 cells (Quintana *et al.*, 2008b). In addition to T cells, the effects of AhR activation in DCs and the subsequent generation of tolerogenic DCs are currently under intense investigation.

AhR Activation in DCs

AhR is constitutively expressed in DCs, while it is only expressed in lymphocytes following activation. It is likely that following exposure to an AhR ligand such as TCDD that DCs will be amongst the first cells in the immune system to respond and display AhR-activated alterations. Studies using murine DCs have shown that AhR activation in DCs causes both phenotypic and functional alterations (Hwang *et al.*, 2007; Vogel *et al.*, 2008; Bankoti *et al.*, 2010b; Simones and Shepherd, 2011). In U937 monocyte-derived DCs, TCDD and FICZ augmented MHC class II and CD86 expression and upregulated the expression of the immunoregulatory enzymes IDO1 and IDO2 (Vogel *et al.*, 2008).

Bankoti and colleagues demonstrated that TCDD exposure in murine inflammatory DCs generated with GM-CSF TCDD exposure altered DC differentiation and increased IDO and TGFβ gene expression, while altering NF-κB signaling in an AhR dependent fashion (Bankoti *et al.*, 2010b). Similar to TCDD, FICZ, BaP, ITE and VAF347 exposure has been shown to disrupt murine DC surface molecule expression and gene expression, inducing a potential tolerogenic DC phenotype (Hwang *et al.*, 2007; Bankoti *et al.*, 2010b; Quintana *et al.*, 2010; Simones and Shepherd, 2011). While a number of studies have demonstrated that AhR activation can modulate DC phenotype and alter intracellular signaling, to date only two studies have shown that AhR activation in DCs leads to the induction of regulatory DCs capable of inducing regulatory T cells and/or dampening autoimmune disease responses.

Studies using VAF347, a high-affinity pharmaceutical ligand for the AhR, have shown that VAF347-treated bone marrow-derived DCs (BMDCs) when injected into mice suppress the rejection of grafted pancreatic islet cells and increases graft survival rates (Hauben *et al.*, 2008). Furthermore, when VAF347-treated BMDCs were injected into mice immunized with ovalbumin (Ova) in CFA they generated anergic T cells that remained refractory to restimulation with Ova (Hauben *et al.*, 2008). These data suggest that AhR activation in DCs alone can suppress T cell mediated immune responses. Corroborating this idea, Quintana and colleagues demonstrated that GM-CSF + IL-4-derived BMDCs exposed to ITE and loaded with MOG-specific peptide and transferred into mice pre-EAE induction, dampened clinical EAE severity and increased the percent of splenic $CD4^+$ $Foxp3^+$ Tregs, while decreasing $CD4^+$ IL-17 and $CD4^+$ IFNγ populations (Quintana *et al.*, 2010). They also showed that splenic DCs from ITE-exposed mice and

ITE-exposed GM-CSF + IL-4-derived BMDCs were capable of inducing $CD4^+$ $Foxp3^+$ Tregs from naïve MOG-specific $2D2^+$ $CD4^+$ T cells (Quintana *et al.*, 2010). These studies demonstrated that AhR activation with ITE can directly generate tolerogenic DCs that are functionally suppressive. To date, it is unknown if TCDD-induced AhR activation in DCs generates tolerogenic DCs and whether DCs are responsible for the observed immune suppression and generation of regulatory T cells following exposure to TCDD.

Hypothesis

The aryl hydrocarbon receptor (AhR) is a steroid-like transcription factor that mediates the toxicity of various environmental pollutants, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and TCDD-like compounds. Exposure to environmentally relevant levels of TCDD and TCDD congeners have been shown to cause immune suppression and influence the course of an immune response by altering effector T cell responses and through the induction of regulatory T cells (Tregs) (Marshall *et al.*, 2008; Quintana *et al.*, 2008b; Quintana *et al.*, 2010). However, the mechanisms underlying TCDD-induced immune suppression and Treg induction remain poorly understood. Dendritic cells (DCs) are professional antigen presenting cells (APCs) that play an integral role in both innate and adaptive immune responses. DCs constitutively express high levels of the AhR and represent a highly sensitive immune cell population to AhR activation by environmental pollutants (Kerkvliet, 2009; Bankoti *et al.*, 2010b; Marshall and Kerkvliet, 2010). DCs can differ in phenotype and function depending on the tissue location, environmental milieu and ongoing immune responses (Banchereau *et al.*, 2000). DCs are commonly classified as steady-state DCs or

inflammatory DCs (Shortman and Naik, 2007b; Naik, 2008b). Steady-state DCs exist in various tissues as sentinels and primary APCs; where as inflammatory DCs arise in response to inflammation and contribute to inflammatory responses. Both DC groups contain various subpopulations with specialized functions, including immune regulation (Naik *et al.*, 2005; Shortman and Naik, 2007b; Naik, 2008b). AhR activation has been characterized in murine inflammatory BMDCs, yet little is known about the consequences of AhR activation in steady-state DCs (Lee *et al.*, 2007; Bankoti *et al.*, 2010b). Using a GM-CSF murine bone marrow-derived DCs (BMDCs) model to generate inflammatory DCs, Bankoti and co-workers demonstrated that TCDD-induced AhR activation altered DC differentiation, NF-κB signaling, TLR-induced cytokine production and antigen uptake (Bankoti *et al.*, 2010b). TCDD-treated inflammatory BMDCs also displayed significant increases in regulatory gene expression, suggesting that AhR-activation leads to the generation of tolerogenic DCs that could potentially alter effector T cell responses (Bankoti *et al.*, 2010b). However, it is unclear whether TCDD-induced AhR activation in inflammatory BMDCs alters their functional capacity to elicit antigen-specific immune responses. Moreover, the effects of AhR activation in steady-state DCs, both *in vitro* and *in vivo*, remain to be defined. **We hypothesize that AhR-activation will alter DC function and that TCDD-induced AhR activation in DCs leads to the induction of tolerogenic DCs capable of dampening T cell responses.** Our studies will investigate the role of AhR activation in both inflammatory and steady-state BMDCs to determine if AhR activation induces tolerogenic DCs *in vitro* and investigate whether exposure to TCDD dampens antigen-specific immune responses *in vivo.* Ultimately our goal is to better understand the underlying mechanisms involved in the

observed immunosuppression following exposure to TCDD and TCDD-like chemicals. This research is important in that it will significantly extend our understanding of how environmental pollutants capable of activating the AhR can disrupt the immune system, and permit better risk identification and exposure characterization.

Specific Aim 1: Characterize the effects of AhR activation in steady-state DCs. (Chapter 2)

We hypothesized that AhR activation alters the differentiation and function of steady-state BMDCs. To test this hypothesis we determined whether AhR activation disrupts steady-state DC surface molecule expression, responsiveness to TLR agonists, NF-κB binding activity, antigen uptake and regulatory gene expression and examined the capacity of AhR-activated steady-state DCs to elicit antigen-specific immune responses *in vitro* and *in vivo*.

Specific Aim 2: Determine whether AhR activation in inflammatory DCs leads to the generation of tolerogenic DCs capable of altering effector T cell responses. (Chapter 3)

We hypothesized that TCDD-induced AhR activation in inflammatory BMDCs induces tolerogenic DCs that are capable of dampening effector T cell responses. To test this hypothesis we examined antigen-specific OTII $CD4^+$ T cell responses following interactions with AhR-activated Ovap-loaded inflammatory BMDCs.

<u>Specific Aim 3:</u> Determine whether *in vivo* exposure to TCDD leads to the generation of Foxp3$^+$ Tregs and tolerogenic DCs during antigen-specific immune responses. (Chapter 4)

We hypothesized that AhR activation by TCDD generates both tolerogenic DCs and Tregs *in vivo*. To test this hypothesis we utilized two separate *in vivo* OTII CD4$^+$ T cell models, in which mice were exposed to vehicle or TCDD and the generation of Ovap-specific Foxp3$^+$ T cells and tolerogenic DCs analyzed following Ovap-DC footpad immunizations or Ovap/adjuvant immunizations.

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CHAPTER 2

Consequences of AhR Activation in Steady-State Dendritic Cells

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ABSTRACT

TCDD is the prototypical AhR ligand and a potent immunotoxicant. However, the mechanisms underlying TCDD-induced immunomodulation remain to be defined. Dendritic cells (DCs) are professional antigen presenting cells (APCs) that constitutively express the AhR and are sensitive to TCDD-induced AhR activation. We hypothesized that AhR activation alters the differentiation and function of steady-state BMDCs. To test this hypothesis, steady-state bone marrow-derived DCs (BMDCs) from C57BL/6 mice were grown in the presence of TCDD or vehicle. TCDD-treated steady-state BMDCs (TCDD-BMDCs) displayed decreased expression of CD11c and CD11a, while increasing the frequency of MHC class II, CD86, CD80, and CD54. Similar phenotypic alterations were observed with the AhR ligands 6-formylindolo[3,2-b]carbazole (FICZ) and 2-(1H-indole-3'-carbonyl)-thiazole-4-carboxylic acid (ITE). TCDD-BMDCs from $AhR^{-/-}$ mice were refractory to TCDD-induced surface marker alterations, whereas TCDD-BMDCs from $AhR^{dbd/dbd}$ mice displayed similar phenotypic alterations as $AhR^{+/+}$ TCDD-BMDCs. Following LPS, CpG or Imiquimod stimulation, TCDD-BMDCs secreted less IL-6, TNFα, IL-10 and IL-12. TCDD also altered NF-κB family member binding activity in unstimulated and LPS- or CpG-stimulated steady-state BMDCs. The internalization of the soluble antigens, ovalbumin and acetylated LDL, was decreased while internalization of latex beads was increased in TCDD-BMDCs when compared to vehicle-BMDCs. TCDD-BMDCs displayed increased mRNA expression of the regulatory gene IDO2 and following LPS stimulation upregulated IDO1, IDO2, TGFβ1 and TGFβ3 gene expression. Additionally, TCDD-BMDCs increased the generation of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs *in vitro* in an IDO-dependent fashion. However, TCDD-treated BMDCs

did not alter antigen-specific T cell activation *in vivo*. Overall, TCDD-induced AhR activation alters the differentiation, activation, innate and immunoregulatory function, but not the T cell-activating capacity of steady-state BMDCs.

INTRODUCTION

Many environmental pollutants exert their toxic effects via activation of the aryl hydrocarbon receptor (AhR). The ligand-activated AhR translocates into the nucleus where it binds the AhR nuclear translocater (ARNT). Subsequently, in the canonical signaling pathway the ligand-bound AhR/ARNT heterodimeric complex binds target genes containing dioxin response elements (DREs) and modulates gene expression. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical AhR ligand and a potent environmental toxicant. Low level TCDD exposure causes immune suppression in a number of animal species and increases susceptibility to infections and cancer (Kerkvliet, 2002). The immunosuppressive effects of TCDD, as well as many other TCDD-like chemicals, are predominantly mediated via the AhR. Within the immune system, TCDD-induced AhR activation affects many cell populations, including antigen presenting cells (APCs) and $CD4^+$ T cells (Vorderstrasse and Kerkvliet, 2001a; Kerkvliet, 2002; Kerkvliet *et al.*, 2002; Funatake *et al.*, 2004; Ruby *et al.*, 2005; Hauben *et al.*, 2008; Lawrence *et al.*, 2008; Jin *et al.*, 2010). Recently, the induction of regulatory T cells (Tregs) has been linked to TCDD-induced AhR activation, and may underlie the immunosuppressive effects of TCDD. (Funatake CJ, 2005; Quintana FJ, 2008). In contrast, the high-affinity natural AhR ligand 6-formylindolo[3,2-b]carbozole (FICZ) has been shown to induce Th17 cells during the course of autoimmune encephalomyelitis in mice (Quintana FJ, 2008). However, Kimura et al. demonstrated that in the presence of TGFβ both TCDD and FICZ induce $Foxp3^+$ Tregs in an AhR-dependent manner (Kimura *et al.*, 2008). While TCDD-induced AhR activation has been characterized in T cell populations, less is known about the role of AhR activation in dendritic cells.

Dendritic cells (DCs) are professional APCs that function in both the innate and adaptive branches of the immune system. DCs act as sentinels to survey and detect foreign pathogens and can elicit multifarious immune responses. As part of their innate functions, DCs recognize pathogen-associated molecular patterns through pathogen recognition receptors, such as Toll-like Receptors (TLRs), and can help clear extracellular pathogens through phagocytosis. Following pathogen recognition, DCs become activated and increase their expression of various accessory molecules including MHC class I and class II, CD80, CD86 and CD54. Activated DCs secrete cytokines that tailor the generation of both innate and adaptive immune responses. DCs are considered "professional APCs" based on their constitutive expression of accessory molecules, mobility, and ability to internalize, process and present antigens to T cells. In unimmunized animals, DCs typically exist as pre-DCs or steady-state DCs (Shotman and Naik, 2007; Geissmann *et al.*, 2010). Pre-DCs do not display a DC phenotype, but have the capacity to develop into DCs upon inflammatory or pathogenic encounter (Shotman and Naik, 2007; Naik, 2008a; Geissmann *et al.*, 2010). These DCs are referred to as "inflammatory DCs" or "TNF and iNOS producing DCs" (Tip DCs) (Naik, 2008a; Geissmann *et al.*, 2010). *In vitro*, GM-CSF is used to generate DCs, which model inflammatory/Tip DCs *in vivo* (Shotman and Naik, 2007; Wu L, 2007; Xu Y, 2007; Kimura *et al.*, 2008; Naik, 2008a). In contrast, immature steady-state DCs consist of both migratory and lymphoid-tissue-resident conventional DC populations (Shotman and Naik, 2007; Naik, 2008a). The generation of steady-state DCs *in vitro* is achieved using the growth factor Fms-like tyrosine kinase 3 ligand (Flt3L) and represent DCs residing in

peripheral immune tissues (Inaba *et al.*, 1992; Brasel *et al.*, 2000; Naik SH, 2007; Vremec D, 2008).

Previously, Vorderstrasse et al. reported that splenic $CD11c^{high}$ DC numbers were reduced and their immunophenotypes altered in mice exposed to immunosuppressive doses of TCDD (Vorderstrasse and Kerkvliet, 2001a; Vorderstrasse *et al.*, 2002). Bankoti and colleagues further characterized AhR activation in naïve DCs, both in the spleen and peripheral lymph nodes, and demonstrated that TCDD selectively affected splenic $CD11c^{high}$ $CD8\alpha^{-}$ $33D1^{+}$ DCs, but not the $CD11c^{high}$ $CD8\alpha^{+}$ $DEC205^{+}$ DCs (Bankoti *et al.*, 2010a). In inflammatory bone marrow-derived DCs (BMDCs) AhR activation induced surface marker alterations, impaired antigen uptake and enhanced secretion of TNFα and IL-6, following stimulation with LPS or CpG (Bankoti *et al.*, 2010c). Moreover, TCDD-induced AhR activation in inflammatory DCs caused NF-κB p65 binding to decrease, while upregulating RelB binding (Bankoti *et al.*, 2010c). Surprisingly, TCDD did not alter the ability of inflammatory BMDCs to activate antigen-specific $CD4^{+}$ T cells *in vivo* (Bankoti *et al.*, 2010c).

In this study, we have evaluated the role of AhR activation in steady-state DCs. We hypothesized that similar to inflammatory BMDCs, AhR activation would modulate the phenotype and function of steady-state BMDCs. Phenotypic status, TLR responsiveness, NF-κB activity, gene expression, antigen uptake and $CD4^{+}$ T cell stimulatory capacity were assessed and steady-state BMDCs from AhR null and AhR DRE-binding deficient mice were used to mechanistically determine the role of the AhR in steady-state BMDC function. Data from this study advance our understanding of how

AhR activation modulates conventional DCs, an effect that undoubtedly contributes to immune modulation following exposure to AhR ligands.

METHODS AND MATERIALS

Animals 6-8 week old male and female C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred at the University of Montana (UM). $AhR^{-/-}$ mice (Schmidt *et al.*, 1996) were a kind gift from Dr. Paige Lawrence (University of Rochester Medical Center, Rochester, NY). $AhR^{dbd/dbd}$ mutant mice (Bunger *et al.*, 2008) were generously provided by Dr. Chris Bradfield and Dr. Ed Glover (University of Wisconsin, Madison, WI). OTII $Foxp3^{eGFP}$ mice were kindly provided by Dr. Randolph Noelle (Dartmouth Medical School, Lebanon, NH) who originally obtained these mice from Dr. Alexander Rudensky (University of Washington School of Medicine, Seattle, WA). All animals were housed and maintained at UM and provided water and chow ad libitum. All animal experiments were approved by the UM IACUC and adhered to the current NIH guidelines for animal usage.

Chemicals and Reagents TCDD was purchased from Cambridge Isotope Laboratories (Andover, MA). FICZ was purchased from BIOMOL International (Plymouth Meeting, PA). 2-(1H-indole-3'-carbonyl)-thiazole-4-carboxylic acid (ITE) was obtained from Tocris Bioscience (Ellisville, MO). All AhR ligands were supplied or suspended in tissue culture grade DMSO (Sigma-Aldrich St Louis, MO). Bone marrow-derived dendritic cells (BMDCs) were grown in complete RPMI (cRPMI): 20mM HEPES, 1.5mM sodium pyruvate, 50ug/ml gentamicin and 10% fetal bovine serum (Hyclone Thermo Fisher Scientific Waltman, MA). BMDC:T cells were cultured in cRPMI or F10 media: F-10 Nutrient Mixture, 20mM HEPES, 50ug/mL gentamicin, and 10% fetal bovine serum (Hyclone). All other media reagents were obtained from Invitrogen (Carlsbad, CA).

Whole chicken ovalbumin (Ova; Grade V) and 1-methyl-DL-tryptophan (1-MT) were purchased from Sigma-Aldrich. Ova-peptide, $Ova_{323-339}$, was obtained from Mimotopes (Clayton, Victoria Australia). LPS (Escherichia coli; 055:B5) was obtained from Sigma-Aldrich and CpG (ODN 1826; type B) and Imiquimod (R837) were purchased from Invivogen (San Diego, CA).

Generation of Steady-State BMDCs BMDCs were generated using methods as previously described (Inaba *et al.*, 1992; Brasel *et al.*, 2000). Briefly, bone marrow cells from the tibia and femur were flushed using cRPMI. Progenitor cells were separated from RBCs and debris by centrifugation using lympholyte M (Cederlane Laboratories; Burlington, NC) and were grown in cRPMI supplemented with 10% conditioned media and 300ng/mL human fms-like tyrosine kinase 3 ligand (Flt3L) purchased from PeproTech (Rocky Hill, NJ). The conditioned media was generated by culturing splenocytes from unmanipulated C57Bl/6 mice in cRPMI for 10 days. Levels of IL-6 in the conditioned media were used standardize each batch and ranged between 2 - 5ng/mL. On day 5, non-adherent cells were removed, washed and reseeded in fresh media containing Flt3L, conditioned media and vehicle or TCDD. On day 10 non-adherent and adherent immature BMDCs were harvested, enumerated and cell viability assessed using Trypan Blue (Sigma-Aldrich). Unless specified, BMDCs were grown in the presence of 10nM TCDD or vehicle control (0.01% DMSO), treatments that caused no significant effects on BMDC viability (data not shown).

Phenotypic Analysis BMDCs were analyzed by flow cytometry as described previously (Shepherd *et al.*, 2001). Briefly, $1x10^5$ - $1x10^6$ cells were washed with PBS containing 1% bovine serum albumin and 0.1% NaN_3 and resuspended in IgG to block non-specific binding (Jackson Laboratory; Bar Harbor, ME). The cells were then stained with fluorochrome-conjugated antibodies for 10min on ice. Appropriate isotype controls were used in conjunction with the primary antibody staining. The following antibodies were used: CD11c-APC (N418) from Invitrogen; MHC class II-PE (M5/114.15.12), CD80-PE (16-10A1), CD86-Pacific Blue (GL-1) from Biolegend (San Diego, CA); and CD54-FITC (3E2), CD11a-PECy7 (2D7) from BDPharmingen (San Diego, CA). Samples were analyzed on a FACSAria flow cytometer using BD FACS Diva software, version 4.0 (BD Biosciences San Jose, CA). Figures were generated using FlowJo, version 8.8.6 (Tree Star Inc, Ashland OR).

TLR Ligand Activation and Cytokine Measurement Immature BMDCs grown in the presence of TCDD or vehicle were aliquoted into six-well plates (Corning St. Louis, MO), at a density of $1x10^6$ cells/mL and stimulated with 1μg/ml LPS (Sigma-Aldrich), 0.5μM CpG (Invivogen) or 30μg/ml Imiquimod (Invivogen) for 24h. Following stimulation, BMDCs were harvested and analyzed via flow cytometry as described above. Supernatants from individual samples were collected and stored at -20^{o}C until further analysis. TNFα, IL-6, IL-10 and IL-12p70 cytokine production was measured using ELISAs per the manufacturer's instructions (BD Biosciences).

NF-κB Activity Steady-state BMDCs ($2x10^6$) were seeded into six-well plates and stimulated with LPS (1μg/mL) or CpG (0.5μM) for 45min. The cells were then harvested washed and nuclear extracts isolated using the Active Motif's Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein levels from the nuclear extracts were quantified using a BCA protein assay (Pierce, Rockford, IL). The binding activity of NF-κB family members (p65, p50, p52, and RelB) was measured using the Active Motif TransAM NF-κB Family kit per the manufacturer's instructions.

Quantitative Real-Time Reverse Transcription-polymerase Chain Reaction (qRT-PCR) To determine the levels of gene expression in BMDCs, qRT-PCR studies were conducted as previously described (Bankoti *et al.*, 2010c). BMDCs were harvested on day 10, washed twice in PBS and resuspended in Trizol (Invitrogen) to isolate RNA. Primers for indoleamine 2,3-dioxygenase 1 (Ido1) and Ido2, transforming growth factor β1 (Tgfβ1), Tgfβ2 and Tgfβ3, latent Tgfβ binding protein (Ltbp3), tissue plasminogen activator (Platzer *et al.*), thrombospondin 1 (Thbs1), aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2), aryl hydrocarbon receptor (Ahr) and toll-like receptor 4 (Tlr4) were purchased from SABiosciences (Frederick, MD). mRNA levels were determined using SYBR green in qRT-PCR reactions (SABiosciences) on a BIO-RAD IQ 5 Light Cycler (BIO-RAD; Hercules, CA).

Antigen Uptake To assess antigen uptake, Alexa Fluor 488 (AF488)-labeled ova, AF488-labeled acetylated-LDL (Invitrogen) or 7-9μm FITC-labeled latex beads (Polysciences, Warrington, PA) were added to $1x10^6$ BMDCs in six-well plates and

incubated at 37°C for 12, 1.5 or 6h, respectively. Following incubation, the BMDCs were harvested, washed 2X and antigen uptake determined by flow cytometry.

BMDC $CD4^+$ T cell Co-cultures $CD4^+$ T cells from the spleen and popliteal and brachial lymph nodes of OTII $Foxp3^{eGFP}$ mice were purified to >75% $CD4^+$ using an autoMACS Cell Separator (Miltenyi Biotec Inc, Auburn, CA) and a $CD4^+$ T cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec Inc.). BMDCs were treated with 100μM 1-MT on day 9 of culture. On day 10, the BMDCs were harvested, washed and cultured with ova-peptide (2μg/mL). After 2.5h exposure to ova-peptide, BMDCs were washed 2X and placed with purified $CD4^+$ T cells at a 1:5 DC to T cell ratio in 96-well plates in cRPMI or F10 media. The co-cultures were harvested on day 3 and the frequency of $CD4^+$ $CD25^+$ $Foxp3^{eGFP}$ T cells determined by flow cytometry.

T Cell Activation The capacity of BMDCs to activate ova-specific $CD4^+$ T cells *in vivo* was assessed using the OTII adoptive transfer model as previously described (Bankoti *et al.*, 2010c). Briefly, $CD45.2^+$ BMDCs were generated in the presence of Flt3L and vehicle or TCDD. On day 10, the BMDCs were harvested, washed and cultured with whole ovalbumin (50μg/mL) to load them with antigen. After overnight exposure to ovalbumin, BMDCs were washed 2X and then injected ($2x10^6$) into each of the hind footpads of $CD45.1^+$ host mice that had received ova-specific OTII $Thy1.1^+$ $CD4^+$ T cells ($2x10^6$) intravenously 24h earlier. The popliteal and brachial lymph nodes were harvested 5 days post-BMDC injection and the ova-loaded donor BMDC ($CD11c^+$ $CD45.2^+$) and OTII T cell ($CD4^+$ $Thy1.1^+$) populations analyzed by flow cytometry.

Statistical Analysis Student's t tests were used to compare two individual samples and fold changes. One-way analysis of variance (ANOVA) was used to analyze data with more than two groups, with Bonferroni post-hoc analysis. p values ≤ 0.05 were considered significant.

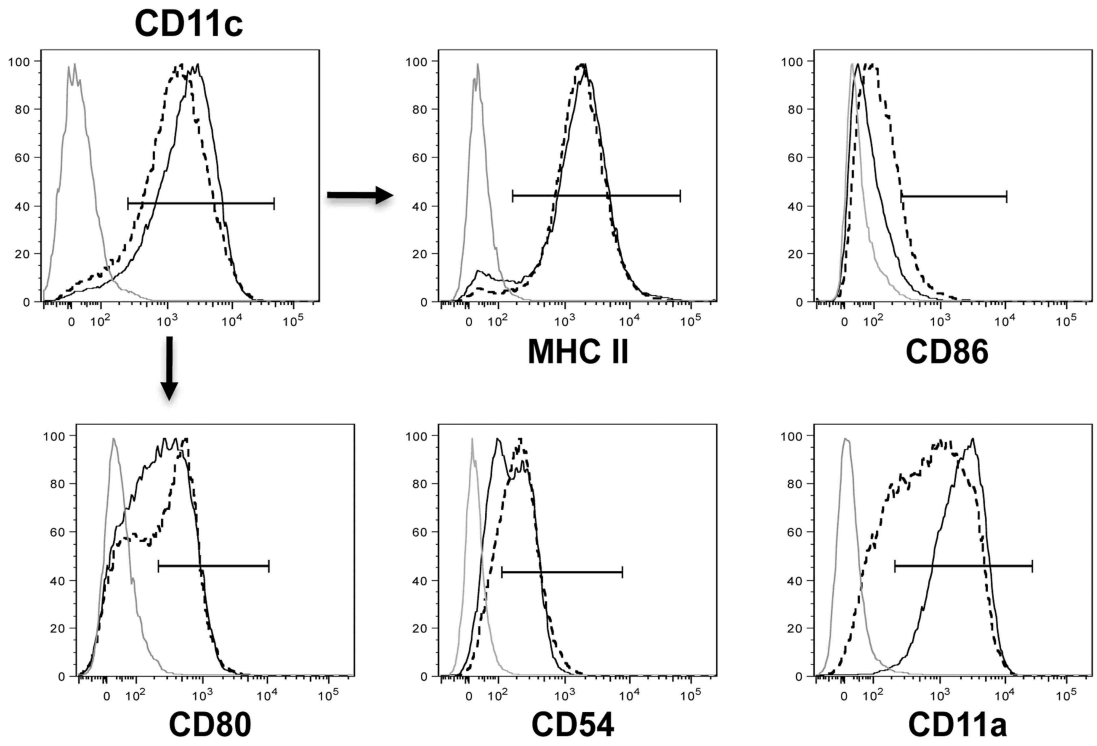
RESULTS

AhR activation alters immature steady-state BMDC growth and differentiation

Exogenous Flt3L when added to bone marrow progenitor cultures *in vitro* has been shown to produce immature DCs, which closely resemble steady-state DCs *in vivo* (Inaba *et al.*, 1992; Brasel *et al.*, 2000; Naik SH, 2007). To assess AhR activation in steady-state DCs, Flt3L-BMDCs were exposed to the prototypical AhR ligand, TCDD. On day 10, the immature (non-adherent) and mature (adherent) BMDC populations were harvested and enumerated. TCDD significantly reduced the number of immature steady-state BMDCs after 10 days in culture (Vehicle = 60 x 10^6 ± 5.3; TCDD = 45 x 10^6 ± 1.3*). However, there was no difference observed in the number of vehicle- and TCDD-treated mature BMDCs (Vehicle = 4.8 x 10^6 ± 0.5; TCDD = 3.7 x 10^6 ± 0.8). Steady-state BMDCs were identified using CD11c, the murine DC lineage marker, and were more than 90 percent $CD11c^+$ (Figure 2.1). Previous studies have shown that TCDD modulates costimulatory surface marker expression on steady-state splenic DCs in unimmunized mice (Vorderstrasse *et al.*, 2002; Bankoti *et al.*, 2010a). TCDD altered the expression of accessory molecules on steady-state BMDCs, as assessed by flow cytometry (Figure 2.1). TCDD decreased the frequency of BMDCs (TCDD-BMDCs) that expressed CD11c and its relative expression when compared to the vehicle-treated controls (vehicle-BMDCs). MHC class II (MHC II) expression was significantly decreased, while the percentage of cells expressing MHC II was slightly increased following TCDD exposure (Figure 2.1). Furthermore, TCDD decreased the expression and frequency of CD11a (Figure 2.1). In contrast, both the expression and frequency of the costimulatory molecules CD80 and CD86, as well as the adhesion molecule CD54, were significantly upregulated on TCDD-

BMDCs (Figure 2.1). To determine if these TCDD-induced effects were concentration dependent, steady-state BMDCs were exposed to 0.1nM, 1nM, and 10nM TCDD. Significant changes in the expression and frequency of CD11c, MHC II, CD86 and CD54 were observed on BMDCs exposed to TCDD concentrations as low as 0.1nM (Figure 2.2). Recently, a number of natural compounds including FIZC and ITE have been shown to bind and activate the AhR with similar affinity as TCDD (Oberg *et al.*, 2005; Henry *et al.*, 2006) To determine if these natural AhR ligands could modulate BMDC differentiation, steady-state BMDCs were exposed to FICZ or ITE. Similar to TCDD, both FICZ and ITE decreased the frequency of steady-state BMDCs expressing CD11c (Figure 2.3). Conversely, FICZ and ITE increased the percentage of MHC II, CD86 and CD54 on steady-state $CD11c^+$ BMDCs when compared to vehicle-treated controls (Figure 2.3).

Figure 2.1



		Vehicle	TCDD
CD11c	Percentage	92.1± 0.3	89.6 ± 0.5 *
	MFI	2718 ± 52	2337 ± 70 *
MHC II	Percentage	91.1 ± 0.7	96.2 ± 0.2 *
	MFI	3055 ± 117	2655 ± 18 *
CD86	Percentage	5.3 ± 0.2	14.3 ± 0.6 *
	MFI	432.7 ± 4.0	489.3 ± 3.9 *
CD80	Percentage	50.3 ± 0.4	55.0 ± 0.7 *
	MFI	560.2 ± 3.6	603.0 ± 4.9 *
CD54	Percentage	64.1 ± 0.6	77.6 ± 0.4 *
	MFI	269.3 ± 2.4	288.0 ± 2.4 *
CD11a	Percentage	98.9 ± 0.07	76.8 ± 0.5 *
	MFI	2536 ± 52	1599 ± 16 *

Figure 2.1. TCDD-induced alteration of steady-state BMDC surface marker expression. Steady-state BMDCs were treated with vehicle (solid black line) or 10nM TCDD (dashed line). Isotype staining is represented by gray lines. Immature vehicle- and TCDD-

BMDCs were harvested on day 10 and surface marker expression analyzed by flow cytometry. MHC class II, CD86, CD80, CD54 and CD11a expression was characterized on $CD11c^+$ BMDCs. Data is represented as a table showing surface marker percentage and mean fluorescent intensity (MFI) values with corresponding SEM values and is representative of three separate experiments with n = 3-6. ***** $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

Figure 2.2

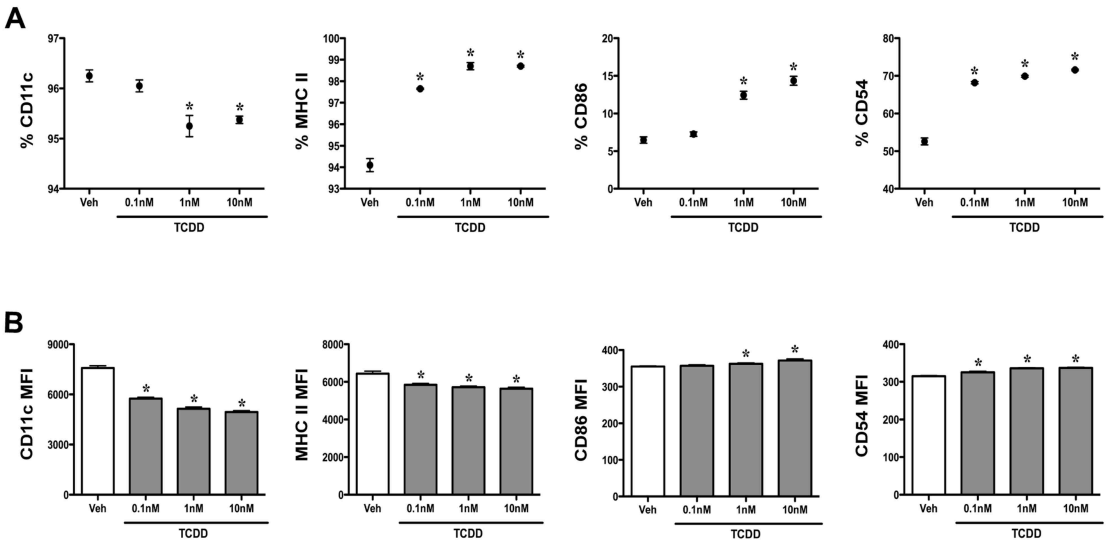


Figure 2.2. TCDD-induced surface marker alterations are concentration-dependent. Steady-state BMDCs were generated in the presence of vehicle control or varying concentrations of TCDD (0.1, 1.0, and 10nM). $CD11c^+$ BMDCs were harvested on day 10 and stained for CD11c, MHC class II, CD86 and CD54 expression as described in the Materials and Methods. Data is representative of three independent experiments with n = 4. ***** $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

Figure 2.3

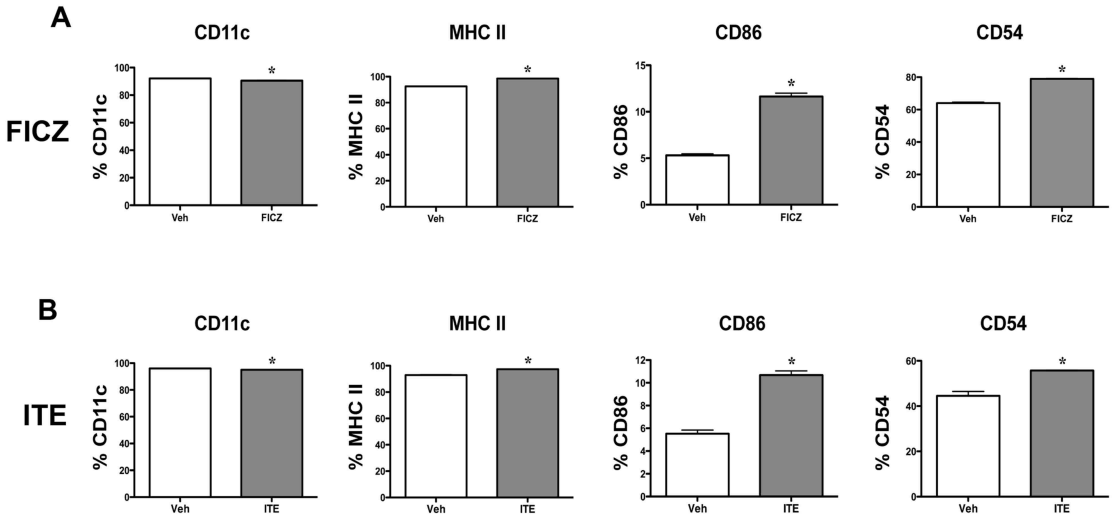


Figure 2.3. Natural AhR ligands alter BMDC accessory molecules. BMDCs were treated with vehicle, (A) 10nM FICZ or (B) 10nM ITE and harvested on day 10. CD11c, MHC II, CD86 and CD54 expression was evaluated on $CD11c^{+}$ BMDCs. (A) Data represents two independent experiments with n = 4-6 and (B) represents one experiment with n = 4. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

TCDD-induced phenotypic alterations are AhR-dependent but not exclusively DRE-mediated

TCDD-induced phenotypic alterations were previously shown to be AhR-dependent in inflammatory BMDCs and steady-state splenic DCs (Vorderstrasse and Kerkvliet, 2001a; Lee *et al.*, 2007; Bankoti *et al.*, 2010a; Bankoti *et al.*, 2010c). As expected, steady-state BMDCs derived from $AhR^{-/-}$ mice were insensitive to the effects of TCDD (Table 2.1). The frequency of BMDCs expressing CD11c, MHC II, CD86 and CD54 remained unchanged, as did the relative expression of CD11c, MHC II, CD86, and CD54 on $AhR^{-/-}$ TCDD-BMDCs, when compared to $AhR^{-/-}$ vehicle-BMDCs (Table 2.1). On the other hand BMDCs derived from $AhR^{dbd/dbd}$ mice, which lack the ability to bind DRE sequences in AhR target genes, were not insensitive to TCDD when compared to the $AhR^{-/-}$ BMDCs (Table 2.1). $AhR^{dbd/dbd}$ TCDD-BMDCs displayed an increased frequency and relative expression of CD11c (Table 2.1). Similar to the effects observed in TCDD-BMDCs from $AhR^{+/+}$ mice, the frequency and relative expression of MHC II, CD86 and CD54 on $AhR^{dbd/dbd}$ TCDD-BMDCs were increased when compared to $AhR^{dbd/dbd}$ vehicle-BMDCs.

Table 2.1

		$AhR^{-/-}$		$AhR^{dbd/dbd}$	
		Vehicle-BMDCs	TCDD-BMDCs	Vehicle-BMDCs	TCDD-BMDCs
CD11c	Percentage	95.9 ± 0.3	95.6 ± 0.1	90.1 ± 0.3	92.1 ± 0.3 *
	MFI	3059 ± 91	3026 ± 28	6040 ±123	6354 ± 80 *
MHC II	Percentage	94.3 ± 0.2	95.0 ± 0.3	89.7 ± 0.3	91.6 ± 0.4 *
	MFI	9994 ± 258	9764 ±194	23910 ± 552	25660 ± 398 *
CD86	Percentage	5.5 ± 0.1	5.4 ± 0.1	14.9 ± 0.3	22.6 ± 0.6 *
	MFI	258 ± 1.9	258 ± 4.0	306 ± 1.1	328 ± 3.0 *
CD54	Percentage	50.8 ± 0.4	50.2 ± 0.5	48.4 ± 0.5	55.1 ± 0.4 *
	MFI	187 ± 0.6	184 ± 0.9	398 ± 1.4	444 ± 1.6 *

Table 2.1. The effects of TCDD on accessory molecule expression on BMDCs from $AhR^{-/-}$ and $AhR^{dbd/dbd}$ mice. Expression of accessory molecules on vehicle- and TCDD-treated steady-state BMDCs derived from $AhR^{-/-}$ or $AhR^{dbd/dbd}$ mice. BMDCs were generated in vehicle or 10nM TCDD as described in the Materials and Methods. BMDCs were harvested on day 10 and CD11c, MHC II, CD86 and CD54 expression evaluated by flow cytometry. $AhR^{-/-}$ data is representative of two experiments with n = 3-4 and $AhR^{dbd/dbd}$ data represents one experiment with n = 5. * indicates $p \leq 0.05$ when compared to the representative vehicle-treated controls.

TCDD disrupts the TLR responsiveness of steady-state BMDCs

Innate immune responses can be initiated via stimulation of TLRs, an event that leads to DC maturation and activation. Subsequently, activated DCs produce inflammatory cytokines that contribute to the development of tailored immune responses. To determine if TCDD-induced AhR activation disrupts steady-state DC activation and/or cytokine production, immature steady-state BMDCs treated with TCDD or vehicle were stimulated for 24h with three TLR agonists; LPS (TLR4), CPG (TLR9) or Imiquimod (TLR7). As expected, TLR activation of immature BMDCs induced increased expression of CD11c, MHC II and CD86 when compared to unstimulated BMDCs (Table 2.2). It should be noted that after growth in cRPMI supplemented with conditioned media and Flt3L, TCDD-BMDCs placed in cRPMI for 24h without growth factors experienced changes in the expression of CD11c and MHC II when compared to the unstimulated vehicle-treated BMDCs (Table 2.2). TCDD-BMDCs stimulated with LPS, CpG or Imiquimod displayed decreased levels of CD11c (Table 2.2). In contrast to CD11c, TCDD-BMDCs expressed higher levels of MHC II, CD86 and CD54 (Table 2.2). In addition, TLR-stimulated TCDD-BMDCs displayed increased frequencies of MHC II, CD86 and CD54 when compared to the TLR-stimulated vehicle-BMDCs. Furthermore, the production of IL-6, TNFα, IL-12 and IL-10 was assessed following TLR stimulation to determine if TCDD disrupts cytokine production by steady-state BMDCs. Small, but detectable amounts of IL-12, were produced by unstimulated BMDCs, while IL-6, TNFα, and IL-10 levels were undetectable (Figure 2.4). Following LPS or CpG stimulation, steady-state BMDCs secreted IL-6, TNFα, IL-10 and IL-12 (Figure 2.4). Only IL-6 and IL-12 were detectable following stimulation with Imiquimod (Figure 2.4). BMDCs

treated with 10nM TCDD produced lower levels of IL-6, TNFα, IL-10 and IL-12 following stimulation with LPS or CpG, while no significant changes were seen in response to Imiquimod at 10nM TCDD (Figure 2.4). Following LPS stimulation, BMDC production of IL-6, TNFα, and IL-10 was decreased with concentrations of TCDD as low as 1nM, while IL-12 production decreased following exposure to concentrations of TCDD as low as 0.1nM (Figure 2.4). Following Imiquimod stimulation, 0.1nM TCDD exposed BMDCs secreted less IL-12 when compared to Imiquimod stimulated vehicle-BMDCs. Similar effects were observed following stimulation with CpG (Figure 2.4). Activated DCs also produce nitric oxide (NO), however, no differences were observed in the secretion of NO between vehicle-BMDCs and TCDD-BMDCs following stimulation with LPS, CpG or Imiquimod (data not shown).

Table 2.2

	% CD11c		CD11c MFI	
	Vehicle	**TCDD**	**Vehicle**	**TCDD**
Unstimulated	89.8 ± 0.1	89.1 ± 1.0	4213 ± 51.7	3570 ± 146 *
LPS	95.9 ± 0.1 #	95.0 ± 0.4 # *	6416 ± 140 #	4268 ± 184 *
CpG	91.9 ± 0.3	90.9 ± 0.3 *	5210 ± 61.2 #	3839 ± 75.3 *
Imiquimod	93.6 ± 0.2 #	91.3 ± 0.2 *	3405 ± 28.8 #	3142 ± 84.0 *

	% MHC II		MHC II MFI	
	Vehicle	**TCDD**	**Vehicle**	**TCDD**
Unstimulated	94.7 ± 0.6	96.7 ± 0.2 *	9157 ± 378	9901 ± 534
LPS	96.6 ± 0.2	97.8 ± 0.1 *	23030 ± 1394 #	22760 ± 851 #
CpG	97.8 ± 0.2 #	98.1 ± 0.1 #	18610 ± 424 #	26070 ± 1444 # *
Imiquimod	94.6 ± 0.4	97.1 ± 0.1 *	13030 ± 554 #	20560 ± 864 # *

	% CD86		CD86 MFI	
	Vehicle	**TCDD**	**Vehicle**	**TCDD**
Unstimulated	16.3 ± 0.8	32.1 ± 0.7 *	306.3 ± 2.3	314.0 ± 2.2 *
LPS	80.2 ± 0.8 #	89.6 ± 0.4 # *	970.0 ± 17.3 #	1538 ± 53.7 # *
CpG	61.7 ± 1.2 #	80.2 ± 0.6 # *	635.0 ± 5.6 #	929.8 ± 24.1 # *
Imiquimod	43.6 ± 0.7 #	70.5 ± 0.8 # *	805.8 ± 14.3 #	1048 ± 29.8 # *

	% CD54		CD54 MFI	
	Vehicle	**TCDD**	**Vehicle**	**TCDD**
Unstimulated	33.1 ± 0.2	46.2 ± 0.6 *	260.8 ± 2.3	297.8 ± 2.4 *
LPS	88.8 ± 0.4 #	89.3 ± 0.1 # *	887.8 ± 13.8 #	988.0 ± 21.3 # *
CpG	72.9 ± 0.9 #	83.1 ± 0.4 # *	665.3 ± 19.7 #	901.3 ± 10.3 # *
Imiquimod	29.6 ± 0.8	55.8 ± 0.8 # *	370.0 ± 4.1 #	524.0 ± 6.2 # *

Table 2.2. Effects of TCDD on the activation of steady-state BMDCs via TLR stimulation. BMDCs treated with vehicle or 10nM TCDD were stimulated with LPS (1μg/mL), CpG (0.5μM) or Imiquimod (30μg/mL) for 24h, harvested and CD11c, MHC II, CD86 and CD54 expression determined by flow cytometry. Mean values of frequency

(%) and mean fluorescent intensity (MFI) are displayed with corresponding SEM. Data represents two independent experiments with n = 4-6 per treatment group. ***** indicates $p < 0.05$ between vehicle- and TCDD-treated samples; # indicates $p < 0.05$ between unstimulated and LPS-, CpG- or Imiquimod-stimulated samples.

Figure 2.4

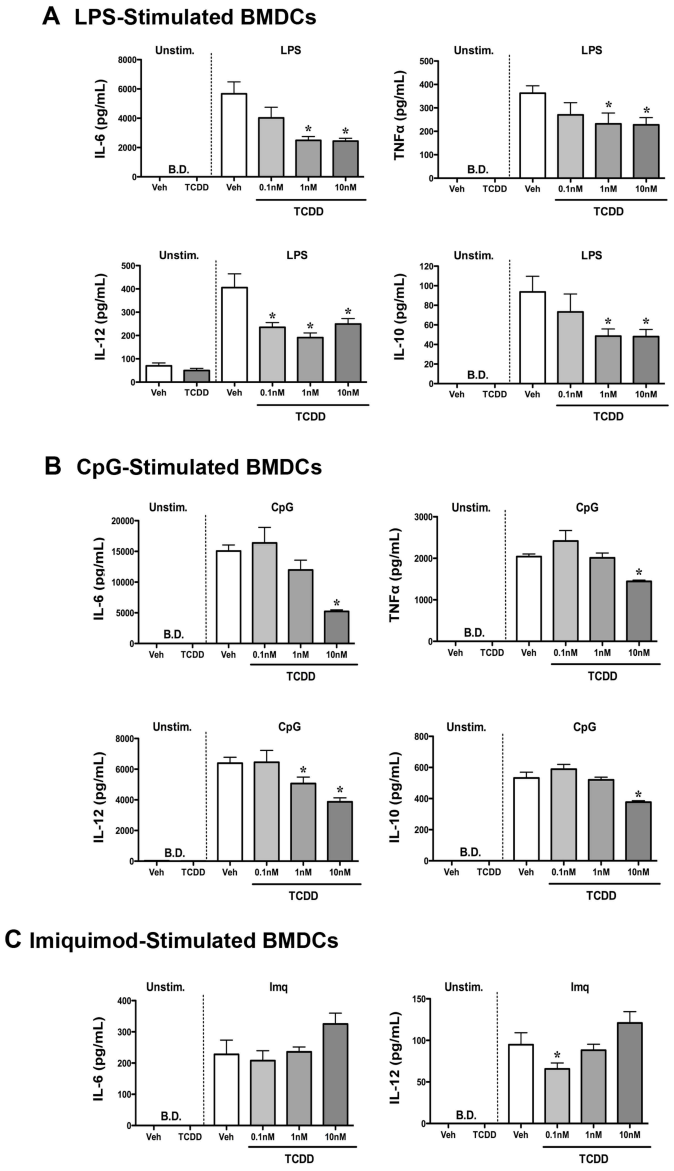


Figure 2.4. TCDD decreases BMDC cytokine production following TLR stimulation. Vehicle- and TCDD-BMDCs were harvested on day 10 and stimulated for 24h with (A) LPS (1μg/mL) or (B) CpG (0.5μM) or (C) Imiquimod (30μg/mL) or unstimulated. Cells were harvested, supernatants collected and IL-6, TNFα, IL-12p70 and IL-10 levels determined by ELISA. B.D. denotes below detection. Data is representative of two separate experiments with n = 4-6. ***** $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

NF-κB activity is altered in TCDD-BMDCs

NF-κB signaling pathways are initiated following TLR ligation in DCs and subsequently mediate DC activation. Recently, it has been demonstrated that the AhR can directly interact with multiple NF-κB family members including RelA, RelB and p52 (Ruby *et al.*, 2002; Vogel *et al.*, 2007a; Vogel *et al.*, 2007b). Therefore, we measured the effects of NF-κB activity in steady-state BMDCs. Following stimulation with LPS or CpG the activity of p65, p52, and p50 was increased, while RelB activity remained constant when compared to vehicle-BMDCs (Figure 2.5). In unstimulated TCDD-BMDCs, RelB binding was increased, while p65 and p52 levels were unchanged when compared to unstimulated vehicle-BMDCs (Figure 2.5). In contrast, p50 activity was decreased in unstimulated TCDD-BMDCs. Following LPS stimulation, TCDD-BMDCs displayed increases in RelB and p52 activity and decreases p65 and p50 activity when compared to LPS-stimulated, vehicle-BMDCs. RelB binding was unchanged following CpG stimulation in TCDD-BMDCs, while p52 binding increased (Figure 2.5). Furthermore, the binding levels of both p65 and p50 were reduced in TCDD-BMDCs following stimulation with CpG when compared to CpG-stimulated vehicle-BMDCs.

Figure 2.5

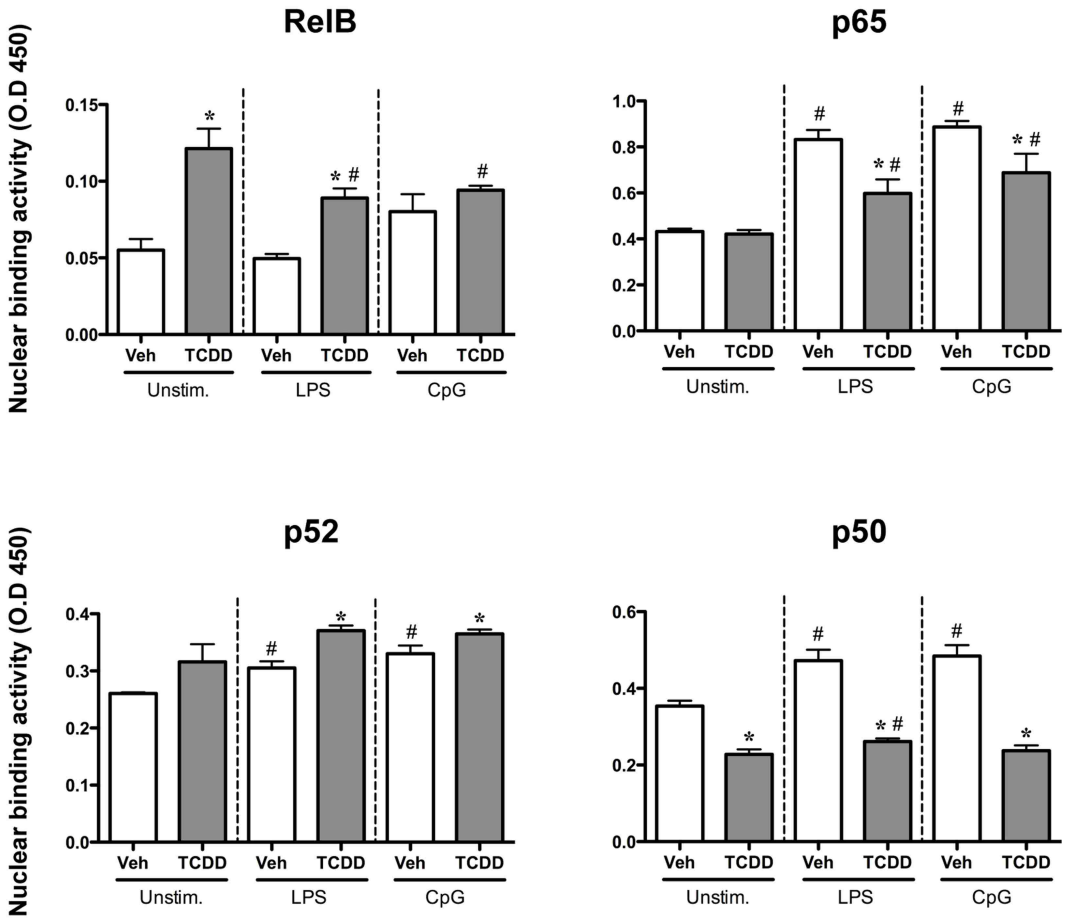


Figure 2.5. TCDD alters NF-kB activity. Vehicle- and TCDD-treated BMDCs were stimulated with LPS (1μg/mL) or CpG (0.5μM) for 45min. Following stimulation, BMDCs were harvested, nuclear lysates isolated and RelB, p65, p52, and p50 binding activity determined as described in the Materials and Methods. Data represents mean ± SEM of 4 samples per treatment group. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups; # indicates significance between unstimulated and LPS- or CpG-stimulated samples, respectively.

AhR activation increases regulatory gene expression in steady-state BMDCs

Inflammatory BMDCs exposed to TCDD increase their expression of various immune regulatory genes, such as TGFβ and indoleamine 2,3-dioxygenase (IDO) (Vogel *et al.*, 2008; Bankoti *et al.*, 2010c). Therefore, the expression of key immunoregulatory genes was measured in steady-state BMDCs following TCDD exposure. While there were no significant fold changes in the expression of Ltbp, Ahr, Thsp1 or TLR4, IDO2 was significantly upregulated in unstimulated TCDD-BMDCs when compared to vehicle-BMDCs (Table 2.3). In contrast, TCDD downregulated TGFβ2 and aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2) expression in unstimulated steady-state BMDCs when compared to vehicle-BMDCs. To determine if TCDD altered the expression of regulatory genes in activated BMDCs, gene expression was measured in vehicle-BMDCs and TCDD-BMDCs stimulated with LPS (Table 2.3). TCDD-BMDCs upregulated IDO1, IDO2, TGFβ1 and TGFβ3 expression following LPS activation while tissue plasminogen activator expression was downregulated (Table 2.3).

Table 2.3

	Unstimulated BMDCs		LPS-stimulated BMDCs	
	Fold change	p value	Fold change	p value
Ido1	-1.18	0.346	2.37 *	0.005
Ido2	6.55 *	0.0008	3.62 *	0.002
TGFβ1	1.1	0.873	1.65 *	0.006
TGFβ2	-2.72 *	0.011	-1.25	0.322
TGFβ3	1.51	0.194	2.37 *	0.013
Plat	-1.49	0.356	-2.82 *	0.007
Ltbp	-1.05	0.863	-1.22	0.639
Ahr	-2.26	0.211	1.03	0.833
Thsp1	-1.12	0.69	1.01	0.981
Aldh1a2	-3.68 *	0.01	2.69	0.059
Tlr4	-3.95	0.199	1.49	0.150

Table 2.3. Vehicle- and TCDD-BMDC regulatory gene expression. Regulatory gene expression in unstimulated or LPS-stimulated BMDCs was determined by qRT-PCR. Gene expression values represent the fold change between vehicle and TCDD treatment from two separate experiments with n = 4 per treatment group. * indicates statistically significant ($p < 0.05$) fold change between vehicle-BMDCs and TCDD-BMDCs. Indoleamine 2,3-dioxygenase 1 (Ido1) and Ido2; transforming growth factor β1 (Tgfβ1), Tgfβ2 and Tgfβ3; tissue plasminogen activator (Platzer *et al.*); latent Tgfβ binding protein (Ltbp3); aryl hydrocarbon receptor (Ahr); thrombospondin 1 (Thbs1); aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2) and toll-like receptor 4 (Tlr4).

TCDD modulates antigen uptake by steady-state BMDCs

Following recognition of antigens such as ovalbumin, low-density lipoprotein (LDL), and latex beads, DCs internalize these compounds via macropinocytosis, receptor-mediated endocytosis, or phagocytosis, respectively. TCDD disrupts antigen uptake by splenic DCs and inflammatory BMDCs (Vorderstrasse and Kerkvliet, 2001a; Vorderstrasse *et al.*, 2002; Bankoti *et al.*, 2010c). To assess whether antigen uptake by steady-state BMDCs is affected by TCDD, BMDCs were exposed to Ova, acetylated LDL, or latex beads and internalization was determined by flow cytometry. On a per cell basis TCDD-BMDCs internalized less Ova and LDL antigen, while increasing the uptake of latex beads when compared to antigen exposed vehicle-BMDCs (Figure 2.6). The overall frequency of Ova-positive and latex bead-positive BMDCs was increased in BMDCs exposed to TCDD (Figure 2.6). The frequency of LDL-positive BMDCs was unchanged between vehicle-BMDCs and TCDD-BMDCs (Figure 2.6).

Figure 2.6

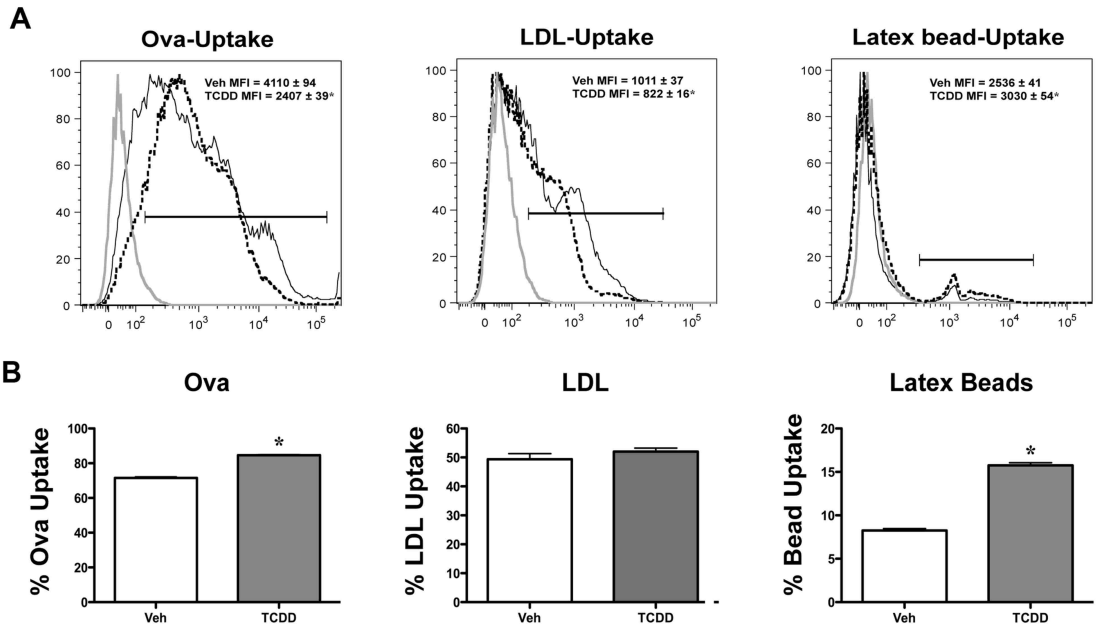


Figure 2.6. TCDD alters antigen uptake by steady-state BMDCs. AF488-Ova, AF488-LDL and FITC-latex beads were utilized to assess antigen uptake by BMDCs. Vehicle- and TCDD-BMDCs were incubated with LDL (1.5h), latex beads (6h) or Ova (12h). Antigen uptake was assessed via flow cytometry as described in the Materials and Methods. Results represent three independent experiments with n = 3-4. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

TCDD-treated BMDCs increase the frequency of CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs in vitro

AhR activation has recently been linked the generation of Tregs in both graft vs. host and autoimmune disease settings (Funatake *et al.*, 2005a; Hauben *et al.*, 2008; Quintana *et al.*, 2008a). To determine whether AhR-activated steady-state BMDCs can alter or induce Tregs *in vitro*, antigen-specific CD4$^+$ OTII T cells from mice expressing Foxp3^{eGFP} were utilized. Following three days in culture, Ova peptide-loaded TCDD-treated BMDCs increased the frequency of CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs approximately 15% when compared to Ova peptide-loaded Vehicle-treated BMDCs (Figure 2.7A). A 23% increase in CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs with TCDD-treated BMDCs was also observed when BMDC:T cells were cultured in F10 media that contains lower levels of tryptophan (Figure 2.7B). To test whether the observed increases in IDO gene expression in TCDD-treated BMDCs correlated with the increased frequency of CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs, vehicle- and TCDD-treated BMDCs were exposed to 1-MT, a tryptophan analog and IDO enzyme inhibitor (Mellor and Munn, 2004). 1-MT pretreatment blocked the observed increase in CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs in TCDD-treated BMDC:CD4$^+$ T cell co-cultures in both the conventional and tryptophan-low media (Figure 2.7).

Figure 2.7

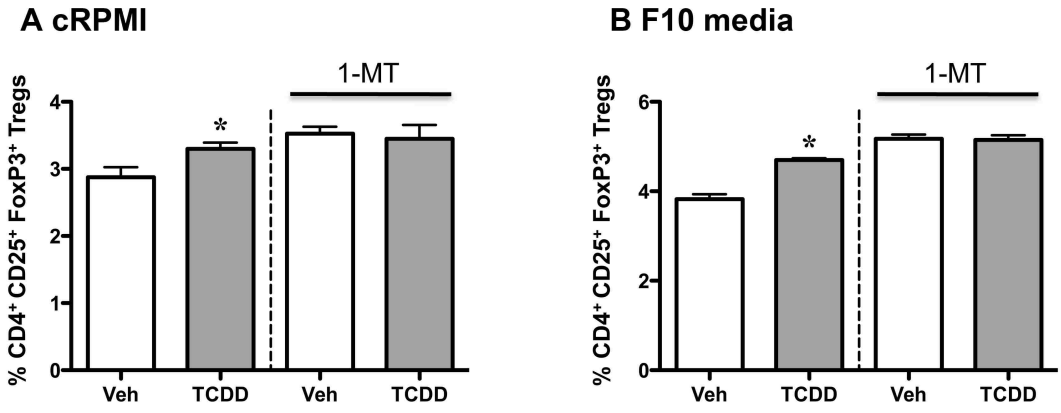


Figure 2.7. TCDD-treated BMDCs increase the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in an IDO-dependent manner. Vehicle- or TCDD-BMDCs were treated with 1-MT 24h pre-harvest. The BMDCs were loaded with Ova-peptide, washed twice and seeded with purified Ova-specific $CD4^+$ T cells from OTII mice expressing $Foxp3^{eGFP}$ in 96-well plates at a 1:5 DC to T cell ratio. The cultures were harvested on day 3 and the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs cultured in (A) cRPMI and (B) F10 media were determined. Data is representative of one experiment with n = 4. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

Steady-state TCDD-BMDCs initiate successful antigen-specific CD4$^+$ T cell activation in vivo

To determine if TCDD-induced AhR activation alters the ability of steady-state DCs to activate CD4$^+$ T cells *in vivo*, we utilized the OTII adoptive transfer model. CD45.1$^+$/Thy1.2$^+$ host mice were seeded with CD4$^+$/ Thy1.1$^+$ OTII T cells and injected with Ova-loaded CD45.2$^+$ vehicle- or TCDD-BMDCs. Distal brachial lymph nodes and proximal draining popliteal lymph nodes were harvested 4 days post-DC immunization. Donor DCs and OTII T cells were identified from host immune cells based on dual expression of CD11c/CD45.2 and CD4/Thy1.1, respectively. Overall, no significant differences were observed in the percent or number of vehicle- or TCDD-treated donor DCs in the draining lymph nodes (Figure 2.8A). TCDD-treated donor DCs displayed an increase in CD86 expression, while MHC II expression was unchanged when compared to vehicle-treated donor DCs (data not shown). The percentage and overall number of CD4$^+$/Thy1.1$^+$ OTII T cells was significantly increased in the popliteal lymph node, when compared to the brachial lymph node, demonstrating expansion of Ova-specific T cells in response to the injection of Ova-loaded BMDCs (Figure 2.8B). However, no differences in the percent or number of OTII$^+$ donor T cells in the draining lymph nodes were detected following the transfer of TCDD-BMDCs when compared to the vehicle-BMDCs. Furthermore, no differences were observed in the activation of donor T cells between mice immunized with vehicle-BMDCs or TCDD-BMDCs as assessed by CD62L and CD44 expression (data not shown).

Figure 2.8

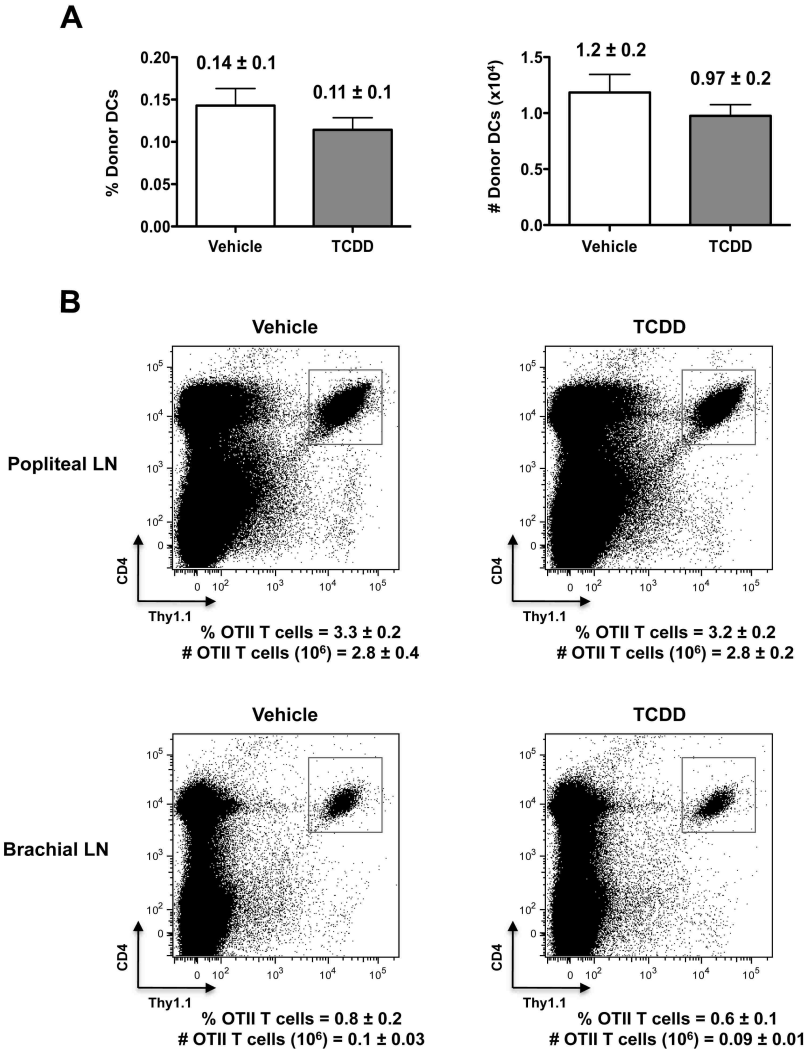


Figure 2.8. *In vivo* activation of Ova-specific $CD4^+$ OTII T cells by Ova-loaded BMDCs. $OTII^+$ $CD4^+$ $Thy1.1^+$ T cells were adoptively transferred into congenic, ($CD45.1^+$) host mice on day −1 relative to immunization. On day 0, adoptively transferred mice were immunized with vehicle- or TCDD-BMDCs loaded with whole ovalbumin. On day 4 post-immunization, popliteal and brachial lymph nodes were harvested from host mice and analyzed by flow cytometry. The frequency and numbers of (A) donor DCs ($CD45.2^+$/$CD11c^+$) and (B) Ova-specific OTII T cells ($CD4^+$/$Thy1.1^+$) were determined. Data is representative of one experiment utilizing seven animals per treatment group.

Table 2.4

	Steady-State BMDCs	Inflammatory BMDCs
Surface Molecule Expression [1]	↓CD11c, ↓MHC II, ↑CD86, ↑CD54, ↓CD11a	↓CD11c, ↑MHC II, ↑CD86, ↑CD54, ↓CD11a
Cytokine Production [2]	↓TNFα, ↓IL-6, ↓IL-12, ↓IL-10	↑TNFα, ↑IL-6, n.s. IL-12, n.s. IL-10
NF-κB Activity	↓p65, ↑RelB	↓p65, n.s. RelB
Antigen Uptake	↑Ova, n.s. LDL, ↑Particulate	↓Ova, ↓LDL, ↑Particulate
Immunoregulatory Gene Expression	n.s. IDO1, ↑IDO2, n.s. TGFβ3	↑IDO1, ↑IDO2, ↑TGFβ3
Treg Generation Capacity (*in vitro*)	↑$CD4^+$ $CD25^+$ $Foxp3^+$ Tregs	↑$CD4^+$ $CD25^+$ $Foxp3^+$ Tregs
Antigen-Specific T cell Activation (*in vivo*)	No effects on OTII $CD4^+$ T cells	No effects on OTII $CD4^+$ T cells

Table 2.4. Comparison of TCDD-induced alterations in steady-state and inflammatory murine BMDCs. [1] Surface molecule expression from unstimulated BMDCs as reported by Bankoti *et al*. 2010 and Lee *et al.* 2007. [2] BMDCs stimulated with LPS for 24h (cytokine production) or 30-45min (NF-κB activity). [3] Unpublished results. n.s. = not significant, ↑ = increase and ↓ = decrease.

DISCUSSION

DCs are integral to the function of both the innate and adaptive branches of the immune system. The generation of an immune response, whether stimulatory or tolerogenic, is largely dependent on DC differentiation, activation and maturation (Banchereau *et al.*, 2000). TCDD-induced AhR activation has been shown to disrupt DC differentiation (Vorderstrasse and Kerkvliet, 2001a; Lee *et al.*, 2007; Bankoti *et al.*, 2010a; Bankoti *et al.*, 2010c). Both naïve splenic DCs and inflammatory (GM-CSF-derived) BMDCs display altered surface molecule expression following TCDD-induced AhR activation (Vorderstrasse and Kerkvliet, 2001a; Ruby *et al.*, 2005; Lee *et al.*, 2007; Bankoti *et al.*, 2010a; Bankoti *et al.*, 2010c). Similar to these studies, our data show that AhR activation modulates the differentiation of Flt3L-derived steady-state BMDCs. DCs constitutively express the murine DC lineage marker CD11c and MHC class II, while costimulation molecule expression including CD80, CD86 and CD54 is lower but present on immature DCs. However, growth in TCDD altered the differentiation of steady-state BMDCs causing immature BMDCs to decrease their expression of CD11c, MHC class II and CD11a and upregulate CD80, CD86 and CD54 costimulation molecule expression in the absence of a maturation signal. Overall, TCDD altered steady-state BMDC differentiation and induced a unique phenotype indicative of partially-matured DCs. Some of the TCDD-induced changes observed in steady-state BMDCs such as the expression of cell surface molecules and immunoregulatory genes were similar to those in inflammatory BMDCs, while others such as LPS-activated cytokine production differed significantly (Table 4). These effects may highlight the biological functions of both steady-state DCs and inflammatory DCs as mediators of T cell immunity and inflammatory responses, respectively. Moreover, altered BMDC differentiation caused by

AhR activation may be relevant to human exposures as AhR activation by B(a)P has been shown to disrupt human monocyte-derived DC differentiation and maturation *in vitro* (Laupeze *et al.*, 2002). Furthermore, AhR activation by the anti-allergenic compound, VAF347, has recently been shown to upregulate AhR target gene expression in human monocyte-derived DCs and alter the differentiation of human myeloid progenitor cells and Langerhan cells (Lawrence *et al.*, 2008; Platzer *et al.*, 2009).

Two natural AhR ligands, FICZ and ITE, which have recently been shown to affect immune cell populations and modulate specific immune responses were evaluated for their ability to alter steady-state BMDC differentiation (Henry *et al.*, 2006; Kimura *et al.*, 2008; Quintana FJ, 2008; Vogel *et al.*, 2008; Bankoti *et al.*, 2010c). FIZC and ITE induced similar phenotypic alterations in steady-state BMDCs when compared to TCDD-BMDCs and vehicle controls. This suggests that high affinity exogenous ligands (TCDD) and high affinity endogenous ligands (FICZ and ITE) can act similarly to alter the differentiation of steady-state BMDCs.

A number of studies have demonstrated that immunomodulation by TCDD and TCDD-like chemicals is dependent on the AhR and AhR signaling events (Vorderstrasse and Kerkvliet, 2001a; Kerkvliet *et al.*, 2002; Lee *et al.*, 2007; Bankoti *et al.*, 2010c; Jin *et al.*, 2010). Steady-state BMDCs derived from $AhR^{-/-}$ mice were refractory to TCDD-induced phenotypic alterations. To determine whether canonical AhR signaling was involved, we investigated the role of dioxin response element (DRE) binding using steady-state BMDCs derived from $AhR^{dbd/dbd}$ mice. $AhR^{dbd/dbd}$ steady-state BMDCs displayed many similar phenotypic changes following exposure to TCDD when compared to $AhR^{+/+}$ steady-state TCDD-BMDCs. These results indicate that canonical

signaling via DREs is not required for TCDD to alter the differentiation of steady-state DCs and suggests that non-canonical AhR signaling may mediate the observed TCDD-induced phenotypic alterations. In inflammatory BMDCs, Bankoti et al. also demonstrated that the effects of TCDD were similar between $AhR^{dbd/dbd}$ and $AhR^{+/+}$ TCDD-BMDCs (Bankoti *et al.*, 2010c), suggesting that non-canonical AhR signaling mediates TCDD-induced defects in both inflammatory and steady-state DCs. Several possible alternatives exist to explain these results, such as the propensity of the activated AhR to physically interact with NF-κB family members or possibly STAT proteins (Ruby *et al.*, 2002; Vogel *et al.*, 2007a; Kimura *et al.*, 2008). However, the possibility that activated AhR physically interacts with NF-κB or STAT proteins in steady-state BMDCs remains to be examined.

As a part of the innate immune system, DCs become activated following pathogenic encounter increasing their expression of various surface molecules and secreting multiple cytokines (Banchereau *et al.*, 2000). Studies have demonstrated that TCDD-induced AhR activation can alter the expression of CD11c, MHC class II and CD86 and cytokine production following TLR stimulation in inflammatory BMDCs (Lee *et al.*, 2007; Bankoti *et al.*, 2010c). Following stimulation of with LPS (TLR4) or CpG (TLR9), $CD11c^{+}$ inflammatory TCDD-BMDCs increased their expression of MHC class II and secreted more IL-6 and TNFα, without affecting their production of IL-10 or IL-12 (Bankoti *et al.*, 2010c). Furthermore, Lee et al. demonstrated that inflammatory BMDCs derived in the presence of GM-CSF, IL-4 and TCDD downregulated CD11c expression and upregulated MHC class II and CD86 expression following LPS stimulation (Lee *et al.*, 2007). Similar to these studies, following stimulation with LPS or CpG, TCDD-

treated steady-state BMDCs displayed altered surface marker expression. Moreover, TCDD-treated steady-state BMDCs produced less IL-6, TNFα, IL-10 and IL-12 following stimulation. This is in contrast to TCDD-treated inflammatory BMDCs that secreted more IL-6 and TNFα following LPS or CpG stimulation (Bankoti *et al.*, 2010c). The differences in cytokine production between AhR-activated inflammatory and steady-state BMDCs is likely due to the fact that they model different DC populations *in vivo*. It is interesting that the levels of each cytokine measured, IL-6, TNFα, IL-12 and IL-10, were reduced by stimulated TCDD-treated steady-state BMDCs, suggesting that AhR activation may disrupt DC maturation. However, the observed increased expression of MHC class II, CD86, and CD54 following stimulation suggests that AhR activation may differentially affect steady-state DC maturation by increasing costimulatory molecule expression, while decreasing the production of key cytokines. The molecular underpinnings and biological significance of such effects remain to be determined.

The altered expression of costimulatory molecules and decreased cytokine production in TCDD-treated steady-state BMDCs following innate recognition of pathogenic components may be due to non-canonical AhR signaling. Recently, a number of non-canonical AhR signaling pathways have been described that include interactions between activated AhR and the NF-κB family member RelB (Vogel *et al.*, 2007a; Kimura *et al.*, 2008). Vogel and colleagues showed that production of the chemokines, BAFF, BLC, and CCL1, from TCDD-treated U937 macrophages was dependent on the expression of both the AhR and RelB and controlled by AhR/RelB complexes binding to promoter elements upstream of the BAFF, BLC, and CCL1 genes (Vogel *et al.*, 2007b). Ruby et al. demonstrated that stimulation of TCDD-exposed DC2.4 cells with TNFα or

anti-CD40 induced a physical association between the AhR and p65, effectively blocking p65 activity (Ruby *et al.*, 2002). To characterize AhR/NF-κB interactions in non-transformed DCs, Lee et al. used inflammatory BMDCs and demonstrated that following LPS stimulation TCDD-treated BMDCs reduce RelB gene expression (Lee *et al.*, 2007). However, Bankoti et al. showed that there was an increasing trend of RelB activity in TCDD-treated inflammatory BMDCs following LPS or CpG stimulation, while p65 activity was significantly reduced (Bankoti *et al.*, 2010c). In unstimulated and stimulated steady-state BMDCs treated with TCDD, NF-κB RelB, p65, p50, and p52 activity was altered. These data suggest that in steady-state BMDCs activated AhR may interact with RelB or p52 to increase DNA binding or alternatively interact with p65 or p50 to block target gene binding. It is currently unknown if activated AhR physically interacts with NF-κB family members to alter target gene binding in steady-state BMDCs or conventional DCs *in vivo*.

DCs contribute to innate immune responses by clearing pathogens via phagocytosis. Two previous studies have evaluated the ability of DCs to internalize antigens following AhR activation (Vorderstrasse *et al.*, 2002; Bankoti *et al.*, 2010c). In the first study, splenic DCs isolated following TCDD exposure showed no difference in their ability to internalize FITC-labeled latex beads (Vorderstrasse *et al.*, 2002; Bankoti *et al.*, 2010c). In the second study, inflammatory BMDCs exposed to TCDD displayed decreases in AF488-labeled Ova and LDL antigen uptake, while increasing FITC-labeled latex bead uptake (Vorderstrasse *et al.*, 2002; Bankoti *et al.*, 2010c). In this study, TCDD altered the ability of steady-state BMDCs to internalize both soluble and particulate antigens. The inconsistencies in these results may be due to differences in the type of

DCs (i.e. ex vivo conventional DCs versus *in vitro*-derived inflammatory or steady-state BMDCs). Nonetheless, defects in antigen uptake following AhR activation in DCs could affect their innate clearance of pathogens and potentially alter antigen processing and presentation and subsequent T cell-mediated immune responses.

Altering DC differentiation and antigen uptake following AhR activation may affect the functional capacity of DCs to induce appropriate antigen-specific T cell responses (Vorderstrasse *et al.*, 2002; Lee *et al.*, 2007). T cell activation not only requires TCR stimulation through antigen recognition in the context of MHC molecules, but also costimulation through interactions between CD86/CD80 and CD28. AhR activation in steady-state BMDCs decreases MHC class II expression, while increasing CD86 and CD80 expression, which could affect T cell activation. Recently, increases in CD86 on inflammatory BMDCs have been attributed to increased T cell activation in mixed lymphocyte reactions (Lee *et al.*, 2007). However, interactions between CD86/CD80 expressed on the DC and CTLA4 on T cells has been shown to inhibit T cell activation and may induce regulatory T cells (Lane, 1997; Funatake CJ, 2005). Furthermore, decreased expression of the adhesion molecule CD11a following AhR activation could affect interactions between T cells and antigen-bearing DCs, leading to suboptimal T cell stimulation. In contrast to inflammatory BMDCs, which display decreased levels of both CD11a and CD54 following TCDD-induced AhR activation, the increased expression of CD54 on steady-state TCDD-BMDCs could enhance T cell adhesion (Bankoti *et al.*, 2010c). However, the TCDD-induced alterations of surface molecules on steady-state BMDCs were modest, and thus, it remains to be determined if these changes are biologically significant.

The induction of tolerance or regulation of immune responses by DCs is dependent on a variety of mediators including TGFβ, IDO as well as other essential amino acid metabolizing enzymes and retinoic acid (Sharma MD, 2007; Yamazaki and Steinman, 2009; Cobbold *et al.*, 2010; Cobbold *et al.*, 2011). Steady-state BMDCs treated with TCDD upregulated the expression of IDO2 approximately 7-fold when compared to vehicle-treated, steady-state BMDCs. Following LPS stimulation, steady-state BMDCs treated with TCDD upregulated expression of IDO1, IDO2, TGFβ1 and TGFβ3. The TCDD-induced induction of IDO2 suggests that TCDD induces a regulatory phenotype in steady-state BMDCs. However, TCDD-treated inflammatory BMDCs displayed greater than 10-fold increases in IDO1, IDO2 and TGFβ3 (Bankoti *et al.*, 2010c). The differences in regulatory gene expression between these two DC populations may be because these BMDCs represent separate DC populations *in vivo* (Shotman and Naik, 2007; Naik, 2008a; Geissmann *et al.*, 2010). Inflammatory DCs are hyper-responsive immune cells and play an integral role in the regulation of inflammation. On the other hand, steady-state BMDCs represent conventional DC populations that are more likely to be refractory to rapid environmental changes, which may account for their overall lower regulatory gene expression when compared to TCDD-treated inflammatory BMDCs.

The regulatory capacity of TCDD-treated steady-state BMDCs is illustrated by their ability to increase the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs *in vitro*. Furthermore, the increased generation of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs was IDO-dependent, as 1-MT treated AhR-activated BMDCs did not generate an increased frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs. These data demonstrate the functional capacity of IDO, which is induced following AhR activation in steady-state BMDCs, to contribute

to the generation of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells. It has yet to be determined if AhR-activated steady-state BMDCs under suboptimal T cell stimulatory conditions induce $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, or other types of regulatory T cell such as Tr1 cells, *in vivo*.

In contrast to our *in vitro* results, TCDD-treated steady-state BMDCs failed to suppress antigen-specific $CD4^+$ T cell responses *in vivo*. This lack of an effect may be due to several plausible outcomes. First, the *in vivo* response may have been oversaturated with antigen (via the ova-loaded BMDCs), essentially "washing out" any TCDD-induced immunoregulatory effects of the BMDCs. Second, the population of BMDCs that are more significantly affected by TCDD may have defects in their ability to migrate to the draining popliteal lymph node and thus never reach that immune tissue to modify the subsequent T cell response. Third, TCDD-treated BMDCs may have to undergo some form of maturation via stimulatory signals such as LPS before they acquire functional immunosuppressive capabilities *in vivo* as was previously observed with VAF347-treated BMDCs (Hauben *et al.*, 2008). Fourth, OTII adoptively transferred mice may need to receive TCDD-treated BMDCs via a different route of administration (ie intravenously and not via the footpad) to realize an immunosuppressive effect. Finally, it could simply be that TCDD-treated, steady-state BMDCs possess only a phenotypic, and not a functional, immunoregulatory capacity. However, as shown in Figure 7, it would appear that TCDD-treated, steady-state BMDCs do indeed possess an immunoregulatory capacity. In these experiments, TCDD-exposed BMDCs were loaded with ovalbumin at concentrations somewhat lower than had been used for the *in vivo* experiments. These BMDCs were then cultured with OTII/$Foxp3^{egfp}$ T cells, leading to a significant increase

in the generation of regulatory T cells *in vitro*. These experiments are consistent with the possibility that in our *in vivo* experiments: (1) the immunosuppressive BMDCs may not have been migrating properly to the draining lymph nodes as previously described for lung DCs (Jin *et al*., 2010); (2) injecting them intravenously may be a more effective "therapeutic" approach (Hauben *et al.*, 2008); and/or (3) the concentration of ovalbumin used to load BMDCs for the *in vivo* experiments may have been inappropriate. On the other hand, our *in vitro* results indicate that TCDD-treated BMDCs are (1) functionally immunoregulatory, and (2) do not need to be LPS-stimulated to obtain this capability. Consequently, additional studies are necessary to define the optimal conditions that are required to translate the effects of TCDD-treated, steady-state BMDCs *in vivo*. These experiments are currently being addressed in our laboratory and will attempt to determine the "therapeutic" potential of AhR-activated BMDCs in several animal models of immune-mediated disease.

In summary, we report here that AhR activation disrupts the differentiation, TLR responsiveness, NF-κB signaling, uptake of antigen, and induction of immunoregulatory gene expression in steady-state BMDCs. Moreover, TCDD-treated, steady-state BMDCs can generate $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs *in vitro* in an IDO-dependent fashion. However, somewhat unexpectedly, TCDD-treated steady-state BMDCs successfully activated $CD4^+$ antigen-specific T cells *in vivo*. Overall, our study offers mechanistic data to further explain the immunomodulatory effects of AhR activation. Moreover, this study defines several specific biomarkers of immunotoxicity following exposure to toxicants that can activate the AhR and identifies selective AhR modulators that may possess therapeutic value.

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CHAPTER 3

Ah Receptor Activation in Inflammatory Murine Bone Marrow-Derived Dendritic Cells Disrupts Antigen-Specific $CD4^+$ T cell Responses *In vitro*

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ABSTRACT

The aryl hydrocarbon Receptor (AhR) mediates the toxic effects of various environmental and dietary compounds, with the immune system being one of the most sensitive mammalian targets of AhR-mediated toxicity. However, the mechanisms underlying immunomodulation following AhR activation remain to be fully defined. Within the immune system, dendritic cells (DCs) express high levels of the AhR and are sensitive to AhR activation. Previously, Bankoti and colleagues demonstrated that AhR activation significantly alters inflammatory bone marrow-derived DC (BMDC) surface molecule expression and increases the expression of known immune regulatory genes such as indoleamine 2,3-dioxygenase-1 and -2 (IDO1, IDO2) and TGFβ3. However, it is unknown whether TCDD-induced alterations in immature inflammatory BMDCs cause functional changes in their ability to interact with $CD4^+$ T cells. Thus, we hypothesized that AhR activation in inflammatory DCs leads to the generation of tolerogenic DCs capable of dampening antigen-specific immune responses. To test this hypothesis, murine GM-CSF BMDCs were generated in the presence of TCDD or a vehicle control, loaded with Ovalbumin peptide (Ovap) and placed with Ovap-specific $OTII^+$ $CD4^+$ T cells *in vitro*. Following 3 to 5 days in culture $CD4^+$ T cell proliferation was assessed and the $CD4^+$ T cell response characterized by cytokine production. While there were minimal changes in $CD4^+$ T cell proliferation, inflammatory AhR-activated BMDCs consistently increased IL-2 levels and decreased IFNγ and IL-10 levels in DC:T cell co-cultures. To explore possible mechanisms underlying these effects, the generation of $Foxp3^+$ Tregs was analyzed in DC:T cell co-cultures, with TCDD-treated BMDCs variably inducing $Foxp3^+$ Tregs.

INTRODUCTION

The AhR is a ligand-activated transcription factor that mediates the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as well as various other halogenated and polycyclic aromatic hydrocarbons. Following ligand-induced AhR activation, increased expression of AhR target genes results in cell- and tissue-specific toxicities. Immune cells are particularly sensitive to ligand-induced AhR activation and environmental pollutants capable of activating the AhR can alter the course on an immune response, most often resulting in immune suppression. However, the mechanisms underlying the observed immunosuppression following AhR activation remain unclear. DCs constitutively express the AhR and are affected by ligand-induced AhR activation. Due to the integral role of DCs in initiating effector T cell responses, it is likely that AhR-activated DCs play a key role in AhR-mediated immunosupression. Previously, Bankoti and colleagues using a GM-CSF-derived inflammatory BMDC model demonstrated that exposure to TCDD during BMDC development altered DC surface molecule expression and significantly up-regulate the immune regulatory genes IDO1 and IDO2 and TGFβ3 (Bankoti *et al.*, 2010b). IDO, an amino acid metabolizing enzyme that catalyzes tryptophan breakdown, is known to play a role in the generation of Tregs and has been shown to be a key element in the modulation of $CD4^+$ T cell responses following AhR activation in both DCs and $CD4^+$ T cells (Vogel *et al.*, 2008; Mezrich *et al.*, 2010; Nguyen *et al.*, 2010; Simones and Shepherd, 2011). In addition to IDO, the increased expression of TGFβ, a potent inducer of Tregs, in TCDD-treated inflammatory BMDCs suggests that AhR activation is inducing a tolerogenic phenotype in DCs. Whether this TCDD-induced tolerogenic phenotype translates into a functional phenotype in which TCDD-exposed

inflammatory DCs are capable of dampening $CD4^+$ T cell responses indirectly via the generation of Tregs is unknown.

Recently, AhR activation has been implicated in the generation of Tregs. Quintana and colleagues demonstrated that $CD4^+$ $Foxp3^+$ Tregs were generated from $CD4^+$ $Foxp3^-$ T cells in the presence of TCDD following anti-CD3/CD28 antibody-mediated activation (Quintana *et al.*, 2008b). These *in vitro* findings were translated into *in vivo* experiments demonstrating that TCDD-induced AhR activation dampened the severity of experimental autoimmune encephalomyelitis (EAE) in mice through the induction of $Foxp3^+$ Tregs in an AhR-dependent fashion (Quintana *et al.*, 2008b). Alternatively, AhR activation with the UV tryptophan breakdown product 6-formylindolo[3,2-b]carbazole (FICZ) caused the polarization of pro-inflammatory Th17 cells, subsequently worsening EAE symptoms in mice (Quintana *et al.*, 2008b; Veldhoen *et al.*, 2008). Interestingly, Bradfield and colleagues observed that AhR activation with the tryptophan catobolite kynurenine generated $Foxp3^+$ Tregs, while FICZ-induced AhR activation polarized Th17 cells following naïve $CD4^+$ T cell activation (Mezrich *et al.*, 2010). In addition to its role in $Foxp3^+$ Treg and Th17 cell polarization, the AhR may also be directly involved in the generation of type 1 regulatory T cells (Tr1) that characteristically produce large amounts of anti-inflammatory IL-10 (Apetoh *et al.*, 2010). The induction of Tr1 cells from naïve $CD4^+$ T cells with IL-27 and TGFβ *in vitro* was greatly enhanced in the presence of the high affinity AhR agonists TCDD or FICZ (Apetoh *et al.*, 2010). In contrast to the induction of $Foxp3^+$ or Tr1 Tregs, Kerkvliet and colleagues demonstrated that TCDD-induced AhR activation generated regulatory $CD4^+$

$CD25^+$ $Foxp3^-$ T cells that were functionally suppressive during graft vs. host responses in mice (Funatake *et al.*, 2005b).

Taken together, AhR clearly plays a role in $CD4^+$ T cell responses. However, naïve T lymphocytes ostensibly express undetectable levels of AhR as compared to immature DCs that constitutively express the AhR and are highly sensitive to AhR activation (Kerkvliet, 2009; Bankoti *et al.*, 2010b; Simones and Shepherd, 2011). While the effects of AhR activation in T cells are well studied, the interactions between TCDD-treated DCs and naïve $CD4^+$ T cells and the resulting effector T cell response are not as well characterized. It is highly plausible that effects seen *in vivo* following exposure to TCDD are mediated in part by DCs rather than direct effects on $CD4^+$ T cell polarization. In this study, we tested the hypothesis that AhR activation in inflammatory BMDCs leads to the generation of tolerogenic DCs capable of dampening antigen-specific immune responses. Overall, we show that AhR-activated inflammatory DCs alter antigen-specific $CD4^+$ T cell responses *in vitro,* as determined by altered cytokine production, and that TCDD-treated inflammatory DCs have the potential to generate $Foxp3^+$ Tregs.

MATERIALS AND METHODS

Animals 6-8 week old male and female C57Bl/6, OTII Thy1.1, Balb/c and DO11.10 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred at the University of Montana. OTII Foxp3^{eGFP} reporter mice were kindly provided by Dr. Randolph Noelle (Dartmouth Medical School, Lebanon, NH) who originally obtained these mice from Dr. Alexander Rudensky (University of Washington School of Medicine, Seattle, WA). All animals were housed and maintained at UM and provided water and chow ad libitum. All animal experiments were approved by the UM IACUC and adhered to the current NIH guidelines for animal usage.

Chemicals and Reagents TCDD and benzo[a]pyrene (BaP) were purchased from Cambridge Isotope Laboratories (Andover, MA). 2-(1H-indole-3'-carbonyl)-thiazole-4-carboxylic acid (ITE) was obtained from Tocris Bioscience (Ellisville, MO). Dexamethasone was purchased from Sigma-Aldrich (St Louis, MO). TCDD, BaP and ITE were supplied or resuspended in tissue culture grade DMSO from Sigma-Aldrich. Dexamethansone was suspended in Endotoxin-free water. Bone marrow-derived dendritic cells (BMDCs) were grown in complete RPMI (cRPMI): 20mM HEPES, 1.5mM sodium pyruvate, 50ug/ml gentamicin and 10% fetal bovine serum (Hyclone Thermo Fisher Scientific Waltman, MA). BMDC:CD4$^+$ T cells were cultured in cRPMI or F10 media: F-10 Nutrient Mixture, 20mM HEPES, 50ug/mL gentamicin, and 10% fetal bovine serum (Hyclone). All other media reagents were obtained from Invitrogen (Carlsbad, CA). 1-methyl-DL-tryptophan (1-MT) were purchased from Sigma-Aldrich. Ova-peptide, Ova$_{323-339}$, was obtained from Mimotopes (Clayton, Victoria Australia).

LPS (Escherichia coli; 055:B5) was obtained from Sigma-Aldrich. Recombinant human IL-2 and TGFβ1 were purchased from Peprotech (Rocky Hill, NJ) and Biolegend (San Diego, CA). Low endotoxin/azide free anti-CD3 (145-2C11) and anti-CD28 (CD28.2) antibodies were purchased from BioLegend.

Generation of Inflammatory BMDCs BMDCs were generated using methods as previously described (Inaba *et al.*, 1992; Bankoti *et al.*, 2010b). Briefly, bone marrow cells from the tibia and femur were flushed using cRPMI. Progenitor cells were separated from RBCs and debris by centrifugation using lympholyte M (Cederlane Laboratories; Burlington, NC) and were grown in cRPMI supplemented with 10% conditioned media and 30ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF) purchased from PeproTech and Biolegend. On days 3 and 5, non-adherent cells were removed, washed and reseeded in fresh media containing GM-CSF and vehicle or TCDD. On day 6 - 7 non-adherent BMDCs were harvested, enumerated and cell viability assessed using Trypan Blue (Sigma-Aldrich). $CD11c^+$ DCs were purified using magnetic separation techniques to ≥ 95% with CD11c-MicroBeads and an autoMACS Cell Separator, Miltenyi Biotec Inc. (Auburn, CA). Unless specified, BMDCs were grown in the presence of 10nM TCDD or vehicle control (0.01% DMSO), treatments that caused no significant effects on BMDC viability (data not shown). For 24h exposure experiments, BMDCs were grown in GM-CSF supplemented media without vehicle or AhR agonists. On day 6, the BMDCs were harvested, purified and cultured in the presence of Ova peptide and vehicle (0.01% DMSO), TCDD (10nM), ITE (100nM) or Dexamethasone (1μM) for 24h.

Flow Cytometry BMDCs and T cells were analyzed by flow cytometry as described previously (Shepherd *et al.*, 2001). Briefly, $1x10^5$ - $1x10^6$ cells were washed with PBS containing 1% bovine serum albumin and 0.1% NaN_3 and resuspended in Fc block, anti-CD16/CD23 antibody generated and purified from 2.4G2 hybridoma cells, to block non-specific binding. The cells were then stained with fluorochrome-conjugated antibodies for 10-15min on ice. Appropriate isotype controls were used in conjunction with the primary antibody staining. Intracellular IL-10 cytokine levels were assessed following paraformaldehyde fixation and 100% methanol or 0.1% saponin permeabilization. Intracellular Foxp3 staining with OTII Thy1.1 $CD4^+$ T cells was performed using a mouse Treg Flow Kit from BioLegend, following manufacturer's instructions. The following antibodies were used for DCs: MHC class II-PE (M5/114.15.12), CD80-PE (16-10A1), CD86-Pacific Blue (GL-1) from BioLegend; and CD11c-APC (HL3), CD54-FITC (3E2), CD11a-PECy7 (2D7) from BDPharmingen (San Diego, CA) and for T cells: CD4 APC, AF700 or Pacific Blue (GK1.5), CD25 APC or PerCpCy5.5 (PC61) and Vα2 PE (B20.1) from BioLegend. All samples were analyzed on a FACSAria flow cytometer using BD FACS Diva software, version 4.0 (BD Biosciences San Jose, CA). Figures were generated using FlowJo, version 8.8.6 (Tree Star Inc, Ashland OR).

DC:$CD4^+$ T cell Co-cultures $CD4^+$ T cells from the spleen and popliteal and brachial lymph nodes of OTII $Foxp3^{eGFP}$ or OTII Thy1.1 mice were purified to ~75% $CD4^+$ using an autoMACS Cell Separator and a $CD4^+$ T cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec Inc.). To obtain naïve $CD4^+$ $CD25^-$ $Foxp3^-$

T cells, enriched $CD4^+$ T cells from OTII $Foxp3^{eGFP}$ mice were stained for CD4 and CD25 expression and naïve $CD4^+$ $CD25^-$ $Foxp3^-$ T cells were FACS-purified (≥ 99%) using a FACSAria flow cytometer (BD Biosciences). $CD11c^+$ BMDCs from Vehicle or AhR-activated cultures were cultured with ova-peptide (Ovap) (2 - 50μg/mL) for 2h-24h. For 24h exposures, $CD11c^+$ BMDCs were cultured with Ovap concurrently with Vehicle, TCDD, ITE or Dexamethasone. After Ovap-loading, BMDCs were washed 2X and placed with enriched $OTII^+$ $CD4^+$ T cells or FACS-purified naïve $OTII^+$ $CD4^+$ $CD25^-$ $Foxp3^-$ T cells at a DC:T cell ratio of 1:5 in 96-well flat bottom plates in cRPMI only (Media Only) or cRPMI supplemented with 100 U/mL IL-2 and 5ng/mL TGFβ1 (+IL-2/TGFβ). As a control enriched $OTII^+$ $CD4^+$ T cells or FACS-purified naïve $OTII^+$ $CD4^+$ $CD25^-$ $Foxp3^-$ T cells were stimulated with plate-bound anti-CD3/anti-CD28 in 96 well plates that were coated with 5μg/mL anti-CD3 and 5μg/mL anti-CD28 overnight at 4^oC. The DC:enriched $OTII^+$ $CD4^+$ T cell and DC:FACS-purified naïve $OTII^+$ $CD4^+$ $CD25^-$ $Foxp3^-$ T cell co-cultures were harvested on day 3 and day 5, respectively, and the frequency of $CD4^+$ $CD25^+$ $Foxp3^{eGFP}$ T cells determined by flow cytometry. $CD4^+$ T cell proliferation was analyzed in DC:FACS-purified naïve OTII $CD4^+$ $CD25^-$ $Foxp3^-$ T cell co-cultures with naïve $CD4^+$ T cells labeled with Cell Trace Violet Dye (Invitrogen) on day 0, following manufacturer's instructions.

Cytokine Measurement Supernatants from individual DC:T cell co-culture experiments were collected and stored at -20^oC until further analysis. IL-2, IL-6, IL-10 and IFNγ cytokine production was measured using ELISAs per the manufacturer's instructions (BD

Biosciences). IL-17, IL-22, and TGFβ1 and TGFβ3 levels were measured using ELISAs from R&D Systems (Minneapolis, MN) according to manufacturer's instructions.

Quantitative Real-Time Reverse Transcription-polymerase Chain Reaction (qRT-PCR) To determine the levels of gene expression in BMDCs, qRT-PCR studies were conducted as previously described (Bankoti *et al.*, 2010b). BMDCs were harvested on day 6-7, washed twice in PBS and resuspended in Trizol (Invitrogen) to isolate RNA. Primers for indoleamine 2,3-dioxygenase 1 (Ido1) and Ido2, aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a1) and Aldh1a2, Arginase (Arg1), branched chain aminotransferase 1, cytosolic (Bcat1), histidine decarboxylase (Hdc), Il-4 induced protein (Il4i1), L-threonine dehydrogenase (Tdh), transforming growth factor β1 (Tgfβ1), Tgfβ2 and Tgfβ3, transforming growth factor beta receptor II (TgfβRII), aryl hydrocarbon receptor (Ahr), aryl hydrocarbon receptor repressor (Ahrr), Cyp1a1, Il-6, Il-10, Il-27 and suppressor of cytokine signaling 1 (SOSC1) and SOCS3 were purchased from SABiosciences (Frederick, MD). mRNA levels were determined using SYBR green in qRT-PCR reactions (SABiosciences) on a BIO-RAD IQ 5 Light Cycler (BIO-RAD; Hercules, CA).

Statistical Analysis Student's t tests were used to compare two individual samples and fold changes. One-way analysis of variance (ANOVA) was used to analyze data with more than two groups, with Bonferroni post-hoc analysis. p values ≤ 0.05 were considered significant.

RESULTS

$CD4^+$ T cells proliferate normally following activation with AhR-activated DCs

In response to appropriate signals provided by stimulatory DCs, $CD4^+$ T cells become activated and clonally expand. Interactions with tolerogenic DCs, however, can dampen $CD4^+$ T cell activation and inhibit proliferation. To determine if TCDD-DCs were providing requisite signals for T cell expansion, the proliferative capacity of $OTII^+$ $CD4^+$ T cells was assessed in DC:T cell co-cultures. Antigen-specific $CD4^+$ T cells expanded in response to Ovap-loaded DCs as compared to DCs without antigen (Figure 3.1A). On day 3, in DC:T cell co-cultures with enriched $CD4^+$ T cells, the number of $CD4^+$ T cells was similar between Veh- and TCDD-DC:T cell groups, whether in media alone or media supplemented with IL-2/TGFβ (Figure 3.1A). Similarly, there was no significant difference in Veh-DC and TCDD-DC induced $CD4^+$ T cell expansion in co-cultures with FACS-purified naïve $CD4^+$ T cells on day 5 (Figure 3.1B). Moreover, DCs exposed to TCDD or ITE for 24h with concurrent Ovap-loading induced $OTII^+$ $CD4^+$ T cell expansion similar to that of Ovap-loaded Veh-DCs. In contrast, DCs treated with dexamethasone (Dex) significantly decreased $CD4^+$ T cell expansion, as assessed by the decreased number of cells on day 5 in DC:T cell co-cultures with FACS-purified naïve $CD4^+$ T cells (Figure 3.1C). Furthermore, in DC:T cell co-cultures with FACS-purified naïve $CD4^+$ T cells labeled with Cell Trace Violet dye to track T cell proliferation no significant differences were observed between Veh-DC and TCDD-DC groups relative to $CD4^+$ T cell proliferation or the ratio of division (Figure 3.1D). In this experiment, only Dex-treated DCs altered T cell proliferation was observed with Dex-DCs (Figure 3.1E). $CD4^+$ T cells proliferated normally in cultures with DCs exposed to TCDD or ITE for

24h and no changes in overall proliferation or the frequency of cells in cycle was noted when these two groups where compared to the Veh-DC:T cell group (Figure 3.1E).

Figure 3.1

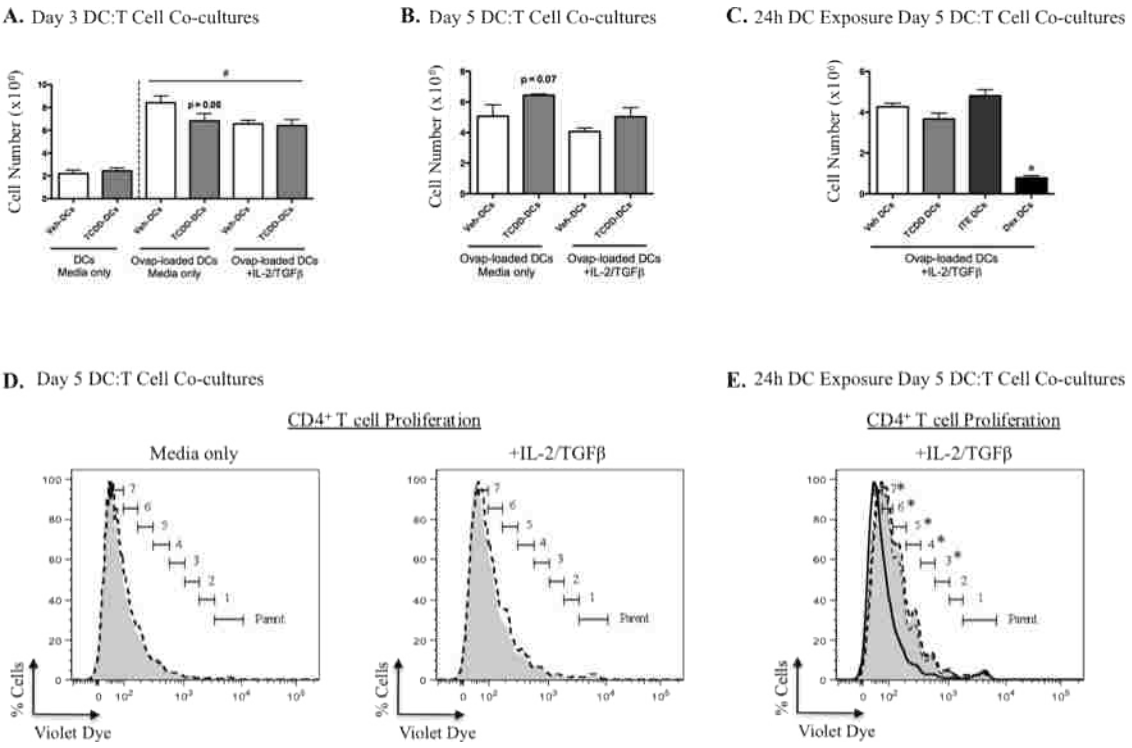

Figure 3.1. $CD4^+$ T cell proliferation in DC:$CD4^+$ T cell co-cultures. (A) DCs were cultured with enriched $OTII^+$ $CD4^+$ T cells in 96-well plates. Co-cultures were harvested and enumerated on day 3. (B and C) DCs were cultured with naïve $OTII^+$ $CD4^+$ $CD25^-$ $Foxp3^{eGFP-}$ T cells which were harvested and enumerated on day 5. Cell proliferation was determined on day 5 in DC:T cell co-cultures with Violet-dye labeled naïve $CD4^+$ T cells (D and E). (D) Solid gray line represents Vehicle-DCs and dashed black line TCDD-DCs, (E) Solid gray line represents Vehicle-DCs, heavy dashed black line TCDD-DCs, dashed line ITE-DCs and solid black line Dex-DCs. Data are representative of (A) five and (B-E) two independent experiments with n = 3 – 4. ***** $p \leq 0.05$ indicates significant difference between Vehicle- and Dex-treated DC:$CD4^+$ T cell groups. # $p \leq 0.05$ indicates significant difference between DC:$CD4^+$ T cells with no antigen and Ovap-loaded DC:$CD4^+$ T cell groups.

AhR-activated DCs alter CD4$^+$ T cell effector cytokine responses

AhR activation can directly disrupt CD4$^+$ T cell differentiation and subsequent cytokine production following T cell activation (Kerkvliet *et al.*, 1996; Quintana *et al.*, 2008b; Mezrich *et al.*, 2010). However, it is unclear whether CD4$^+$ T cell responses are affected following antigen-specific interactions and activation with AhR-activated DCs. To better understand how AhR-activated DCs influence antigen-specific T cell responses, BMDCs grown in the presence of Vehicle, TCDD or BaP were Ovap-loaded and cultured with Ova-specific OTII$^+$ CD4$^+$ T cells and cytokine production measured as a readout of the CD4$^+$ T cell effector response. It is important to note that no AhR ligands, other than what may be produced by the DCs in the DC:T cell co-cultures, were present in the co-cultures, thus, any AhR-induced alterations on the CD4$^+$ T cell response are due solely to the DC. In DC:T cell co-cultures with enriched CD4$^+$ T cells, ~ 60 – 85% OTII$^+$ CD4$^+$ T cells, IL-2 levels were significantly increased in co-cultures with TCDD- or BaP-DCs (Figure 3.2A). IFNγ and IL-10 levels were significantly decreased in co-cultures with AhR-activated DCs when compared to Veh-DC:T cell co-cultures (Figure 3.2A). TGFβ1 and TGFβ3 levels were below detection in the DC:T cell co-cultures (data not shown). The pattern of increased IL-2 and decreased IFNγ and IL-10 production was observed in TCDD- and BaP-DC:T cell co-cultures with FACS-purified OTII$^+$ CD4$^+$ CD25$^-$ Foxp3^{eGFP-} naïve T cells (Figure 3.2B). Altered cytokine responses were observed in nearly all Veh- and TCDD-DC co-culture experiments (Appendix Table A.4). However, DCs exposed to either TCDD or ITE for 24h failed to alter IL-2 or IFNγ production in DC:FACS-purified OTII$^+$ CD4$^+$ CD25$^-$ Foxp3^{eGFP-} T cell co-cultures, while IL-10 levels were decreased when compared to Veh-DC:T cell co-cultures. In contrast, Dex-DCs

significantly decreased IL-2 levels, while having no effect on IFNγ or IL-10 production (Figure 3.2C). Similar changes in cytokine levels were seen in DC:T cells cultured in IL-2/TGFβ supplemented media (Figure 3.2).

Figure 3.2

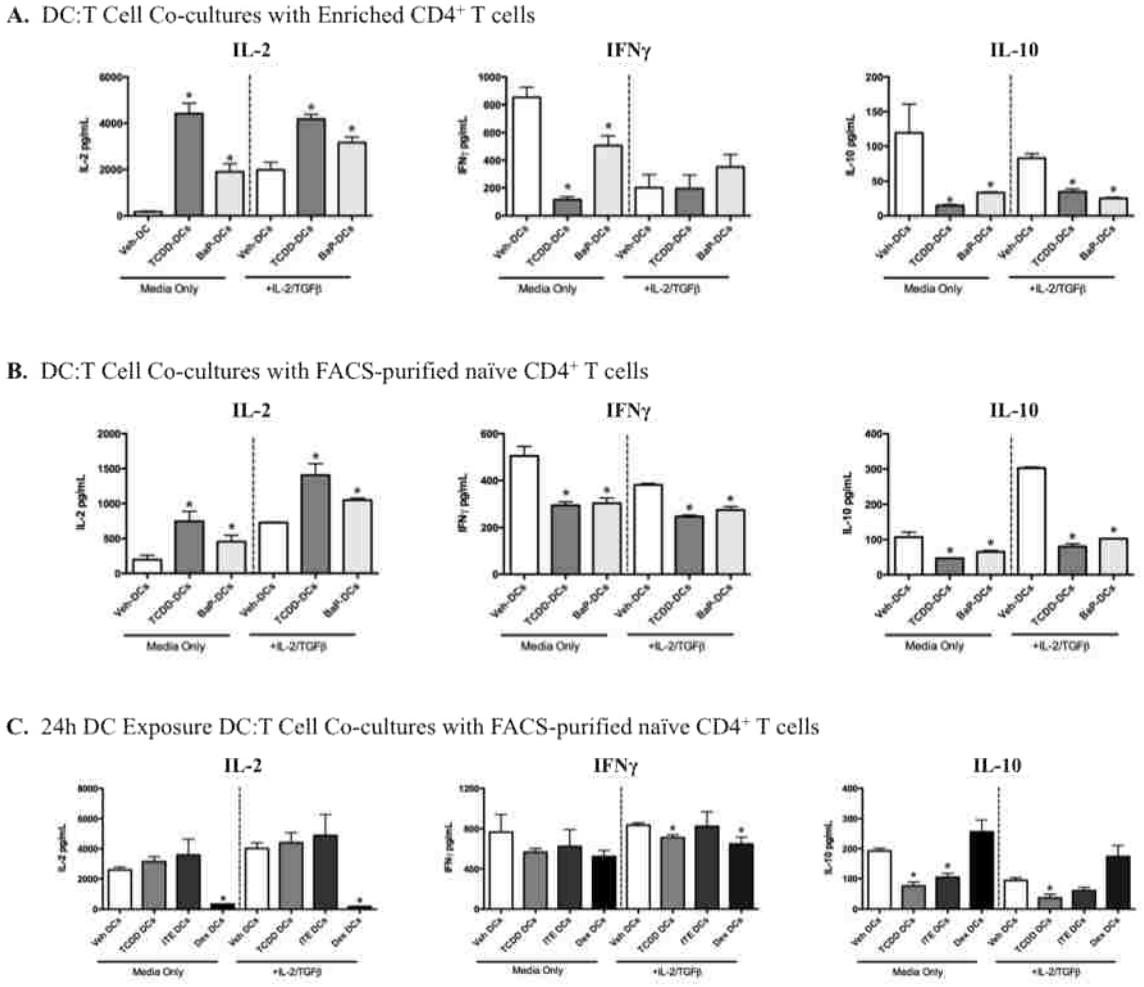

Figure 3.2. Cytokine levels in DC:CD4$^+$ T cell co-cultures. (A) DCs were cultured with ~80% pure CD4$^+$ T cells enriched from OTII Foxp3^{eGFP} reporter mice and harvested on day 3. (B and C) DCs were cultured with ~99% naïve CD4$^+$ CD25$^-$ Foxp3$^-$ T cells from OTII Foxp3^{eGFP} reporter and harvested on day 5. IL-2, IFNγ and IL-10 levels in culture supernatants determined by ELISA. Data are representative of (A) five (B), three and (C) two independent experiments with n = 3 – 4. * $p \leq 0.05$ indicates significant difference between Vehicle and AhR-activated groups.

TCDD-induced AhR activation induces immunoregulatory genes in inflammatory BMDCs

Previously, TCDD-induced AhR activation has been shown to induce both IDO1 and IDO2 gene expression in inflammatory BMDCs (Table 2.1) (Bankoti *et al.*, 2010b). IDO mediates tryptophan catabolism and increased expression can alter immune responses (Mellor and Munn, 2004; Vogel and Matsumura, 2009). To determine if additional amino acid metabolizing enzymes are affected following AhR activation, Arginase (Arg1), Bcat1, Hdc, Il4i1, and Tdh mRNA levels were measured in TCDD-treated BMDCs (TCDD-DCs). Arginase gene expression was significantly up-regulated in TCDD-DCs, while there were no changes in Bcat1, Il4i1 and Tdh expression when compared to Vehicle-treated BMDCs (Veh-DCs) (Table 3.1). In contrast to IDO, Hdc gene expression was significantly reduced in TCDD-DCs. AhR activation with ITE in BMDCs has been shown to increase Aldh1a1 gene expression and contribute to retinoic acid production (Quintana *et al.*, 2010). However, TCDD-induced AhR activation failed to increase Aldh1a1, however, Aldh1a2 expression was significantly up-regulated in TCDD-DCs (Table 3.1, Appendix Table A.5). TCDD-induced AhR activation has also been shown to increase TGFβ3 expression in inflammatory BMDCs (Bankoti *et al.*, 2010b). However, TGFβ1 and TGFβ2 gene expression in inflammatory BMDCs is relatively unaffected, where as TGFβ receptor II (TGFβRII) gene expression is significantly decreased following growth in TCDD (Table 3.1). In addition to producing TGFβ, tolerogenic DCs secrete various cytokines to help polarize T cell effector responses. Therefore, IL-6, IL-10 and IL-27 gene expression was determined in Veh- and TCDD-DCs. TCDD did not affect BMDC IL-6, IL-10 or IL-27 gene expression, although

there was trend towards increased IL-6 expression (Table 3.1). As expected, there was a significant increase in the expression of prototypical AhR-inducible genes CYP1A1 and AhRR in TCDD-DCs (Table 3.1).

Environmental pollutants other than TCDD, such as BaP, have also been shown to alter DC gene expression (Hwang *et al.*, 2007; Lee *et al.*, 2007). Similar to TCDD, inflammatory BMDCs derived in the presence of BaP displayed significant increases in IDO1 and IDO2, as well as characteristic CYP1A1 gene expression when compared to Veh-DCs (Table 3.1). However, BaP failed to induce TGFβ3 gene expression and significantly down-regulated Aldh1a2 expression (Table 3.1). BaP-induced AhR activation in BMDCs did not alter IL-6, IL-10, or IL-27 gene expression (Table 3.1).

The induction of IDO is highly sensitive to AhR activation. For example, immature $CD11c^+$ BMDCs exposed to TCDD or ITE for 24h significantly up-regulated both IDO1 and IDO2 gene expression (Table 3.1). There was no induction of IDO in immature $CD11c^+$ BMDCs exposed to the glucocorticoid Dex for 24h. In fact, IDO2 gene expression was significantly down-regulated in Dex-DCs (Table 3.1). The induction of TGFβ3 was less sensitive than IDO in 24h exposure experiments, as only Dex exposure induced TGFβ3 to appreciable levels (Table 3.1). 24h exposure to TCDD, ITE or Dex significantly decreased the gene expression of IL-6 and IL-10 in immature BMDCs. Similar to growth in TCDD or BaP, 24h exposure to TCDD or ITE significantly up-regulated CYP1A1 gene expression in immature BMDCs (Table 3.1). Unexpectedly, 24h exposure to Dex caused a significant increased in CYP1A1 gene expression.

Table 3.1

	TCDD-treated		BaP-treated		24h TCDD exposure		24h ITE exposure		24h Dex exposure	
Gene	Fold Change	p-value	Fold Change	p-value	Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Ido1	6.5*	0.00004	2.7*	0.0005	3.3*	0.0002	2.4*	0.00003	0.6	0.9
Ido2	8.1*	0.00008	3.5*	0.003	4.3*	0.002	4.2*	0.0009	0.6*	0.03
Aldh1a1	0.4	0.09	1.7	0.6	1.2	0.6	1.4	0.2	7.9*	0.001
Aldh1a2	2.8*	0.0004	0.4*	0.01	0.6*	0.01	0.6	0.07	0.7*	0.03
Arginase	2.4*	0.009	1.0	0.9	–	–	–	–	–	–
Bcat 1	2.2	0.2	–	–	–	–	–	–	–	–
HdC	0.1*	0.008	–	–	–	–	–	–	–	–
Il4i1	0.5	0.07	–	–	–	–	–	–	–	–
Tdh	0.6	0.2	–	–	–	–	–	–	–	–
Tgfβ1	1.0	0.8	0.8	0.6	1.1	0.6	1.1	0.6	1.2	0.4
Tgfβ2	0.9	0.9	0.8	0.5	0.5*	0.04	0.6	0.09	0.9	0.6
Tgfβ3	13.8*	0.00003	0.8	0.4	1.1	0.1	1.1	0.6	8.0*	0.0002
TgfβrII	0.4*	0.02	–	–	–	–	–	–	–	–
AhR	0.8	0.7	–	–	–	–				
AhRR	3.1*	0.009	–	–	–	–	–	–	–	–
Cyp1a1	42.7*	0.0003	11.0*	0.0001	26.4*	0.0001	14.8*	0.00002	7.6*	0.0001
Il-6	1.9	0.06	0.5	0.2	0.8*	0.02	0.7*	0.002	0.4*	0.001
Il-10	1.1	0.9	1.3	0.6	0.6*	0.04	0.5*	0.001	0.6*	0.005
Il-23	–	–	–	–	1.2	0.1	1.3	0.3	7.0*	0.0001
Il-27	1.3	0.5	2.1	0.2	1.4*	0.005	1.1	0.1	1.1	0.06

Table 3.1. Gene expression in immature AhR-activated inflammatory BMDCs. Gene expression was evaluated with qRT-PCR. Results are expressed as AhR ligand-induced fold change in comparison to Vehicle-BMDCs after normalization to housekeeping gene β-actin expression, with n = 3 – 4 per treatment group. * $p \leq 0.05$ indicates significance between Vehicle-treated and AhR ligand-treated BMDCs.

AhR-activated DCs effect the generation of regulatory T cells

Recently it has been shown that AhR activation with TCDD can lead to the generation of Tregs during inflammatory autoimmune responses (Quintana *et al.*, 2008b). In addition, exposure to ITE was shown to generate tolerogenic DCs capable of inducing Tregs and dampening EAE pathology (Quintana *et al.*, 2010). However, it is unknown whether TCDD-induced AhR activation in DCs leads to the generation of tolerogenic DCs that are capable of inducing Tregs. To address this question, the induction of $Foxp3^+$ Tregs was followed in DC:T cell co-cultures with $CD4^+$ T cells isolated from OTII $Foxp3^{eGFP}$ reporter mice (Fontenot *et al.*, 2005). On day 3, there was a slight, albeit significant, increase in the overall frequency of $CD4^+$ $CD25^+$ $Foxp3^{eGFP+}$ Tregs in DC:T cell co-cultures with antigen-specific enriched $CD4^+$ T cells (Figure 3.3A). The percent Treg increase with TCDD-DCs was observed in media alone as well as in cultures containing optimal Treg induction conditions with IL-2/TGFβ supplemented media (Figure 3.3B). Interestingly, this effect on TCDD-treated inflammatory BMDCs was not consistently inducible over all experiments (Table 3.2; Appendix Table A.1). In total, 17 Veh- and TCDD-DC:T cell co-culture experiments of similar design including enriched $OTII^+$ $CD4^+$ T cells that contained both naïve $CD4^+$ T cells and natural Treg populations were conducted and the frequency of Tregs analyzed on day 3 to 4. The purity of the $OTII^+$ $CD4^+$ T cells in these cultures varied between 59 and 88%. Out of these 17 experiments, 6 displayed significant increases in the percent of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs with TCDD-DCs when compared to Veh-DC:T cell co-cultures, while 4 displayed a significant reduction in the percent of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs (Appendix Table A.1). In the remaining 7 experiments there was no significant difference in the frequency

of Tregs induced on day 3 or 4 between Veh-DC and TCDD-DC groups (Appendix Table A.1). Throughout the course of these experiments different conditions such as the addition of TCDD, kynurenine or anti-IL-6 into the DC:T cell co-cultures were included to evaluate the potential for TCDD-DCs to induce $Foxp3^+$ Tregs. However, only the addition of kynurenine was observed to induce $Foxp3^+$ Tregs in TCDD-DC:T cell co-cultures when compared to kynurenine Veh-DC:T cell co-cultures (Appendix Table A.1). Surprisingly, DCs grown in the presence of increasing concentrations of TCDD (10, 50 and 100nM) and LPS-stimulated or TGFβ1-treated TCDD-DCs failed to significantly increase $Foxp3^+$ Tregs in DC:T cell co-cultures when compared to control Veh-DC:T cell groups (Appendix Table A.1). BMDCs grown in the presence of BaP displayed similar Treg induction capacities as BMDCs grown in the presence of TCDD (Appendix Table A.2).

Although the induction of Tregs with TCDD-DCs was somewhat inconsistent, we investigated potential mechanisms by which TCDD-DCs were inducing Tregs. To determine if the induction of Tregs by TCDD-treated BMDCs was dependent upon IDO or TGFβ, we performed blocking studies with 1-methyltryptophan (1-MT) or anti-TGFβ (α-TGFβ) blocking antibody, respectively. In two independent experiments with 1-MT and α-TGFβ, there was no significant increase in the percent of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs with TCDD-DCs on day 3 in media only (Appendix Figure A.2). However, there was an increase in the number of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in TCDD-DC media only conditions and in the presence of either 1-MT or α-TGFβ there was no change in the number of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs when compared to Veh-DC:T cell controls (Appendix Figure A.2).

To alleviate any inconsistencies in DC:T cell co-cultures with enriched $CD4^+$ T cells, which contained a small fraction of cells that were not $CD4^+$, and to determine whether AhR-activated DCs were capable of generating iTregs, DCs were cultured with FACS-purified naïve $CD4^+$ $CD25^-$ $Foxp3^-$ T cells from OTII $Foxp3^{eGFP}$ reporter mice. In these cultures, increases in $Foxp3^+$ Tregs would be attributable exclusively to iTregs and not via an expansion of nTregs. In these DC:T cell co-cultures TCDD-DCs failed to induce a significant percent of iTregs in media alone or with IL-2/TGFβ when compared to Veh-DC:T cell co-cultures on day 5 (Figure 3.4). In three independent experiments, two displayed no changes in the percent and number of iTregs induced by TCDD-DCs, while one experiment displayed a small but a significant increase in the percent of iTregs with TCDD-DC:T cells in the presence of IL-2/TGFβ when compared to Veh-DC:T cell co-cultures controls (Table 3.2 and Appendix Table A.1).

To determine if immature DCs exposed to AhR ligands become tolerogenic as reported by Quintana *et. al.*, immature BMDCs on day 6 were purified and cultured with Ovap in the presence of Veh, TCDD, ITE or Dex. The Ovap-loaded, AhR agonist-treated DCs were harvested after 24h in culture, washed and cultured with FACS-purified naïve $CD4^+$ $CD25^-$ $Foxp3^-$ T cells. In these co-cultures neither TCDD-DCs nor ITE-DCs induced iTregs on day 5 when compared to Veh-DC:T cell co-cultures (Figure 3.5). Under optimal Treg generation conditions with IL-2/TGFβ both TCDD-DCs and ITE-DCs caused a significant reduction in the percent of iTregs present on day 5 (Figure 3.5B).

Overall, the induction of antigen-specific Tregs in TCDD-DC:T cell co-cultures with $OTII^+$ $CD4^+$ T cells from OTII $Foxp3^{eGFP}$ reporter mice was observed, but with

inconsistent reproducibility (Appendix Table A.3). On the other hand, in a separate experiment utilizing Veh- and TCDD-DCs generated from Balb/c mice and Ovap-specific $CD4^+$ T cells from DO11.10 mice, a significant increase in the percent of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells was observed on day 5 as determined by intracellular Foxp3 staining (Appendix Figure A.1).

Figure 3.3

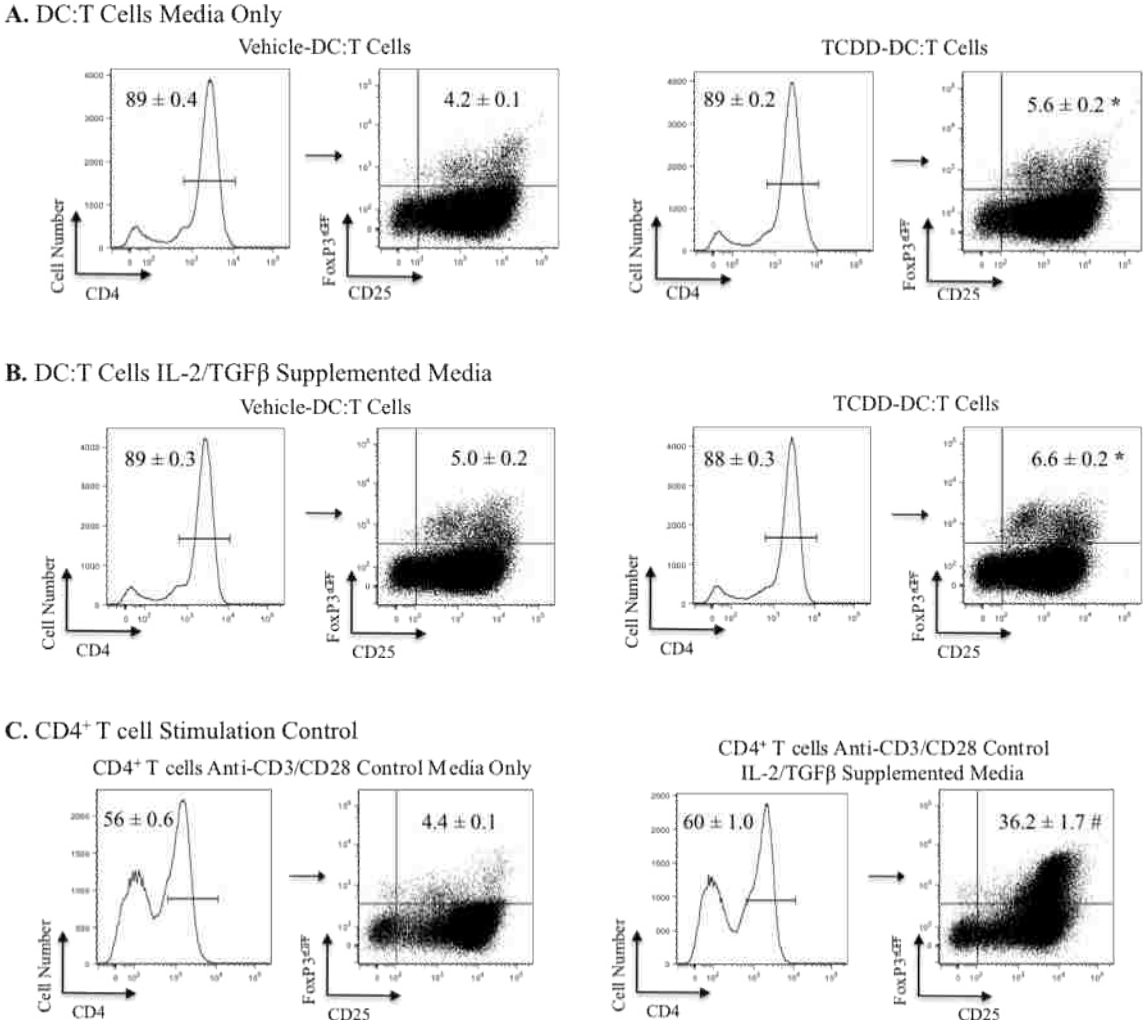

Figure 3.3. $CD4^+$ $CD25^+$ $Foxp3^+$ Treg generation in DC:T cell co-cultures. Vehicle-DC and TCDD-DCs were co-cultured with enriched $OTII^+$ $CD4^+$ T cells and the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in (A) media alone or (B) media supplemented with IL-2/TGFβ assessed on day 3. (C) Plate-bound anti-CD3/CD28 stimulation controls with enriched $OTII^+$ $CD4^+$ T cells in media only or IL-2/TGFβ supplemented media. * $p \leq 0.05$ indicates significant difference between Vehicle-DC:T cell and TCDD-DC:T cell groups. # $p \leq 0.05$ indicates significant difference between anti-CD3/CD28 stimulated $CD4^+$ T cells in media alone and IL-2/TGFβ supplemented media.

Figure 3.4

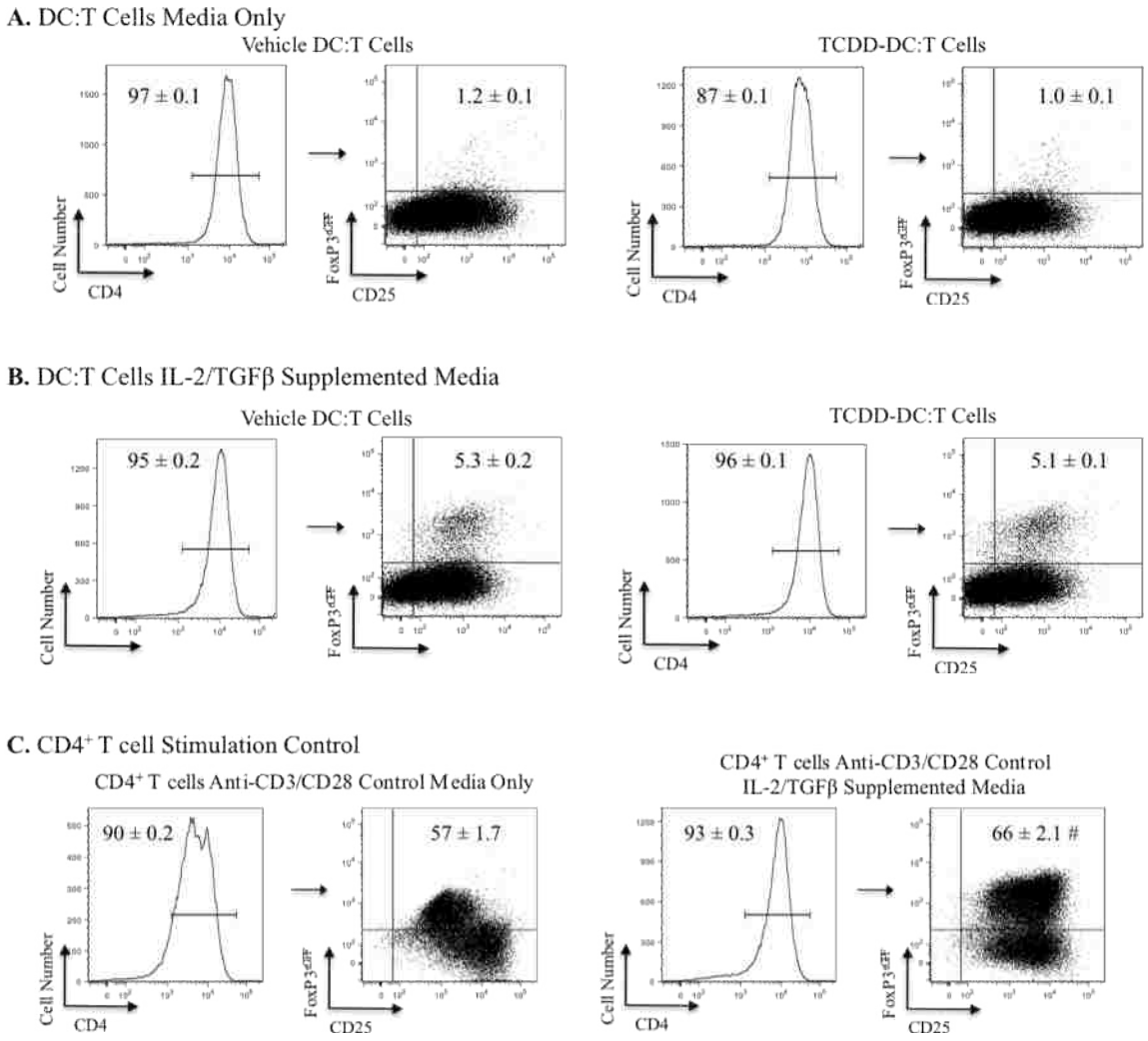

Figure 3.4. $CD4^+$ $CD25^+$ $Foxp3^+$ iTreg generation in DC:T cell co-cultures. Vehicle-DC and TCDD-DCs were co-cultured with FACS-purified naïve $OTII^+$ $CD4^+$ $CD25^-$ $Foxp3^{eGFP-}$ T cells and the percent of iTregs in (A) media alone or (B) media supplemented with IL-2/TGFβ assessed on day 5. (C) Plate-bound anti-CD3/CD28 stimulated FACS-purified OTII $CD4^+$ T cells in media only or media supplemented with IL-2/TGFβ. * $p \leq 0.05$ indicates significant difference between Vehicle-DC:T cell and TCDD-DC:T cell groups. # $p \leq 0.05$ indicates significant difference between $CD4^+$ T cell anti-CD3/CD28 stimulation in media alone and media with IL-2/TGFβ.

Table 3.2

A. DC:CD4$^+$ T cell Co-cultures with Enriched CD4$^+$ T Cells

Parameter		Independent Experiment Media Only DC:T cells			Independent Experiment +IL-2/TGFβ DC:T cells		
		# 1	# 2	# 3	# 1	# 2	# 3
Cell Numbers x10^5	Veh	8.4 ± 0.6	2.3 ± 0.2	7.7 ± 1.1	6.6 ± 0.3	3.8 ± 0.5	12.1 ± 0.8
	TCDD	6.8 ± 0.7	4.3 ± 0.2 *	6.6 ± 0.4	6.4 ± 0.5	4.9 ± 0.2 *	11.4 ± 0.5
% CD4$^+$ CD25$^+$ FoxP3$^+$ Tregs	Veh	4.2 ± 0.1	3.9 ± 0.1	2.7 ± 0.5	5.0 ± 0.2	7.7 ± 0.2	2.0 ± 0.1
	TCDD	5.5 ± 0.2 *	3.3 ± 0.1 *	2.4 ± 0.2	6.6 ± 0.2 *	5.3 ± 0.2 *	1.7 ± 0.2
Treg Cell Number x10^4	Veh	3.5 ± 0.2	12 ± 1.1	21 ± 3.5	3.3 ± 0.1	29 ± 3.3	24 ± 1.8
	TCDD	3.8 ± 0.3	14 ± 0.7 *	16 ± 2	4.3 ± 0.4 *	26 ± 1.9	20 ± 2.7

B. DC:CD4$^+$ T cell Co-cultures with FACS-purified CD4$^+$ CD25$^-$ FoxP3^{eGFP-} T Cells

Parameter		Independent Experiment Media Only DC:T cells			Independent Experiment +IL-2/TGFβ DC:T cells		
		# 1	# 2	# 3	# 1	# 2	# 3
Cell Numbers x10^6	Veh	1.4 ± 0.4	–	5.0 ± 0.8	1.4 ± 0.3	15 ± 2.0	4.1 ± 0.2
	TCDD	2.1 ± 0.2	–	6.4 ± 0.1	1.8 ± 0.2	15 ± 0.3	5.0 ± 0.6
% CD4$^+$ CD25$^+$ FoxP3$^+$ Tregs	Veh	0.6 ± 0.1	2.4 ± 0.7	1.2 ± 0.1	5.0 ± 0.2	6.8 ± 0.2	5.3 ± 0.2
	TCDD	0.5 ± 0.1	3.0 ± 0.6	1.0 ± 0.1	6.6 ± 0.2 *	6.5 ± 0.2	5.1 ± 0.1
Treg Cell Number x10^4	Veh	1.0 ± 0.4	–	5.7 ± 0.6	4.8 ± 0.8	100 ± 0.2	22 ± 1.2
	TCDD	1.0 ± 0.1	–	6.2 ± 0.5	8.0 ± 1.4	90 ± 0.1	26 ± 2.9

Table 3.2. Total cell numbers, percent CD4$^+$ CD25$^+$ FoxP3$^+$ Tregs and Treg numbers in three independent experiments. * $p \leq 0.05$ indicates significant difference between Vehicle-DC:T cells and TCDD-DC:T cells groups.

Figure 3.5

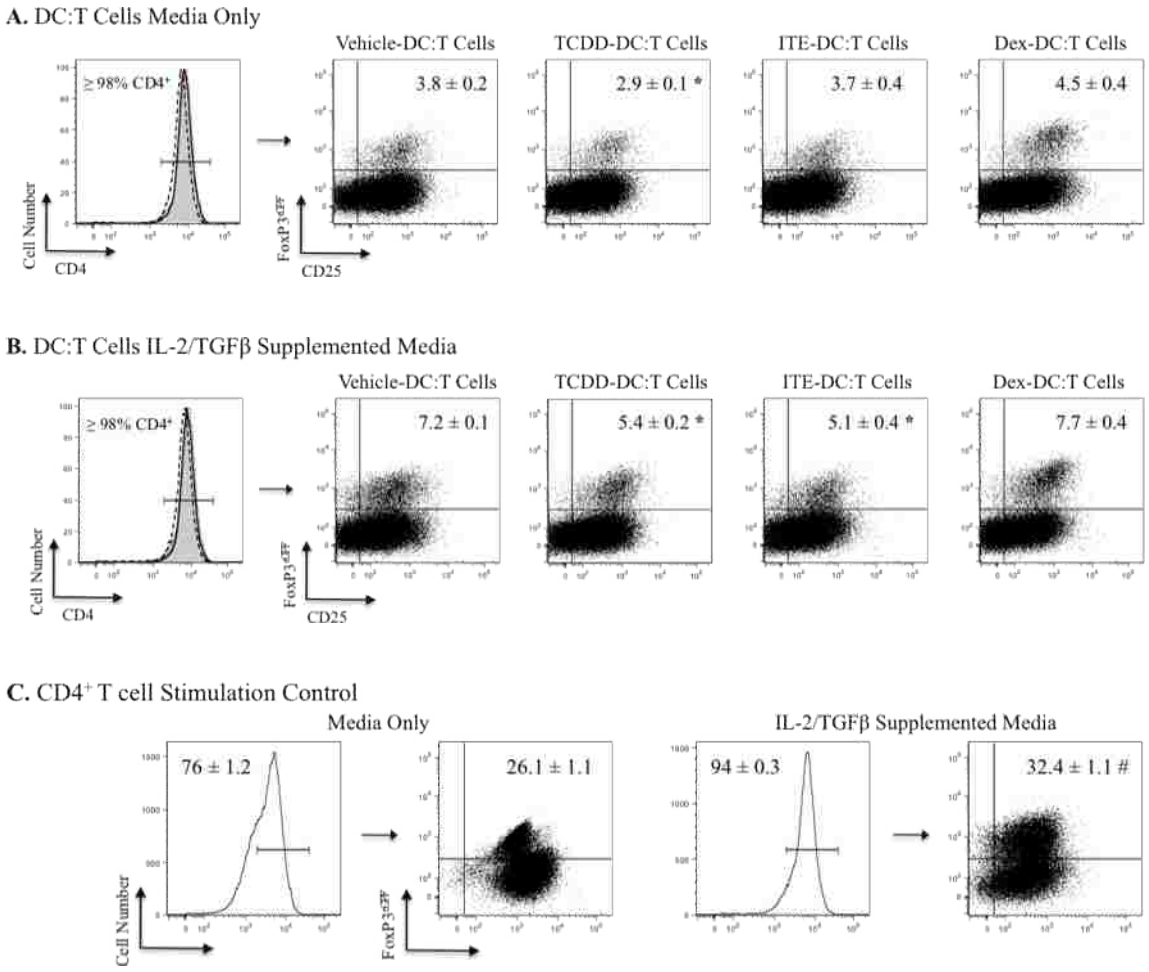

Figure 3.5. $CD4^+$ $CD25^+$ $Foxp3^+$ iTreg generation with 24h Vehicle-, TCDD-, ITE- or Dex-exposed DCs. 24h-exposed Ovap-DCs were co-cultured with FACS-purified naïve $OTII^+$ $CD4^+$ $CD25^-$ $Foxp3^{eGFP-}$ T cells and the percent of iTregs in (A) media alone or (B) media supplemented with IL-2/TGFβ assessed on day 5. $CD4^+$ T cells in DC:T cell co-cultures displayed as: Vehicle-DCs (solid gray), TCDD-DCs (solid line), ITE-DCs (dotted line), and Dex-DCs (long dashed line). (C) Plate-bound anti-CD3/CD28 stimulated FACS-purified OTII $CD4^+$ T cells in media only or media supplemented with IL-2/TGFβ. ***** $p \leq 0.05$ indicates significant difference between Vehicle-DC:T cell and exposed-DC:T cell groups. # $p \leq 0.05$ indicates significant difference between $CD4^+$ T cell anti-CD3/CD28 stimulation in media alone and media with IL-2/TGFβ.

iTregs generated in TCDD-DC:CD4$^+$ T cell co-cultures are suppressive

Because of the potential for TCDD-treated inflammatory BMDCs to induce Foxp3$^+$ Tregs, albeit inconsistently, it was necessary to evaluate the suppressive capacity of Tregs induced by TCDD-DCs when compared to Veh-DCs. Therefore, the suppressive capacity of iTregs generated in both Veh- and TCDD-DC:T cell co-cultures was determined. CD4$^+$ CD25$^+$ Foxp3$^+$ iTregs were FACS-purified from DC:T cell co-cultures on day 5 and placed into fresh cultures with FACS-purified Cell Trace-labeled naïve CD4$^+$ T cells (T responder cells) in the presence of anti-CD3/CD28. iTregs from TCDD-DC:T cell co-cultures slightly, but significantly inhibited the proliferation of responder CD4$^+$ T cells when compared to iTregs from Veh-DC:T cell co-cultures (Figure 3.6). However, CD4$^+$ Foxp3$^-$ T cells from TCDD-DC:T cell co-cultures displayed a similar capacity to suppress responder T cell proliferation (Figure 3.6).

Figure 3.6

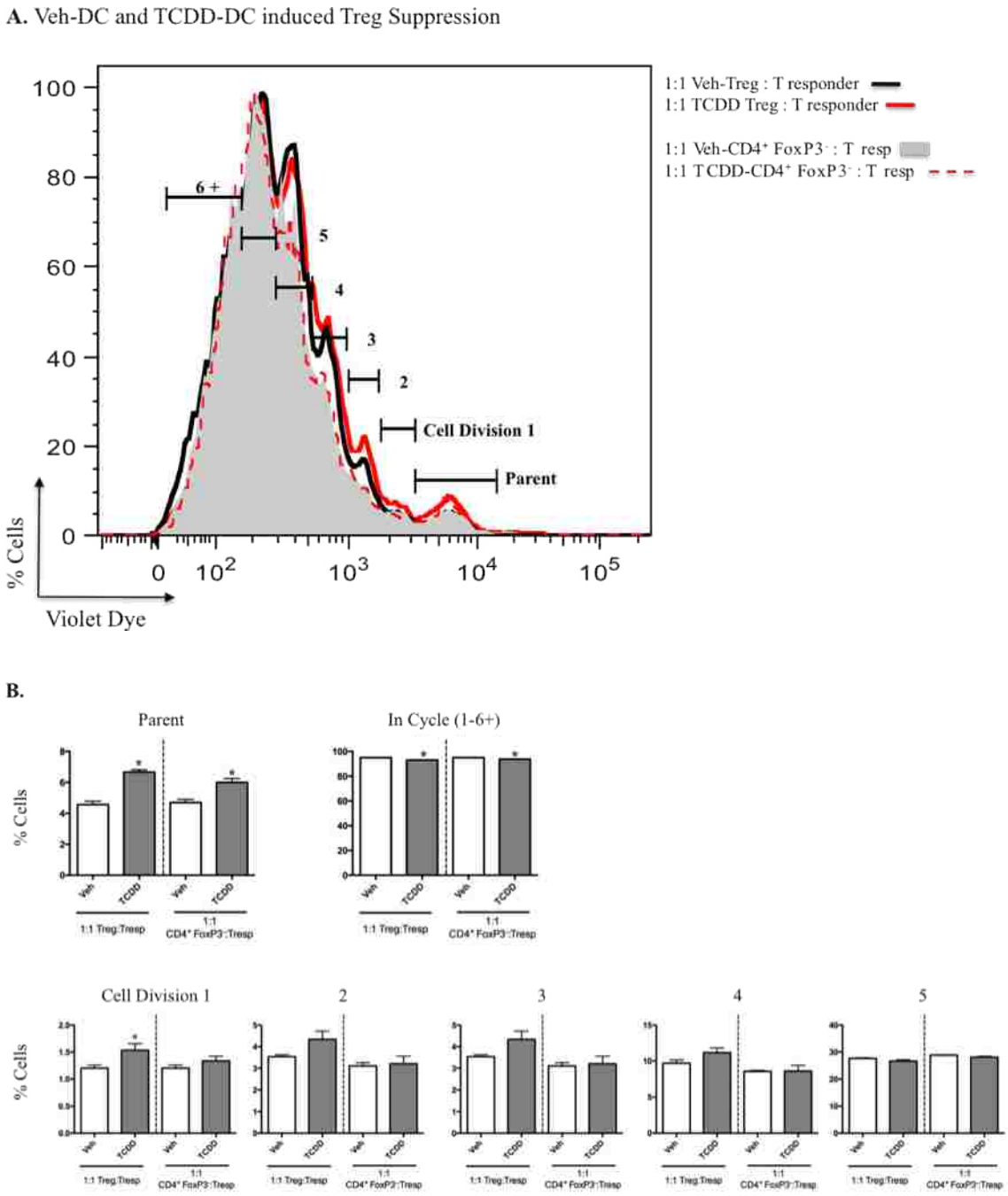

Figure 3.6. Treg suppressive capacity. iTregs (Tregs) generated and purified on day 5 from Vehicle-DC and TCDD-DC:T cell co-cultures with FACS-purified naïve $CD4^+$ T cells and placed with fresh naïve $CD4^+$ T cell responders (Tresp) at a 1:1 Treg:Tresp ratio. $CD4^+$ $Foxp3^-$ T cells purified from DC:T cell co-cultures were also placed with

Tresp. Tresp proliferation was assessed on day 4. * $p \leq 0.05$ indicates significant difference between Vehicle-DC:T cell and TCDD-DC:T cell groups.

AhR-activated DCs do not generate Tr1 regulatory T cells

Another possible outcome following the co-culture of TCDD-DCs with $OTII^+$ $CD4^+$ T cells is that instead of classical $CD4^+$ $CD25^+$ $Foxp3^+$ Treg induction, Tr1 regulatory cells may be induced. To assess this possibility, DC:T cell co-cultures were generated using with $OTII^+$ $CD4^+$ cells from OTII mice and the induction of $CD4^+$ $IL\text{-}10^+$ T cells in Veh- and TCDD-DC:T cell co-cultures was determined by intracellular staining. There was no induction of $CD4^+$ $IL\text{-}10^+$ T cells in either Veh- or TCDD-DC:T cell co-cultures. In contrast, anti-CD3/CD28 stimulated $CD4^+$ T cell controls did induce $CD4^+$ $IL\text{-}10^+$ T cells (Figure 3.7). Moreover, the addition of IL-27, which has been demonstrated to induce Tr1 cells in an AhR-dependent fashion, failed to induce appreciable levels of $CD4^+$ $IL\text{-}10^+$ T cells in our DC:T cell co-cultures (Appendix Table A.3) (Apetoh *et al.*, 2010).

Figure 3.7

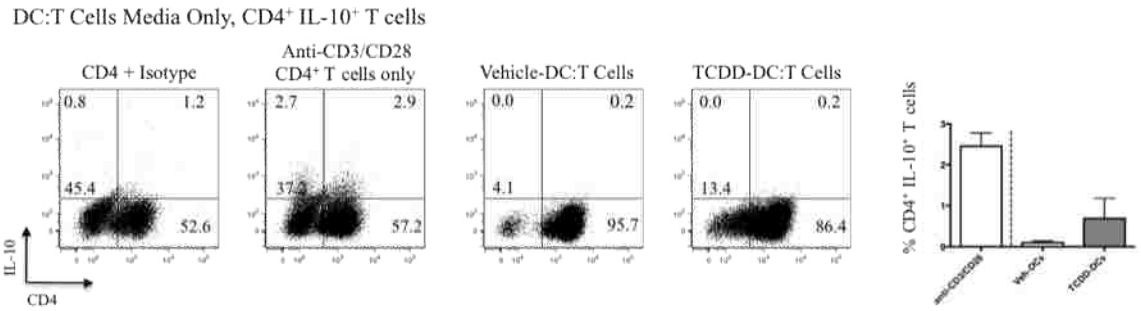

Figure 3.7. Generation of $CD4^+$ $IL\text{-}10^+$ T cells in DC:T cell co-cultures. Vehicle-DC and TCDD-DCs were co-cultured with enriched $OTII^+$ $CD4^+$ T cells and the percent of $CD4^+$ $IL\text{-}10^+$ T cells assessed on day 3.

DISCUSSION

Exposure to the prototypical AhR ligand and environmental pollutant TCDD is well known to cause immune suppression in various animal models (Harris *et al.*, 1973; Kerkvliet, 2002). However, the exact mechanisms underlying TCDD-induced immune suppression remain unclear. DCs are professional antigen presenting cells that are imperative to T cell activation and polarization. DCs also express high levels of the AhR, making them sensitive targets to environmental pollutants capable of activating the AhR. In previous studies we have characterized the effects of TCDD-induced AhR activation in inflammatory BMDCs (Bankoti *et al.*, 2010b; Simones and Shepherd, 2011). In these studies it was demonstrated that AhR activation disrupted BMDC differentiation in an AhR-dependent fashion assessed by altered CD11c, MHC II, CD86, CD54 and CD11a surface molecule expression (Bankoti *et al.*, 2010b; Simones and Shepherd, 2011). Furthermore, immature inflammatory BMDCs exposed to TCDD displayed unique changes in gene expression of known immune regulatory proteins (Bankoti *et al.*, 2010b; Simones and Shepherd, 2011). Inflammatory DCs were especially sensitive to TCDD-induced gene alterations, as IDO1 and IDO2 expression was increased more than 20-fold over Vehicle-BMDC expression (Bankoti *et al.*, 2010b). TGFβ3 expression was also significantly up-regulated following TCDD exposure in inflammatory BMDCs (Bankoti *et al.*, 2010b). The significant increases in IDO1, IDO2 and TGFβ3 coupled with the changes in surface molecule expression made TCDD-treated inflammatory BMDCs ideal candidates to test whether AhR-activated DCs could alter $CD4^+$ T cell responses *in vitro.*

There was little difference in the proliferative capacity of $OTII^+$ $CD4^+$ T cells in response to Ovap-loaded AhR-activated DCs or Vehicle-DCs. As expected, DCs exposed

to the glucocorticoid dexamethasone did inhibit $CD4^+$ T cell proliferation. While the proliferative capacity of $CD4^+$ T cells was unaffected, there were significant changes in the cytokines produced by Veh- and TCDD-DC-activated $CD4^+$ T cells, with increased IL-2 and decreased IFNγ and IL-10 levels being the typical pattern (Appendix Table A.4). These data suggest that TCDD-DCs may directly effect $CD4^+$ effector T cells and alter the course of an immune response. While IL-2 in association with TGFβ causes the generation of Tregs, IL-2 by itself can help drive the expansion of Th1 and Th2 cells as well as initiate activation induced cell death (AICD) in T cells (Liao *et al.*, 2011). The later effect of IL-2, AICD, may not be pertinent in our DC:T cell co-cultures as there was no difference in the number of viable $CD4^+$ T cells between Veh-DC and TCDD-DC groups, even though IL-2 levels were significantly increased in TCDD-DC:T cell co-cultures. The observed effects on IL-2 production in DC:T cell co-cultures with TCDD-DCs may be an indirect effect through AhR activation in the $CD4^+$ T cells by kynurenine produced in the DC:T cell co-cultures. IL-2 contains a number of DREs in its promoter/enhancer region and its production is increased in T cells following AhR activation in various model systems (Kerkvliet, 2009). To explain the reduction in IFNγ and/or IL-10 levels in TCDD-DC:T cell co-cultures, suppressor of cytokine signaling (SOCS) gene expression was measured in $CD4^+$ $Foxp3^-$ T cells from Veh- and TCDD-DC:T cell co-cultures on day 5 (data not shown). However, there were no changes in either SOCS1 or SOCS3 expression in the purified $CD4^+$ $Foxp3^-$ populations expanded by Veh- or TCDD-DCs (data not shown). Thus, mechanisms underlying the increased production of IL-2 and decreased production of INFγ and IL-10 by $CD4^+$ T cells following antigen-specific stimulation with TCDD-DCs remain to be elucidated.

Recently, TCDD-induced AhR activation has been shown to play a role in the polarization of regulatory T cells (Quintana *et al.*, 2008b; Mezrich *et al.*, 2010; Quintana *et al.*, 2010). The generation of Tregs may be an important consequence of AhR activation and may underlie the immunosuppressive effects of TCDD. It is possible that in our DC:T cell co-culture system that the altered cytokine levels are due to increases in $Foxp3^+$ Tregs that are subsequently altering the $CD4^+$ effector T cell response. We did observe an increase in $Foxp3^+$ Tregs in TCDD-DC:$CD4^+$ T cell co-cultures; however, we often observe inconsistent induction of $Foxp3^+$ Tregs. There are a number of explanations that may have contributed to the inconsistent $Foxp3^+$ Tregs readout. First, as compared to steady-state DCs, inflammatory DCs while being highly responsive to TCDD, via AhR activation, may not fully differentiate into tolerogenic DCs due to their inherent programming as inflammatory cells. However, others have shown that AhR activation in inflammatory BMDCs with ligands other than TCDD (ITE, I3C and Indirubin) effectively induces $Foxp3^+$ Tregs *in vitro* (Quintana *et al.*, 2010; Benson and Shepherd, 2011). Therefore, it is likely that ligand-specific AhR effects exist relative to the generation of functionally tolerogenic DCs. Second, the Ova-specific OTII model may be less sensitive than other immune models for the generation of $Foxp3^+$ Tregs. For example, recent studies have reported significant induction of $Foxp3^+$ Tregs in a myelin oligodendrocyte glycoprotein (MOG)-specific 2D2 $CD4^+$ T cell model using ITE-treated inflammatory BMDCs (Quintana *et al.*, 2010). However, we were unable to replicate these results with ITE-treated inflammatory BMDCs using the Ova-specific DC:T cell model, suggesting that inherent differences may exist in the generation of $Foxp3^+$ Tregs between these antigen-specific DC:T cell models *in vitro*. It is also possible that there are

strain-specific differences. For example, when DC:T cell co-cultures were set up using inflammatory BMDCs from Balb/c mice and Ovap-specific $CD4^+$ T cells from DO11.10 mice, we observed a significant increase in the percent of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs following intracellular staining (Appendix Figure A.5). Thus, further investigations into possible antigen- and strain-specific effects on the generation of $Foxp3^+$ Tregs with TCDD-treated inflammatory BMDCs *in vitro* are warranted. Third, AhR-activated inflammatory BMDCs may not induce $Foxp3^+$ Tregs, but rather generate Tr1 or Th3 type regulatory T cells. However, the induction of $CD4^+$ IL-10^+ Tr1 cells was not observed in our studies and little to no TGFβ1 or TGFβ3 was detected in the DC:T cell co-cultures containing AhR-activated inflammatory BMDCs. There remains the possibility that AhR-activated BMDCs can also induce regulatory or suppressive T cells that are not $Foxp3^+$, Tr1 or Th3-type Tregs. This is evident by the fact that in suppression assays $CD4^+$ $Foxp3^-$ T cells from AhR-activated DC:T cell co-cultures had a similar suppressive capacity as $CD4^+$ $Foxp3^+$ Tregs. In addition, in a graft vs. host model, functional $CD4^+$ $CD25^+$ $Foxp3^-$ Tregs were generated following TCDD exposure rather than $Foxp3^+$ Tregs (Funatake *et al.*, 2005b; Marshall *et al.*, 2008). Forth, both DCs and T cells may require TCDD-induced AhR activation for full commitment towards Tregs. While this scenario better emulates *in vivo* exposures, no increases were observed in the generation of Tregs when TCDD was present throughout the course of a 3 day DC:T cell co-culture experiment. Fifth, there may be a timing issue in the generation of $Foxp3^+$ Tregs, in that AhR-activated BMDCs may induce Tregs in the first 24 to 48 hours in culture. These time points were not assessed in our DC:T cell co-cultures; however, due to the stability of $Foxp3^+$ Tregs in the presence of IL-2 and TGFβ it is unlikely that $Foxp3^+$ Tregs

generated in the initial 24 to 48 hours of the DC:T cell co-cultures would not be present on days 3 through 5 (time points that were assessed). Thus, it is unlikely that a temporal component exists for the detection and retention of $Foxp3^+$ Tregs in DC:T cell co-cultures utilizing AhR-activated BMDCs. Overall, it is likely that TCDD-treated inflammatory BMDCs can induce $Foxp3^+$ Tregs in an antigen-specific fashion; however, further studies will need to address potential AhR ligand-, antigen- and stain-specific differences that may influence the generation of regulatory T cells *in vitro*.

Another possible explanation for the observed TCDD-DC:Treg induction inconsistencies is that the effects of TCDD in BMDCs, i.e. changes in surface molecule expression and increases in regulatory gene expression, were different between experiments. However, in nearly all of the DC:T cell co-culture experiments we observed the standard TCDD-induced phenotypic surface marker alterations which consisted of decreases in CD11c, CD11a, CD54 and increased CD86 expression on a per cell basis (Appendix Table A.1). Moreover, there were minimal changes in expression of other markers thought to be associated with tolerogenic DCs such as CD103 and TGFβ in BMDCs exposed to TCDD (data not shown). In DC:T cell experiments in which gene expression was analyzed in Veh- and TCDD-DCs as a quality control measure, there was consistent up-regulation of IDO1, IDO2 and TGFβ in TCDD-DCs (Appendix Table A.5). There were changes, although inconsistent, in the expression of TGFβ1 and TGFβ2 as well as Aldh1a2 between experiments, with significant decreases in TGFβ1 and significant increases in TGFβ2 and Aldh1a2 observed from experiment to experiment (Appendix Table A.5). Furthermore, it is unclear whether any of these changes in gene expression translated into increased protein expression and/or protein activity. In one case

where protein levels of IDO and TGFβ1 and TGFβ3 were measured in Veh- and TCDD-DCs, there was no significant change in their protein expression levels in TCDD-DCs when compared to Veh-DC (data not shown). While IDO gene expression may have been increased in TCDD-DCs there may be no difference in its ability to catalyze the breakdown of tryptophan between Veh- and TCDD-DCs. In addition, IDO activity in DCs has been shown to be dependent on the activation status of DCs, with IDO enzymatic activity achieved only after additional activation with TNFα or TLR ligands (Braun *et al.*, 2005; Pallotta *et al.*, 2011). However, LPS-stimulated BMDCs exposed to TCDD did not display a greater ability to induce Tregs than LPS-stimulated Veh-DCs or unstimulated Veh- or TCDD-DCs (Appendix Table A.1). Furthermore, natural levels of tryptophan in culture media have been shown to influence the generation of Tregs vs. Th17 cells and increased Treg induction has been observed in F10 media that lacks tryptophan (Veldhoen *et al.*, 2009; Mezrich *et al.*, 2010). However, there was no increase in the percent of Tregs generated following interactions with TCDD-DCs in F10 media in our DC:T cell co-culture system with inflammatory BMDCs (Appendix Table A.1). Taken together our data suggests that while inflammatory BMDCs exposed to TCDD significantly up-regulate immune regulatory gene expression, they are not always capable of generating $Foxp3^+$ or Tr1 Tregs.

In summary, we demonstrated that inflammatory AhR-activated BMDCs altered antigen-specific $CD4^+$ T cell responses by affecting $CD4^+$ effector T cell cytokine production. The altered cytokine levels may be attributed to the generation of $Foxp3^+$ Tregs rather than Tr1 Tregs in TCDD-DC:T cell co-cultures. The inconsistent generation of $Foxp3^+$ Tregs in our *in vitro* DC:T cell system may highlight the fact that there are

likely additional signals required for AhR-activated DCs to become tolerogenic and that the *in vitro* system lacks these additional signals or is too insensitive to detect to replicate the effects that occur *in vivo*.

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CHAPTER 4

Consequences of TCDD-Induced AhR Activation During Antigen-Specific Immune Responses *In vivo*

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ABSTRACT

Environmental pollutants capable of activating the aryl hydrocarbon receptor (AhR) cause immunosuppression. AhR activation can induce regulatory T cells (Tregs), which may contribute to the observed immunosuppression. However, the mechanisms underlying Treg induction and immune modulation following AhR activation are unclear. We hypothesized that AhR activation by TCDD generates both regulatory dendritic cells (DCs) and Tregs *in vivo*. To determine if AhR-activation induces antigen-specific Tregs *in vivo*, $CD4^+$ T cells isolated from OTII $Foxp3^{eGFP}$ mice were transferred iv into vehicle- or TCDD-exposed mice followed by footpad immunization with ovalbumin (ova) peptide-loaded bone marrow-derived BMDCs. The antigen-specific immune response in the draining popliteal lymph node was dampened in TCDD-exposed mice as determined by a reduction in the number of ova-specific $CD4^+$ T cells and an increase in ova-specific $CD4^+$ $Foxp3^+$ Tregs. In addition, $CD4^+$ T cells from the draining lymph node produced significantly less IL-2, IFNγ and IL-10 when restimulated with ova peptide. Similar results were observed in a second Ovap-specific model in which Vehicle- and TCDD-exposed mice were immunized with Ovap in adjuvant and DCs and $CD4^+$ T cells analyzed in the spleen and poptileal and brachial lymph nodes 10 days later. Overall, these results indicate that *in vivo* exposure to the prototypical AhR ligand, TCDD, induces both regulatory DCs and $Foxp3^+$ Tregs, which contribute to the generation of an immunosuppressive environment.

INTRODUCTION

It is well known that exposure to the environmental pollutant and prototypical aryl hydrocarbon receptor (AhR) ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes immune suppression in mice (Kerkvliet, 2002). Numerous studies have implicated TCDD-induced AhR activation in decreased antibody production, altered $CD8^+$ and $CD4^+$ T cell responses and disrupted dendritic cell (DC) fate and function, all of which ultimately lead to increased susceptibility to bacterial and viral infections in animal models (Kerkvliet *et al.*, 1996; Sulentic *et al.*, 2000; Vorderstrasse *et al.*, 2003; Bankoti *et al.*, 2010a). On the other hand, the immunosuppressive effects of TCDD have recently been demonstrated to dampen autoimmune disease responses in a murine model of multiple sclerosis, in which researchers uncovered that TCDD exposure causes the induction of $Foxp3^+$ regulatory T cells (Tregs) (Quintana *et al.*, 2008b). The immunosuppressive nature of TCDD may be at least in part due to its ability to induce Tregs. However, AhR activation with 6-formylindolo[3,2-b]carbazole (FICZ), an endogenous AhR ligand, can induce pro-inflammatory Th17 cells rather than Tregs during experimental autoimmune encephalomyelitis (EAE) in mice (Quintana *et al.*, 2008b; Veldhoen *et al.*, 2008). In addition, AhR activation has been implicated in the control of Th17 cells that produce copious amounts of IL-22 (Veldhoen *et al.*, 2008; Alam *et al.*, 2010; Stockinger *et al.*, 2011). Somewhat in contrast to studies, Kerkvliet and co-workers discovered a novel population of suppressive $CD4^+$ $CD25^+$ $Foxp3^-$ T cells that arose during graft vs. host responses in the presence of TCDD (Funatake *et al.*, 2005b). These *in vivo* studies have demonstrated that the AhR plays an integral role in $CD4^+$ T cell polarization. However, is unclear whether AhR activation induces murine

Tregs or Th17 cells in models other than EAE or whether TCDD-exposure induces Foxp3$^-$ suppressor T cells in various antigen-specific immune response models.

Here we show that TCDD exposure decreases antigen-specific CD4$^+$ T cell responses in two separate murine models designed to better understand the role of AhR activation in both DCs and T cells and the subsequent generation of Tregs. First, we studied the effects of TCDD exposure during Ovalbumin-specific CD4$^+$ T cell responses initiated by Ovalbumin peptide-loaded DCs transferred into host mice through the hind footpad. In this adoptive transfer model CD4$^+$ T cell responses are decreased in mice exposed to TCDD and Tregs generated, but only when a mixed population containing Ovap-specific naïve CD4$^+$ T cells and natural Treg populations are transferred into host mice prior to TCDD exposure. When purified Ovap-specific CD4$^+$ CD25$^-$ Foxp3$^-$ populations are transferred into host mice, no iTregs are generated, although the localized CD4$^+$ T cell response is decreased following TCDD exposure. Second, in a systemic Ovalbumin-specific adjuvant model, TCDD exposure decreased antibody production, reduced the number of splenic CD4$^+$ T cells and CD11c$^+$ MHC II$^+$ DCs and increased the frequency of CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs in the spleen and lymph nodes. Moreover, TCDD-exposure in this model increased the frequency of iTregs generated from antigen-specific naïve CD4$^+$ CD25$^-$Foxp3$^-$.

MATERIALS AND METHODS

Animals 6-8 week old male and female C57Bl/6 and CD45.1 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred at the University of Montana. OTII Foxp3^{eGFP} reporter mice were kindly provided by Dr. Randolph Noelle (Dartmouth Medical School, Lebanon, NH) who originally obtained these mice from Dr. Alexander Rudensky (University of Washington School of Medicine, Seattle, WA). All animals were housed and maintained at UM and provided water and chow *ad libitum*. All animal experiments were approved by the UM IACUC and adhered to the current NIH guidelines for animal usage.

Chemicals and Reagents TCDD was purchased and supplied in anisole from Cambridge Isotope Laboratories (Andover, MA). Bone marrow-derived DCs (BMDCs) were grown in complete RPMI (cRPMI): 20mM HEPES, 1.5mM sodium pyruvate, 50ug/ml gentamicin and 10% fetal bovine serum (Hyclone Thermo Fisher Scientific Waltman, MA). All other media reagents were obtained from Invitrogen (Carlsbad, CA). Ova-peptide, $Ova_{323-339}$, was obtained from Mimotopes (Clayton, Victoria Australia). Recombinant human IL-2 and TGFβ1 were purchased from Peprotech (Rocky Hill, NJ) and Biolegend (San Diego, CA). Violet Dye Cell Trace was purchased from Invitrogen.

Flow Cytometry DCs and T cells were analyzed by flow cytometry as described previously (Shepherd *et al.*, 2001). Briefly, $1x10^5$ - $1x10^6$ cells were washed with PBS containing 1% bovine serum albumin and 0.1% NaN_3 and resuspended in Fc block, anti-CD16/CD23 antibody generated and purified from 2.4G2 hybridoma cells, to block non-

specific binding. The cells were then stained with fluorochrome-conjugated antibodies for 10-15min on ice. Appropriate isotype controls were used in conjunction with the primary antibody staining. Intracellular Foxp3 staining was performed using a mouse Treg Flow Kit from BioLegend, following manufacturer's instructions. Antibodies utilized for DC sating: CD45.2 PerCpCy5.5 (30-F11), MHC class II-PE (M5/114.15.12), CD80-PE (16-10A1), CD86-Pacific Blue (GL-1), CD103 (2E7) from BioLegend; and CD11c-APC (HL3) and CD40 (3/23) from BDPharmingen (San Diego, CA). For T cell staining: CD45.2 PerCpCy5.5 (30-F11), CD4 APC, AF700 or Pacific Blue (GK1.5), CD25 APC or PerCpCy5.5 (PC61) and Vα2 PE (B20.1) from BioLegend. All samples were analyzed on a FACSAria flow cytometer using BD FACS Diva software, version 4.0 (BD Biosciences San Jose, CA). Figures were generated using FlowJo, version 8.8.6 (Tree Star Inc, Ashland OR).

Bone Marrow-Derived Dendritic cells (BMDCs) BMDCs were generated using methods as previously described (Inaba *et al.*, 1992; Bankoti *et al.*, 2010b). Briefly, bone marrow cells from the tibia and femur were flushed using cRPMI. Progenitor cells were separated from RBCs and debris by centrifugation using lympholyte M (Cederlane Laboratories; Burlington, NC) and were grown in cRPMI supplemented with 30ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF) purchased from PeproTech or Biolegend. On days 3 and 5, non-adherent cells were removed, washed and reseeded in fresh media containing GM-CSF. On day 6-7 non-adherent BMDCs were harvested, enumerated and cell viability assessed using Trypan Blue (Sigma-Aldrich). $CD11c^+$ DCs were purified using magnetic separation techniques to ≥ 95% with CD11c-MicroBeads

and an autoMACS Cell Separator (Miltenyi Biotec Inc. Auburn, CA). $CD11c^+$ BMDCs were loaded with 50μg/mL Ova-peptide (Ovap) in cRPMI for 2-24h. Ovap-loaded BMDCs were washed twice with cRPMI to remove excess Ovap and resuspended in Hanks balanced salt solution (HBSS) (Invitrogen).

Ovap-DC Footpad Immunization Adoptive Transfer Model Antigen-specific Treg generation *in vivo* was assessed using an OTII adoptive transfer model as previously described (Bankoti *et al.*, 2010b; Simones and Shepherd, 2011). Briefly, $CD45.2^+$ $OTII^+$ $CD4^+$ T cells were isolated from the spleen and popliteal and brachial lymph nodes of OTII $Foxp3^{eGFP}$ reporter mice. $OTII^+$ $CD4^+$ T cells were enriched to ≥ 75% $CD45.2^+$ $OTII^+$ $CD4^+$ with a $CD4^+$ T cell Isolation kit and an autoMACS Cell Separator (Miltenyi Biotec Inc). Enriched $OTII^+$ $CD4^+$ T cells were washed, resuspended in HBSS and $1x10^6$ cells transferred into CD45.1 host mice intravenously. In separate experiments to assess the generation of iTregs, enriched $OTII^+$ $CD4^+$ T cells were further purified to ≥ 99% $CD4^+$ $CD25^-$ $Foxp3^{eGFP-}$ via fluorescence cell activated cell sorting (FACS) with a FACSAria flow cytometer and $CD24.2^+$ $CD4^+$ $CD25^-$ $Foxp3^{eGFP-}$ T cells ($1x10^6$) transferred intravenously into CD45.1 host mice. 24h post-$CD4^+$ T cell transfer, CD45.1 host mice were gavaged with vehicle (anisole in peanut oil) or TCDD (15μg/kg). Ovap-loaded $CD45.2^+$ $CD11c^+$ BMDCs were then injected ($1x10^6$ - $2x10^6$) into each hind footpad of host mice 24h after vehicle and TCDD exposure. The popliteal and brachial lymph nodes were harvested 4-5 days post-BMDC immunization and the Ovap-loaded donor BMDCs ($CD45.2^+$ $CD11c^+$) and antigen-specific $OTII^+$ T cell ($CD45.2^+$ $CD4^+$) populations analyzed by flow cytometry. One hundred thousand cells from the popliteal

lymph node were restimulated in 96-well plates with Ovap (100μg/mL) or with anti-CD3 (5μg/mL) and anti-CD28 (5μg/mL) antibody-coated wells.

Ovap Sigma Adjuvant System Model The generation of tolerogenic DCs and Tregs following TCDD exposure was assessed in an adjuvant-induced inflammatory model. $CD45.1^+$ host mice were gavaged with vehicle or TCDD (15μg/kg). Twenty four hours post-vehicle and TCDD exposure, host mice were immunized i.p. with Ovap (1mg/mL) suspended in Sigma Adjuvant System following manufacturer's instructions (Sigma Aldrich). Mice were euthanized 10 days post-immunization and blood, spleen and popliteal and brachial lymph nodes collected. Single cell suspensions were prepared from the spleen and combined popliteal and brachial lymph nodes and DC and $CD4^+$ T cell phenotypes assessed by flow cytometry. $Foxp3^+$ Tregs were quantified via intracellular Foxp3 staining. One hundred thousand cells from the spleen or lymph nodes were restimulated with Ovap (100μg/mL) or plate-bound anti-CD3 (5μg/mL) and anti-CD28 (5ug/mL) and IL-2, IFNγ and IL-10 production assessed via ELISA. Splenic $CD11c^+$ DCs were enriched using a mouse CD11c DC isolation kit according to manufacturer's instructions (Invitrogen) and were further purified by FACS to a purity of 50-90% $CD11c^+$. A fraction of purified DCs were used for qRT-PCR studies and the remainder were loaded with Ovap (50μg/mL) and cultured with enriched Violet Dye Cell Trace-labeled $OTII^+$ $CD4^+$ T cells from naïve OTII $Foxp3^{eGFP}$ reporter mice in the presence of IL-2 (100U/mL) and TGFβ (5ng/mL). Splenic Ovap-DC:T cell co-cultures were harvested on day 5 and $CD4^+$ T cell proliferation and $CD4^+$ $CD25^+$ $Foxp3^+$ generation determined by flow cytometry.

In a separate Ovap/adjuvant experiment, FACS-purified (≥ 98%) $OTII^+$ $CD4^+$ $CD25^-$ $Foxp3^{eGFP-}$ T cells were transferred into $CD45.1^+$ host mice 24h before vehicle and TCDD exposure. The $CD45.1^+$ host mice were gavaged and immunized as described above. On day 10 post-immunization, antigen-specific $CD45.2^+$ $CD4^+$ $CD25^+$ $Foxp3^{eGFP+}$ Tregs were assessed from the spleen and popliteal and brachial lymph nodes. Host $CD45.1^+$ $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs were also analyzed via intracellular Foxp3 staining.

Antibody and Cytokine Measurement Total IgG, IgG2a and IgM and Ovap-specific IgG, IgG2a and IgM levels were measured in the plasma of individual mice by ELISA following manufacturer's instructions (Southern Biotech, Birmingham AL). Ova-specific IgG, IgG2a and IgM levels were quantified using whole ovalbumin (50μg/mL) coated plates. Supernatants DC:T cell co-cultures were collected and stored at -20^{o}C until further analysis. IL-2, IFNγ and IL-10 cytokine production was measured using ELISAs per the manufacturer's instructions (BD Biosciences). IL-22 levels were measured using ELISA from R&D Systems (Minneapolis, MN) according to manufacturer's instructions.

Quantitative Real-Time Reverse Transcription-polymerase Chain Reaction (qRT-PCR) Splenic DC gene expression was assessed by qRT-PCR as previously described (Bankoti *et al.*, 2010b). FACS-purified splenic DCs were washed twice in PBS and resuspended in Trizol (Invitrogen) to isolate RNA. Primers for indoleamine 2,3-dioxygenase 1 (Ido1) and Ido2, aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a1) and Aldh1a2, Arginase (Arg1), transforming growth factor β1 (Tgfβ1), Tgfβ2 and Tgfβ3, transforming growth factor beta receptor II (TgfβRII), aryl hydrocarbon

receptor (Ahr), aryl hydrocarbon receptor repressor (Ahrr), Cytochrome P450 1a1 (Cyp1a1) and interleukin-6 (IL-6), IL-10, IL-23 and IL-27 were purchased from SABiosciences (Frederick, MD) or IDT (San Diego CA). mRNA levels were determined using SYBR green in qRT-PCR reactions (SABiosciences) on a BIO-RAD IQ 5 Light Cycler (BIO-RAD; Hercules, CA).

Statistical Analysis Student's t tests were used to compare two individual samples. The delta delta Ct method was used to calculate fold changes in qRT-PCR studies, with Student's t test used to measure significance. p values ≤ 0.05 were considered significant.

Figure 4.1

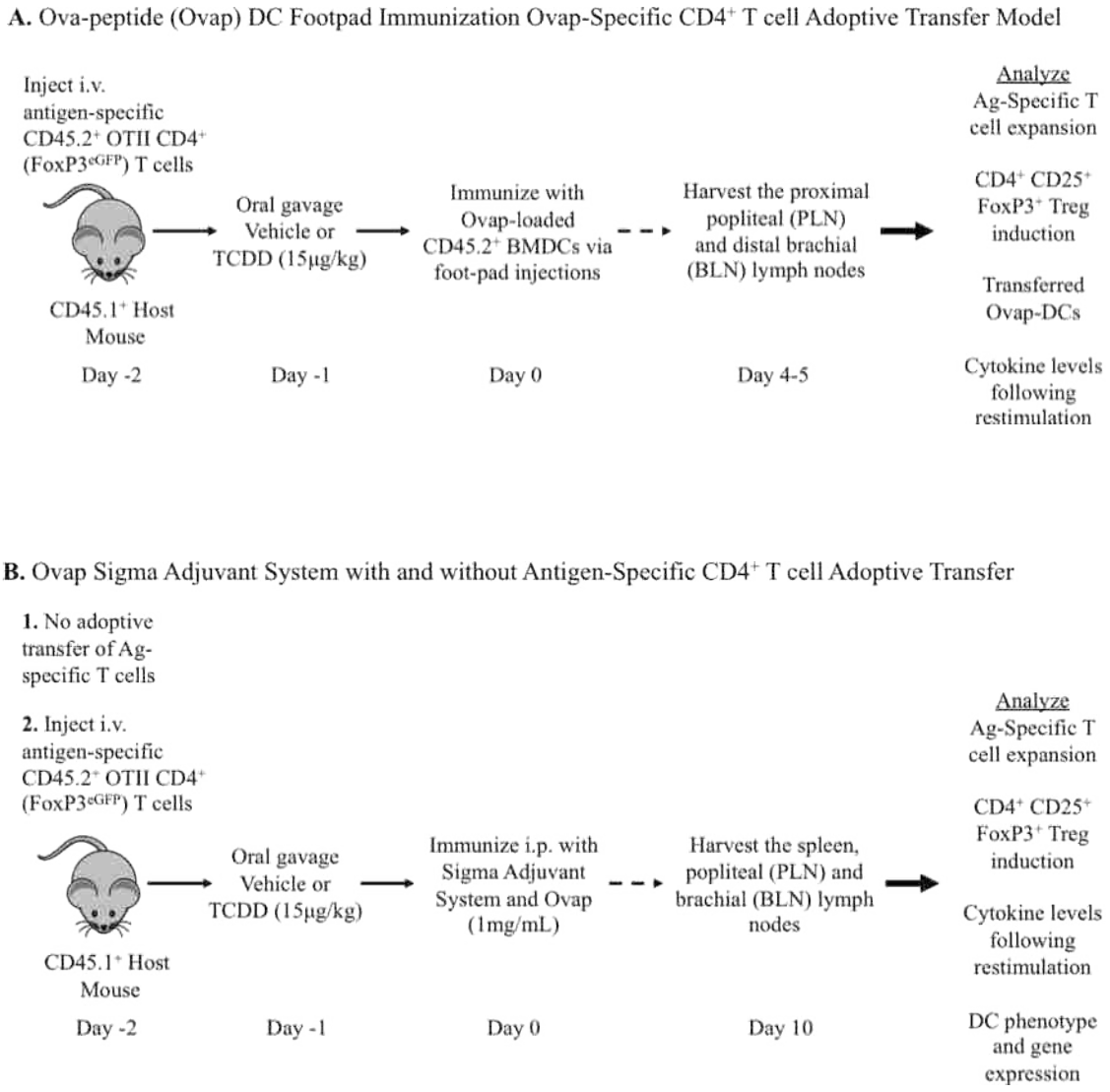


Figure 4.1. *In vivo* adoptive transfer models.

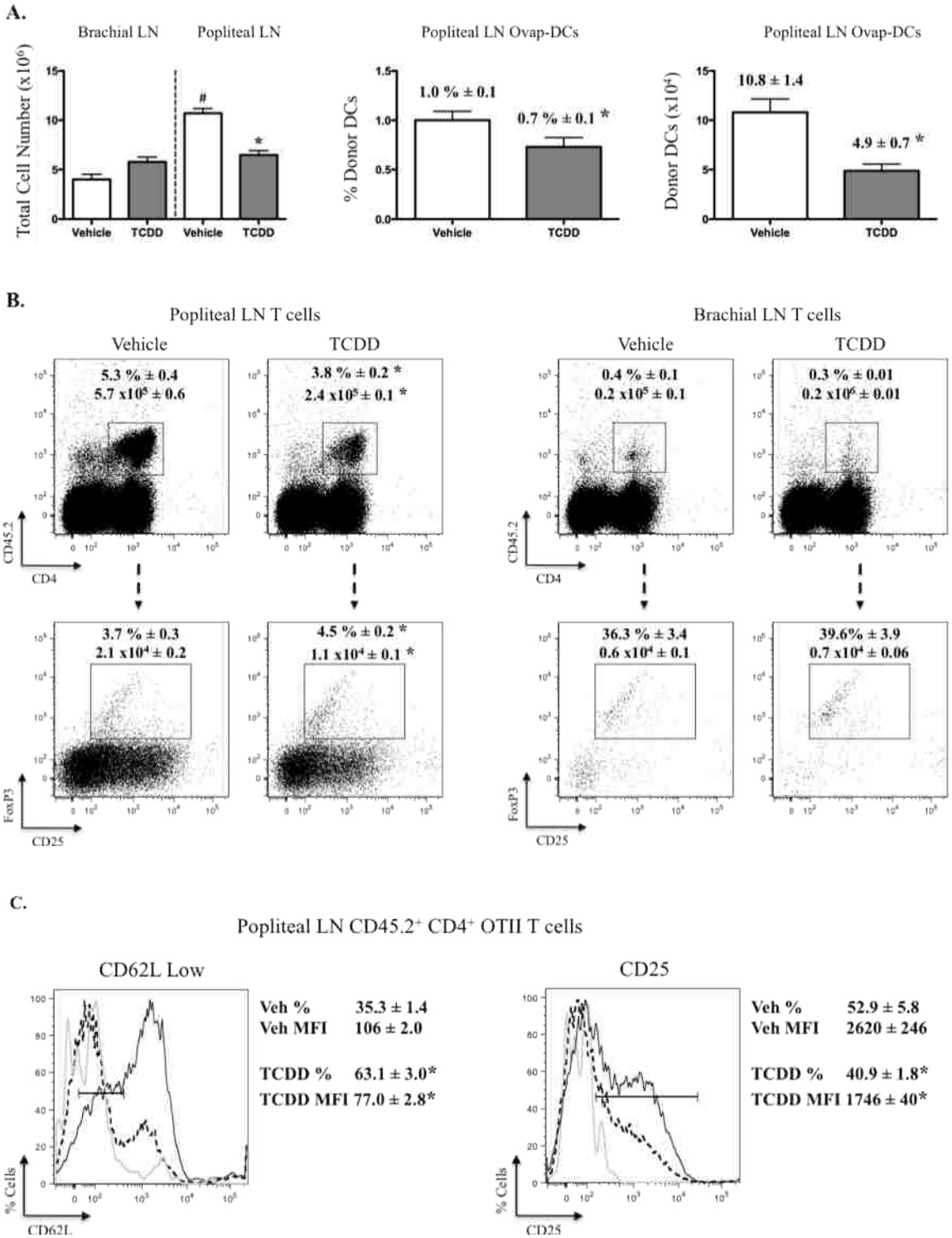
RESULTS

TCDD-induced AhR activation decreases antigen-specific CD4$^+$ T cell responses

Antigen-specific interactions between DCs and T cells were assessed *in vivo* to determine whether TCDD-induced AhR activation causes the generation of tolerogenic DCs and Tregs. Initially, CD45.1$^+$ host mice were seeded with enriched ~75% CD45.2$^+$ OTII$^+$ CD4$^+$ T cells from naïve OTII Foxp3$^+$ reporter mice (Figure 4.1A). This Ovap-specific CD4$^+$ T cell population contained both naïve CD4$^+$ T cells as well as ~5% Foxp3^{eGFP+} natural Tregs. CD45.1$^+$ host mice were exposed to vehicle or TCDD 24h after the CD4$^+$ T cell transfer. Following transfer of CD4$^+$ T cells, Ovap-loaded CD11c$^+$ BMDCs were injected into the hind footpads of host mice to elicit an antigen-specific immune response primarily in the draining popliteal lymph nodes (PLNs) when compared to the distal brachial lymph nodes (BLNs) (Figure 4.1A). On day 4 post-DC footpad immunization, a significant increase in the total number of cells in the PLNs was observed, which correlated with a significant increase in the frequency and number of Ovap-specific CD45.2$^+$ CD4$^+$ OTII T cells when compared to BLN samples (Figure 4.2). In TCDD-exposed mice there was a significant reduction in both the percent and number of Ovap-loaded donor DCs and CD45.2$^+$ CD4$^+$ T cells in the PLNs when compared to Vehicle-treated samples from the PLN (Figure 4.2). However, there was a significant increase in the frequency of CD45.2$^+$ CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs in the PLNs of mice exposed to TCDD (Figure 4.1B). Because there was a significant reduction in total cell numbers in the PLNs of TCDD-exposed mice, the increased Treg frequency did not correlate with an increased number of CD45.2$^+$ CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs.

Antigen-specific CD45.2$^+$ CD4$^+$ OTII T cells from the PLNs of TCDD-exposed mice displayed significantly reduced levels of both CD62L and CD25 surface molecules when compared to expression levels on CD45.2$^+$ CD4$^+$ T cells from PLNs of Vehicle-mice. To assess the CD4$^+$ T effector response in the draining lymph node, cells from the PLNs of Vehicle- and TCDD-exposed mice were restimulated with Ovap or anti-CD3/CD28 for 48h and cytokine levels measured. As expected there was an increased production of IL-2, IFNγ and IL-10 following Ovap or anti-CD3/CD28 stimulation when compared to unstimulated PLN samples (Figure 4.2D). Cells from the PLNs of TCDD-exposed mice produced significantly less IL-2, IFNγ and IL-10 following restimulation when compared to cells from Vehicle-treated mice (Figure 4.2D).

Figure 4.2


A.
Brachial LN
Popliteal LN
Total Cell Number (x10⁶)
Vehicle
TCDD
Vehicle
TCDD
Popliteal LN Ovap-DCs
% Donor DCs
1.0 % ± 0.1
0.7 % ± 0.1 *
Vehicle
TCDD
Popliteal LN Ovap-DCs
Donor DCs (x10⁴)
10.8 ± 1.4
4.9 ± 0.7 *
Vehicle
TCDD
B.
Popliteal LN T cells
Vehicle
TCDD
5.3 % ± 0.4
5.7 x10⁵ ± 0.6
3.8 % ± 0.2 *
2.4 x10⁵ ± 0.1 *
CD45.2
CD4
3.7 % ± 0.3
2.1 x10⁴ ± 0.2
4.5 % ± 0.2 *
1.1 x10⁴ ± 0.1 *
FoxP3
CD25
Brachial LN T cells
Vehicle
TCDD
0.4 % ± 0.1
0.2 x10⁵ ± 0.1
0.3 % ± 0.01
0.2 x10⁶ ± 0.01
CD45.2
CD4
36.3 % ± 3.4
0.6 x10⁴ ± 0.1
39.6% ± 3.9
0.7 x10⁴ ± 0.06
FoxP3
CD25
C.
Popliteal LN CD45.2⁺ CD4⁺ OTII T cells
CD62L Low
Veh % 35.3 ± 1.4
Veh MFI 106 ± 2.0
TCDD % 63.1 ± 3.0*
TCDD MFI 77.0 ± 2.8*
% Cells
CD62L
CD25
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TCDD % 40.9 ± 1.8*
TCDD MFI 1746 ± 40*
% Cells
CD25

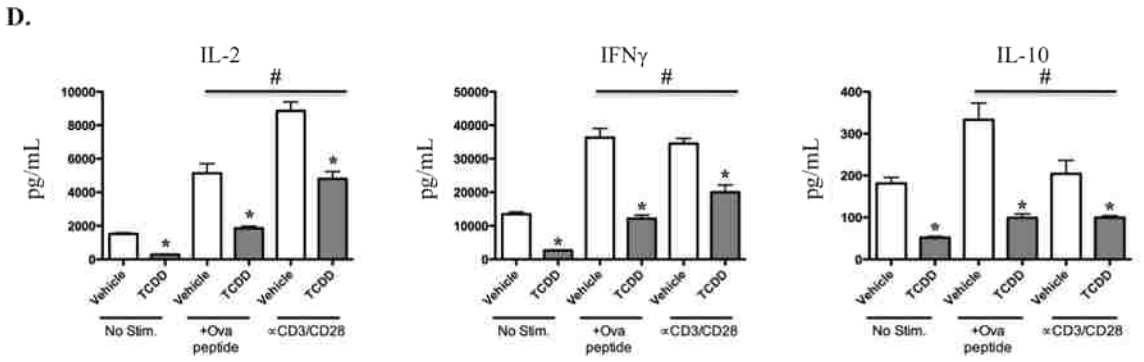


Figure 4.2. TCDD exposure disrupts antigen-specific immune responses in adoptively transferred mice. CD45.1^{+} host mice were intravenously seeded with CD45.2^{+} OTII^{+} CD4^{+} T cells enriched from OTII CD4 Foxp3^{eGFP} reporter mice and gavaged with vehicle or TCDD (15μg/kg) 24h later. Ovap-loaded CD11c^{+} BMDCs were injected into host mouse hind footpads 24h post-Vehicle or TCDD exposure. The distal brachial lymph nodes (BLNs) and proximal popliteal lymph nodes (PLNs) were harvested 4 days after Ovap-DC footpad immunizations. (A) Total PLN and BLN cell numbers were assessed and the frequency of CD45.2^{+} CD4^{+} Ovap-specific T cells and CD45.2^{+} CD11c^{+} DCs determined via flow cytometry. (B) Overall frequency and calculated numbers of Ovap-specific CD45.2^{+} CD4^{+} T cells and CD45.2^{+} CD4^{+} CD25^{+} Foxp3^{eGFP+} Tregs in the PLNs and BLNs of Vehicle- and TCDD-exposed mice. (C) Activation status of CD45.2^{+} CD4^{+} T cells in the PLNs, percent and mean fluorescence intensity (MFI). Samples represented as isotype; staining grey line, Vehicle-mice; solid black line, TCDD-exposed mice; heavy black dashed line. (D) Cytokine levels in 48h restimulation cultures with 100,000 cells from the PLNs of Vehicle- and TCDD-treated mice. No stim denotes cells in media alone. ***** indicates significance with $p \leq 0.05$ between Vehicle and TCDD groups. # indicates significant difference between (A) BLN and PLN samples and (D) No stim and stimulation samples. Data are represented as mean ± standard error of the mean (SEM) with n = 6 from one experiment.

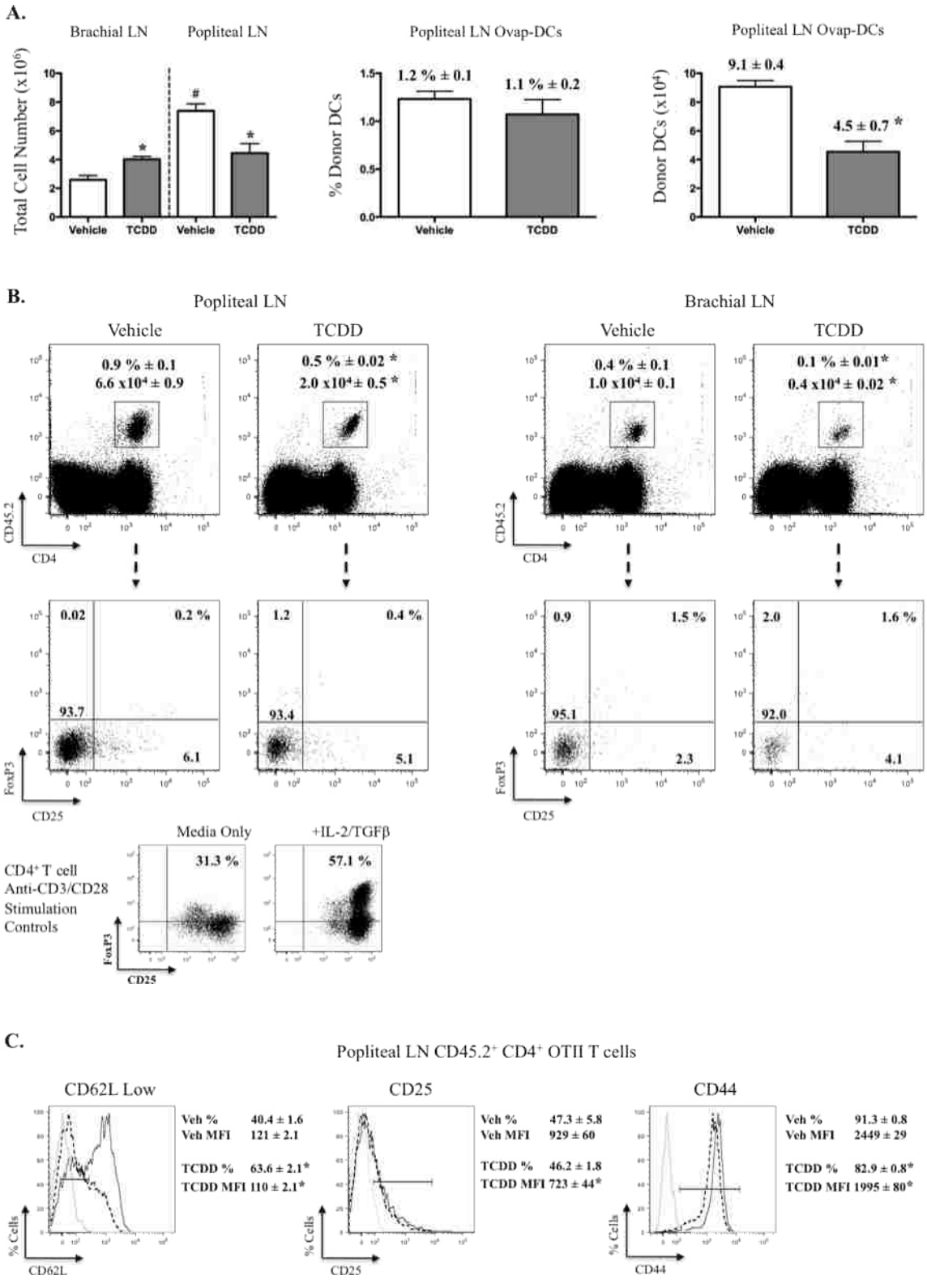
TCDD does not induced antigen-specific iTregs in the Ovap-DC model

Expansion of Foxp3$^+$ regulatory T cells in the periphery can be attributed to either expansion of nTregs and/or the generation of iTregs from naïve CD4$^+$ T cells. While AhR activation can induce both Treg and Th17 cells during autoimmune inflammatory responses, it is unclear whether AhR activation leads to the generation of iTregs or the expansion on nTregs in other immune models (Quintana *et al.*, 2008b; Veldhoen *et al.*, 2008; Quintana *et al.*, 2010). In the first Ovap-DC adoptive transfer experiment, host mice were seeded with an enriched population of antigen-specific CD4$^+$ T cells, which contained a small fraction ($\leq$ 5%) of CD4$^+$ CD25$^+$ Foxp3$^+$ nTregs. To determine if TCDD-induced AhR activation causes the induction of antigen-specific iTregs following interactions with Ovap-DCs, CD45.1$^+$ host mice were seeded with FACS-purified CD45.2$^+$ CD4$^+$ CD25$^-$ Foxp3$^-$ T cells from OTII Foxp3^{eGFP} mice. In this experimental design, the generation of Foxp3$^+$ Tregs in the draining PLN is attributable exclusively via the induction of iTregs due to the absence of nTregs. Similar to the first Ovap-DC footpad immunization adoptive transfer experiment, there was a significant increase in total cell numbers in the PLNs when compared to the BLNs on day 5 post-DC footpad immunizations (Figure 4.3A). There was no significant difference in the frequency of Ovap-loaded donor DCs in the PLNs between vehicle- and TCDD-exposed mice (Figure 4.3A). However, there was a significant reduction in total PLN cell numbers in TCDD-exposed mice when compared to the Vehicle-treated controls. This reduction in overall cell number corresponded to a significant reduction in the number of Ovap-loaded donor DCs (Figure 4.3A). Additionally, the frequency and number of Ovap-specific CD45.2$^+$ CD4$^+$ T cells was significantly reduced in the PLNs following TCDD-exposure when

compared to the Vehicle-treated controls (Figure 4.3B). Although we expected to see an increase in the frequency of $Foxp3^+$ iTregs in TCDD-exposed mice, $Foxp3^+$ iTregs were not observed in the PLNs of either Vehicle- or TCDD-exposed mice (Figure 4.3B). This may have been due to lack of antigen-specific $CD4^+$ T cell responsiveness. However, FACS-purified $CD4^+$ $CD25^-$ $Foxp3^-$ T cells were responsive to anti-CD3/CD28 simulation *in vitro*, and under optimal Treg induction conditions with IL-2 and TGFβ there was a 57% increase in $Foxp3^+$ iTregs (Figure 4.3B).

While there was no induction of iTregs in TCDD-exposed mice, $CD4^+$ effector cells from TCDD-exposed mice displayed significantly reduced expression of CD62L, CD25 and CD44 (Figure 4.3C). Furthermore, $CD4^+$ T cell responses were diminished following *ex vivo* restimulation. In response to Ovap, cells from the PLNs of TCDD-exposed mice produced significantly less IL-2 and IL-10, while there was trend toward decreased IFNγ production when compared to stimulated PLN cells from Vehicle-mice (Figure 4.3C). There were no significant differences between Vehicle and TCDD PLN groups following stimulation with anti-CD3/CD28 (Figure 4.3C).

Figure 4.3


A.
Brachial LN
Popliteal LN
Total Cell Number (x10^6)
Vehicle
TCDD
Vehicle
TCDD
Popliteal LN Ovap-DCs
% Donor DCs
1.2 % ± 0.1
1.1 % ± 0.2
Vehicle
TCDD
Popliteal LN Ovap-DCs
Donor DCs (x10^4)
9.1 ± 0.4
4.5 ± 0.7 *
Vehicle
TCDD
B.
Popliteal LN
Brachial LN
Vehicle
TCDD
Vehicle
TCDD
0.9 % ± 0.1
6.6 x10^4 ± 0.9
0.5 % ± 0.02 *
2.0 x10^4 ± 0.5 *
0.4 % ± 0.1
1.0 x10^4 ± 0.1
0.1 % ± 0.01*
0.4 x10^4 ± 0.02 *
CD45.2
CD4
0.02
0.2 %
93.7
6.1
1.2
0.4 %
93.4
5.1
0.9
1.5 %
95.1
2.3
2.0
1.6 %
92.0
4.1
FoxP3
CD25
Media Only
+IL-2/TGFβ
CD4+ T cell
Anti-CD3/CD28
Stimulation
Controls
31.3 %
57.1 %
FoxP3
CD25
C.
Popliteal LN CD45.2+ CD4+ OTII T cells
CD62L Low
CD25
CD44
Veh % 40.4 ± 1.6
Veh MFI 121 ± 2.1
TCDD % 63.6 ± 2.1*
TCDD MFI 110 ± 2.1*
Veh % 47.3 ± 5.8
Veh MFI 929 ± 60
TCDD % 46.2 ± 1.8
TCDD MFI 723 ± 44*
Veh % 91.3 ± 0.8
Veh MFI 2449 ± 29
TCDD % 82.9 ± 0.8*
TCDD MFI 1995 ± 80*
% Cells
CD62L
% Cells
CD25
% Cells
CD44

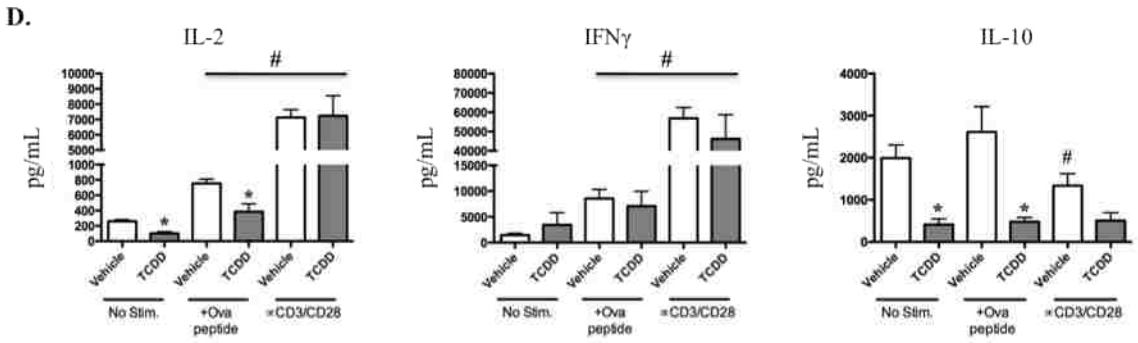


Figure 4.3. TCDD does not induce iTregs during an Ovap-specific immune response in adoptively transferred mice. CD45.1^{+} host mice were intravenously seeded with FACS-purified CD45.2^{+} CD4^{+} CD25^{-} Foxp3^{-} T cells from OTII CD4 Foxp3^{eGFP} reporter mice and gavaged with vehicle or TCDD (15μg/kg) 24h later. Ovap-loaded CD11c^{+} BMDCs were injected into host mouse hind footpads 24h post-Vehicle and TCDD exposure. The BLNs and PLNs were harvested 5 days after Ovap-DC footpad immunizations. (A) Total PLN and BLN cell numbers were assessed and the frequency of CD45.2^{+} CD4^{+} Ovap-specific T cells and CD45.2^{+} CD11c^{+} DCs determined by flow cytometry. (B) Overall frequency and calculated numbers of Ovap-specific CD45.2^{+} CD4^{+} T cells and CD45.2^{+} CD4^{+} CD25^{+} Foxp3^{eGFP+} Tregs in the PLNs and BLNs of Vehicle- and TCDD-treated mice. (C) Activation status of CD45.2^{+} CD4^{+} T cells in the PLNs, percent and mean fluorescence intensity (MFI). Samples represented as isotype; staining grey line, Vehicle-mice; solid black line, TCDD-exposed mice; heavy black dashed line. (D) Cytokine levels in 48h restimulation cultures with 100,000 cells from the PLNs of Vehicle- and TCDD-exposed mice. No stim denotes cells in media alone. ***** indicates significance with $p \leq 0.05$ between Vehicle and TCDD groups. # indicates significant difference between (A) BLN and PLN samples and (D) No stim and stimulation groups. Data is representative of two independent experiments and represented as mean ± SEM of n = 6.

TCDD exposure suppresses Ovap/adjuvant responses and induces Foxp3$^+$ Tregs

The Ovap-DC footpad immunization adoptive transfer model is useful for analyzing antigen-specific CD4$^+$ T cell activation, polarization and expansion under relatively low inflammatory circumstances in the draining lymph node. However, to evaluate the effects of TCDD on the generation of regulatory T cells during the course of an antigen-specific immune response in an inflamed environment, we employed a model in which mice were exposed to Vehicle or TCDD and then immunized with Ovap suspended in Sigma Adjuvant System. DCs and CD4$^+$ T cells were characterized 10 days post-immunization in the spleen and combined popliteal and brachial lymph nodes (PBLNs). This experimental approach better models antigen-specific immune responses under inflammatory conditions in which TCDD exposure has been shown to cause Treg polarization (Quintana *et al.*, 2008b).

To assess the antigen-specific T cell-mediated humoral immune response on day 10, total IgG, IgG2a and IgM and Ovap-specific IgG, IgG2a and IgM levels were measured in the plasma of Vehicle- and TCDD-exposed following immunization with Ovap/adjuvant. When compared to non-immunized mice, an increase in total Ig levels was detected in immunized mice (Table 4.2). Total IgG, IgG2a and IgM and Ovap-specific IgG and IgM antibody levels were significantly reduced in Ovap/adjuvant immunized mice exposed to TCDD when compared to Vehicle-treated mice (Table 4.2). Ovap-specific IgG2a levels were below detection in both Vehicle- and TCDD-exposed immunized mice. Decreased antibody production following TCDD exposure is a well-characterized effect in Ova adjuvant models and well as other antigen-specific mouse models (Shepherd *et al.*, 2000; Sulentic *et al.*, 2000; Ito *et al.*, 2002; Nohara *et al.*, 2002;

Inouye *et al.*, 2003). We observed similar decreases in overall antibody production in our Ovap/adjuvant system.

In Ovap/adjuvant immunized mice total cellularity in the spleen was significantly decreased, while it was increased in the PBLNs of TCDD-exposed mice (Figure 4.4A). The frequency of $CD4^+$ T cells in the spleen and PBLNs remained unchanged between immunized Vehicle- and TCDD-exposed mice (Figure 4.4B). However, there was a significant reduction in the number of splenic $CD4^+$ T cells in TCDD-exposed mice when compared to the number of splenic $CD4^+$ T cells in Vehicle-treated mice (Figure 4.4B). To determine if TCDD had any effect on $Foxp3^+$ Tregs, the frequency and number of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells were assessed in the spleen and PBLNs. In the spleens of immunized TCDD-exposed mice the percent of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs was significantly increased while the number of Tregs did not significantly differ when compared to Vehicle-treated mice (Figure 4.4B). In the PBLNs of immunized TCDD-exposed mice there was a significant increase in both the frequency and number of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs when compared to the PBLNs of Vehicle-treated mice (Figure 4.4B).

In both the spleen and PBLNs there was an increased frequency of $CD4^+$ T cells expressing CD25 in TCDD-mice (Figure 4.4C). However, on a per cell basis CD25 expression was significantly decreased on $CD4^+$ T cells from TCDD-exposed mice, while there was no change in CD44 expression when compared to Vehicle-treated controls. Moreover, the expression of GITR on $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs was not significantly altered in the spleen, while it was increased on Tregs in the PBLNs of TCDD-exposed mice (Figure 4.4C). To further assess the $CD4^+$ T cell response, cells from the spleen and

PBLNs were restimulated *ex vivo* with Ovap or anti-CD3/CD28. IL-2 levels were significantly increased in splenic samples from immunized TCDD-exposed mice in response to anti-CD3/CD28 stimulation (Figure 4.4C). IFNγ levels in response to Ovap were significantly decreased in both splenic and PBLN samples from immunized TCDD-exposed mice, while IL-10 levels were below detection in Ovap restimulation groups (Figure 4.4C). IL-10 was detectable in anti-CD3/CD38 cultures with a significant reduction only seen in anti-CD3/CD28-stimulated PBLN samples from immunized TCDD-exposed mice (Figure 4.4C).

Table 4.1

A.

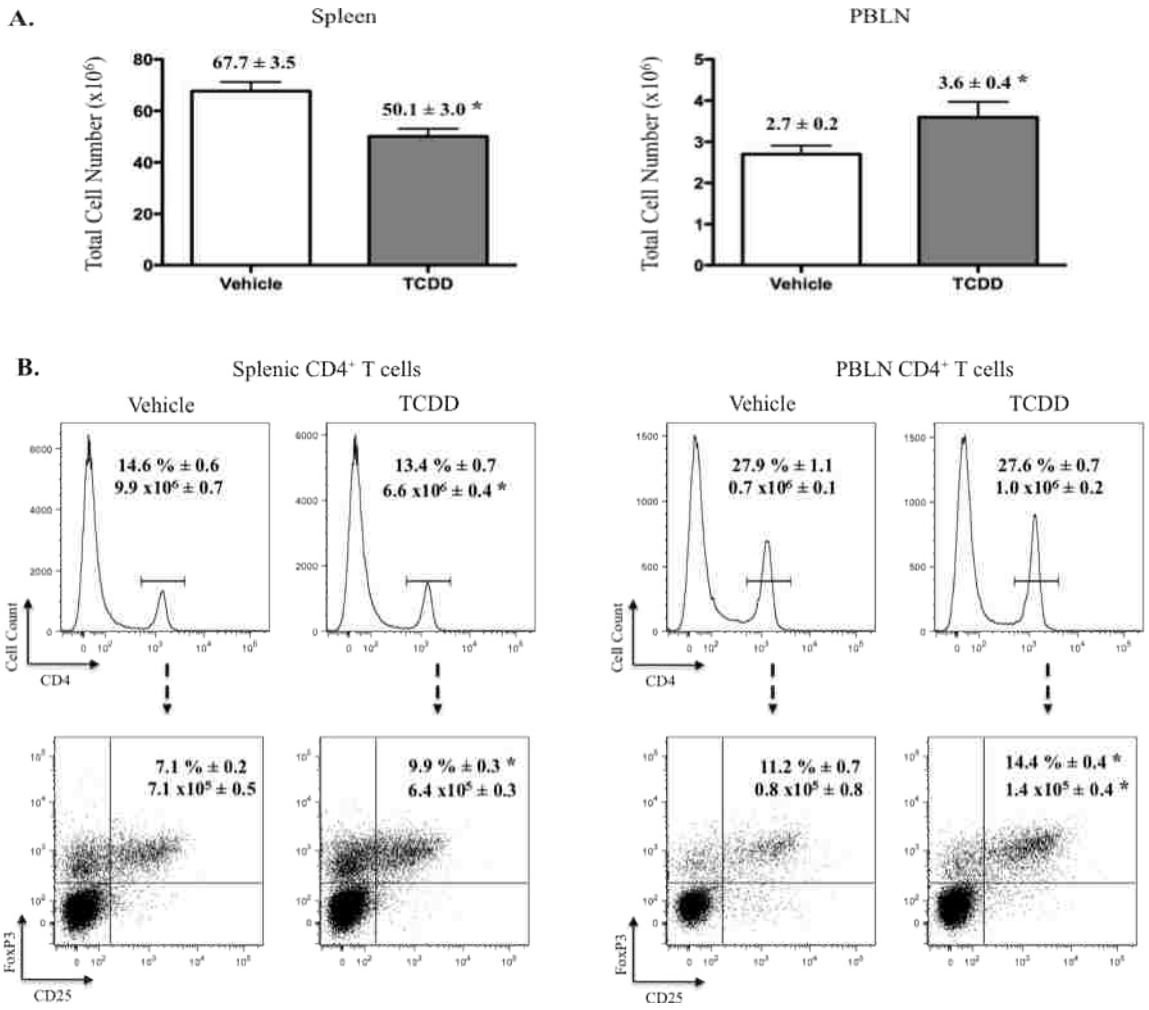
Antibody (µg/mL)	Naïve	Vehicle	TCDD
Total IgG	407 ± 14	1653 ± 246	634 ± 152 *
Total IgG2a	15 ± 0.4	2308 ± 17	1066 ± 19 *
Total IgM	207 ± 17	773 ± 169	445 ± 104 *
Ovap-specific IgG	54 ± 2.2	1022 ± 400	345 ± 62 *
Ovap-specific IgG2a	B.D.	B.D.	B.D.
Ovap-specific IgM	222 ± 0.9	4171 ± 907	1653 ± 246 *

B.

Antibody (µg/mL)	Naïve	Vehicle	TCDD
Total IgG	407 ± 14	814 ± 171	288 ± 88 *
Total IgG2a	15 ± 0.4	11.7 ± 1.3	2.2 ± 0.3 *
Total IgM	207 ± 17	725 ± 162	435 ± 149
Total IgG1	–	20.8 ± 0.8	19.0 ± 1.8
Ovap-specific IgG	54 ± 2.2	123 ± 18	76.9 ± 22
Ovap-specific IgG2a	B.D.	B.D.	B.D.
Ovap-specific IgM	222 ± 0.9	1304 ± 433	951 ± 437

Table 4.1. TCDD-exposure reduces antibody levels following Ovap/adjuvant immunization. (A) Day 10 plasma Ig levels in mice exposed to Vehicle or TCDD and immunized with Ovap in Sigma Adjuvant System. (B) Day 10 plasma Ig levels in host mice exposed to Vehicle or TCDD, seeded with Ovap-specific $CD4^+$ T cells and immunized with Ovap in Sigma Adjuvant System. Naïve non-exposed non-immunized mice plasma Ig levels were measured for comparison. Data is represented as mean ± standard error of the mean (SEM) with (A) vehicle n = 9 and TCDD n = 12 and (B) Vehicle and TCDD n = 6. * indicates significance with $p \leq 0.05$ between Vehicle and TCDD samples. B.D. below detection.

Figure 4.4


A.
Spleen
PBLN
Total Cell Number (x10^6)
67.7 ± 3.5
50.1 ± 3.0 *
2.7 ± 0.2
3.6 ± 0.4 *
Vehicle
TCDD
B.
Splenic CD4+ T cells
PBLN CD4+ T cells
Vehicle
TCDD
14.6 % ± 0.6
9.9 x10^6 ± 0.7
13.4 % ± 0.7
6.6 x10^6 ± 0.4 *
27.9 % ± 1.1
0.7 x10^6 ± 0.1
27.6 % ± 0.7
1.0 x10^6 ± 0.2
Cell Count
CD4
7.1 % ± 0.2
7.1 x10^5 ± 0.5
9.9 % ± 0.3 *
6.4 x10^5 ± 0.3
11.2 % ± 0.7
0.8 x10^5 ± 0.8
14.4 % ± 0.4 *
1.4 x10^5 ± 0.4 *
FoxP3
CD25

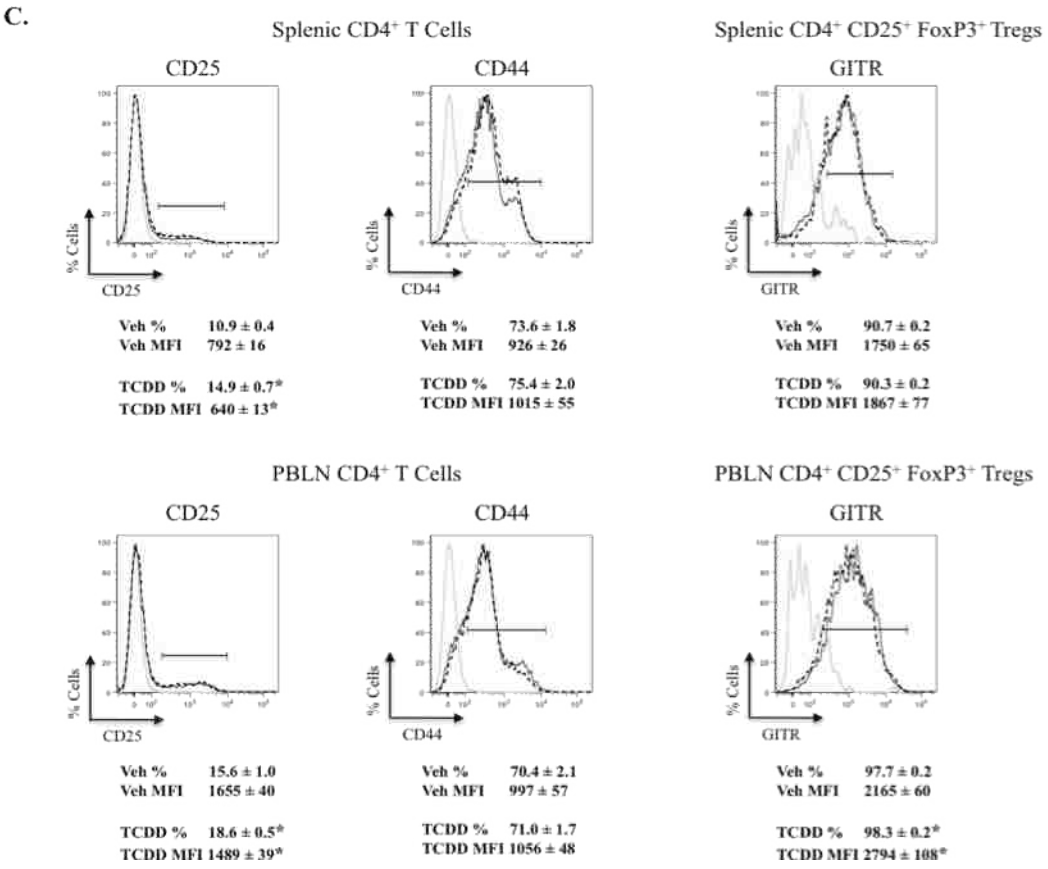


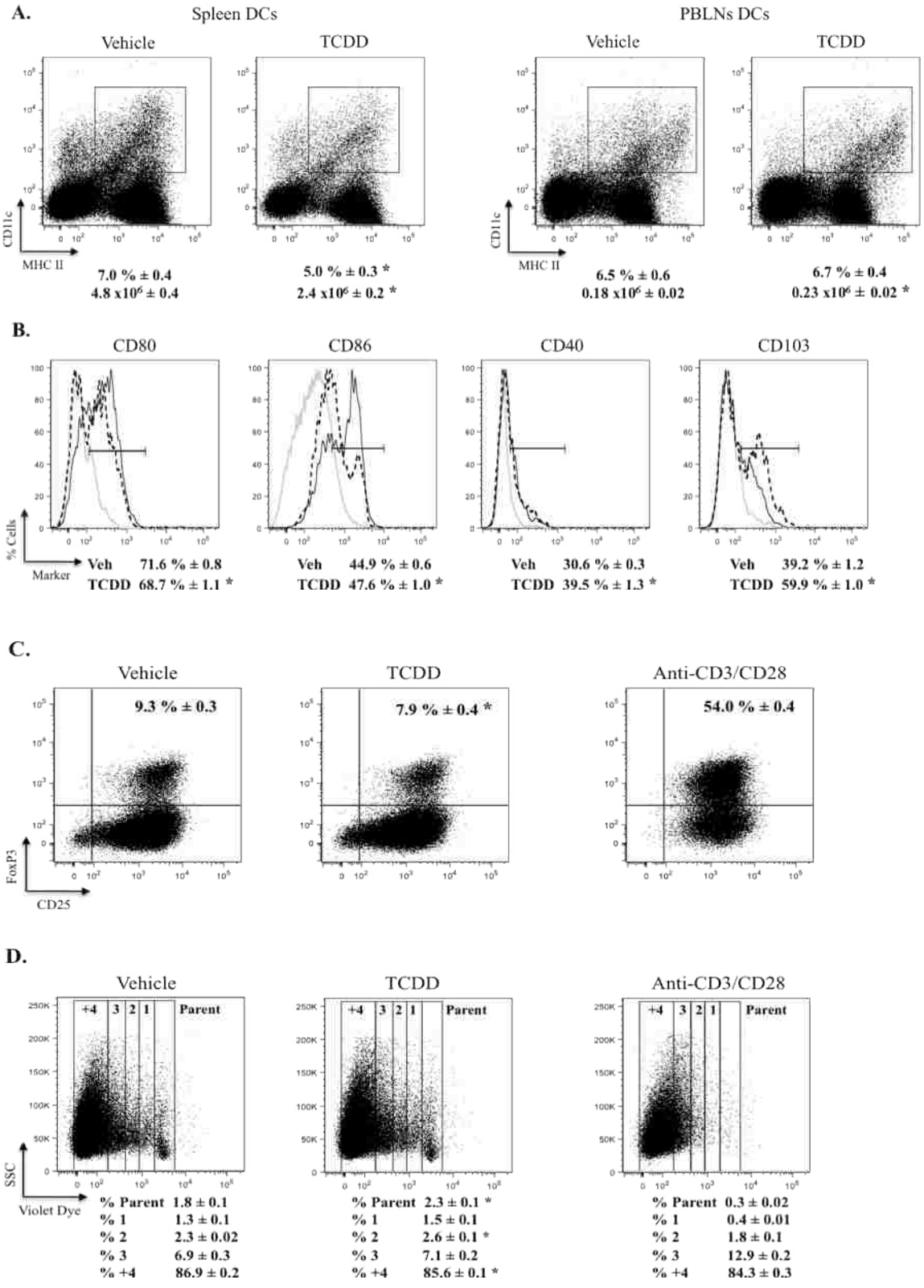
Figure 4.4. $CD4^+$ T cell characterization in TCDD-treated Ovap/Adjuvant immunized mice. Mice were gavaged with Vehicle or TCDD (15µg/mL) and 24h later immunized with Ovap (1mg/mL) suspended in Sigma Adjuvant System. (A) Spleens and popliteal and brachial lymph nodes (PBLNs) were harvested on day 10 post-immunization and cells enumerated. (B) $CD4^+$ T cells and $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs were assessed. Foxp3 expression was detected by intracellular staining. (C) Activation status of $CD4^+$ T cells in the spleen and PBLNs and GITR expression on $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, percent and

mean fluorescence intensity (MFI). Samples represented as isotype; staining grey line, Vehicle-mice; solid black line, TCDD-exposed mice; heavy black dashed line. (D). Cytokine levels in 5 day restimulation cultures with 100,000 cells from the spleen or PBLNs of immunized Vehicle- and TCDD-exposed mice. ***** indicates significance with $p \leq 0.05$ between Vehicle and TCDD groups. Data are represented as mean ± SEM of six samples from two independent experiments.

TCDD reduces splenic DCs in Ovap/adjuvant immunized mice without altering their APC function ex vivo

TCDD exposure in naïve mice causes the deletion of stimulatory $CD11c^{high}$ $33D1^{+}$ DCs in the spleen, while tolerogenic $CD11c^{low}$ $DEC205^{+}$ DCs are retained (Bankoti *et al.*, 2010a). The retention of the $CD11c^{low}$ $DEC205^{+}$ tolerogenic DC population in the spleen could contribute to the induction of $Foxp3^{+}$ Tregs and subsequent immune suppression in TCDD-exposed mice following Ovap/adjuvant immunization. In immunized mice exposed to TCDD there was a significant reduction in the frequency and number of $CD11c^{+}$ MHC II^{+} DCs (Figure 4.5A). In the $CD11c^{+}$ MHC II^{+} DC fraction present in mice exposed to TCDD there was an increased frequency of DCs expressing CD86 CD40 and CD103 when compared to $CD11c^{+}$ MHC II^{+} DCs present in the spleen of Vehicle-treated mice (Figure 4.5B). Furthermore, splenic $CD11c^{+}$ DCs purified from TCDD-exposed mice had elevated gene expression of IDO1, IDO2 and Aldh1a2 when compared to $CD11c^{+}$ DCs from Vehicle-treated mice (Table 4.2). However, purified splenic $CD11c^{+}$ DCs from immunized TCDD-exposed mice failed to induce $Foxp3^{+}$ Tregs in *ex vivo* co-cultures with naïve Ovap-specific $CD4^{+}$ T cells enriched from naïve OTII $Foxp3^{eGFP}$ reporter mice when compared to $CD11c^{+}$ DCs from Vehicle-treated mice (Figure 4.5D). There was a slight reduction in overall $CD4^{+}$ T cell proliferation following interactions with $CD11c^{+}$ DCs from TCDD-exposed mice (Figure 4.5C). In addition, IL-2 and IL-10 cytokine production in these $CD11c^{+}$ DC:$OTII^{+}$ $CD4^{+}$ T cell co-cultures was similar between Vehicle- and TCDD-DC groups, while IFNγ levels were significantly reduced in TCDD-DC:T cell co-cultures (Figure 4.5E).

Figure 4.5


A.
Spleen DCs
PBLNs DCs
Vehicle
TCDD
Vehicle
TCDD
CD11c
MHC II
7.0 % ± 0.4
4.8 x10⁶ ± 0.4
5.0 % ± 0.3 *
2.4 x10⁶ ± 0.2 *
6.5 % ± 0.6
0.18 x10⁶ ± 0.02
6.7 % ± 0.4
0.23 x10⁶ ± 0.02 *
B.
CD80
CD86
CD40
CD103
% Cells
Marker
Veh 71.6 % ± 0.8
TCDD 68.7 % ± 1.1 *
Veh 44.9 % ± 0.6
TCDD 47.6 % ± 1.0 *
Veh 30.6 % ± 0.3
TCDD 39.5 % ± 1.3 *
Veh 39.2 % ± 1.2
TCDD 59.9 % ± 1.0 *
C.
Vehicle
TCDD
Anti-CD3/CD28
9.3 % ± 0.3
7.9 % ± 0.4 *
54.0 % ± 0.4
FoxP3
CD25
D.
Vehicle
TCDD
Anti-CD3/CD28
+4
3
2
1
Parent
SSC
Violet Dye
% Parent 1.8 ± 0.1
% 1 1.3 ± 0.1
% 2 2.3 ± 0.02
% 3 6.9 ± 0.3
% +4 86.9 ± 0.2
% Parent 2.3 ± 0.1 *
% 1 1.5 ± 0.1
% 2 2.6 ± 0.1 *
% 3 7.1 ± 0.2
% +4 85.6 ± 0.1 *
% Parent 0.3 ± 0.02
% 1 0.4 ± 0.01
% 2 1.8 ± 0.1
% 3 12.9 ± 0.2
% +4 84.3 ± 0.3

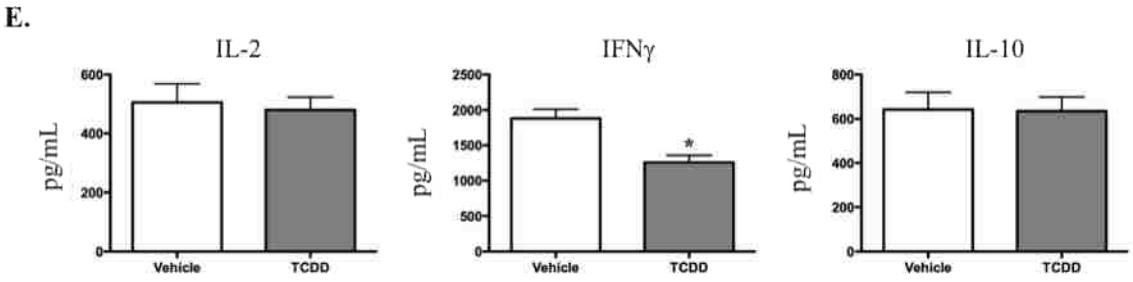


Figure 4.5. DC characterization in TCDD-trated Ovap/adjuvant immunized mice. Mice were gavaged with Vehicle or TCDD (15μg/mL) and 24h later immunized with Ovap in Sigma Adjuvant System. (A) $CD11c^+$ MHC II^+ DC in the spleens and popliteal and brachial lymph node (PBLN) day 10-post immunization. (B) Activation marker expression on $CD11c^+$ MHC II^+ splenic DCs. (C and D) Enriched Cell Trace-labeled $CD4^+$ T cells from naïve OTII $Foxp3^{eGFP}$ reporter mice were co-cultured with $CD11c^+$ Ovap-loaded splenic DCs isolated and purifed from immunized Vehicle and TCDD-exposed mice. Co-cultures were harvested on day 5 and the percent of $Foxp3^+$ Tregs and $CD4^+$ T cell proliferation assessed by flow cytometry. (E) Cytokine levels in $CD4^+$ T cell:DC co-cultures. ***** indicates significance with $p \leq 0.05$ between Vehicle and TCDD groups. Data are represented as mean ± SEM of six samples from one experiment.

Table 4.2

	A. Naïve mice 48h TCDD exposure		**B.** Naïve mice 10-day TCDD exposure		**C.** TCDD-exposed Ovap/adjuvant immunized mice day 10	
Gene	Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Ido1	6.8 *	0.007	9.1 *	0.002	11.3 *	0.001
Ido2	28.2 *	0.004	15.4 *	0.03	76.0 *	0.001
Aldh1a1	0.4	0.1	–	–	0.7	0.4
Aldh1a2	0.4	0.053	–	–	4.0 *	0.0001
Arginase	–	–	–	–	1.0	0.9
Tgfβ1	0.9	0.8	0.6 *	0.01	0.8 *	0.02
Tgfβ2	3.4 *	0.03	0.4 *	0.04	1.4	0.6
Tgfβ3	7.1 *	0.02	0.7	0.11	1.2	0.8
TgfβrII	–	–	–	–	0.7	0.2
AhR	–	–	–	–	0.4 *	0.01
AhRR	–	–	–	–	12.7 *	0.002
Cyp1a1	1.6	0.4	1.0	1.0	0.9	0.9
Il-1β	–	–	–	–	0.3 *	0.02
Il-6	–	–	–	–	1.4	0.2
Il-10	1.1	0.9			0.9	0.2
Il-23	–	–	–	–	0.8	0.6
Il-27	–	–	–	–	0.6	0.2

Table 4.2. Gene expression in purified $CD11c^+$ splenic DCs. Gene expression was evaluated with qRT-PCR. (A) DCs isolated from naive mice 48h after Vehicle or TCDD exposure. (B) DCs isolated from naïve mice 10 days post-Vehicle or TCDD exposure. (C) DCs isolated from mice exposed to Vehicle or TCDD and immunized with Ovap/Sigma Adjuvant System on day 10 post-immunization. Results are expressed as TCDD-induced fold change in comparison to Vehicle-treated mice after normalization to housekeeping gene β-actin expression, with (A and B) n = 3 per treatment group and (C) Vehicle n = 3 and TCDD n = 2. * $p \leq 0.05$ indicates significance between Vehicle and TCDD-exposed mice. Data is representative of one experiment for each group A-C.

Antigen-specific iTregs are generated in response to TCDD-induced AhR activation in an Ovap/adjuvant OTII^{+} CD4^{+} adoptive transfer model

To better track the induction of Foxp3^{+} iTregs in the Ovap/adjuvant model, CD45.2^{+} CD4^{+} CD25^{-} Foxp3^{eGFP-} T cells purified from OTII Foxp3^{eGFP} reporter mice were transferred into CD45.1^{+} host mice. These naïve CD45.2^{+} CD4^{+} T cells were allowed to seed the spleen and lymph nodes of host mice for 24h before oral exposure to Vehicle or TCDD (15μg/mL). Mice were then immunized with Ovap/adjuvant and the induction of iTregs assessed in the spleen and PBLNs 10 days post-immunization. Similar to the Ovap/adjuvant system without the transfer of Ovap-specific CD4^{+} T cells, a significant reduction in the number of cells in the spleen was observed in immunized TCDD-exposed mice when compared to Vehicle-treated mice (Figure 4.6A). There was no difference in the overall cell number in the PBLNs between immunized Vehicle-mice and TCDD-exposed mice (Figure 4.6A). In the spleen, TCDD-induced AhR activation caused a significant reduction in both the frequency and number of Ovap-specific CD45.2^{+} CD4^{+} T cells (Figure 4.6B). The frequency of CD45.2^{+} CD4^{+} T cells was greatly reduced in the PBLNs as compared to the frequency of CD45.2^{+} CD4^{+} T cells in the spleen of immunized mice (Figure 4.6B). The percent of splenic antigen-specific CD45.2^{+} CD4^{+} CD25^{+} Foxp3^{eGFP+} iTregs was significantly increased when compared to splenic iTregs in immunized Vehicle-treated mice (Figure 4.6C). In splenic *ex vivo* restimulation cultures with Ovap or anti-CD3/CD28, cells from TCDD-exposed mice produced less IFNγ and IL-10 in response to Ovap or anti-CD3/CD28 stimulus, respectively (Figure 4.6C).

Consistent with previous Ovap/Adjuvant experiments, the frequency and number of splenic $CD11c^+$ MHC II^+ DCs were significantly decreased in immunized TCDD-exposed mice (Figure 4.7A). Furthermore, splenic DCs from TCDD-treated mice expressed increased levels of CD86 and CD103 (Figure 4.7B). In the PBLNs there was an increase in the number of $CD11c^+$ MHC II^+ DCs, but the expression of CD86 and CD103 on DCs did not differ significantly between TCDD-treated and Vehicle-treated mice (Figure 4.7B).

Figure 4.6

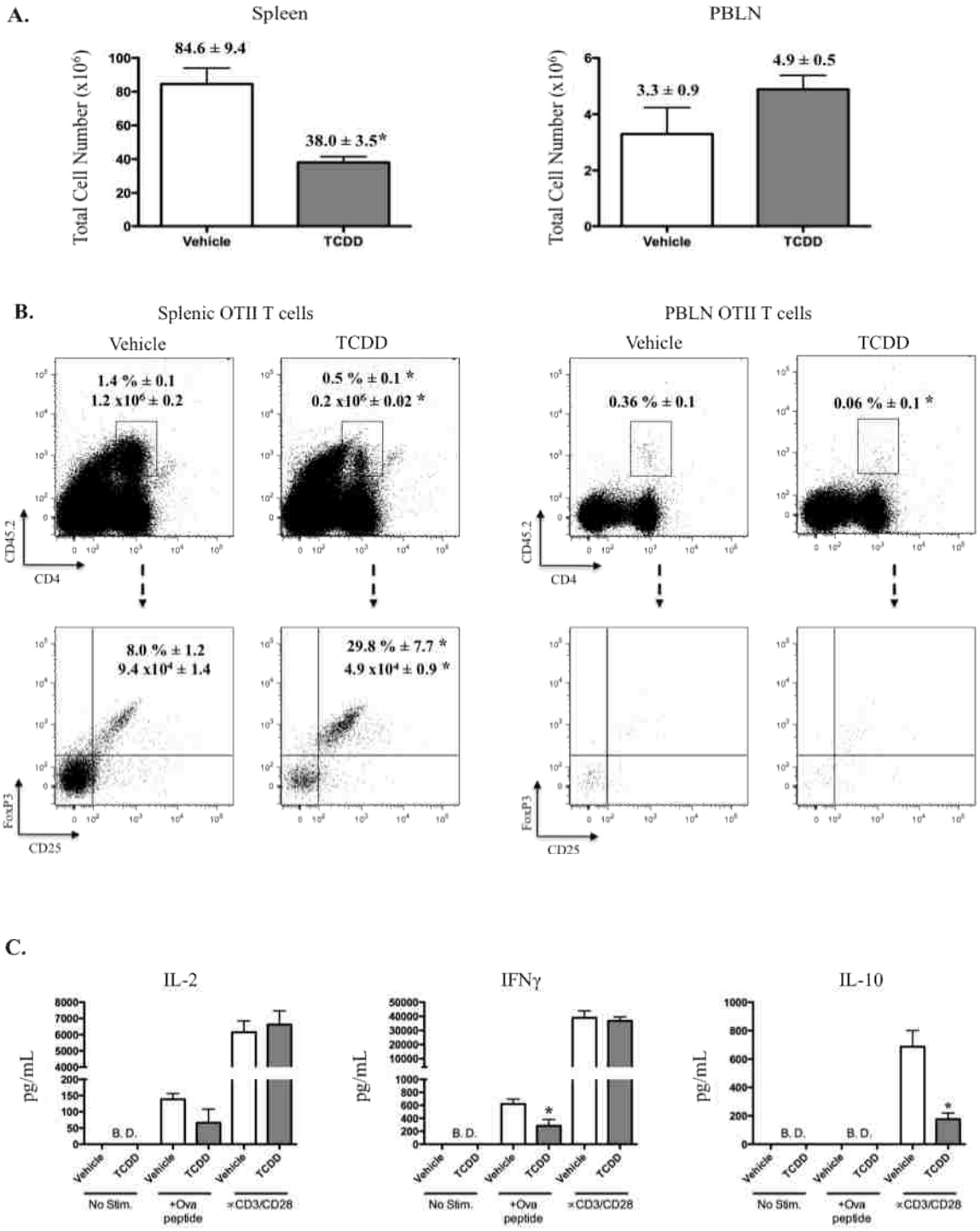


Figure 4.6. Antigen-specific $CD4^+$ T cell responses in adoptively transferred TCDD-treated and immunized mice. $CD45.1^+$ host mice were intravenously seeded with $1x10^6$ FACS-purified $CD45.2^+$ $CD4^+$ $CD25^-$ $Foxp3^-$ T cells from OTII $Foxp3^{eGFP}$ reporter mice.

24h after the transfer of antigen-specific naïve $CD4^+$ T cells, host mice were gavaged with Vehicle or TCDD and 24h later immunized with Ovap in Sigma Adjuvant System. (A) Spleens and PBLNs were harvested on day 10 post-immunization and cells enumerated. (B) $CD45.2^+$ $CD4^+$ and $CD4^+$ $CD25^+$ $Foxp3e^{eGFP+}$ T cells were assessed. (C) Cytokine levels in 48h restimulation cultures with 100,000 cells from the spleen or PBLNs of immunized Vehicle- and TCDD-exposed mice. ***** indicates significance with $p \leq 0.05$ between Vehicle and TCDD groups. Data are displayed as mean ± SEM with n = 6 from one experiment.

Figure 4.7

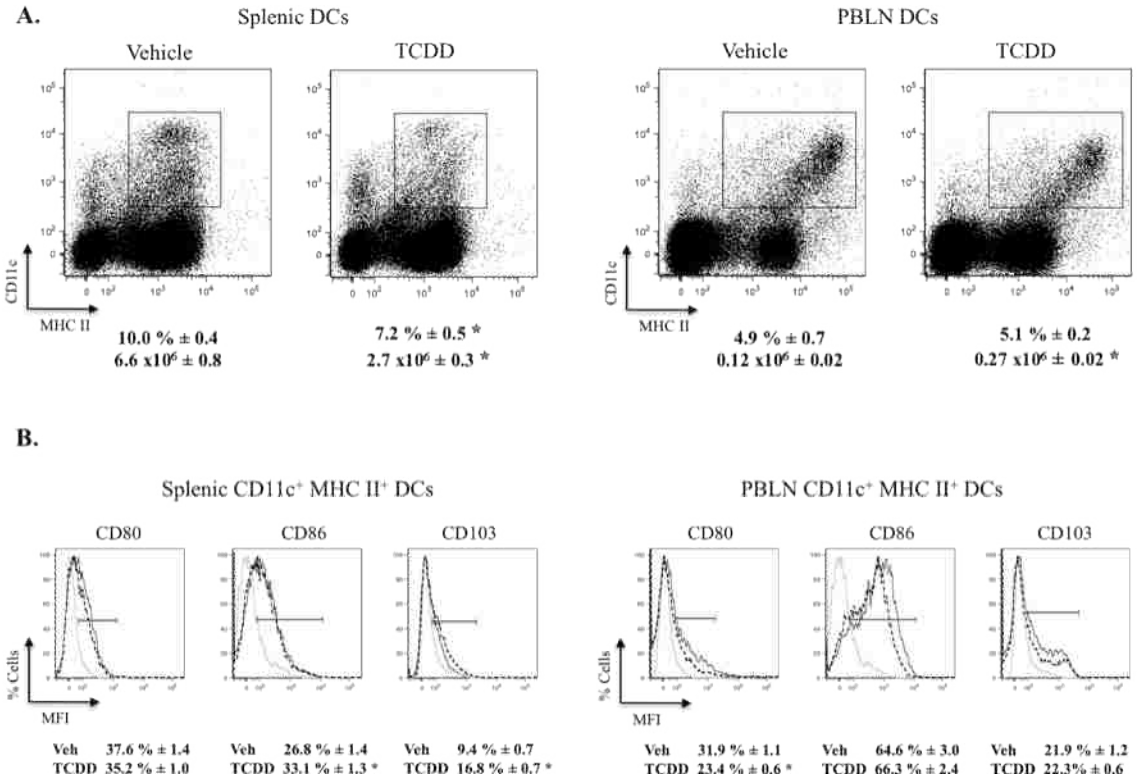


Figure 4.7. DC characterization in adoptively transferred TCDD-treated immunized mice. (A) $CD11c^+$ $MHC\ II^+$ DC in the spleens and PBLNs day 10 post-immunization. (B) Activation marker expression on $CD11c^+$ $MHC\ II^+$ splenic DCs. Data is representative of one experiment. ***** indicates significance with $p \leq 0.05$ between Vehicle and TCDD groups. Data are displayed as mean ± SEM with n = 6.

DISCUSSION

The AhR, while being a well-known steroid-like transcription factor in toxicology has recently come into the limelight for its potential role in the polarization of Tregs and Th17 effector cells in mice (Quintana *et al.*, 2008b; Veldhoen *et al.*, 2008; Stockinger *et al.*, 2011). However, few studies have examined the role of TCDD-induced AhR activation in DCs and whether AhR activation *in vivo* causes the generation of tolerogenic DCs capable of inducing regulatory T cells *ex vivo*. In previous Ovap-DC footpad immunization adoptive transfer experiments we have shown that TCDD exposure significantly dampens the Ovap-specific $CD4^+$ T cell response as determined by a reduction in both the frequency and number of Ovap-specifc $CD4^+$ T cells in the PLNs of TCDD-treated mice (our unpublished data). Furthermore, when cells from the PLNs of TCDD-exposed mice were restimulated with Ovap they produced significantly less IL-2, IFNγ and IL-10 when compared to cells from Vehicle-treated control mice. In these studies, TCDD exposure could be causing the generation $Foxp3^+$ Tregs during the initial course of the Ovap-sepcific $CD4^+$ T cell immune response in the draining lymph node, which could subsequently dampen the overall $CD4^+$ T cell effector response as well as DC function. To examine this possibility, Ova-specific $CD4^+$ T cells from OTII $Foxp3^{eGFP}$ reporter mice were used to track the generation of $Foxp3^+$ Tregs in the draining lymph node following TCDD exposure and Ovap-DC footpad immunizations. In the first experiment, $CD4^+$ T cells containing a small fraction of nTregs were transferred into $CD45.1^+$ host mice and on day 4 there was a significant increase in $Foxp3^+$ Tregs in the draining lymph nodes of mice exposed to TCDD. Similar to previous experiments we observed a reduction in both Ovap-DCs and $OTII^+$ $CD4^+$ T cells in TCDD-exposed mice.

It is possible that the increased frequency of Foxp3$^+$ Tregs is creating a suppressive environment in the draining lymph node that is directly inhibiting the Ovap-specific CD4$^+$ T cell effector response. To determine whether the induction of Tregs in TCDD-exposed mice was a preferential expansion of nTregs or iTregs, the Ovap-DC footpad immunization adoptive transfer experiments were repeated, this time utilizing FACS-purified OTII$^+$ CD4$^+$ CD25$^-$ Foxp3$^-$ T cells. Unexpectedly, on day 4 there was no induction of Foxp3$^+$ iTregs, in either Vehicle- or TCDD-exposed mice, yet there was still a significant reduction in the frequency and number of OTII$^+$ CD4$^+$ T cells in TCDD-exposed mice. One explanation for the lack of Foxp3$^+$ iTregs in TCDD-exposed mice that received naïve OTII$^+$ CD4$^+$ T cells is that TCDD-induced AhR activation causes the expansion or retention of nTregs rather than the generation of iTregs from naïve CD4$^+$ T cells. It may also be possible that the transferred Ovap-specific naïve CD4$^+$ T cells were unresponsive following *ex vivo* isolation, enrichment, FACS purification and transfer into host mice. However, as a control, a fraction of FACS-purified OTII$^+$ CD4$^+$ CD25$^-$ Foxp3$^-$ T cells were stimulated for 4 days with plate-bound anti-CD3/CD28 in media alone or media supplemented with IL-2 and TGFβ. The CD4$^+$ T cells in these *ex vivo* control cultures were responsive to anti-CD3/CD28 stimulation and in the presence of IL-2/TGFβ there was a significant induction of Foxp3 iTregs. These results likely rule out that the transferred OTII$^+$ CD4$^+$ CD25$^-$ Foxp3$^-$ T cells were unresponsive.

In addition to the possible expansion or retention of nTregs, TCDD significantly reduced the number of Ovap-DCs in the PLNs. The reduction of Ovap-DCs could be responsible for the observed decreases in antigen-specific CD4$^+$ T cells in the draining lymph nodes of TCDD-exposed mice. Furthermore, it is likely that AhR activation in the

transferred Ovap-DCs is altering their functional capacity to present antigen and stimulate $CD4^+$ T cells as we have previously shown *in vitro* that TCDD-induced AhR activation disrupts BMDC differentiation and induces an immature DC phenotype (Bankoti *et al.*, 2010b). It is also possible that TCDD is directly affecting the $OTII^+$ $CD4^+$ T cells either leading to apoptosis or altering their responsiveness to antigen presented by DCs. It is interesting that in both Ova-DC footpad immunization adoptive transfer experiments that the $CD4^+$ effector T cells down-regulated CD62L and CD25 expression. Activated $CD4^+$ effector cells typically down-regulate CD62L expression while increasing CD25 expression. It is possible that TCDD-induced AhR activation in $OTII^+$ $CD4^+$ T cells is causing emigration from the draining lymph node in a unique semi-activated state following interactions with AhR-activated DCs. Taken together, TCDD exposure affects both DC and $CD4^+$ effector T cell populations, and TCDD-exposure in the context of an antigen-specific $CD4^+$ T cell response under minimal inflammatory conditions may lead to the expansion, retention or increased functional capacity of nTregs rather than the induction of iTregs.

The lack of effects on regulatory T cells in the Ovap-DC footpad immunization adoptive transfer experiments may be due to a lack of inflammation. In instances when Treg and Th17 polarization has been observed following TCDD-induced AhR activation, it has usually been with a highly inflammed disease state (Quintana *et al.*, 2008b; Veldhoen *et al.*, 2008). This is supported by the observation that following Ovap/adjuvant immunization there was a significant increase in host $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs as well as a substantial increase in antigen-specific $Foxp3^+$ iTregs when Ovap-specific $CD4^+$ $CD25^-$ $Foxp3^-$ T cells were transferred into host mice before TCDD

exposure and Ovap/adjuvant immunization. The induction of regulatory T cells may be mediated by tolerogenic DCs induced following TCDD exposure. Previously, Bankoti and colleagues demonstrated that TCDD exposure in naïve mice preferentially causes the deletion of stimulatory $CD11c^{high}$ $CD8^{-}$ $33D1^{+}$ and retention of tolerogeinc $CD11c^{intermedate}$ $CD8^{+}$ $DEC205^{+}$ splenic DCs (Bankoti *et al.*, 2010a). In the Ovap/adjuvant immunization model we observed a similar decrease in $CD11c^{high}$ DCs in the spleen following TCDD exposure. While we did not fully characterize the DC subsets in the spleen or PBLNs in this model, we did observe an increase in $CD11c^{+}$ MHC II^{+} $CD103^{+}$ DCs in the spleens and PBLNs of TCDD-exposed immunized mice. $CD103^{+}$ DCs in mucosal sites as well as the spleen have been shown to be highly tolerogenic and can directly induce Tregs (Coombes *et al.*, 2007; Yamazaki *et al.*, 2008; Del Rio *et al.*, 2010). Furthermore, splenic DCs from TCDD-exposed immunized mice displayed increased gene expression of IDO1 and IDO2 and Aldh1a2. These particular proteins have been associated with tolerogenic DC function and the induction of Tregs in an AhR-dependent manner (Vogel *et al.*, 2008; Mezrich *et al.*, 2010; Quintana *et al.*, 2010). However, when splenic DCs were isolated from mice exposed to TCDD they failed to induce antigen-specific $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ iTregs in *in vitro* DC:T cell co-cultures. Thus, there may be additional signals that are required for the generation of iTregs *in vitro.* It is also possible that AhR activation by TCDD is required in $CD4^{+}$ T cells for Treg polarization following interactions with AhR-activated DCs.

To date this is the first study to assess the generation of Tregs in response to TCDD-induced AhR activation and Ovap/adjuvant immunization. However, previous studies by Ito and co-workers showed that oral TCDD exposure followed by immediate

i.p. immunization with whole Ovalbumin absorbed to Alum suppressed Ova-specific IgG1 production and decreased the total number of cells in the spleen as well as splenic $CD3^+$ T cell numbers (Ito *et al.*, 2002). They also showed that splenic cells from TCDD-exposed mice when restimulated with Ova for 48h produced less IL-5, while IL-2, IL-4 and IL-6 levels remained unchanged when compared to Vehicle restimualtion groups (Ito *et al.*, 2002). Moreover, Shepherd and colleagues using a DO11.10 adoptive transfer model in which naïve Balb/c mice were orally exposed to Vehicle or TCDD and seeded with Ovap-specific DO11.10 $CD4^+$ T cells then immunized with Ova emulsified in CFA demonstrated that TCDD exposure reduced total IgG and IgM and Ova-specific IgG2a levels on day 10 post-immunization (Shepherd *et al.*, 2000). The frequency of $DO11.10^+$ $CD4^+$ T cells in the spleen of TCDD-exposed mice were also significantly reduced, yet there was no change in IL-2 or IFNγ production following a 3 day *ex vivo* restimulation with Ova (Shepherd *et al.*, 2000). Similar to these studies we demonstrated that B6 mice exposed to TCDD and immunized with Ovap suspended in Sigma Adjuvant System, similar to CFA, produced less total IgG and IgM and Ovap-specific IgG and IgM, displayed reduced numbers of splenic $CD4^+$ T cells and disrupted $CD4^+$ T cell activation status. Following restimulation with Ovap, splenic cells from TCDD/immunized mice produce less IFNγ. In our Ovap/adjuvant immunization adoptive transfer model with $OTII^+$ $CD4^+$ $CD25^-$ $Foxp3^-$, similar results were observed including a reduction in total Ig levels, reduced antigen-specific T cell populations in the spleen and altered cytokine production following restimulation.

In conclusion, we have demonstrated that the generation of regulatory T cells following TCDD-induced AhR activation is likely dependent on an inflammatory

environment. With natural Tregs expanding in the presence of AhR activation under minimal inflammation conditions and antigen-specific inducible Tregs being generated during adjuvant-induced inflammatory responses. TCDD-induced tolerogenic DCs may be responsible for the induction of iTregs in our adoptive transfer immunization model; however, further studies are required to fully evaluate the role of TCDD-induced tolerogenic DCs *in vivo.*

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CHAPTER 5

CONCLUSIONS

In the last half-century the understanding of AhR biology has grown immensely. Since the discovery of the AhR numerous studies utilizing different animal models have shown that low-dose exposure to TCDD, the prototypical AhR ligand, causes immune suppression. However, the mechanisms underlying the observed immune suppression have been difficult to define and remain to be fully elucidated. TCDD-induced AhR activation impairs both innate and adaptive immune responses by effecting the fate and function of the cells involved in generating successful immune responses. To date, AhR research has been primarily centered on the effects in lymphocytes. In comparison, relatively little research has been conducted on how AhR activation in DCs affects their fate and function. Immature DCs, in contrast to naïve lymphocytes, constitutively express high levels of the AhR and due to their sentinel and APC functions in the immune system are likely to be key cells mediating the immunosuppressive effects of TCDD. The studies in this dissertation have (1) examined the effects of AhR-activation in steady-state BMDCs; (2) investigated the capacity of AhR-activated inflammatory BMDCs to dampen $CD4^+$ effector T cell responses and induce regulatory T cells *in vitro*; and (3) examined the effects of TCDD-induced AhR activation on the generation of tolerogenic DCs and regulatory T cells *in vivo*.

AhR-Activation in Steady-State BMDCs

The seminal study examining the fate and function of DCs demonstrated that naïve mice exposed to TCDD displayed a reduced number of splenic DCs, with the remaining DCs displaying an altered surface molecule phenotype (Vorderstrasse and

Kerkvliet, 2001b). Subsequent studies by Bankoti and colleagues demonstrated that the loss of splenic DCs was to due to a selective loss of $CD11c^{High}$ DCs in AhR-dependent manner (Bankoti *et al.*, 2010a). Furthermore, both splenic DCs and lymph node DCs in TCDD-treated mice displayed altered expression of key surface molecules (Bankoti *et al.*, 2010a). To better understand the consequences of AhR activation in steady-state DCs we utilized a Flt3L-BMDC model, which generates DCs *in vitro* that are similar to steady-state DCs found in peripheral immune tissues such as the spleen and lymph nodes (Brasel *et al.*, 2000; Naik *et al.*, 2007; Shortman and Naik, 2007a). Continual AhR activation with TCDD altered steady-state BMDC differentiation and generated a phenotypically distinct semi-mature BMDCs. These effects on DC differentiation were AhR-dependent, but not entirely DRE-dependent, highlighting the possibility that these changes may occur via non-canonical AhR signaling in DCs. These alterations were observed with the prototypical AhR ligand TCDD; however, the classification of AhR as an orphan receptor has been challenged recently by the discovery of a variety of endogenous AhR ligands. Steady-state BMDCs grown in the presence of FICZ or ITE, two endogenous AhR ligands, displayed similar surface molecule alterations as those grown in the presence of TCDD. While ligand-specific effects have been characterized in different cell types, AhR activation by the endogenous ligands FICZ and ITE caused similar changes in surface molecule expression as TCDD in steady-state BMDCs.

In response to several TLR agonists including LPS, CpG and Imiquimod, AhR-activated steady-state BMDCs produced less IL-6, TNFα, IL-10 and IL-12. The reduced cytokine production may be in part due the altered NF-κB signaling, as TCDD-exposure increased RelB binding activity, while reducing p65 activity in response to LPS or CpG

stimulation. In light of these observations, steady-state BMDCs grown in the presence of TCDD did not alter antigen-specific $CD4^+$ T cell immune responses *in vivo* in an Ovap-DC adoptive transfer model. However, *in vitro* AhR-activated steady-state BMDCs increased the frequency of antigen-specific $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in co-cultures with $CD4^+$ T cells, isolated from OTII $Foxp3^{eGFP}$ reporter mice, in an IDO-dependent fashion. This was not surprising as gene expression studies revealed that TCDD-induced AhR activation in steady-state BMDCs up-regulated the expression of IDO2, a well-characterized immunoregulatory enzyme involved in the generation of regulatory T cells.

This study was novel because it was the first study to examine the effects of AhR activation in an *in vitro* model of Ftl3L-derived steady-state BMDCs. Extrapolating these results to an *in vivo* exposure situation, it is possible that TCDD-induced AhR activation alters steady-state DC differentiation, either in the bone marrow during DC development from common myeloid and lymphoid progenitor cells or in the spleen where pre-DCs help contribute to the maintenance of resident DC populations (Shortman and Naik, 2007a; Wu and Liu, 2007). AhR activation could effectively induce semi-mature AhR-activated steady-state DCs that are not only less responsive to danger signals from microbial pathogens, but are able to induce Tregs in the periphery. Taken together, the decreased innate responsiveness and Treg-induction capabilities of AhR-activated steady-state BMDCs offer plausible mechanisms underlying the immunosuppressive effects of TCDD.

AhR Activation in Inflammatory BMDCs

The effects of AhR activation in GM-CSF-derived inflammatory BMDCs have been previously described (Lee *et al.*, 2007; Bankoti *et al.*, 2010b). In contrast to Flt3L-

derived steady-state BMDCs, AhR activation in GM-CSF-derived inflammatory BMDCs induces the expression of IDO1 and TGFβ3 along with the expression of IDO2. Because AhR activation induces immunoregulatory gene expression as well as alters expression of key immune proteins on inflammatory BMDCs, we hypothesized that AhR-activated inflammatory BMDCs would suppress antigen-specific T cell-mediated immune responses *in vitro*.

While AhR-activated inflammatory BMDCs had no effect on $CD4^+$ T cell proliferation, they consistently altered IL-2, IFNγ and IL-10 production by T cells in DC:T cell co-cultures. The observed alterations in cytokine secretion demonstrate that AhR-activated inflammatory BMDCs are capable of altering $CD4^+$ T cell immune responses *in vitro.* Interestingly, similar alterations in cytokine production were observed almost a decade earlier following *ex vivo* restimulation with $CD4^+$ T cell samples from mice exposed to TCDD and immunized with Ova antigen and adjuvant (Shepherd *et al.*, 2000; Ito *et al.*, 2002). Our studies demonstrated that TCDD-induced AhR activation in inflammatory BMDCs dampens effector $CD4^+$ T cell responses *in vitro*. These data coupled with *in vivo* studies suggest that AhR activation in DCs may cause immune deviation and/or inhibit the generation of successful effector T cell responses, ultimately resulting in immune suppression. In addition, the observed immune suppression following exposure to TCDD may be mediated by the generation of tolerogenic DCs that are capable of inducing regulatory T cells in the periphery.

AhR Activation In vivo and Altered Immune Responses

The AhR following ligand-induced activation plays a role in the polarization of regulatory T cells and Th17 cells during inflammatory autoimmune responses *in vivo*

(Quintana et al., 2008b; Veldhoen et al., 2008; Stockinger et al., 2011). Our *in vitro* studies demonstrated that AhR activation leads to the generation of tolerogenic DCs in two distinct DC populations, both of which were capable of suppressing antigen-specific $CD4^+$ T cell responses. Knowing that the AhR plays a role in the generation of regulatory T cells and the fact that *in vitro* AhR activation disrupts DC function we sought to examine the generation of both tolerogenic DCs and Tregs *in vivo* during antigen-specific immune responses. Unexpectedly, $Foxp3^+$ Tregs were generated only when naïve $CD4^+$ T cells and nTregs were transferred into host mice exposed to TCDD and immunized with Ova-DCs. These results suggest that in a less inflamed environment AhR activation may help to maintain or expand nTreg populations rather than generate iTregs. In contrast, during periods of increased inflammation, AhR activation may instead lead to the generation of iTregs rather than the expansion of nTregs, as we observed a significant increase in the frequency of $Foxp3^+$ Tregs in our Ovap/adjuvant model with FACS-purified $CD4^+$ $CD25^-$ $Foxp3^-$ T cell transfers. Furthermore, *ex vivo* restimulation of cells from TCDD-exposed mice in both models caused altered cytokine responses, similar to those seen in our *in vitro* experiments. Regardless of the generation of $Foxp3^+$ Tregs, TCDD-induced AhR activation dampened *in vivo* immune responses in our antigen-specific exposure experiments. While TCDD-induced AhR activation may play a role in the generation of Tregs it is also directly affecting effector T cell responses. Further studies are needed to fully assess the role and requirement of AhR-induced tolerogenic DCs and regulatory T cells *in vivo.*

Overall, we have demonstrated that AhR activation alters the differentiation of steady-state BMDCs and induces a tolerogenic phenotype in inflammatory BMDCs,

which dampens antigen-specific $CD4^+$ effector T cell responses *in vitro*. *In vivo* TCDD exposure suppresses antigen-specific immune responses and induces $Foxp3^+$ regulatory T cells. Collectively, these data enhance our understanding of how TCDD exposure causes immune suppression.

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APPENDIX

Abbreviations

Std. Standard

N.C. No change

N.A. Not available

− No data collected

⇧ Significant increase

🡻 Significant decrease

Appendix Table A.1

Date	DC Phenotype	DC Gene Profile	Ova peptide	$CD4^+$ T cell purity	Days in Culture/ well type	Cell Numbers	Proliferation	% $CD4^+$ $FoxP3^+$	**% $CD4^+$ $CD25^+$ $FoxP3^+$**	% $CD4^+$ $CD25^+$ $FoxP3^{neg}$
08.09.10	Std.	–	2μg/ml	63%	3, 96-well	N.C.	–	Increase	**Increase**	Decrease
08.30.10	Std.	–	2μg/ml	77%	3, 96-well	N.C.	–	Increase	**Increase**	Decrease
09.19.10 w/TCDD	Std.	–	2μg/ml	85%	3, 96-well	–	–	N.C.	**N.C.**	N.C.
	Std.	–	2μg/ml	85%	3, 96-well	–	–	Decrease	**Decrease**	Decrease
09.25.10	Std.	–	2μg/ml	86%	3, 6-well	Decrease	–	Increase	**Increase**	Decrease
10.15.10 w/F10 media w/$AhR^{-/-}$ DCs	Std.	–	2μg/ml	80%	3, 6-well	N.C.	–	Decrease	**Decrease**	n.a.
	Std.	–	2μg/ml	80%	3, 6-well	N.C.	–	Decrease	**Decrease**	n.a.
	Altered	–	2μg/ml	80%	3, 6-well	N.C.	–	Decrease	**Decrease**	n.a.
12.03.10	Std.	Std.	2μg/ml	51%	3, 96-well	N.C.	–	Decrease	**Decrease**	n.a.
12.13.10	Std.	Std.	2μg/ml	76%	3, 96-well	Increase	–	Decrease	**Decrease**	n.a.
01.12.11	Std.	–	2μg/ml	69%	3, 96-well	N.C.	–	N.C.	**N.C.**	n.a.
02.18.11	Std.	–	2μg/ml	84%	4, 96-well	N.C.	–	N.C.	**N.C.**	n.a.
03.18.11 w/TCDD w/kynurenine	Std.	Std.	1μg/ml	76%	3, 96-well	–	–	Increase	**Increase**	n.a.
	Std.	Std.	1μg/ml	76%	3, 96-well	–	–	N.C.	**N.C.**	n.a.
	Std.	Std.	1μg/ml	76%	3, 96-well	–	–	Increase	**Increase**	n.a.
05.13.11	Std.	Altered	1μg/ml	81%	4, 96-well	–	–	N.C. *Increase in media only*	**N.C. *Increase in media only***	n.a.
06.03.11 Male	Std.	Altered	1μg/ml	77%	4, 96-well	–	–	N.C.	**N.C.**	n.a.
Female	Std.	–	1μg/ml	88%	4, 96-well	–	–	Increase	**Increase**	n.a.
06.24.11 w/anti-IL-6	Std.	–	1μg/ml	75%	4, 96-well	–	–	N.C.	**N.C.**	n.a.
	Std.	–	1μg/ml	75%	4, 96-well	–	–	Decrease	**Decrease**	n.a.
07.25.11	Std.	–	1μg/ml	81%	4, 96-well	–	–	Increase	**Increase**	n.a.
08.05.11	Std.	–	1μg/ml	78%	3, 96-well	–	–	Decrease	**Decrease**	n.a.
08.26.11 10nM	Std.	Std.	1μg/ml	74%	4, 96-well	–	–	N.C.	**N.C.**	n.a.
50nM	Std.	Std.	1μg/ml	74%	4, 96-well	–	–	N.C.	**N.C.**	n.a.
100nM	Std.	Std.	1μg/ml	74%	4, 96-well	–	–	N.C.	**N.C.**	n.a.
09.21.11 +LPS	Altered	Altered	50μg/ml	84%	5, 95-well	–	–	Decrease	**N.C.**	Decrease
+TGFβ1	Altered	Altered	50μg/ml	84%	5, 95-well	–	–	N.C.	**N.C.**	Decrease
11.01.11 +LPS	Std.	Std.	50μg/ml	97%	5, 12-well	N.C.	N.C.	Increase	**Increase**	N.C.
	Altered	Altered	50μg/ml	97%	5, 12-well	Increase	Increase	Decrease	**Decrease**	N.C.
11.22.11	Std.	Altered	50μg/ml	99.7%	5, 6-well	N.C.	–	N.C.	**N.C.**	Decrease
12.06.11	Std.	Altered	2μg/ml	99.6%	5, 6-well	N.C.	N.C.	N.C.	**N.C.**	Decrease

Appendix Table A.1. 1:5 Veh/TCDD-DC:$CD4^+$ (OTII $FoxP3^{GFP}$) T cell Co-culture Set-up and Results.

Appendix Table A.2

A.

Date/Expt. #	DC Phenotype	DC Gene Profile	Ova peptide	$CD4^+$ T cell purity	Days in Culture/ well type	Cell Numbers	Proliferation	% $CD4^+$ $FoxP3^+$	% $CD4^+$ $CD25^+$ $FoxP3^+$	% $CD4^+$ $CD25^+$ $FoxP3^{neg}$
07.25.11	Std.	–	1μg/ml	81%	4, 96-well	–	–	N.C. *decrease in media only*	N.C. *decrease in media only*	n.a.
08.05.11	Std.	–	1μg/ml	78%	3, 96-well	–	–	N.C. *increase in media only*	N.C. *increase in media only*	n.a.
11.01.11 +LPS	Std.	Std.	50μg/ml	97%	5, 12-well	Increase	N.C.	N.C.	Decrease	N.C.
	Altered	Altered	50μg/ml	97%	5, 12-well	Increase	Increase	Decrease	Decrease	Increase

B.

Date	IL-2 Media only	IFNγ Media only	IL-10 Media only		IL-2 +IL-2/TGFβ1	IFNγ IL-2/TGFβ1	IL-10 IL-2/TGFβ1
07.25.11	Increase	Increase	N.C.		Decrease	N.C.	N.C.
08.05.11	Decrease	Increase	Decrease		Decrease	N.C.	Decrease
11.01.11 +LPS	Decrease	Decrease	Decrease		Decrease	Decrease	Decrease
	Increase	N.C.	N.C.		Increase	N.C.	Decrease

Appendix Table A.2. (A) 1:5 Veh- and BaP-DC:$CD4^+$ (OTII $FoxP3^{GFP}$) T cell co-culture results (B) 1:5 Veh/BaP-DC:$CD4^+$ (OTII $FoxP3^{GFP}$) T cell co-culture cytokines.

Appendix Table A.3

A.

Date/Expt. #	DC Phenotype	DC Gene Profile	Ova peptide	$CD4^+$ T cell purity	Days in Culture/ well type	Cell Numbers	Proliferation	% $CD4^+$ $FoxP3^+$	% $CD4^+$ $CD25^+$ $FoxP3^+$	% $CD4^+$ $CD25^+$ $FoxP3^{neg}$
11.15.10 I	Std.	–	2μg/ml	78%	3, 6-well	N.C.	–	Decrease	Decrease	n.a.
OTII $AhR^{-/-}$	Std	–	2μg/ml	81%	3, 6-well	N.C.	–	Decrease	Decrease	n.a.
12.03.10 II	Std.	–	2μg/ml	78%	3, 96-well	N.C.	–	N.C.	N.C.	n.a.
06.03.11 Male	Std.	Altered	1μg/ml	89%	4, 96-well	–	–	Decrease	Decrease	n.a.
Female	Std.	–	1μg/ml	85%	4, 96-well	–	–	N.C.	N.C.	n.a.

B.

Date/Expt. #	DC Phenotype	DC Gene Profile	Ova peptide	$CD4^+$ T cell purity	Days in Culture/ well type	Cell Numbers	Proliferation	%$CD4^+$ $IL\text{-}10^+$	% $CD4^+$ $IFN\gamma^+$
02.18.11	Std.	–	2μg/ml	80%	4, 96-well	N.C.	–	N.C.	N.C.
w/IL-27	Std.	–	2μg/ml	80%	4, 96-well	N.C.	–	N.C.	N.C.
06.03.11 Male	Std.	Altered	1μg/ml	89%	4, 96-well	–	–	Increase	Increase
Female	Std.	–	1μg/ml	85%	4, 96-well	–	–	N.C.	N.C.

Appendix Table A.3. (A) 1:5 Veh- and TCDD-DC:$CD4^+$ (OTII) T cell co-cultures intracellular FoxP3 staining results (B) 1:5 Veh- and TCDD-DC:$CD4^+$ (OTII) T cell co-cultures intracellular IL-10 and IFNγ staining results.

Appendix Table A.4

Date	IL-2 Media only	IFNγ Media only	IL-10 Media only		IL-2 +IL-2/TGFβ1	IFNγ IL-2/TGFβ1	IL-10 IL-2/TGFβ1
09.25.10	Decrease	Decrease	Decrease		Decrease	Decrease	Decrease
05.13.11	Increase	Decrease	Decrease		Increase	Decrease	Decrease
07.25.11	Increase	N.C.	Decrease		Decrease	N.C.	N.C.
08.05.11	Increase	Decrease	Decrease		Increase	N.C.	Decrease
09.21.11 +LPS +TGFβ1	Increase	Decrease	Decrease		Increase	Decrease	Decrease
	Increase	Decrease	B.D.		N.C.	Decrease	Decrease
11.01.11 +LPS	Increase	Decrease	Decrease		Increase	Decrease	Decrease
	Increase	Decrease	Decrease		Increase	N.C.	N.C.
11.22.11	Increase	Decrease	Decrease		Increase	Decrease	N.C.
12.06.11	N.C.	Decrease	Decrease		N.C.	Decrease	Decrease

Appendix Table A.4. 1:5 Veh/TCDD-DC:$CD4^+$ (OTII $FoxP3^{GFP}$) T cell co-culture cytokines.

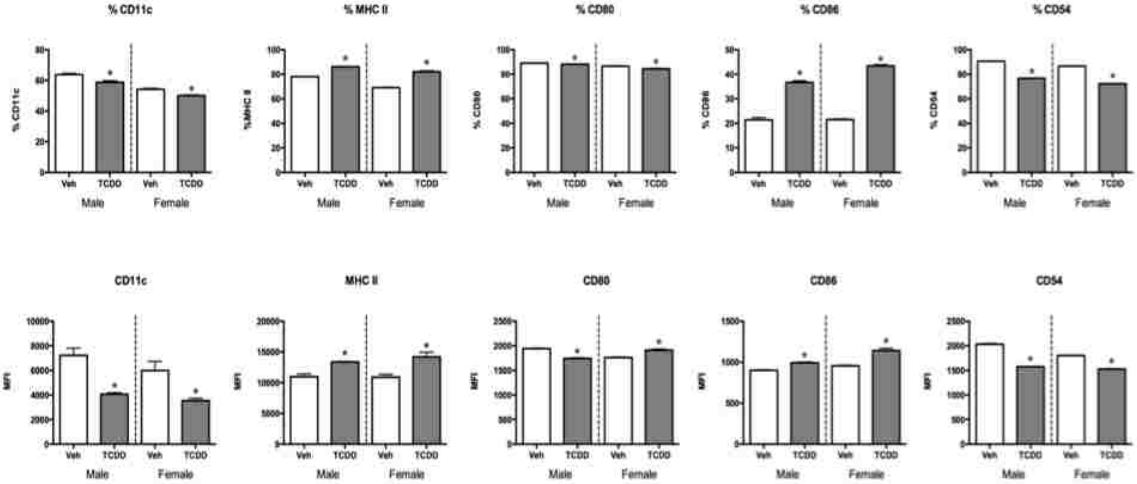
Appendix Table A.5

Date	DC Gene Profile	*Cyp1a1*	*Ido1*	*Ido2*	*Tgfβ1*	*Tgfβ2*	*Tgfβ3*	*Aldh1a1*	*Aldh1a2*	*Arginase*	*Il-6*	*Il-10*	*Il-27*	*AhRR*
12.03.10	Std.	⇧	⇧	⇧	↓	N.C.	⇧	N.C.	–	–	–	N.C.	–	N.C.
12.13.10	Std.	⇧	⇧	⇧	↓	⇧	⇧	–	–	–	–	–	–	–
03.18.11	Std.	⇧	⇧	⇧	↓	⇧	⇧	N.C.	N.C.	–	–	–	–	–
05.13.11	Altered	⇧	N.C.	–	–	–	–	–	–	–	N.C.	–	–	–
06.03.11 Male	Altered	N.C.	⇧	–	–	–	–	–	–	–	N.C.	–	–	–
08.26.11 10nM	Std.	⇧	⇧	⇧	N.C.	⇧	⇧	N.C.	⇧	–	–	N.C.	–	–
50nM	Std.	⇧	⇧	⇧	N.C.	⇧	⇧	N.C.	⇧	–	–	N.C.	–	–
100nM	Std.	⇧	⇧	⇧	↓	⇧	⇧	N.C.	⇧	–	–	N.C.	–	–
09.21.11	Std.	⇧	⇧	⇧	N.C.	N.C.	⇧	↓	⇧	–	N.C.	N.C.	–	–
+LPS	Altered	⇧	⇧	⇧	⇧	⇧	⇧	⇧	N.C.	–	N.C.	N.C.	–	–
+TGFβ1	Altered	⇧	⇧	⇧	N.C.	N.C.	N.C.	N.C.	⇧	–	↓	N.C.	–	–
11.01.11 TCDD	Std.	⇧	⇧	⇧	–	–	–	–	–	–	–	–	–	–
BaP	Altered	⇧	⇧	⇧	–	–	–	–	–	–	–	–	–	–
11.22.11	Altered	⇧	⇧	⇧	N.C.	N.C.	⇧	N.C.	⇧	N.C.	N.C.	N.C.	⇧	⇧
12.06.11	Altered	⇧	⇧	⇧	N.C.	⇧	⇧	N.C.	⇧	N.C.	N.C.	N.C.	⇧	⇧

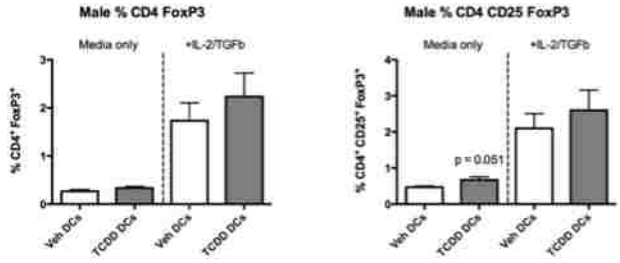
Appendix Table A.5. Gene expression of Vehicle- TCDD- or BaP-treated BMDCs used in DC:T cell co-culture assays.

Appendix Figure A.1

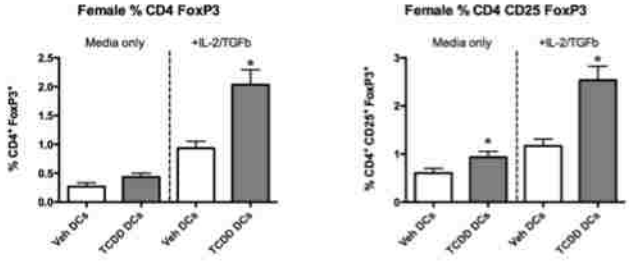
A. Male and Female Balb/c Vehicle- and TCDD-DCs



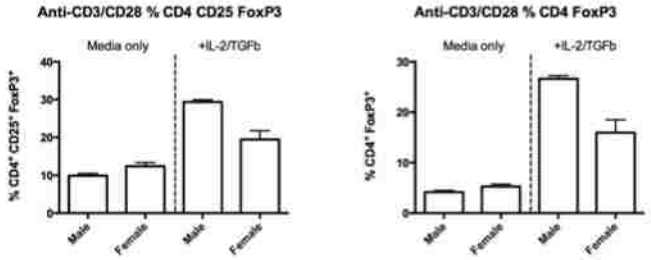
B. Male Balb/c DC:DO11.10^{+} CD4^{+} T Cells



C. Female Balb/c DC:DO11.10^{+} CD4^{+} T Cells

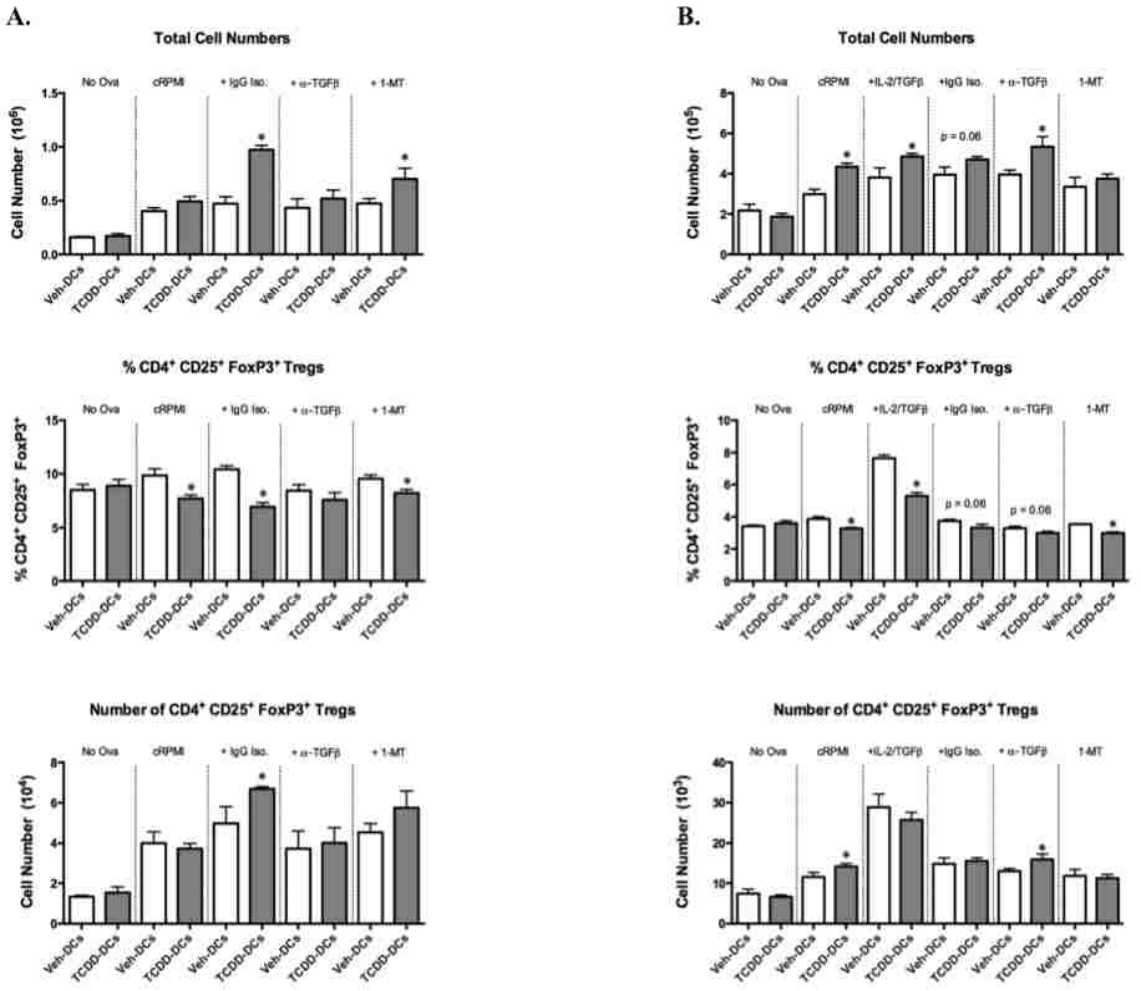


D. CD4^{+} T cell Stimulation Control



Appendix Figure A.1. DC:T cell co-cultures with Vehicle- and TCDD-DCs generated from Balb/c mice and $CD4^+$ T cells from DO11.10 mice. Ovap-DCs were cultured with FACS-purified naïve $CD4^+$ $CD25^-$ T cells as described in Chapter 3 Methods and Materials. DC:T cell co-cultures were harvested on day 5. (A) Male and female BMDC surface molecule phenotype following 7 days in growth with Vehicle or TCDD. (B) Male and (C) Female Vehicle- and TCDD-DC:T cell co-cultures, frequency of $CD4^+$ $Foxp3^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs assessed on Day 5. (D) Male and Female $DO11.10^+$ $CD4^+$ T cells stimulated with plate-bound anti-CD3/CD28 in media only or media supplemented with IL-2/TGFβ. Data representative of one experiment with n = 3. * indicates, $p \leq 0.05$, significant difference between Vehicle and TCDD groups.

Appendix Figure A.2



Appendix Figure A.2. Veh- and TCDD-DC:T cell co-cultures with 1-MT and anti-TGFβ Treg inhibitors. Vehicle-DC and TCDD-DCs were co-cultured with enriched OTII$^+$ CD4$^+$ T cells and the percent of CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs assessed on day 3. The number of Tregs in culture was calculated from the total cell number and the percent CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs present on day 3. (A) and (B) are two independent experiments with n = 4. ***** $p \leq 0.05$ indicates significant difference between Vehicle-DC:T cell and TCDD-DC:T cell groups.