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Consequences of Aryl Hydrocarbon Receptor Activation in Crohn's Disease

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CONSEQUENCES OF ARYL HYDROCARBON RECEPTOR ACTIVATION IN CROHN'S DISEASE

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CONSEQUENCES OF ARYL HYDROCARBON RECEPTOR ACTIVATION
IN CROHN'S DISEASE

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Activation of the aryl hydrocarbon (AhR), a ligand-activated transcription factor present in many immune cells, can trigger immunosuppressive responses through the generation of regulatory cells. Several AhR ligands exist in the diet including environmental contaminants, such as its prototypical ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and natural sources, such as indole-3-carbinol (I3C) and indirubin. Crohn's disease, a chronic inflammatory state of the gastrointestinal tract, affects millions worldwide; however, the exact cause is unknown and treatments do not cure the disease. The role of the AhR in mucosal immune responses is not well understood to date so we aimed to understand the consequences of AhR activation in gut inflammatory responses. *The overall hypothesis of this project is that AhR agonists found in the diet have the potential to dampen inflammation associated with Crohn's disease.* The first goal of this project was to examine the role of AhR activation in the development and progression of colitis using TCDD and the natural ligand I3C. TCDD suppressed TNBS-induced colitis, as demonstrated by the decreased disease severity and dampened inflammation in the gut. TCDD-treated mice exhibited decreased inflammatory mediator production and increased frequency of regulatory cells, both Foxp3+ Tregs and dendritic cells (DCs) in gut immune tissues. In comparison, I3C suppressed disease severity in females but not males. Sex-specific effects on colonic cytokine production and gene expression were observed. The second goal of this project was to define the cellular mechanisms by which AhR ligands elicit their effects on important immune cell populations present in the gut. Therefore, we investigated the immunomodulatory effects of the dietary AhR ligands I3C and indirubin in bone marrow derived DCs (BMDCs). I3C- and indirubin-treated BMDCs upregulated the expression of immunoregulatory genes, such as *ALDH1A, IDO*, and *TGFβ*, and also drove the generation of Foxp3+ Tregs. Following LPS stimulation, I3C- and IO-treated BMDCs suppressed the LPS-induced production of TNF-α, IL-1β, IL-6 and IL-12. Anti-inflammatory effects were also observed in intestinal epithelial cells (IECs) treated with AhR ligands. Thus, I3C and indirubin possess immunosuppressive and anti-inflammatory effects in BMDCs and IECs. Taken together, our data demonstrate that the AhR is a therapeutic target that warrants further investigation, as natural AhR ligands may effectively prevent the onset of chronic inflammatory diseases or more effectively induce and maintain remission when combined with conventional medicine.

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CHAPTER 1: INTRODUCTION

This study describes the effects of aryl hydrocarbon receptor (AhR) activation on the initiation and progression of inflammation associated with Crohn's disease. The introductory chapter will: 1) describe the basic functions of the immune system with a focus on the gut mucosal immune system 2) provide information regarding the role of the AhR and its numerous ligands in immune responses 3) explain the immune dysregulation contributing to Crohn's disease and 4) describe the potential role of dietary AhR ligands to modulate intestinal inflammation.

Immune system

Overview: functions and components

The immune system is a complex network of tissues, cells, and soluble mediators that work together to effectively protect the body against infection and disease. There are four primary functions of immune responses: proper recognition of infection, containment of infection, self-regulation to inactivate the robust protective response, and protection from recurring disease due to the same pathogen. Successful immune responses typically involve both the innate and adaptive branches of the immune system. Innate responses occur within hours of pathogenic invasions via non-specific recognition of non-self molecules while the adaptive responses take days due to the recognition specificity of foreign antigens, which are substances capable of activating the immune system. Several factors contribute to the effectiveness of these immune responses including genetics, stress, diet, age, and medications among many others.

All cells involved in these immune responses arise from hematopoietic stem cells in the bone marrow and further differentiate into various populations of leukocytes. Innate cells, including neutrophils, macrophages, and dendritic cells (DCs), are the first responders to initiate immune and inflammatory responses. T cells and B cells are the lymphocytes responsible for mounting antigen-specific adaptive immune responses. These leukocytes produce and secrete soluble mediators (such as cytokines, chemokines, antibodies, and cytotoxic granules) that influence surrounding cells and thus the ensuing immune response. A more detailed discussion is provided below regarding two critical immune cell populations, DCs and T cells, and their specific roles in immunity and inflammation.

Antigen presenting cells (APCs)

APCs are essential to properly mounting innate and initiating adaptive immune responses that effectively fight infectious agents. Although DCs, macrophages, and B cells all function as APCs, DCs are the professional APCs. DCs are unique in that they are involved in the induction of both protective and regulatory responses, especially in the gut. As shown in Figure 1.1, the life cycle of DCs involves antigen capture in the periphery, migration to lymph nodes, and presentation to T cells, which results in lymphocyte activation necessary to trigger adaptive responses (Banchereau *et al.*, 2000). During this process, DCs change from immature DCs in the periphery to mature DCs in the lymphoid tissue. Consequently, alterations in cell surface phenotype and functions of DCs also occur (Figure 1.2).

Figure 1.1
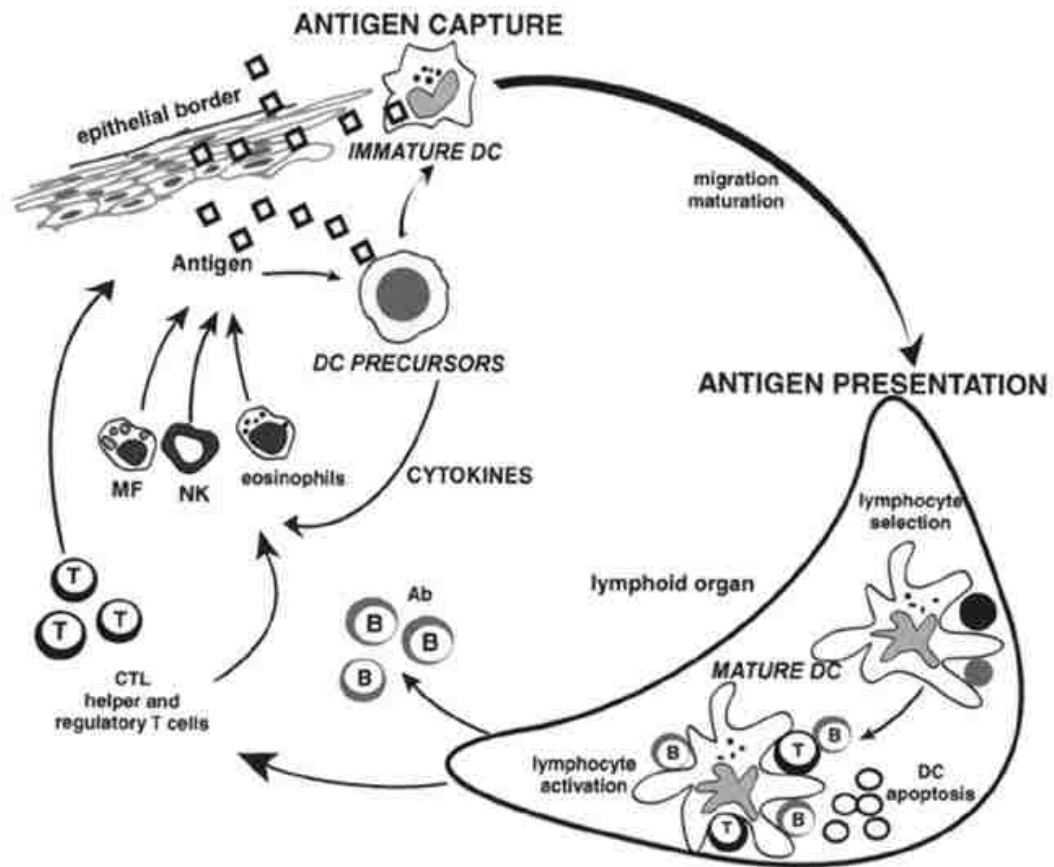
Figure 1.1. Life cycle of dendritic cells (DCs). Immature DCs survey the periphery for foreign antigens that have penetrated mechanical barriers and entered tissues. Upon recognition, DCs take up antigen, process it, migrate to lymphoid organs, and present antigen to lymphocytes, which results in their activation. Activated lymphocytes then return to the site of infection to clear it from the body (Banchereau *et al.*, 2000).

Figure 1.2

Immature DC
Low CD11c
Low MHC II
Low CD86
Low CD40
High Ag uptake
Poor migration
Poor T cell activator

Mature DC
High CD11c
Medium MHC II
Medium CD86
Medium CD40
Low Ag uptake
Good migration
Good T cell activator

Activated DC
High CD11c
High MHC II
High CD86
High CD40
No Ag uptake
Poor migration
Great T cell activator
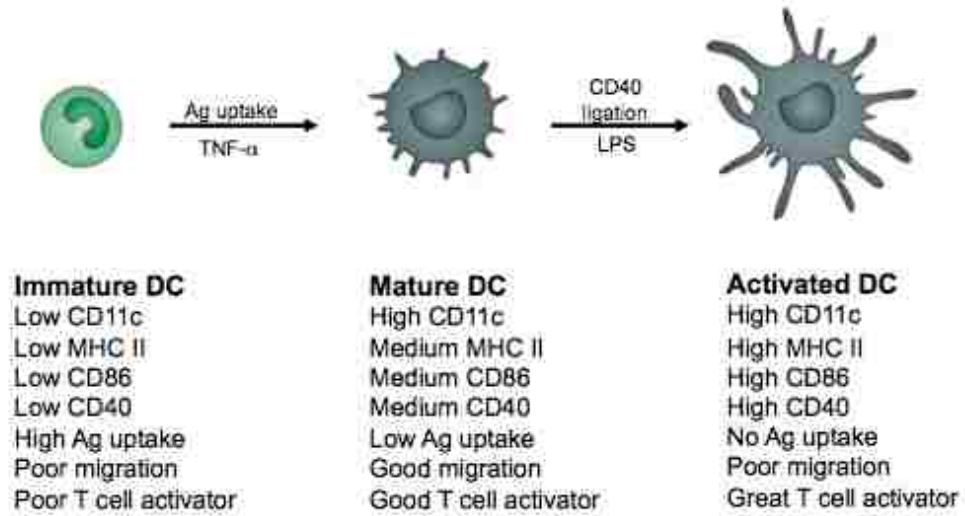
Figure 1.2. Stages of DC maturation. Following antigen uptake, immature DCs undergo a maturation process to activated DCs, which results in several immunophenotypic and function changes.

Immature DCs are particularly efficient at antigen uptake but have poor abilities to migrate to lymph nodes and activate T cells. Conversely, activated DCs do not take up antigen but are very effective T cell activators.

Successful antigen processing and presentation by DCs first begins with proper uptake of antigens via receptor-mediated phagocytosis or macropinocytosis. The recognition of pathogen associated molecular patterns (PAMPs) occurs through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and scavenger receptors, which are expressed on the surface of DCs. The antigen is then internalized, processed, and displayed on the cell surface via major histocompatibility complexes (MHC). Finally, to successfully activate T cells, three signals are required between the APC and T cell (Figure 1.3). Signal 1 is an activation signal involving the MHC:peptide complex with the T cell receptor (TCR) complex. Signal 2 is a co-stimulatory signal between the B7 molecules on DCs with CD28 or CTLA4 on T cells. Signal 3 involves cytokines and other secreted molecules that act as a differentiation signal. However, if one of these signals is missing, T cells are not effectively activated. When T cells only receive signal 1 they become unresponsive (anergic), which allows for peripheral tolerance induction. These unresponsive cells are particularly important in avoiding autoimmune responses to tissue-specific antigens. Furthermore, when signal 2 is received in the absence of an antigen, no effects occur on T cells.

Figure 1.3
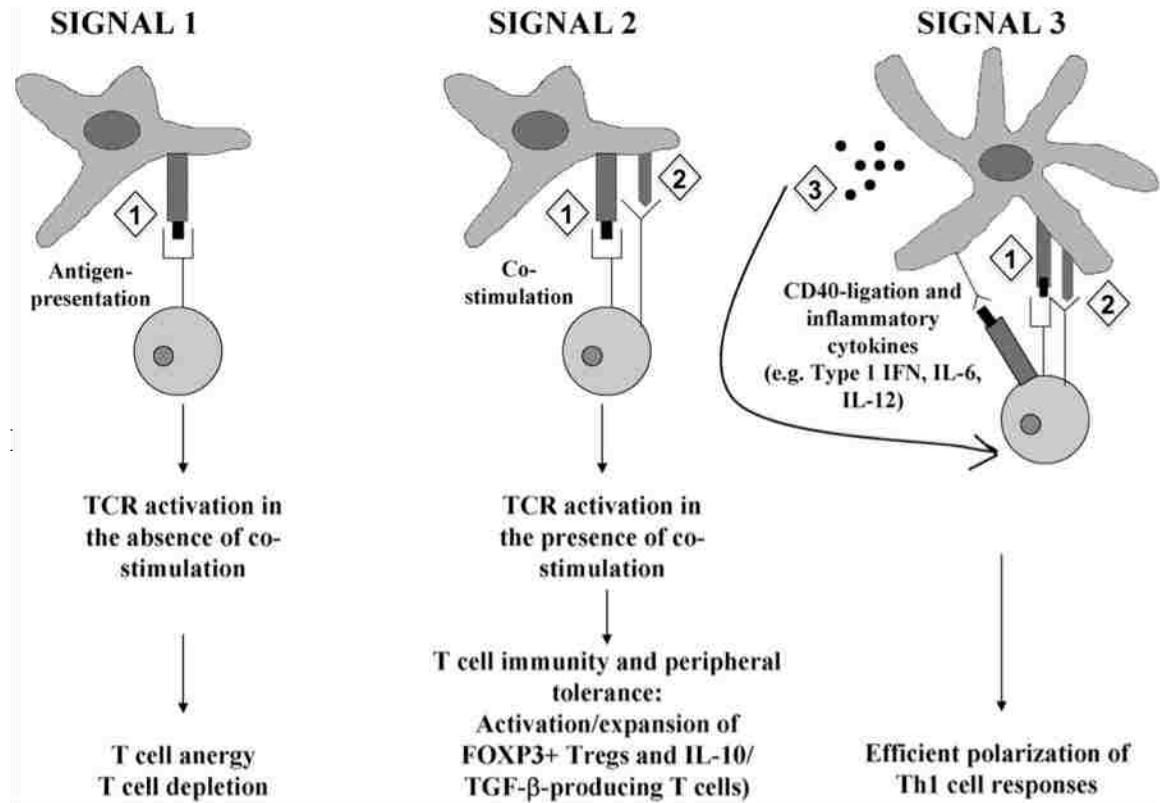
Figure 1.3. Three signal hypothesis of T cell activation. To effectively activate T cells APCs, such as DCs, deliver three signals including (1) binding of the MHC2:peptide complex to the TCR, (2) costimulation, and (3) cytokines (adapted from Cools *et al.*, 2007).

T cells

T cells are important adaptive immune cells that function to kill pathogens as well as to activate and regulate other cells in the body. The two broad classes of T cells include CD4+ helper T cells (Th cells), which can activate APCs and B cells that ultimately produce antibodies, and CD8+ cytotoxic T cells (CTLs), which kill tumor cells or virus-infected cells. Following multiple signals from APCs, naïve T cells can differentiate into a variety of effector or regulatory cells (Figure 1.4). In particular, naïve CD4+ T cells can become Th1, Th2, Th17, Th22, or regulatory T cells (Tregs), which each have distinct roles in the ensuing immune response. Each effector cell type is characterized by the cytokine profile that leads to differentiation of that subtype and the primary cytokines produced. Th1, Th17, and Th22 cells are known for their roles in inflammatory and autoimmune responses whereas Th2 cells are best recognized for their involvement in allergic responses. Tregs are vital to regulating immune responses by suppressing effector cell proliferation and secretion of anti-inflammatory cytokines. Finally, since these are adaptive cells, memory cells are generated in the course of the immune response. These memory T cells will rapidly differentiate into effector cells upon subsequent exposure to the same antigen.

The balance of effector and Tregs is necessary to maintain homeostasis within the immune system. Defects leading to robust effector cell responses and/or weak regulatory cell responses contribute to the immune dysregulation found in numerous chronic inflammatory diseases, such as inflammatory bowel disease.

Figure 1.4
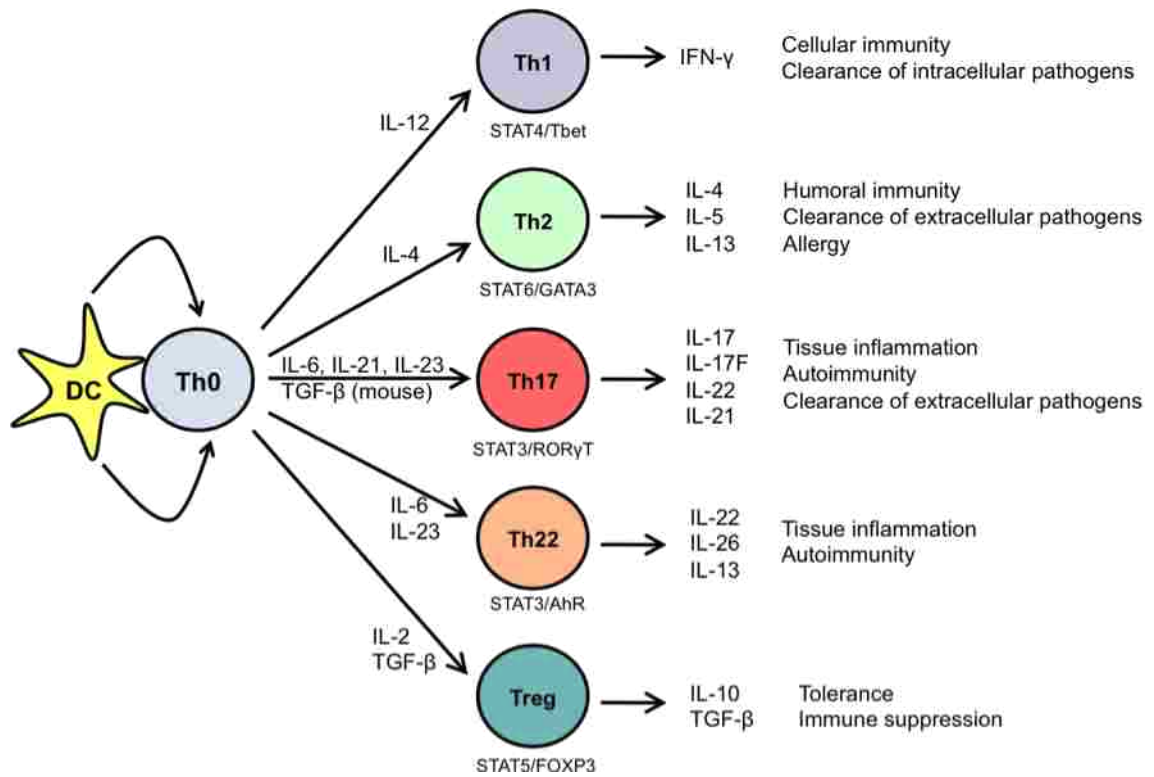
Figure 1.4. Characterization of CD4+ T cell populations. Upon activation, naïve CD4+ T cells (Th0) can differentiate into several subtypes based on cytokine production and function in immune responses. Transcription factors necessary for each population is shown below the cell, and function is listed on the right (adapted from Jetten, 2009).

Inflammation

One of the essential steps in combating infection is inflammation. Innate cells, such as the first responding neutrophils and macrophages, trigger the recruitment of other cells to the site of infection by releasing pro-inflammatory cytokines and chemokines (secreted mediators that direct cellular trafficking) through signaling cascades that activate transcription factors, such as nuclear factor kappa B (NF-kB). Furthermore, several acute response proteins are produced including C-reactive protein (CRP), serum amyloid A (SAA), cyclooxygenase (COX), and nitric oxide (NO). The local accumulation of cells and soluble mediators causes redness, swelling, heat, and pain, which are the hallmark clinical signs of an inflammation. Later in the response, effectors of the adaptive branch, such as effector T cells and antibodies, contribute to the containment and clearance of infection.

Inflammation is generally a beneficial immune response when it is localized and tightly regulated. Acute inflammation leads to resolution, healing, and priming of the adaptive responses. Signaling cascades are eventually inactivated to ensure that the attack does not spread to nearby tissues. Chronic inflammation and systemic inflammation (sepsis), however, are damaging conditions that can lead to many diseases including cancer and potentially death. Under chronic inflammatory conditions, transcription factors, such as NF-kB, remain activated thereby promoting the transcription of many pro-inflammatory cytokine genes, such as IL-6, IL-12, and TNF-α. Tissues are often damaged as constant activation of inflammatory pathways leads to granulomas, fibrotic scar tissue, fluid accumulation, and abscesses. Thus, moderation is essential to reaping the benefits of the inflammatory immune response.

Gut mucosal immunity

Exposure to a diverse and extensive range of antigens and unusual effector and regulatory responses makes the mucosal immune system distinctly different than the rest of the immune system. It must properly distinguish between pathogens and innocuous antigens including food particles and commensal bacteria, which are essential for the clearance of toxins, xenobiotic metabolism, nutrient absorption, and prevention of pathogenic bacteria colonizing the gut. The gut associated lymphoid tissues (GALT), which include the Peyer's patches of the small intestine, isolated lymphoid follicles, and the intraepithelial lymphocytes (IELs), are responsible for mounting the appropriate immune response to this vast array of antigens.

Unique features

There are two primary compartments of the intestines: the epithelium and the lamina propria (Figure 1.5). The epithelium is the first line of defense in the gut, as the tight junctions and mucus produced by Goblet cells are critical to preventing transfer of luminal contents into the underlying tissue. Microfold cells (M cells) are another type of specialized epithelial cells unique to the gut. These cells are the route by which antigen enters the Peyer's patch from the lumen. Most of the immune cells are found in the lamina propria, which acts as the interface between the epithelium and lymphoid tissue below and is where most immune cells are found in the intestines. In fact, large numbers of effector cells reside in the gut tissue even in the absence of infection. Furthermore, plasma cells (highly differentiated B cells) in the lamina propria that receive a transforming growth factor-beta (TGF-β) switch factor signal produce copious amounts

Figure 1.5
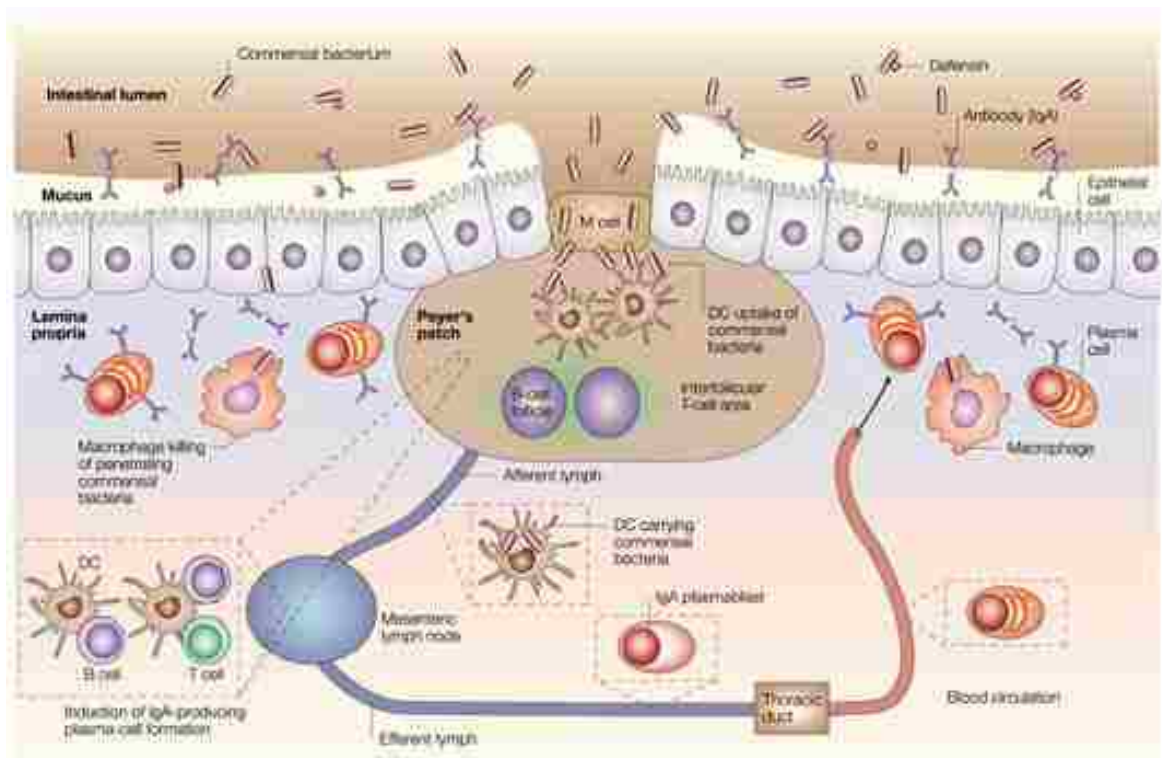
Figure 1.5. Components of the intestinal immune system. The epithelium and lamina propria are the two major compartments in the intestines that contribute to protective and tolerant immune responses. Intraepithelial lymphocytes, intestinal epithelial cells (IECs), DCs, M cells, Paneth cells (not shown), and goblet cells (not shown) are found in the epithelial layer lymphocytes, whereas DCs, macrophages, and other innate cells reside in the lamina propria. Following antigen uptake, APCs travel to the mesenteric lymph nodes to generate an adaptive response that includes IgA production by plasma cells (Macpherson and Harris, 2004).

of immunoglobulin A (IgA), which is present as a dimer and the predominant antibody isotype produced in the gut. In the periphery, the IgG isotype dominates, and IgA is present as a monomer. There are several functions of IgA at the epithelial surface: neutralizing pathogens in the mucus layer to prevent penetration, neutralizing antigens internalized in endosomes in the epithelial layer, and exporting pathogens from the lamina propria.

Important cellular players of intestinal immune responses

As with the peripheral immune system, APCs play a critical role in mounting appropriate mucosal immune responses. In the gut, both the intestinal epithelial cells (IECs) and DCs play important roles in antigen recognition, processing, and presentation. These cell populations sample luminal contents via pattern recognition receptors (PRRs) expressed on their surfaces. IECs sample antigens directly whereas DCs sample the luminal contents by projecting extensions through the epithelial layer ("snorkeling") or indirectly via antigen processed by IECs. Both of these cell populations secrete mediators, such as IL-10 and TGF-β, that can influence surrounding cells and the ensuing immune response. Upon antigen uptake, DCs travel to the mesenteric lymph nodes (MLNs) to trigger subsequent adaptive responses. The activated T cells then home back to the gut primarily via expression of the surface marker $\alpha 4\beta 7$, an integrin that mediates the migration of cells to the intestines and associated immune tissues.

Based on the antigen sampled, DCs and IECs produce mediators to generate either protective immunity, which is similar to peripheral responses, or oral tolerance, which is an unresponsiveness to harmless food, commensal bacteria, or non-pathogenic

bacteria. In the presence of pathogenic organisms, DCs become activated and express strong costimulatory ligands that drive the differentiation of naïve CD4 T cells into Th1, Th2, Th17, or Th22 cells. Alternatively, in the presence of commensal bacteria, which lack the virulence factors necessary for penetrating the epithelium, immature DCs give weak costimulatory signals and induce Tregs. Furthermore, in the absence of infection, IECs produce prostaglandin-E2 (PGE-2), TGF-β, and thymic stromal lipoprotein (TSLP) that inhibit DC maturation. These regulatory DCs express CD103 on the cell surface and produce protective mediators, such as TGF-β and IL-10. Therefore, the activation state of local DCs determines either protective immunity or tolerance, which is the default immune status of the gut.

As previously mentioned, effector T cells are prevalent in the gut immune tissues, which is typically indicative of an inflammatory phenotype; however, regulatory cells play a prominent role in preventing potentially harmful responses by effector cells. Following exposure to oral antigens, several classes of Tregs are induced including thymic-derived Foxp3+ Tregs, mucosally-induced Foxp3+ Tregs, Tr1 cells, and Th3 cells (reviewed in Weiner *et al.*, 2011). These different classes likely differ in antigen specificity, development, and mechanism of immune regulation. Interestingly, the frequency of these Treg populations changes along the gastrointestinal tract due to the nature of the different microenvironments, as the colon carries a significantly greater bacteria load than the small intestine where the primary function is nutrient absorption. Most IL-10 producing Tregs in the colon lamina propria are Foxp3+ Tregs while Foxp3- Tr1 cells are more prevalent in the small intestine (Maynard *et al.*, 2007). Although there are several classes of Tregs responding, in general the primary function of Tregs is to

induce/maintain tolerance and decrease inflammation in the gut. Therefore, perturbations in this regulatory state can lead to disease involving intestinal inflammation including inflammatory bowel diseases.

Factors that alter mucosal immune responses

Several factors including age, stress, and medications can modulate immune responsiveness. Perhaps one of the most influential factors in the mucosal immune system is the diet since dietary compounds directly contact the tissues in the gastrointestinal tract. Numerous studies have identified dietary constituents that can either promote or suppress inflammation in the gut. These compounds or their metabolite can act directly on cells and tissues to alter inflammatory mediator production or indirectly by altering the intestinal microbial communities.

More specifically, intake of certain fats and carbohydrates drastically affects immune responses by promoting inflammation while intake of other nutrients suppresses inflammatory responses. For example, fatty diets promote inflammation in the intestine as well as in other peripheral tissues because they change the intestinal microflora such that Gram negative bacteria, which contain endotoxin, predominate instead of Gram positive bacteria (Cani *et al.*, 2007). This increased presence of lipopolysaccharide (LPS) in the gut is recognized by immune cells, such as DCs and IECs, which triggers inflammation not only in the gut but also in other tissues and contributes to the development of metabolic disease. Moreover, increased intake of linoleic acid, an omega-6 fatty acid, alters the balance between omega-3 fatty acids and omega-6 fatty acids such that omega-6 fatty acids dominate and promote inflammation (Wall *et al.*, 2009). Finally,

refined carbohydrates, from which fiber and essential nutrients have been removed, have been shown to increase circulating levels of pro-inflammatory cytokines including IL-6 and TNF-α (Esposito *et al.*, 2002).

In contrast, many dietary supplements including probiotics, fiber, fish oil, and vitamins have the potential to dampen inflammation in the gut. Probiotics, or the beneficial bacteria found in many commercial food products, alter intestinal immune responses in several ways (extensively reviewed in Sanders, 2011). They decrease pathogen binding, produce antibacterial substances, and decrease pro-inflammatory cytokine production. Additionally, barrier integrity is improved with increased production of mucins and defensins. When this "good" bacteria is recognized by DCs, they produce regulatory signals, such as IL-10, that drive the proliferation of Tregs, which ultimately promotes tolerance and suppresses gut inflammation. Soluble fiber, also known as the prebiotic non-starch carbohydrate, is also anti-inflammatory in nature. Its main fermentation byproduct, butyrate, inhibits NF-kB signaling and therefore the production of pro-inflammatory cytokines (Segain *et al.*, 2000; Luhrs *et al.*, 2002). Omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), also inhibit the production of several inflammatory mediators including pro-inflammatory cytokines and eicosanoids (Issa and Saeian, 2011). Foods also contain many vitamins that can generate an immunoregulatory environment. Vitamin A is metabolized into retinoic acid (RA) by CD103+ regulatory DCs in the gut. In turn, RA can contribute to the differentiation of naïve T cells into Tregs while also inhibiting the generation of Th17 cells. Vitamin D has also been shown to increase Foxp3+ regulatory T cells (Hardenberg *et al.*, 2011).

Clearly there are several sensitive pathways critical to maintaining mucosal immune homeostasis, and the commensal bacteria play an important role in this process. It is essential to understand the factors that are necessary to maintain a properly functioning intestinal immune system. A better understanding of these mechanisms will help us better understand intestinal immune disorders and how to effectively treat them.

Aryl hydrocarbon receptor

Many factors contribute to the immune responsiveness of cells. The aryl hydrocarbon receptor (AhR) is a cytosolic, ligand-activated transcription factor that is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) protein family and binds a variety of natural and synthetic compounds. The AhR is present and inducible in immune cells but is constitutively expressed in DCs. Furthermore, AhR expression is dramatically increased in Th17 cells compared to other subsets of Th cells, such as Th1 and Th2 cells (Veldhoen *et al.*, 2008).

As shown in Figure 1.6, the canonical pathway involves ligand binding to the AhR in the cytosol to activate the receptor. Following binding, accessory proteins (hsp90 and XAP2) dissociate and reveal the nuclear localization signal (nls) allowing the ligand:AhR complex to translocate into the nucleus. There it complexes with the AhR nuclear translocator (ARNT), binds to dioxin response elements (DREs), and modulates transcription of several genes, including Phase I drug metabolizing enzymes, such as CYP1A1. AhR activation is believed to modulate immune and inflammatory responses via transcriptional regulation as there exist multiple (putative) DREs in the promoter regions of several cytokine genes, such as those implicated in Tregs (TGF-β and IL-10), Th1 cells (IL-12), as well as Th17 cells (IL-21 and IL-23) (Kerkvliet, 2009). A non-

Figure 1.6
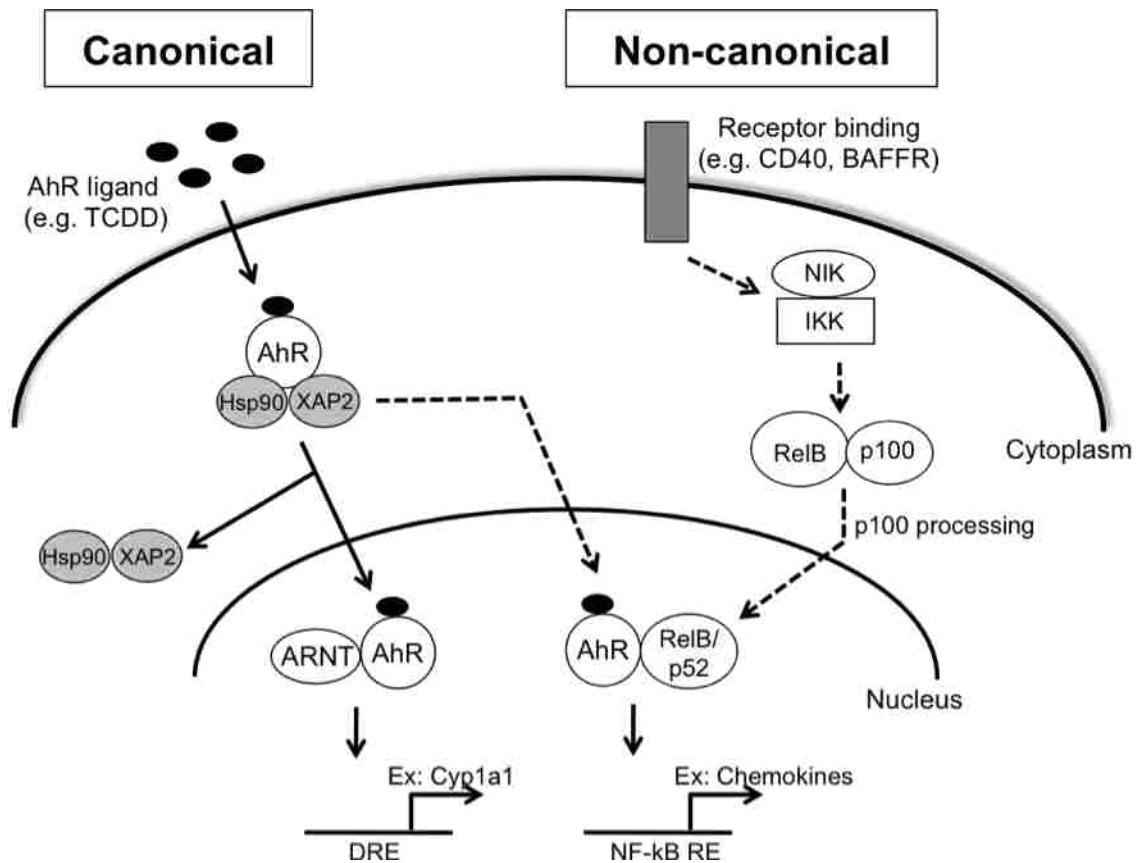
Figure 1.6. Canonical and non-canonical AhR signaling pathways. In the canonical AhR signaling pathway, a ligand diffuses into the cell and binds cytosolic AhR complex, which triggers dissociation of chaperone proteins. Upon entering the nucleus, the ligand-bound AhR binds ARNT before binding DREs thereby triggering transcription of the AhR gene battery. In the non-canonical signaling pathway, the ligand-bound AhR binds RelB in the nucleus thereby allowing binding to NF-kB response elements (adapted from Tian *et al.*, 2002).

canonical AhR signaling pathway also exists, and involves binding of the ligand-bound AhR to the nuclear factor-κB (NF-κB) family member, RelB, which subsequently activates transcription of genes controlled by both the AhR and NF-κB (Tian *et al.*, 1999; Tian *et al.*, 2002; Vogel and Matsumura, 2009). Therefore, both of these AhR signaling pathways have the potential to elicit immunomodulatory effects following AhR activation.

The prototypical ligand of the AhR is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a byproduct of various industrial processes. TCDD is the most popular chemical to study the effects of AhR activation because it is the highest affinity ligand for this receptor and is resistant to metabolism (Ward, 1985; Davila *et al.*, 1995; Mann *et al.*, 1999). Several adverse effects, including those on the immune system, are observed following exposure to TCDD. Studies show that AhR null animals do not experience the immunotoxic effects associated with administration of TCDD thereby indicating that the AhR is necessary to mediate the immunosuppressive effects of TCDD and many TCDD-like chemicals. Since many immune cells express the AhR and many immune genes contain DREs (Kerkvliet, 2009), it is critical to understand how AhR activation can affect the function of the immune system.

Effects of the AhR on inflammatory responsiveness

Prior to discussing the role of the AhR in mediating the effects of various ligands, it must be acknowledged that, independent of exogenous ligands, the AhR plays an important role in controlling several physiological processes including inflammatory responses. Recently, several studies have revealed that mice lacking the AhR are more

sensitive to inflammatory responses. AhR null mice display increased sensitivity to LPS-induced septic shock, which was primarily due to macrophage dysfunction (Sekine *et al.*, 2009). Furthermore, these investigators found that LPS-stimulated bone marrow-derived macrophages secreted more pro-inflammatory cytokines than the wildtype AhR cells. Kimura and colleagues extended these results by demonstrating that AhR forms a complex with Stat1 and NF-kB in macrophages to ultimately inhibit IL-6 activity (Kimura *et al.*, 2009). Similar effects have been observed in disease models. In murine models of inflammatory bowel diseases, several investigators have revealed that AhR deficiency results in a more severe disease phenotype (Arsenescu *et al.*, 2011; Furumatsu *et al.*, 2011; Monteleone *et al.*, 2011). These data suggest that the AhR plays an important role in regulating inflammation so intentional and targeted activation of the AhR may be a promising therapeutic approach for the treatment chronic inflammatory diseases, such as inflammatory bowel disease.

Immunoregulatory effects of TCDD
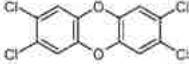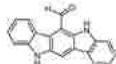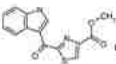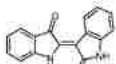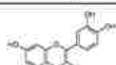
Although early reports suggested that TCDD exacerbates some inflammatory responses, more recent studies have defined cellular mechanisms for its potent immunosuppressive effects on both humoral and cell-mediated responses. TCDD affects humoral immunity by suppressing CD40L-activated B cell proliferation (Ito *et al.*, 2002; Allan and Sherr, 2005). T cells were initially believed to be the primary target of TCDD in the immune system due to thymic involution, which results in thymocyte loss, arrest of T cell proliferation, and premature emigration of T cell progenitors (Laiosa *et al.*, 2003; Temchura *et al.*, 2005; McMillan *et al.*, 2007). In addition to suppressing CD4+ Th cell

differentiation, TCDD also enhances the development of Tregs, as it has been demonstrated that CD4+CD25+ Tregs producing suppressive cytokines are induced following TCDD exposure (Funatake *et al.*, 2005; Marshall *et al.*, 2008). There are several mechanisms by which the AhR can influence the differentiation and expansion of Tregs: (1) the AhR can directly regulate Foxp3 expression due to the DREs present in the Foxp3 promoter (Hauben *et al.*, 2008; Kimura *et al.*, 2008; Quintana *et al.*, 2008); (2) the AhR signaling pathway can affect TGF-β production via cross-talk with the TGF-β signaling pathway (Guo *et al.*, 2004; Santiago-Josefat *et al.*, 2004; Gomez-Duran *et al.*, 2006; Thomae *et al.*, 2006); and (Miller) cell-cell interactions and cytokine milieu produced by DCs drastically alter the fate of naïve Th cells (Thorstenson and Khoruts, 2001; Akbari *et al.*, 2002; Yamazaki *et al.*, 2006; Bankoti *et al.*, 2010a; Bankoti *et al.*, 2010b; Benson and Shepherd, 2011; Simones and Shepherd, 2011). In particular, AhR activation in DCs triggers the induction of IL-10, TGF-β, aldehyde dehydrogenase, and indoleamine-2,3-dioxygenase (IDO), an enzyme that catalyzes the essential amino acid tryptophan via the kynurenine pathway.

Immunomodulatory effects of other AhR ligands

Being a promiscuous orphan receptor, the AhR can bind a structurally diverse range of compounds including various environmental contaminants, endogenous compounds, dietary components, and therapeutic agents. Many of these compounds act as AhR agonists and have been shown to alter immune responses (Table 1.1). These ligands, however, do not typically bind the AhR as strongly as TCDD and are usually quickly metabolized, which can alter their effects on the immune system.

Table 1.1. AhR ligands and their role in immunity[a]

Class	Compound	Structure	Immunomodulatory effects
Environmental contaminants	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)		• Immunosuppressive and induces FoxP3+ Tregs
	Benzo[a]pyrene (BaP)		• AhR agonist
Endogenous	6-formylindolo(3,2-b) carbazole (FICZ)		• Induces Th17 differentiation (Veldhoen et al, 2008) • Enhances EAE development
	2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE)		• AhR agonist but does not induce TCDD-induced toxicity
Dietary	Indole-3-carbinol (I3C)		• AhR agonist primarily used for anti-cancer and anti-inflammatory effects
	Indirubin		• AhR agonist primarily used for anti-cancer and anti-inflammatory effects
	Quercetin		• AhR agonist primarily used for anti-cancer and anti-inflammatory effects
Drugs	VAF347		• Anti-inflammatory and anti-allergenic (Hauben et al, 2008; Lawrence et al, 2008)

[a]A diverse range of compounds that bind the AhR and exert a variety of effects on immune cells to modulate immune responsiveness.

It has been suggested that by binding to the AhR in CD4+ T cells, tryptophan metabolites can elicit their immunosuppressive effects by inducing a Treg phenotype (Fallarino *et al.*, 2002; Frumento *et al.*, 2002; Terness *et al.*, 2002; Belladonna *et al.*, 2006; Fallarino *et al.*, 2006). One of the most studied metabolites is 6-formylindolo[3,2-b]carbazole (FICZ) due to its potent AhR agonistic activity. It has been suggested that AhR activation occurs in a ligand-specific manner as activation via TCDD results in Treg production while activation via FICZ results in the production of Th17 cells, which may be due to differences in the timing and duration of AhR activation (Ho and Steinman, 2008). Quintana and colleagues recently reported that prolonged activation of AhR via TCDD resulted in the production of suppressive Treg cells while brief AhR activation via FICZ generated inflammatory Th17 cells in a murine model of autoimmune encephalitis (Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). It could be hypothesized that significant upregulation of the AhR in Th17 cells makes these cells more susceptible to endogenous AhR ligands that could subsequently mount immunosuppressive Treg responses to dampen the Th17-mediated responses.

In contrast to exacerbating autoimmunity, FICZ was recently reported to suppress the severity of several models of murine colitis, which was partly dependent on IL-22 (Monteleone *et al.*, 2011). FICZ-treated mice experienced decreased production of IFN-γ, IL-17A, and TNF-α and increased IL-22 production. As previously mentioned, IL-22 is a cytokine produced by Th22 and Th17 cells in an AhR-dependent manner (Veldhoen *et al.*, 2008; Trifari *et al.*, 2009; Veldhoen *et al.*, 2009). This cytokine is often associated with autoimmunity since it promotes inflammation by acting synergistically with IL-17, INF-γ, and TNF-α. In the gut, however, IL-22 exhibits a protective role because it

induces antimicrobial peptides that help maintain mucosal barrier integrity. Thus, the effects of IL-22 production induced by AhR activation seem to be dependent on the microenvironment of the inflammatory response.

It must be emphasized that, to date, it is not well understood why some AhR ligands have the potential to promote the development of a Th17-mediated inflammatory response while others promote the development of a Treg-mediated suppressive response. Due to the critical role that these T cell populations play in the generation of immune and inflammatory responses in the gut mucosa, it is necessary to examine the effects of AhR activation in this compartment.

Crohn's disease

Inflammatory bowel disease (IBD), comprised of Crohn's disease and ulcerative colitis, is a chronic inflammatory state of the gastrointestinal tract. This disease typically affects young Caucasians aging from 15 to 35 years old that live in urbanized, developed countries (Head and Jurenka, 2004). It is estimated that the average number of cases currently ranges from 100-200 cases per 100,000 individuals in North America, northern Europe, and the United Kingdom (Loftus *et al.*, 1998; Bernstein *et al.*, 1999; Loftus *et al.*, 2000; Loftus, 2004; Cho, 2008). Unlike ulcerative colitis, Crohn's disease affects all layers of the intestinal wall anywhere along the gastrointestinal tract, but especially in the ileum and colon. Due to a severe inflammatory response, Crohn's disease patients experience many unpleasant symptoms, such as frequent diarrhea, abdominal pain, and rectal bleeding. Many painful and potentially life-threatening intestinal and extraintestinal complications can also affect patients. Since the exact cause of Crohn's

disease remains unknown, conventional treatments typically consist of a combination of corticosteroids and immunomodulatory agents, azathioprine and 6-mercaptopurine, to diminish flare-ups and induce remission. In addition to these conventional therapies, many patients use various complementary and alternative therapies to enhance the effects of conventional therapies or reduce the side effects caused by conventional medicine.

Intestinal immune dysregulation

As previously described, epithelial and immune cells sample antigens in the lumen of the gut to induce a state of tolerance such that an immune response is not elicited towards commensal bacteria under homeostatic conditions. There is a balanced differentiation of Th0 cells into effector cells (Th1, Th2, Th17, Th22) and regulatory cells (Tregs). In Crohn's disease, this state of oral tolerance appears to be disrupted, as demonstrated in Figure 1.7 (Sanchez-Munoz *et al.*, 2008). Several factors have been implicated in the pathogenesis of the disease, including a defective mucosal epithelium, imbalance between effector T cells and Tregs, oxidative stress, and the hygiene hypothesis, which suggests that lack of antigen exposure at a young age triggers the onset of Crohn's disease (Head and Jurenka, 2004). In the mucosal immune system, the epithelium is the first line of defense, and defects in this barrier have been implicated in the pathogenesis of Crohn's disease (Baumgart and Dignass, 2002). Moreover, altered innate immune mechanisms, including genetic polymorphisms associated with intracellular bacterial processing and autophagy, could also lead to an inappropriate immune response to commensal bacteria (Hugot *et al.*, 2001; Ogura *et al.*, 2001; Baumgart and Carding, 2007; Cho, 2008). These environmental and genetic factors shift the balance between effector

Figure 1.7
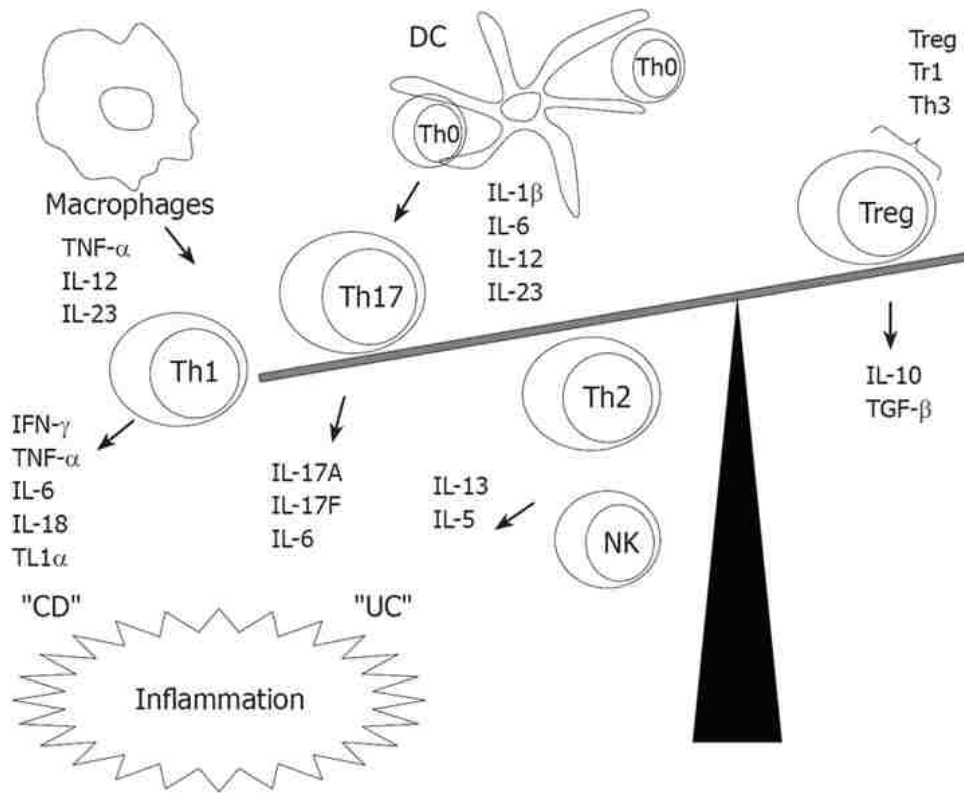
Figure 1.7. **Immune dysregulation contributing to Crohn's disease (CD) and ulcerative colitis (UC).** In IBD, the balance between effector T cells and Tregs is disrupted. Th1 and Th17 cells drive the immune dysregulation in CD whereas UC is a Th2-mediated response.

and regulatory cells such that Th1 inflammatory cells predominate over Tregs. Th1-mediated immune responses are implicated in the development of colitis as Crohn's disease patients show elevated levels of IL-12 (Monteleone *et al.*, 1997; Parronchi *et al.*, 1997; Liu *et al.*, 1999). Furthermore, decreased levels of IL-10, a potent immunoregulatory cytokine, also play a role in this disease. It should be noted that recently, Th17 cells, in addition to Th1 cells, have been implicated in the development of Crohn's disease. The differentiation, expansion, and stabilization of Th17 cells involves the cytokine IL-23, which numerous genome-wide association studies have linked to the development of Crohn's (Abraham and Cho, 2008). Clearly the pathogenesis of Crohn's disease is very complex as it involves a combination of genetic and environmental factors that alter multiple populations of innate and adaptive immune cells.

Complementary therapies for Crohn's disease management

Numerous Crohn's disease patients seek complementary therapies to alleviate symptoms of the disease not relieved by conventional medicine and/or to reduce the side effects from conventional medicines. Many of these therapies include dietary supplements, such as omega-3-fatty acids and probiotics, which have the potential to alter inflammation. Since AhR activation can result in suppression of Th1-mediated immune responses, dietary components that are AhR ligands would be expected to alter the inflammatory response associated with Crohn's disease. Cruciferous vegetables, such as broccoli, brussel sprouts, and cabbage, contain glucosinolates, which are sulfur-containing compounds that possess chemoprotective effects (Hayes *et al.*, 2008). The hydrolysis of glucobrassican forms indole-3-carbinol (I3C), which is sold as a

commercially available dietary supplement. In the acidic environment of the stomach, I3C forms a condensation product, diindolomethane (DIM). The oral consumption of I3C has been shown to induce Phase I and Phase II enzymes similar to TCDD (Higdon *et al.*, 2007; Hayes *et al.*, 2008). Additionally, the beneficial effects of this supplement may also involve NF-kB as DIM has been shown to inhibit the activation of NF-κB (Hayes *et al.*, 2008).   Most recently, Kim and colleagues revealed that DIM decreased inflammatory damage and inflammatory mediator production induced by the dextran sodium sulfate model of colitis in Balb/c mice (Kim *et al.*, 2009). However, the cellular signaling events responsible for this effect were not examined. In addition to dietary supplements, traditional Chinese medicines (TCMs) may also alter AhR signaling. Indirubin, a TCM used to treat chronic myelocytic leukemia and other inflammatory conditions, increased CD4+CD25+Foxp3+ T cells in indirubin-treated mice (Zhang *et al.*, 2007).   Collectively, these results suggest that common dietary constituents may modulate inflammation and immunity via activation of the AhR.

Since CD affects millions of Americans, it is important to study the mechanisms underlying this disease so that new and more effective therapeutics can be developed. Moreover, because AhR activation can effectively modulate both immune and inflammatory responsiveness, studying the effects of both synthetic and natural AhR ligands on the generation of Crohn's disease is warranted. Therefore, the goal of the studies described in this dissertation is to elucidate the role of AhR activation by dietary supplements and TCM in the development and progression of inflammation associated with Crohn's disease. The results of this project will provide novel information about gut mucosal immunity.

Hypothesis and specific aims

The goal of the proposed research is to elucidate the role of aryl hydrocarbon receptor activation (AhR) in the development and progression of inflammation associated with Crohn's disease. This proposal is novel in that the role of AhR activation in mucosal immunity has not been investigated. Ultimately this research will lead to a better understanding of Crohn's disease, how dietary factors can affect it, and potential therapeutic modalities that may be useful for humans.

Crohn's disease, one of the conditions encompassing inflammatory bowel disease (IBD), is a chronic inflammatory disorder of the gastrointestinal tract typically affecting younger patients of European descent. There is no known cause; however, it is suspected that the disease likely results from various genetic and environmental factors triggering an inappropriate immune response to normal gut bacteria. Currently, the most common therapies for Crohn's disease include a combination of corticosteroids and immunomodulatory drugs. Since these drugs are often accompanied by unpleasant and severe side effects, it is important to investigate the potential prophylactic and therapeutic effects of natural products since it has been suggested that dietary components can alter the inflammatory state of the gut. The AhR is a ligand-activated transcription factor most recognized for its involvement in the toxicity of the prototypical AhR ligand TCDD, an environmental contaminant that typically affects people through the diet. Activation of the AhR by TCDD results in the regulation of transcription, an effect that is primarily mediated via Dioxin Response Elements (DREs) in the promotor/enhancer regions of

specific target genes, such as CYP1A1 (a xenobiotic metabolizing enzyme) and IL-2 (an important immune cytokine). Moroever, TCDD is an extremely potent chemical that has recently been shown to suppress immune responses via the generation of regulatory T cells. In addition to TCDD, other potent dietary AhR ligands have the potential to modulate the immune system presumably via activation of the AhR and DRE-mediated gene regulation. Crohn's disease patients often seek natural, complementary therapies in addition to the conventional therapeutics. Importantly, some compounds present in dietary supplements are also potent AhR ligands. Therefore, the potential exists for these chemicals to alter the generation of immune responses and inflammation associated with Crohn's disease. Common dietary supplements, such as indole-3-carbinol (I3C), a compound found in cruciferous vegetables, and tryptophan, an essential amino acid, can be potent natural AhR ligands. Moreover, tryptophan and its metabolites, such as 6-formylindolo[3,2-b]carbazole (FICZ), have been shown to have immunomodulatory effects following AhR activation. Furthermore, plants used in traditional Chinese medicine, such as indirubin, also have a critical role in influencing inflammatory responses. **We hypothesize that AhR agonists found in the diet have the potential to dampen inflammation associated with Crohn's disease.** This central hypothesis will be tested by the following specific aims:

Specific Aim 1: Examine the role of AhR activation in development and progression of inflammation associated with Crohn's disease.

Specific Aim 2: Investigate the cell-specific mechanism(s) by which dietary AhR ligands exert their effects inflammatory responses.

Specific Aim 1

The purpose of these studies is to specifically investigate the role of AhR activation by various ligands during the inflammatory response associated with Crohn's. Since the murine model of TNBS-induced colitis (described below) resembles human Crohn's disease in the histological outcome as well as the cytokine profile, we will utilize this model. The approach to this Specific Aim involves 3 key experiments. To fully characterize the role of AhR activation in Crohn's, we will first examine the readouts of inflammation using the prototypical AhR ligand, TCDD, in $AhR^{+/+}$ mice. Secondly, we will definitively show that the AhR is involved in this response by using $AhR^{-/-}$ mice. Finally, we will then examine the effects of other dietary AhR ligands throughout the development of Crohn's disease. Overall, the results of Specific Aim 1 will define the immunomodulatory effects of TCDD and other dietary AhR ligands in mucosal immunity. Chapters 2-4 describe the results of this Aim.

Experimental approach: murine colitis models

Completion of Specific Aim 1 requires use of a murine model of Crohn's disease. There exist many animal models to investigate the development, progression and maintenance of colitis (Bouma and Strober, 2003). Although none of these animal models perfectly represent human Crohn's disease, each model has its own advantages for studying various aspects of inflammation in the gut (Pizarro *et al.*, 2003). For example, the CD4+CD45RB^{high} SCID transfer model elicits a chronic Th1 transmural response and defines specific effector and regulatory cell populations while the IL-10 knock out model elicits an acute Th1 transmural response and defines the role of pro-

inflammatory and regulatory cytokines as well as specific cell populations in intestinal inflammation. Perhaps the most commonly used animal model to study Crohn's disease is the TNBS-induced model of colitis that was first described by Neurath and colleagues in 1995 (Neurath *et al.*, 1995). This model shares similar immunological pathways as human Crohn's disease and is useful for testing new potential therapeutic interventions (Pizarro *et al.*, 2003).   More specifically, in this model the use of an ethanolic vehicle disrupts the mucosal barrier, allowing TNBS to haptenate colonic proteins and leading to a Th1-mediated immune response driven by IL-12 and other pro-inflammatory cytokines including TNF-α, IL-6, IFN-γ, and IL-17 (Kawada *et al.*, 2007).   Deficiencies in regulatory cytokines, including IL-10, and TGF-β, have also been reported. Therefore, we have chosen the TNBS-induced model of colitis primarily because the innate immune response induced by this chemical most closely resembles the effects observed in people, which is useful for investigating potential prophylactic therapeutics. Furthermore, in the TNBS model, a sensitization step can be added such that T cells are primed and subsequently elicit a more potent T cell response upon a secondary exposure during the TNBS enema. Therefore, based on the potential for TCDD and other AhR ligands to modulate inflammatory and immune responses by altering the differentiation of T cells, the effects of AhR activation may be more pronounced in this model of colitis that involves greater contributions from T cells.

Specific Aim 2

The toxicity of TCDD is primarily due to binding of ligand:AhR:ARNT complex to the DRE in the regulatory region of various target genes, including those involved in innate and adaptive immune responses. However, it has also been suggested that the effects of AhR activation may be due to DRE-independent events. It has been reported that the ligand-bound AhR can interact with the Rel component of NF-κB, which would allow for altered transcription of immune genes that are under the control of the DRE or NF-κB. It is therefore important to evaluate the potential DRE-dependent and independent events leading to the immunosuppressive effects of various AhR ligands. The results obtained from this Specific Aim will provide data that will mechanistically define the role of AhR activation by various ligands in immunity and inflammation. Chapter 5 describes the results of this Aim.

Experimental approach

For this Aim, murine BMDCs were generated from the bone marrow of AhR+/+ and AhR-/- mice (both on a C57Bl/6 background) using the growth factor GM-CSF in the presence of the DMSO vehicle or the AhR ligands, I3C and indirubin. Serveral endpoints were evaluated in these studies including cell surface molecule expression via flow cytometry, cytokine production via ELISAs, and gene transcription via qRT-PCR. The role of NF-kB was determined with the TransAM NF-kB colorimetric assay, and co-cultures with naïve antigen-specific T cells were generated to evaluate the immuneregulatory potential of the ligand-treated DCs.

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CHAPTER 2

Aryl hydrocarbon receptor activation by TCDD reduces inflammation associated with Crohn's disease

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ABSTRACT

Crohn's disease results from a combination of genetic and environmental factors that trigger an inappropriate immune response to commensal gut bacteria. The aryl hydrocarbon receptor (AhR) is well known for its involvement in the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an environmental contaminant that affects people primarily through the diet. Recently, TCDD was shown to suppress immune responses by generating regulatory T cells (Tregs). We hypothesized that AhR activation dampens inflammation associated with Crohn's disease. To test this hypothesis, we utilized the 2,4,6-trinitrobenzenesulfonic acid (TNBS) murine model of colitis. Mice were gavaged with TCDD prior to colitis induction with TNBS. Several parameters were examined including colonic inflammation via histological and flow cytometric analyses. TCDD-treated mice recovered body weight faster and experienced significantly less colonic damage. Reduced levels of IL-6, IL-12, IFN-γ, and TNF-α demonstrated suppression of inflammation in the gut following TCDD exposure. Foxp3$^{\text{egfp}}$ mice revealed that TCDD increased the Foxp3+ Treg population in gut immune tissue following TNBS exposure. Collectively, these results suggest that activation of the AhR by TCDD decreases colonic inflammation in a murine model of colitis in part by generating regulatory immune cells. Ultimately, this work may lead to the development of more effective therapeutics for the treatment of Crohn's disease.

KEY WORDS

INTRODUCTION

Crohn's disease, one of the conditions encompassed by inflammatory bowel disease, is a chronic inflammatory disease of the gastrointestinal tract that primarily affects younger European Americans (Head and Jurenka, 2004). The exact cause of Crohn's disease remains unknown; however, it is suspected that both genetics and environmental factors contribute to an inappropriate immune response to commensal bacteria. Normally there exists a state of tolerance in the gut such that effector and regulatory cell differentiation is balanced and immune responses are not mounted against commensal bacteria or food substances. In Crohn's disease, however, imbalanced differentiation of T effector cells (Teffs) and T regulatory cells (Tregs) results in Th1- and Th17-dominated inflammatory responses (Sanchez-Munoz *et al.*, 2008). Since current medical treatment can be associated with severe side effects and life-threatening complications, it is essential to better understand this immune dysfunction so that novel and more effective therapeutics can be developed.

The aryl hydrocarbon receptor (AhR) is a member of the bHLH/PAS protein family and acts as a cytosolic, ligand-activated transcription factor that binds a variety of synthetic and natural compounds. The AhR is perhaps most recognized in the immune system for its role in mediating the immunotoxic effects of the prototypical ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Cannonical signaling involves ligand-activated AhR interaction with dioxin response elements to modulate gene transcription. Many immune cells express the AhR and several cytokine genes possess dioxin response elements so AhR activation can significantly impact immune responses. Recently, it has been shown that the generation of CD4+CD25+ Tregs is the likely mechanism

responsible for the immunosuppressive effects following TCDD exposure (Funatake *et al.*, 2005; Marshall *et al.*, 2008). Moreover, AhR-deficient mice do not experience immunosuppression following exposure to TCDD and TCDD-like compounds, thus demonstrating that the effects of these chemicals are AhR-mediated.

To examine the potential of new therapeutic agents to treat Crohn's disease, the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced mouse model resembles inflammatory responses in human Crohn's disease so it is commonly used for this purpose (Neurath *et al.*, 1995; Pizarro *et al.*, 2003). In this model, TNBS binds colonic proteins to trigger a Th1-mediated response primarily driven by the pro-inflammatory cytokine IL-12 as well as a Th17-mediated response driven by IL-17. Excessive amounts of TNF-α and IFN-γ along with deficiencies in the regulatory cytokines IL-10 and TGF-β have also been observed (Kawada *et al.*, 2007). Based on the potential for TCDD and other AhR ligands to modulate inflammatory and immune responses by altering the differentiation of T cells, the effects of AhR activation may be quite pronounced in this model of colitis involving Th1 and Th17 cells.

Since Crohn's disease affects millions of people worldwide, it is essential to investigate the underlying mechanisms of pathogenesis so that new and more effective therapeutics can be developed. Additionally, activation of the AhR modulates immune and inflammatory responses so studying the effects of AhR activation on the pathogenesis of Crohn's disease is warranted. Therefore, we explored the effects of TCDD on the generation of TNBS-induced colitis in mice. We hypothesized that AhR activation reduces the inflammation generated in a murine model of Crohn's disease by promoting an immunosuppressive environment in the gut. Collectively, our data

demonstrate for the first time that AhR activation by TCDD in the gut suppresses inflammation and generates regulatory cells in the TNBS model of colitis in mice. These results also provide novel information regarding gut mucosal immunity and suggest the AhR may be a potential therapeutic target for Crohn's disease.

MATERIALS AND METHODS

Laboratory animals. Six to eight week old male and female AhR$^{+/+}$, AhR$^{-/-}$, and Foxp3^{egfp} mice (all on the C57Bl/6 background) were bred and maintained in the animal research facility at the University of Montana. C57Bl/6 AhR$^{+/+}$ were originally obtained from The Jackson Laboratories (Bar Harbor, ME) and bred in-house. C57Bl/6 AhR$^{-/-}$ (B6.AhR^{tm1Bra}) mice were obtained from Dr. Paige Lawrence (University of Rochester Medical College, Rochester, NY) and bred as previously described (Schmidt and Bradfield, 1996). Foxp3^{egfp} mice were kindly provided by Dr. R. Noelle (Dartmouth Medical School, Lebanon, NH) who originally obtained these mice from Dr. A. Rudensky (University of Washington School of Medicine, Seattle, WA). All mice were housed under specific pathogen-free conditions and maintained on 12 h dark/light cycles. Throughout each experiment animals were individually caged, and standard laboratory food and water were provided *ad libitum*. All protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee and adhered to the current NIH guidelines for animal usage.

Chemicals for in vivo use. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories Inc., (Woburn, MA), initially dissolved in anisole

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(Sigma-Aldrich, St. Louis, MO), and further diluted in peanut oil, which was used as a vehicle control for gavages. 2,4,6-trinitrobenzenesulfonic acid (TNBS) was also obtained from Sigma-Aldrich and dissolved in acetone/olive oil for pre-sensitization or 40% ethanol for enema administration.

Colitis induction. As described in Figure 2.1, mice were gavaged on day -1 and day 6 with 15 μg TCDD per kg body weight (~200-250 μl total volume) or a comparable volume of peanut oil vehicle control prior to pre-sensitization and colitis induction. Pre-sensitization on day 0 involved an application of 150 μl 5% TNBS in a 4:1 acetone:olive oil solution between the shoulder blades such that mice were not orally exposed to TNBS. Colitis was then induced via an intrarectal injection with TNBS on day 7, as previously described with modifications (Wirtz *et al.*, 2007). Briefly, mice were anesthetized with isoflurane and received an enema of 2.5 mg TNBS in 40% ethanol (50 μl total volume) via a 3.5 F catheter inserted 3 cm into the colon. To ensure distribution of TNBS in the colon, mice were held in a vertical position for 90 s before being returned to their cages. Body weight loss and the severity of clinical symptoms (overall body condition, stool consistency, and dehydration state via a skin pinch test) was monitored daily, as fully described in Table 2.1A. Briefly, the severity of each clinical sign was scored on a scale 0-3 and combined to determine the total severity score. On days 10-12, mice were euthanized via CO_2 asphyxiation followed by cervical dislocation.

Figure 2.1
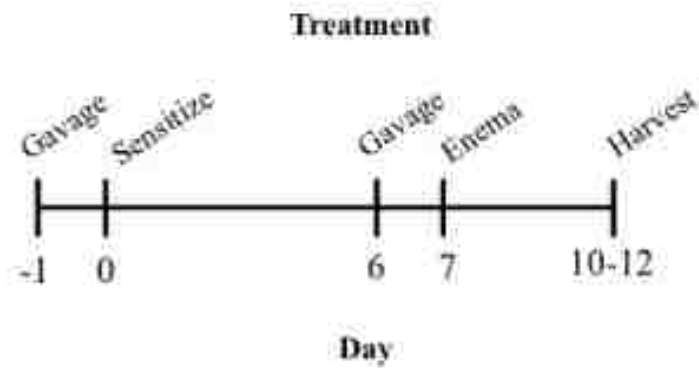
Figure 2.1. Experimental design. This schematic represents the experimental design utilized for the studies in which animals were gavaged with 15 μg/kg TCDD, peripherally sensitized with 5% TNBS, intrarectally injected with 2.5 mg TNBS, and subsequently harvested for evaluation.

Table 2.1. Scoring criteria for severity of clinical symptoms and microscopic damage

A. Clinical severity

Score	I. Stool Consistency	II. Dehydration	III. Body Condition
0	Well-formed	None	Normal
1	Semi-formed	Mild	Slower movements
2	Loose	Moderate	Significant weight loss or abdominal distension
3	Liquid	Severe	Emaciated and very slow movements

B. Microscopic damage

Score	I. Inflammatory Cell Infiltration	II. Tissue Lesions
0	Normal	Normal
1	Increased numbers of inflammatory cells in the lamina propria	Surface epithelial degeneration and/or mucosal hyperplasia
2	Inflammatory cells extending into the submucosa	Focal/multifocal ulcer and/or crypt loss
3	Transmural extension of infiltrate	Extensive mucosal damage and ulceration

(A) Clinical severity in terms of stool consistency, dehydration, and overall body condition was documented during disease progression. Total severity score represents a combination of all three categories (I-III) such that clinical severity scores range from 0 to 9. (B) Hematoxylin and eosin (H&E)-stained tissue sections were scored microscopically based on inflammatory cell infiltration and tissue lesions. The total damage score represents a combination of scores from both categories (I and II) such that damage scores range from 0 to 6.

Colon processing and histology. Colons were excised from each mouse and washed with PBS to remove debris. For histological analysis, a 1 cm section from the distal colon was fixed in 2% paraformaldehyde, processed in the Shandon Citadel 2000 Automated Tissue Processor (Thermo Fisher Scientific, Waltham, MA) with vacuum unit, embedded using the Shandon Histocentre 2 Embedding unit, sectioned at 7 μm using the Thermo Shandon Finesse 325 microtome, and stained with hematoxylin and eosin (H&E) in the Shandon Veristain 24-4 Slide Stainer. Colons were assessed microscopically by a blinded individual to determine the severity of inflammation based on the modified scale shown in Table 2.1B (Hartmann *et al.*, 2000). Briefly, the extent of inflammatory cell infiltration and tissue lesions was scored on a scale 0-3 and combined to determine a total damage score. Slides were imaged using the Nikon E800 Microscope with Cambridge Research Instrumentation, Inc. and Nuance camera software version 1.62 at 4X magnification. The remaining colon was divided into two sections for cell isolation, as described below, and homogenization in lysis buffer from which the supernatants were analyzed for cytokine production using commercially available ELISA kits.

Cytokine assays. Supernatants from homogenized colon tissue were examined for the levels of IL-6, IL-10, IL-12, IL-17, IFN-γ, and TNF-α via enzyme-linked immunosorbent assay (ELISA). Samples were analyzed according to the manufacturer's instructions using mouse cytokine-specific BD OptEIA ELISA kits (BD Biosciences, San Diego, CA) or R&D ELISA kits (Minneapolis, MN).

Antibody production. IgA production was assessed in the colon, feces, and serum. Five days after colitis induction, colons were harvested and homogenized in lysis buffer, and supernatants from tissue were collected. Fecal pellets were collected from the distal colon and homogenized in 0.01% NaN_3 (1 μl 0.01% NaN_3 per 1 mg feces), as previously described (Kato *et al.*, 2001). Supernatants were collected for antibody production analysis. Finally, immediately after euthanization, a cardiac puncture was performed to collect serum. All samples were stored at -20°C until subsequent evaluation of antibody levels using a commercially available ELISA kit per the manufacturer's instructions (SouthernBiotech, Birmingham, AL).

qRT-PCR. RNA was isolated from colon tissue with Trizol reagent (Invitrogen, Carlsbad, CA) followed by RNA clean-up using the Total RNA Kit with optional DNase treatment (Omega Bio-Tek, Norcross, GA) according to the manufacturers' instructions. First strand cDNA synthesis was performed using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Resulting cDNA was subjected to qRT-PCR using commercially obtained primers (SABiosciences, Frederick, MD) including aryl hydrocarbon receptor repressor (*AhRR*), aldehyde dehydrogenase family 1, subfamily A2 (*Aldh1a2*), a proliferation-inducing ligand (*APRIL*), B cell activating factor (*BAFF*), cytochrome P450, family 1, subfamily a, polypeptide 1 (*Cyp1a1*), forkhead box P3 (*Foxp3*), indoleamine-2,3-dioxygenase 1 (*IDO1*), indoleamine-2,3-dioxygenase 2 (*IDO2*), interleukin 17A (*IL17A*), interleukin 17F (*IL17F*), RAR-related orphan receptor gamma (*Rorc*), transforming growth factor, beta 1 (*Tgfb1*), transforming growth factor, beta 2 (*Tgfb2*), and transforming growth factor, beta 3 (*Tgfb3*). Reactions were performed

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with PerfeCTa SYBR Green Supermix for iQ (Quanta Biosciences) on a Bio-Rad iQ™5 Multicolor RT-PCR Detection System. Resulting data were normalized to β-actin, and fold changes were calculated using the ΔΔCt method, which compares threshold values of the samples of interest for a particular gene relative to a housekeeping gene.

Preparation of cells from mesenteric lymph nodes (MLNs) and colon. Mesenteric lymph nodes (MLNs) were removed, washed in media, homogenized using the end of a syringe plunger, and subsequently filtered through a 40 μm nylon cell strainer, as previously described (Bankoti *et al.*, 2010a). Lamina propria mononuclear cells were also isolated from the colon tissue, as previously described (Weigmann *et al.*, 2007). Briefly, colons were excised and washed with PBS, digested with collagenase/DNase (Sigma-Aldrich/Invitrogen), filtered, and subjected to Percoll (GE Healthcare, Pittsburg, PA) density centrifugation. Cells from both tissues were counted via Trypan blue exclusion on a hemacytometer, and $2x10^6$ cells were subsequently washed with buffer prior to cell staining.

Flow cytometry. Expression of accessory molecules on isolated cells was determined by flow cytometry, as previously described (Shepherd *et al.*, 2001). Briefly, cells were washed with PAB (1% bovine serum albumin and 0.1% sodium azide in PBS). To eliminate non-specific staining, cells were blocked with 30 μg purified rat and/or hamster IgG (Jackson ImmunoResearch, West Grove, PA). Optimal concentrations of fluorochrome-conjugated monoclonal antibodies were used to stain cells for an additional 10 min. Antibodies used in these experiments included CD4-AlexaFluor 700 (GK1.5),

CD25-PerCP/Cy5.5 (PC61), CTLA4-APC (UC10-4B9), GITR-PECy7 (YGITR), α4β7-PE (DATK32), CD103-Pacific Blue (2E7), CD11c-PE (HL3), MHC2-PECy7 (M5/114.15.2), CD86-APC (GL-1), and their corresponding isotype controls, all of which were purchased from BioLegend (San Diego, CA) or BD Biosciences (San Jose, CA). One to five hundred thousand events were collected using a BD FACSAria flow cytometer and analyzed using FACSDiva (Version 6.1.2, BD Biosciences, San Jose, CA) and FlowJo (Version 8.7.1, TreeStar, Inc., Ashland, OR) software programs.

Immunohistochemistry. Using the embedded colon tissue prepared as described above, 5 μm sections were mounted on slides and allowed to air dry prior to being deparaffinized and coverslipped using the ProLong Gold anti-fade reagent with DAPI mounting media (Invitrogen Molecular Probes). Slides were imaged at 40X magnification using the Olympus FluoView 1000 LSC on an inverted 1x81 microscope with spectral detection and TIRF module (Version 2.1b software with 405, 458, 488, 515, 559, and 635 laser lines available).

Chemicals for cell culture. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) in DMSO was obtained from Cambridge Isotope Laboratories Inc. (Woburn, MA). For in vitro cultures, TCDD was used at a final concentration of 1 nM, and the vehicle control DMSO (Sigma-Aldrich) was used at a final concentration of 0.01%. These concentrations of TCDD and DMSO were not cytotoxic.

Cell culture. CMT-93 cells (ATCC, Manassas, VA) were cultured in complete RPMI media (cRPMI) consisting of RPMI (GibcoBRL, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT), 50 μM mercaptoethanol, 20 mM HEPES, 10 mM sodium pyruvate, and 50 μg/ml gentamicin (GibcoBRL). Cells were maintained at 37°C and 5% CO_2 with weekly passage and utilized for experimentation at 80-90% confluency.

Cell activation and treatment. CMT-93 cells were incubated overnight in T25 flasks to facilitate attachment prior to experimentation. Prior to activation, cells were 80% confluent. Cells were then concomitantly stimulated with 1 μg/ml LPS (*Escherichia coli* (055:B5), Sigma-Aldrich) and treated with 1 nM TCDD or the DMSO (0.01%) vehicle control. After 24 h, cells were harvested for qRT-PCR analysis and supernatants were collected for analysis of IL-6, IL-10, IL-12, IFN-γ, and TNF-α levels.

Statistics. All statistical analyses were performed using GraphPad Prism 4.0a for Macintosh (GraphPad Software, San Diego, CA). Student's *t*-test was used to compare vehicle-treated groups and TCDD-treated groups while 2-way analysis of variance (ANOVA) with Bonferroni's post-hoc test was used to compare multiple groups. Values $p \leq 0.05$ were determined to be significant.

RESULTS

AhR activation by TCDD decreases the severity of clinical symptoms in TNBS-treated mice. In order to characterize the effects of TCDD on the progression of TNBS-induced colitis in C57Bl/6 AhR^{+/+} mice, clinical symptoms were monitored throughout the course

of the experiment. Crohn's disease is a progressive wasting disease so body weight loss is a hallmark indicator of clinical severity. As shown in Figure 2.2A, vehicle-treated mice rapidly lost approximately 17% of their initial body weights following TNBS exposure and did not recover by the end of the study. Conversely, TCDD-treated mice lost only 8% of their initial weights and recovered weight rapidly. The protective effect of TCDD was also observed in the clinical severity of colitis, as measured by stool consistency, dehydration, and body condition (Table 2.1A). Vehicle-treated mice typically had loose stools, mild dehydration, and slower body movements while the TCDD-treated mice had semi-formed stools initially but recovered quite rapidly (Figure 2.2B). Thus, TCDD decreases the severity of clinical symptoms of mice in the TNBS-induced model of Crohn's disease.

Inflammation and tissue damage in the colon decreases following treatment with TCDD. Crohn's disease results in transmural inflammation and ulceration of the colon tissue. To examine the degree of inflammatory cell infiltration and tissue lesions, cross sections of the distal colon were stained with H&E and assessed microscopically to determine damage (Table 2.1B and Figure 2.3A). Transmural infiltration of inflammatory cells and extensive mucosal damage was frequently observed in vehicle-treated mice following TNBS exposure, which resulted in an average damage score of 5.5. However, the extent of inflammatory cell infiltration and lesions was significantly less in TCDD-treated mice, as represented by an average damage score of 3.2. Representative images of each treatment group are shown in Figure 2.3B. These results demonstrate that TCDD

Figure 2.2

A.

B.
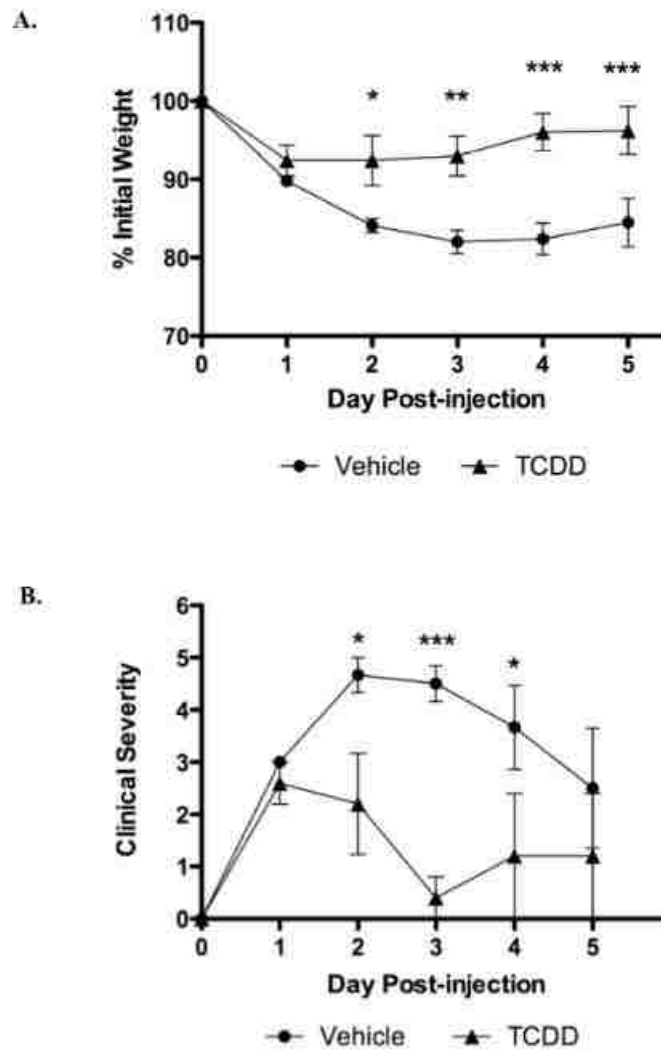
Figure 2.2. TCDD reduces wasting disease and clinical symptoms associated with TNBS-induced colitis. Animals were monitored daily after disease induction for body weight loss (A) and other clinical symptoms including body condition, dehydration state, and stool consistency (B). Clinical severity was determined by combining the symptom scores for a total possible severity score of 9, as described in Table 2.1A. Results are shown as mean ± SEM (n=6) and representative of three separate experiments. *indicates significances of $p \leq 0.05$, **indicates significance of $p \leq 0.01$, and ***indicates significance of $p \leq 0.0001$.

exposure prior to colitis induction significantly suppress colonic inflammation following TNBS insult.

AhR activation decreases the production of inflammatory mediators in colitis.

TNBS-induced colitis is driven by pro-inflammatory cytokines including IL-6, IL-12, and IL-17 (Kawada *et al.*, 2007). Accordingly, cytokine production in supernatants from homogenized colon tissue was evaluated. As shown in Figure 2.4, TCDD decreased the production of IL-6, IFN-γ, and TNF-α by approximately 45% each as well as the production of IL-12 by 18%. In a separate preliminary experiment, neither IL-6 (TCDD=363±18 pg/ml; vehicle=382±22 pg/ml) nor IL-12 (TCDD=2763±141 pg/ml; vehicle=2633±190 pg/ml) colonic levels were significantly reduced in TCDD-treated AhR null mice when compared to the vehicle-treated wild-type controls. Furthermore, production of IL-10 and IL-17 in the colon was unaffected by TCDD in TNBS-treated animals. Together, these results suggest that TCDD can alter the gut immune environment by suppressing pro-inflammatory cytokines in an AhR-dependent manner and decrease inflammation associated with TNBS-induced colitis.

The effects of TCDD on antibody production were also assessed because IgA plays an important role in regulating immune responses in the gut. Following TCDD treatment, IgA levels in the supernatants from homogenized colon tissue and feces increased from 38.89 μg/ml to 54.99 μg/ml and from 5.85 μg/ml to 15.46 μg/ml, respectively (Table 2.2), an effect that was not observed in AhR null mice treated with TCDD (data not shown). While a trend was observed for a TCDD-induced increase in

Figure 2.3
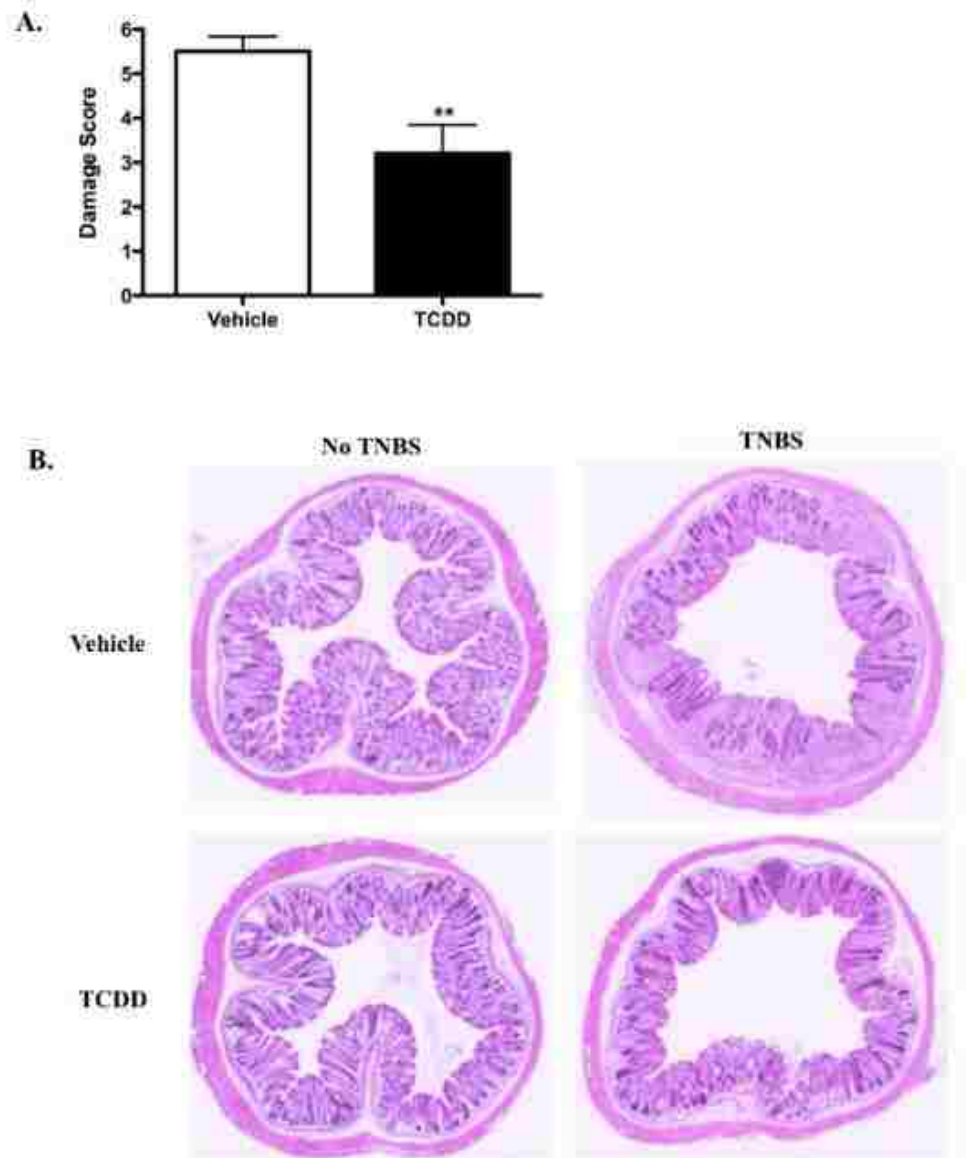
Figure 2.3. Colonic inflammation is reduced following exposure to TCDD. Animals were harvested five days after disease induction, and a section of the distal colon assessed microscopically. Damage score (A) was determined by combining the scores for inflammatory cell infiltration (0-3) and tissue damage (0-3), as described in Supplemental Table 1B. Representative images of H&E-stained tissues sections of vehicle-treated and TCDD-treated animals are shown in B. Results are shown as mean ± SEM (n=6) and are representative of three separate experiments. **indicates significance of p≤0.01.

Figure 2.4
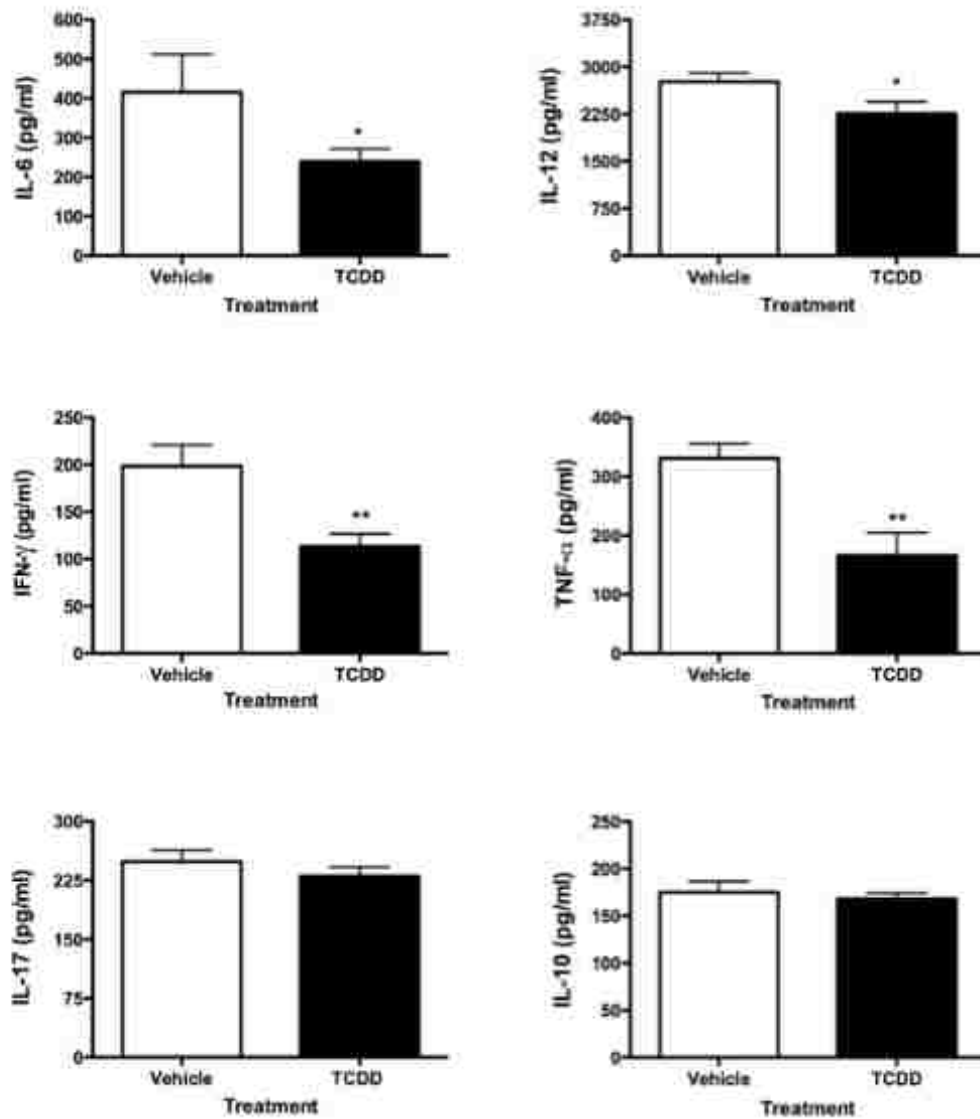
Figure 2.4. Modulation of cytokine production by TCDD in colon tissue. On day five following disease induction, colons were excised, washed, and homogenized. The supernatants were assessed for cytokine production, as described in the Materials and Methods. Results are shown as mean ± SEM (n=6) and representative of three separate experiments. *indicates significance of $p \leq 0.05$ and **indicates significance of $p \leq 0.01$.

IgA in the serum of TNBS-exposed mice, this effect was not statistically significant. TCDD also increased total Ig production in the colon from 48.67 μg/ml to 113.1 μg/ml but did not alter total Ig levels in the feces or serum. Interestingly, without the TNBS-triggered inflammatory response, TCDD did not induce significant increases in IgA production in the gut (data not shown). Thus, TCDD modulates humoral immune responses in the gut of TNBS-treated mice.

We also examined the potential for intestinal epithelial cells (IECs) to contribute to the suppression of inflammation following TCDD treatment since these cells are important in maintaining the mucosal barrier against gut flora and mounting immune responses by acting as antigen presenting cells. CMT-93 cells, a murine intestinal epithelial cell line, were stimulated with 1 μg/ml LPS and concurrently treated with TCDD. The LPS-induced production of IL-6, as determined by ELISA, was suppressed by TCDD treatment (Table 2.3A). Similar effects were observed when cells were stimulated with CpG and concomitantly treated with TCDD (data not shown). IL-10, IL-12, and IFN-γ were not detected in either of these experiments (data not shown).

TCDD alters transcription of immunoregulatory genes in colonic tissue of TNBS-treated mice. To gain further insight into factors that may be responsible for the immunomodulatory effects of TCDD in colitis, intestinal tissue was evaluated by qRT-PCR to assess changes in gene transcription. Three days after colitis induction, which was the peak of clinical disease severity, TCDD enhanced the expression of *AhRR, Cyp1a1,* and *IDO1* by 6.74, 216.40, and 2.91 fold, respectively (Table 2.4). Conversely, mRNA levels of *APRIL, IDO2,* and *IL17A* were decreased. Five days after enema

Table 2.2. Effects of TCDD on antibody production in TNBS-exposed mice[a]

| | IgA (μg/ml) | | Total Ig (μg/ml) | |
	Vehicle	TCDD	Vehicle	TCDD
Colon	39.89 ± 3.75	54.99 ± 8.39*	48.67 ± 4.52	113.1 ± 16.43***
Feces	5.85 ± 1.40	15.46 ± 3.31***	8.38 ± 1.52	9.51 ± 1.80
Serum	147.20 ± 7.11	173.70 ± 14.53	$3.056 \pm 0.28^{\S}$	$2.43 \pm 0.35^{\S}$

[a]Supernatants from colon tissue, feces, and blood were obtained five days after colitis induction from vehicle- and TCDD-treated mice, as described in Supplemental Materials and Methods, and subsequently analyzed for IgA and total Ig production. Data is shown as mean $\pm$ SEM, which is representative of three separate experiments (n=6). *indicates significance of $p \leq 0.05$ when compared to vehicle-treated controls and ***indicates significance of $p \leq 0.0001$. §indicates mg/ml.

Table 2.3. Effects of TCDD on cytokine production and gene transcription in cultured

IECs

A. TCDD decreases the LPS-induced production of IL-6

Treatment	IL-6 (pg/ml)
DMSO	BD
1 nM TCDD	BD
LPS	485.6 ± 15.8
1 nM TCDD + LPS	133.2 ± 6.9***

B. TCDD alters gene transcription in IECs

Gene	LPS		1nM TCDD		1nM TCDD + LPS	
	Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
APRIL	**4.53**	0.045	**2.83**	0.006	**6.85**	0.001
BAFF	3.12	0.092	1.04	0.397	**2.45**	0.002
Cyp1a1	-1.10	0.237	**124.80**	<0.0001	**195.80**	<0.0001
IL6	**11.90**	0.0003	**-3.15**	0.0009	**2.68**	0.015
IDO1	-1.76	0.274	-1.00	0.495	-1.06	0.454
IDO2	**1.92**	0.035	1.27	0.102	2.00	0.053
Tgfb1	1.27	0.058	1.01	0.466	**1.98**	0.017
Tgfb2	**2.00**	0.013	**-1.91**	0.006	1.48	0.070
Tgfb3	-1.09	0.322	-1.26	0.105	1.27	0.175

(A) Supernatants were harvested 24 h after concurrent treatment of LPS and TCDD and subsequently assessed for cytokine production, as fully described in the Supplemental Materials and Methods. ***indicates significance of $p \leq 0.0001$ when compared to the LPS-stimulated samples. BD represents below detection. (B) Cells were treated as described in A and lysed in Trizol for qRT-PCR analysis. Fold change is relevant to vehicle-treated mice and normalized to β-actin. Genes analyzed include: a proliferation-inducing ligand (*APRIL*), B cell activating factor (*BAFF*), cytochrome P450, family 1, subfamily a, polypeptide 1 (*Cyp1a1*), indoleamine-2,3-dioxygenase 1 (*IDO1*), indoleamine-2,3-dioxygenase 2 (*IDO2*), interleukin 6 (*IL6*), transforming growth factor, beta 1 (*Tgfb1*), transforming growth factor, beta 2 (*Tgfb2*), and transforming growth factor, beta 3 (*Tgfb3*). Data shown are representative of one experiment (n=6). Bolded p-values indicate significance of $p \leq 0.05$.

administration, a significant upregulation of *AhRR, Cyp1a1*, and *IDO* was observed; however, mRNA levels of *IL17A* and *IL17F* were unaffected by TCDD, which was consistent with the results obtained from the ELISA. Although not significant, a modest trend for increased *TGFβ3* mRNA following AhR activation by TCDD was observed in wild type animals. These effects, with the exception of *Cyp1a1* and AhRR induction, were specific to the inflamed colonic tissue and did not affect tissue from the small intestine.

The ability of TCDD to also alter inflammatory responses by IECs was assessed via qRT-PCR. TCDD induced *Cyp1a1* mRNA and downregulated the LPS-induced expression of *IL6* (Table 2.3B). Moreover, TCDD treatment of LPS-stimulated IECs resulted in a 6.85- and 2.45-fold upregulation in *APRIL* and *BAFF*, respectively. Thus, IECs may be directly affected by TCDD to help mount immunosuppressive responses in the inflamed gut by decreasing the production of pro- inflammatory cytokines and enhancing the activity of B cells and their subsequent IgA responses.

Immunoregulatory cells in the MLNs and colon are induced following AhR activation. AhR activation by TCDD promotes the generation of Tregs in several disease models so we hypothesized that TCDD dampened inflammation in TNBS-treated mice by increasing regulatory cell populations in the gut. Therefore, Foxp3^{egfp} mice that contain a knock-in allele encoding a green fluorescent protein (GFP)-Foxp3 fusion protein were utilized to specifically assess the presence of Foxp3+ Tregs. Initial experiments with these mice revealed no significant changes in regulatory cell populations when mice were harvested five days after TNBS enema administration (data not shown). Since the

Table 2.4. TCDD alters gene transcription in the intestinal tissue of TNBS-exposed mice[a]

Gene	Small Intestine Day 3		Colon Day 3		Colon Day 5	
	Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
AhRR	**2.17**	0.026	**6.74**	0.009	**25.49**	<0.0001
Aldh1a2	1.01	0.494	1.42	0.088	1.43	0.179
APRIL	-1.01	0.475	**-2.05**	0.024	-1.29	0.179
BAFF	-1.06	0.413	-1.62	0.115	-1.22	0.191
Cyp1a1	**50.56**	0.0009	**216.40**	0.0002	**1807**	<0.0001
Foxp3	1.26	0.331	-1.07	0.450	-1.17	0.366
IDO1	1.65	0.109	**2.91**	0.006	**2.15**	0.026
IDO2	-1.27	0.271	**-1.67**	0.067	-1.05	0.440
IL17A	2.89	0.255	**-9.40**	0.009	-1.47	0.265
IL17F	1.64	0.165	1.46	0.220	1.25	0.337
Rorc	1.24	0.143	1.23	0.198	-1.38	0.236
Tgfb1	1.08	0.328	1.36	0.116	-1.19	0.299
Tgfb2	-1.25	0.270	1.00	0.495	1.16	0.335
Tgfb3	-1.05	0.400	1.16	0.274	1.71	0.051

[a]Three or five days following colitis induction, colon tissue from wild type mice treated with TCDD was homogenized in Trizol and subjected to qRT-PCR to assess alterations in gene transcription. Fold change is relevant to vehicle-treated mice and normalized to β-actin. Genes analyzed include: aryl hydrocarbon receptor repressor (*AhRR*), aldehyde dehydrogenase family 1, subfamily A2 (*Aldh1a2*), a proliferation-inducing ligand (*APRIL*), B cell activating factor (*BAFF*), cytochrome P450, family 1, subfamily a, polypeptide 1 (Cyp1a1), forkhead box P3 (*Foxp3*), indoleamine 2,3-dioxygenase (*IDO1*), 1 indoleamine 2,3-dioxygenase 2 (*IDO2*), interleukin 17A (*IL17A*), interleukin 17F (*IL17F*), RAR-related orphan receptor gamma (*Rorc*), transforming growth factor, beta 1 (*Tgfb1*), transforming growth factor, beta 2 (*Tgfb2*), and transforming growth factor, beta 3 (*Tgfb3*). Bolded values indicate significance of $p \leq 0.05$. Data are representative of a single experiment with n=6 mice per treatment group.

peak of clinical disease severity occurred three days after disease induction, Foxp3^{egfp} mice were evaluated three days following the TNBS enema. Populations of regulatory cells, both Tregs and dendritic cells (DCs), following TCDD treatment were analyzed in the colon tissue and MLNs (Table 2.5). Several markers were used to define regulatory cell populations: Tregs were characterized by the expression of CD4, CD25, Foxp3, CTLA4, GITR, CD103, and α4β7 while DCs were characterized by the expression of CD11c, MHC class II, CD86, and CD103.

In the MLNs, TCDD increased the percentage of CD4+CD25+Foxp3+ cells from 6.34% to 7.74%, and the percentage of these Tregs that expressed CD103 increased by 6.5% (Table 2.5A), an effect that did not occur in AhR null mice treated with TCDD (data not shown). Additionally, the CD4+CD25-Foxp3+ Tregs were also evaluated since it has recently been shown that TCDD-induced CD25- Tregs can effectively suppress immune responses (Funatake *et al.*, 2009). The percentage of CD103+ and α4β7+ cells increased in the CD4+CD25-Foxp3+ Treg population by approximately 10% and 3%, respectively. In the colon, TCDD increased the number of cells expressing CTLA4 from 4.05% to 13.80% as well as the relative expression (MFI) of GITR from 386 to 710 in the CD4+CD25+Foxp3+ Tregs, even though this population was significantly decreased following TCDD exposure. Additionally, this population of Tregs also exhibited a 12% increase in cells expressing α4β7. AhR activation by TCDD also increased the percentage of CD4+CD25-Foxp3+ cells expressing CTLA4 as well as the relative expression of GITR from 325 to 458, CD103 from 1639 to 1915, and α4β7 from 155 to 185. Immunohistochemistry performed on the colon tissue revealed an increased Treg population in TNBS-treated mice, as more Foxp3+ expression was observed in mice

treated with TCDD (Figure 2.8). It should be noted that TCDD treatment in the absence of the TNBS enema did not induce an increased frequency of Foxp3+ cells in the gut (data not shown).

Regulatory DCs were also examined in these experiments because these cells can promote the generation of Tregs. TCDD increased the percentage of CD11c+MHC2+CD103+ DCs in the MLNs from 8.673% to 11.88% (Table 2.5B), an effect that was also not observed in AhR null mice (data not shown). However, a greater frequency of CD11c+ DCs isolated from the colon expressed decreased levels of MHC class 2. Collectively, these results suggest that AhR activation by TCDD induces regulatory immune cells in the gut that may reduce the inflammation in mice experiencing TNBS-induced colitis.

Table 2.5. Effects of TCDD on immune cells and their regulatory cell surface marker expression during TNBS-induced colitis in Foxp3[egfp] mice[a]

A. Tregs in the gut

	MLNs			Colon		
	Vehicle	TCDD	p-value	Vehicle	TCDD	p-value
CD4+CD25+Foxp3+	6.34	7.74	**0.036**	26.8	15.98	**0.017**
CTLA4	11.78	12.30	0.411	4.05	13.80	**0.001**
GITR	83.86	85.52	0.274	386	710	**<0.0001**
CD103	45.36	51.90	**0.008**	1945	2072	0.104
α4β7	18.12	20.04	0.263	235	263	**0.029**
CD4+CD25-Foxp3+	3.90	3.98	0.444	16.78	17.02	0.445
CTLA4	2.20	2.94	0.151	0.62	1.42	**0.033**
GITR	78.66	78.88	0.472	325	458	**0.005**
CD103	32.90	42.74	**<0.0001**	1639	1915	**0.007**
α4β7	5.60	8.36	**0.009**	155	185	**0.013**

B. DCs in the gut

	MLNs			Colon		
	Vehicle	TCDD	p-value	Vehicle	TCDD	p-value
CD11c+	3.43	2.74	**0.036**	9.56	11.65	**0.030**
MHC2	81.73	82.4	0.369	20.56	11.07	**0.001**
CD86	652	592	0.228	338	364	0.288
CD103	24.43	33.14	**0.005**	88.54	92.43	0.160
MHC2+CD103+	8.673	11.88	**0.024**	6.595	4.910	0.089

[a]Leukocytes from colon tissue and mesenteric lymph nodes (MLNs) were isolated three days after disease induction and immunophenotypically evaluated by flow cytometry to determine the presence of regulatory T cells (Tregs) and dendritic cells (DCs). Values are listed as the percent of the bolded parent population indicated in the table, except for GITR, CD103, and α4β7 expression on colon T cells and CD86 expression on DCs, which indicate the Median Fluorescence Intensity (MFI) values. Bolded p-values indicate significance of p≤0.05. Data are representative of a single experiment (n=6). Representative histograms are shown in Figures 2.5-2.7.

Figure 2.5
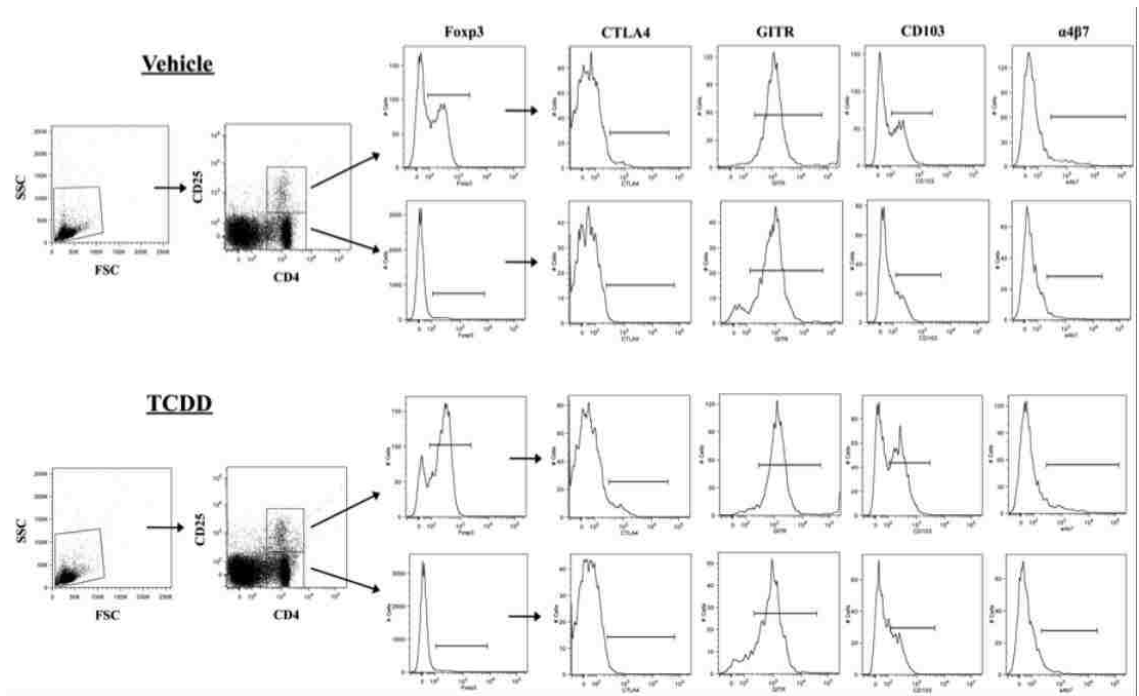
Figure 2.5. Representative histograms for T cells isolated from the MLNs. Leukocytes were harvested from MLNs three days after disease induction, as described in Materials and Methods. Gating strategy is displayed in a linear manner to define regulatory characteristics of CD4+ T cells.

Figure 2.6
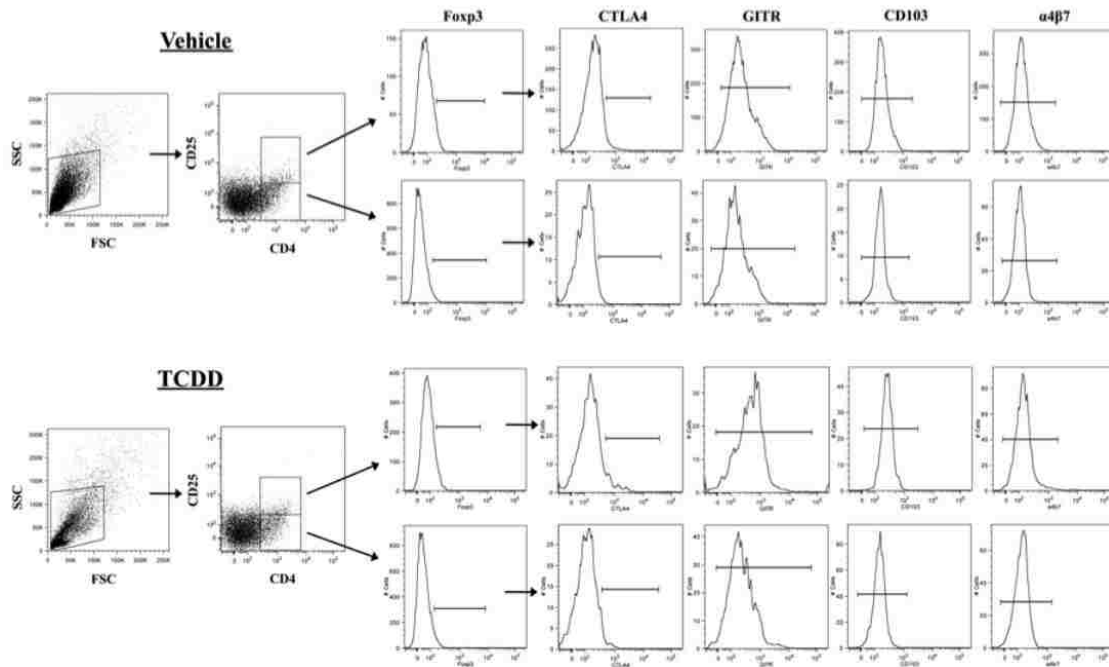
Figure 2.6. Representative histograms for T cells isolated from colonic tissue.
Leukocytes were harvested from the colon three days after disease induction, as
described in Materials and Methods. Gating strategy is displayed in a linear manner to
define regulatory characteristics of CD4+ T cells.

Figure 2.7
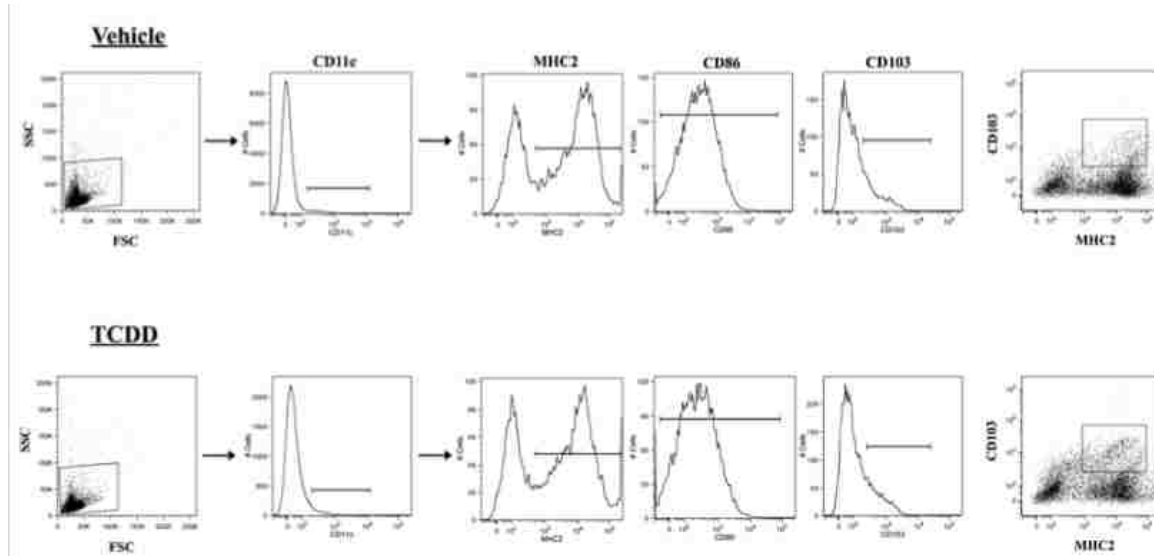
Figure 2.7. Representative histograms for DC isolated from the MLNs and colonic tissue. Leukocytes were harvested from MLNs and the colon three days after disease induction, as described in Materials and Methods. Gating strategy is displayed in a linear manner to define surface phenotype of CD11c+ DCs.

Figure 2.8

<div align="center">Vehicle   TCDD</div>
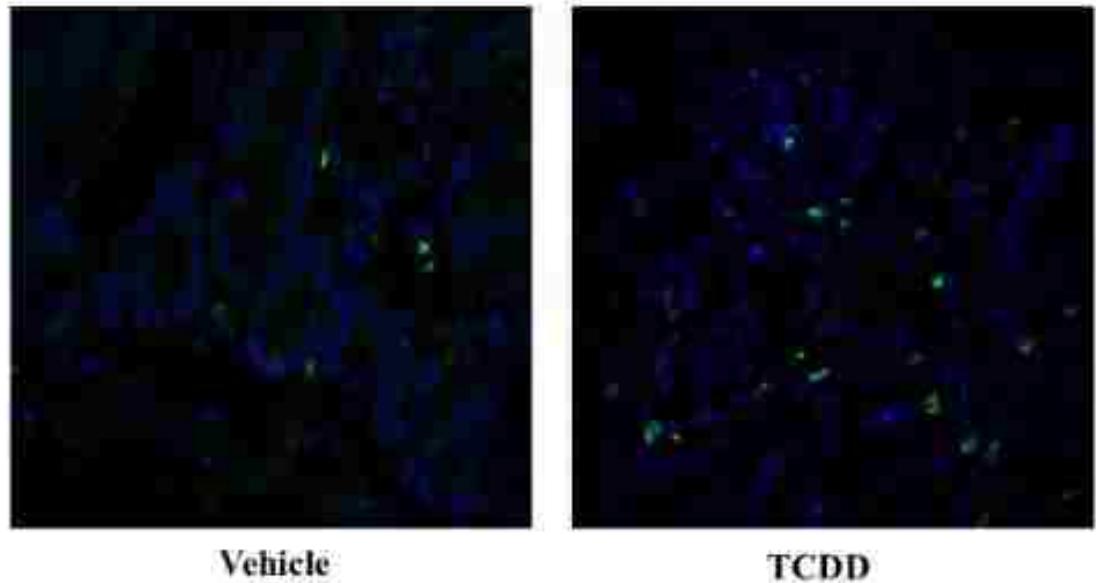
Figure 2.8. TCDD increases Foxp3 expression in colon tissue. Colons were harvested from Foxp3^{egfp} mice three days after disease induction. Tissues were sectioned, mounted on slides, and stained with DAPI to visualize the nucleus. Slides were imaged at 40X magnification using the Olympus FluoView 1000 LSC on an inverted 1x81 microscope with spectral detection and TIRF module. Images are representative of a single experiment (n=6).

DISCUSSION

Crohn's disease arises from a combination of genetic and environmental factors that trigger an inappropriate immune response against commensal bacteria. This disease is characterized by progressive weight loss, transmural inflammation in the intestines, and infiltration of inflammatory cells that produce inflammatory mediators. More specifically, many pro-inflammatory cytokines, such as IL-12, are upregulated in the inflamed intestinal mucosa of Crohn's disease patients (Monteleone *et al.*, 1997; Parronchi *et al.*, 1997; Liu *et al.*, 1999; Abraham and Cho, 2008; Eastaff-Leung *et al.*, 2010). Thus, the first goal of this study was to characterize the effects of TCDD treatment in gut mucosal inflammation associated with TNBS-induced colitis. When compared to vehicle-treated mice, TCDD treatment significantly reduced disease severity, as indicated by decreased body weight loss, recovery periods, and colonic inflammation. TCDD also altered the cytokine environment by decreasing production of the inflammatory mediators IL-6, IL-12, IFN-γ, and TNF-α. Notably, five days after colitis induction, TCDD did not appear to affect IL-10 or IL-17 protein levels, which have both been implicated in human IBD. Although the TNBS-induced colitic response in mice is mediated primarily by Th1 cells and may not correlate exactly with the human condition that involves both Th17 and Th1 cells, no animal model perfectly represents human IBD. However, TNBS-induced colitis in mice does induce many of the inflammatory sequelae that contribute to human IBD, and thus this model is very useful for assessment of these early events.

Recently, Takamura and colleagues utilized the dextran sodium sulfate (DSS) model of ulcerative colitis, another type of inflammatory bowel disease, to study the effects of TCDD on gut inflammation (Takamura *et al.*, 2010). In their model, pre-

treatment with TCDD ameliorated disease severity, inflammation, and TNF-α production in colonic tissue of DSS-treated mice. Although these results were obtained in a model that elicits a slightly different immune response, our results are consistent with those obtained in this independent study. Thus, TCDD treatment suppresses pro-inflammatory cytokine production that may be responsible for propagating inappropriate effector T cell responses in the gut. Interestingly, the TCDD dose (15 ug/kg) that was used in our studies was slightly higher than the TCDD dose (5 μg/kg) used by Takamura and colleagues. Selection of the dose of TCDD in our experiments was based on the reproducible, but not overtly toxic, effects of 15 ug/kg in many models of immunity performed in C57Bl/6 mice. To date, we have not performed dose-response studies; however, it would be of interest to evaluate the potential for both higher and lower doses to affect inflammation associated with the colitic response in TNBS-treated mice.

The intestinal immune system is comprised of organized lymph tissue, various innate and adaptive immune cells, and intestinal epithelial cells (IECs). In addition to the cells located in the lamina propria, the mesenteric lymph nodes (MLNs) are essential sites for mounting successful adaptive immune responses against pathogens in the gut. In the healthy gut, there is a balance between effector and regulatory cells such that no response is elicited against commensal organisms. In Crohn's disease, this balance is disrupted and many cellular components contribute to the exaggerated immune and inflammatory responses. In the inflamed mucosa of Crohn's disease patients, there is a significant decrease in Foxp3 expression in CD4+CD25+ cells along with decreased numbers of CD4+CD25+Foxp3+ cells, which may contribute to enhanced Th1- and Th17-mediated immune responses (Eastaff-Leung *et al.*, 2010). Moreover, TCDD has been reported to

induce CD4+CD25+ T cells that express elevated levels of GITR and CTLA4 and suppress proliferation of effector T cells (Funatake *et al.*, 2005). Thus, we investigated potential alterations in several key immune cell populations in the context of gut mucosal immunity. Pre-treatment of Foxp3^{egfp} mice with TCDD increased Foxp3+ cells in the gut of TNBS-treated mice. These cells displayed increased levels of GITR, CTLA4, and CD103, which indicate a regulatory potential for these cells. It must be acknowledged that our flow cytometry data revealed a decreased frequency of Foxp3+ cells while the immunohistochemistry images revealed increased Foxp3+ expression in the colonic tissue. This discrepancy is potentially due to the fact that the colon section removed for histology was at the site of TNBS administration, and therefore possessed the most severe inflammation, while surrounding tissue was used to isolate leukocytes for flow cytometric analyses. Even though the GFP fluorescence was not quantified in these images, the increase of Foxp3+ Tregs at the site of inflammation in TCDD-treated mice most likely contributes to decreased inflammation. Furthermore, Treg induction by TCDD may primarily occur in the context of an inflammatory environment since we did not observe increased *Foxp3* expression with TCDD treatment alone. Regulatory DCs were also present in the MLNs, as indicated by increased frequencies of CD103+ DCs. Although not directly assessed by flow cytometry, downregulation of *IL17A* mRNA in the colon suggests that TCDD may decrease Th17 cells; however, no corresponding changes in IL-17 protein levels were observed. We expect that performing these studies in Rorγt reporter mice will fully define the contribution of Th17 cells in this model and how TCDD might affect this population of inflammatory T cells. Taken together, our

results demonstrate that TCDD generates regulatory cells that may contribute to decreased inflammation in the gut of TNBS-treated mice.

Successful antibody responses in the gut are essential to limiting inappropriate responses against commensal bacteria. IgA is the primary antibody responsible for limiting bacterial penetration in the gut (Macpherson and Uhr, 2004), and its production is promoted by TGF-β. In the normal intestinal immune environment DCs maintain a tolerogenic environment, as they are conditioned by TGF-β and PGE-2 to secrete IL-10 and/or TGF-β (Iwasaki and Kelsall, 1999; Chirdo *et al.*, 2005). In addition, IECs play an important role in the innate and adaptive immune responses in the gut. The epithelium is not only the first line of defense against bacteria, but IECs can also act as antigen presenting cells capable of influencing intestinal immune function. Defects in the epithelial barrier have been associated with CD pathogenesis (Baumgart and Dignass, 2002). In our experiments, IgA production was increased in the colon tissue and feces suggesting that TCDD may be acting on DCs and/or IECs to produce switch factors necessary to induce IgA production by B cells in the gut. Additionally, *BAFF* and *APRIL* are expressed in many immune cells and play important roles in lymphocyte homeostasis (Ng *et al.*, 2005). Although *BAFF* transcript levels were reduced in the colon, *TGFβ* upregulation was observed, which may be responsible in part for the increased IgA production in the gut following TCDD treatment in TNBS-exposed mice. Moreover, stimulated IECs upregulated *BAFF* and *APRIL* mRNA levels following TCDD exposure. Thus, IECs may contribute to the amelioration of disease severity in the TNBS-induced colitis model by contributing to increased production of IgA. Additional studies focused on further defining the effects of TCDD on IgA production in the gut and the

mechanisms responsible for these effects are warranted. It must be emphasized that TCDD may be acting on T cells, B cells and other immune and non-immune cells to reduce inflammation in the gut, and we believe that these events need not be mutually exclusive. Indeed, it is very likely that the effects of TCDD on all of these cells collectively may be necessary to generate a regulatory environment during TNBS-induced colonic inflammation. Thus, to definitively determine if TCDD is acting directly on T cells or indirectly on B cells, DCs or IECs to mount protective immune responses during the generation of colitis, it would be necessary to perform adoptive transfer experiments or generate TNBS-induced colitis in AhR conditional knockout mice. Our laboratory is currently pursuing these experimental possibilities.

The effects of TCDD notably occur via activation of the AhR, which is a ligand-activated transcription factor present in many immune cells including T cells, B cells, DCs, and macrophages (Marshall and Kerkvliet, 2010). As such, activation of the AhR by TCDD results in the suppression of immune and inflammatory responses. Since it has been demonstrated in AhR-deficient mice that the immunosuppressive effects of TCDD are AhR-dependent, we anticipate that TCDD is also acting through the AhR to suppress colitis. Although our preliminary study using AhR null mice suggested that many of our observed TCDD-induced effects are dependent on the AhR, additional studies are currently underway in our laboratory to fully examine the role of the AhR in mediating the effects of TCDD as well as endogenous AhR ligands in TNBS-induced colitis.

Indoleamine-2,3-dioxygenase (IDO) has been implicated in promoting the generation of Tregs. Tryptophan, an essential amino acid, is primarily catabolized via induction of IDO, an enzyme that has been linked to immune tolerance. Furthermore,

tryptophan metabolites can activate the AhR (Fallarino *et al.*, 2002; Frumento *et al.*, 2002; Terness *et al.*, 2002; Denison and Nagy, 2003; Belladonna *et al.*, 2006; Fallarino *et al.*, 2006). It has been suggested that by binding to the AhR in activated CD4+ T cells, tryptophan metabolites can elicit their immunosuppressive effects by inducing a Treg phenotype. Moreover, Bankoti and colleagues recently demonstrated that TCDD upregulated *IDO1, IDO2, TGFβ1*, and *TGFβ2* in LPS-stimulated inflammatory bone marrow-derived DCs (Bankoti *et al.*, 2010b). In our study, TCDD treatment increased *IDO1* transcript levels in the colon of TNBS-treated mice. It is therefore possible that TCDD induces *IDO* in the intestinal DCs and/or CD4+ T cells subsequently leading to a significant induction of Tregs in mice exposed to TNBS. Furthermore, tolerogenic DCs capable of promoting Treg differentiation can be generated following the interaction of ligand-activated AhR with RelB via the non-canonical NF-kB signaling pathway, an effect that is mediated via IDO induction in DCs and subsequent Foxp3 expression in T cells (Mellor *et al.*, 2004; Belladonna *et al.*, 2007; Curti *et al.*, 2007; Tas *et al.*, 2007; Vogel *et al.*, 2008; Bankoti *et al.*, 2010b).

Current therapeutic strategies for Crohn's disease involve a combination of corticosteroids and immunomodulatory drugs to rapidly reduce inflammation and induce remission. These therapeutics, however, often possess severe adverse effects. Although TCDD suppressed colonic inflammation in this model, it is recognized that TCDD would not serve as a useful therapeutic agent for Crohn's disease patients due to its potential toxic side effects. There are numerous other AhR ligands present in the diet, such as indole-3-carbinol in cruciferous vegetables and resveratrol in grapes that have documented anti-inflammatory properties and have little or no associated toxicity (Leibelt

et al., 2003; Aggarwal and Shishodia, 2006; Cottart *et al.*, 2010). Thus, it is possible that AhR agonists found naturally in the diet can activate the AhR to reduce disease severity similar to TCDD. Recent studies have suggested a potential protective effect in the colitic response for 3,3'-diindolylmethane, the acid condensation product of indole-3-carbinol (Kim *et al.*, 2009) and resveratrol (Martin *et al.*, 2006; Larrosa *et al.*, 2009; Cui *et al.*, 2010). Although these studies were not performed in TNBS-treated mice, they demonstrated decreased disease severity and gut inflammation during a colitic response; however, the role of the AhR in mediating the effects in each of these studies was not evaluated. Conversely, AhR activation by 6-formylindolo[3,2-b]carbazole (FICZ), a typtophan metabolite, exacerbated inflammatory responses by promoting Th17 differentiation in some murine disease models, such as experimental autoimmune encephalitis (Martin *et al.*, 2006; Ho and Steinman, 2008; Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). Although ligand-specific effects may be due to the timing and duration of AhR activation, it must be emphasized that, to date, it is not well understood why some AhR ligands, such as FICZ, have the potential to promote the development of Th17-mediated inflammatory responses while others, such as TCDD, promote the development of Treg-mediated suppressive responses. Therefore, it is critical to continue to evaluate the effects of AhR activation by multiple ligands in the context of gut inflammation.

Collectively, our results demonstrate for the first time that the potent AhR ligand TCDD suppresses gut inflammation and disease severity in the murine model of TNBS-induced colitis, an effect that correlates with an induction of Tregs. Further investigations are needed to elucidate the signaling events responsible for Treg generation in the gut, as

TCDD may induce Tregs directly or alternatively via the generation of regulatory DCs. It would also be interesting to evaluate other AhR ligands, as these compounds may prove to represent a new class of therapeutics to treat Crohn's disease in humans.

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CHAPTER 3

Indole-3-carbinol exerts sex-specific effects in murine colitis

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ABSTRACT

Due to the severe adverse effects that can accompany conventional therapies for Crohn's disease, the search for natural complementary therapies has increased dramatically in recent years. Indole-3-carbinol (I3C), a constituent of cruciferous vegetables, possesses anti-inflammatory properties; however, its effects on intestinal inflammation have yet to be evaluated. To test the hypothesis that I3C dampens intestinal inflammation, C57Bl/6 mice were treated with I3C and exposed to 2,4,6-trinitrobenzenesulfonic acid (TNBS) to induce colitis. Several parameters of disease severity and inflammation were subsequently evaluated. I3C dampened the disease severity, as indicated by decreased body weight loss and decreased severity of clinical signs. Interestingly, this effect was observed in female but not male mice, which displayed a trend towards exacerbated colitis. Differential effects were observed in the profiles of cytokine production, as the production of pro-inflammatory cytokines was increased in males. The sex-specific effect of I3C in TNBS-induced colitis is a novel finding and warrants further investigation since this is a common dietary compound and is also available commercially.

KEY WORDS

Indole-3-carbinol (I3C); inflammation; Crohn's disease

INTRODUCTION

The desire to identify natural products to treat and prevent various diseases has increased dramatically in recent years due to the decreased satisfaction with conventional therapeutics. One compound of interest is indole-3-carbinol (I3C), a prevalent constituent of cruciferous vegetables including broccoli, brussel sprouts, and cabbage. I3C is primarily recognized for its potent anti-cancer properties since it has the unique ability to eliminate tumor cells while being protective in normal cells. Importantly, no immediate or long-term severe adverse effects have been documented following human consumption in several cancer clinical trials (Bradlow *et al*., 1994; Reed *et al*., 2005; Rosen and Bryson, 2004; Wong *et al*., 1997) thereby emphasizing its usefulness as a therapeutic agent.

Several molecular targets have been identified for I3C and its metabolites, such as the primary acid condensation product diindolylmethane (DIM). Activities contributing the observed effects of I3C include decreased proliferation, increased apoptosis, and decreased metastasis (reviewed in Aggarwal and Ichikawa, 2005). Furthermore, I3C can bind several cytosolic receptors and alter several important signaling pathways including aryl hydrocarbon receptor (AhR), estrogen receptor (ER), androgen receptor (AR), and nuclear factor-kappa B (NF-κB). A complex signaling cascade is likely induced following I3C exposure due to crosstalk between these pathways including physical interaction of the ligand-bound AhR with NF-κB (Vogel *et al.*, 2007a; Vogel *et al.*, 2007b) and the ER (Matthews *et al*., 2005; Ohtake *et al*., 2003; Wormke *et al*., 2003).

While I3C has been primarily investigated for its anti-cancer properties, there has been increased interest in evaluating its immunomodulatory properties. It has been

documented that I3C can suppresses inflammation *in vitro* and *in vivo* in part by inhibiting NF-κB activation and decreasing production of inflammatory mediators (Benson and Shepherd, 2011b; Chang *et al.*, 2011; Takada *et al.*, 2005; Tsai *et al.*, 2010). Therefore, the potential exists for this compound to be effective in reducing the severity of chronic inflammatory diseases including Crohn's disease and ulcerative colitis, which are inflammatory bowel diseases. In fact, the I3C acid condensation product DIM suppressed disease severity of dextran sodium sulfate (DSS)-induced colitis, which is an animal model of ulcerative colitis (Kim *et al.*, 2009). However, the potential beneficial effects of its parent compound, I3C, on intestinal inflammation have yet to be investigated.

In this study we assessed the potential of I3C to suppress inflammation in a murine model of Crohn's disease. We hypothesized that I3C would exert anti-inflammatory effects in the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis. Our results demonstrate for the first time that I3C exerts sex-specific effects in TNBS colitis, as it ameliorates disease severity and inflammation in female, but not male, mice. I3C clearly has potential to be a useful therapeutic for intestinal inflammation; however, future studies are necessary to further define the sex-specific effects of this compound in the gut.

MATERIALS AND METHODS

Laboratory animals. Six to eight week old male and female C57Bl/6 mice, originally obtained from The Jackson Laboratories (Bar Harbor, ME), were bred and maintained in the animal research facility at the University of Montana. All mice were housed under

specific pathogen-free conditions and maintained on 12 h dark/light cycles. Throughout each experiment animals were individually caged, and standard laboratory food and water were provided *ad libitum*. All protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee and adhered to the current NIH guidelines for animal usage.

Chemicals. Indole-3-carbinol (I3C) was obtained from Sigma-Aldrich (St. Louis, MO), and suspended in peanut oil, which was used as a vehicle control for gavages, at 10 mg/ml. The purity and stability of I3C was verified in house via NMR. 2,4,6-trinitrobenzenesulfonic acid (TNBS) was also obtained from Sigma-Aldrich and dissolved in acetone/olive oil for pre-sensitization or 40% ethanol for enema administration.

Colitis induction. As described in Figure 3.1, mice were gavaged daily starting on day -1 and ending on day 6 with 100 mg I3C per kg body weight (~200-250 μl total volume) or a comparable volume of peanut oil vehicle control prior TNBS exposures. Pre-sensitization on day 0 included an application of 150 μl 5% TNBS in a 4:1 acetone:olive oil solution between the shoulder blades. Colitis was then induced via an enema containing TNBS on day 7, as previously described (Benson and Shepherd, 2011). Body weight loss and the severity of clinical symptoms (overall body condition, stool consistency, and dehydration state) was monitored daily and scored as previously described in detail (Benson and Shepherd, 2011a). On day 12, mice were euthanized via CO_2 asphyxiation followed by cervical dislocation.

Figure 3.1
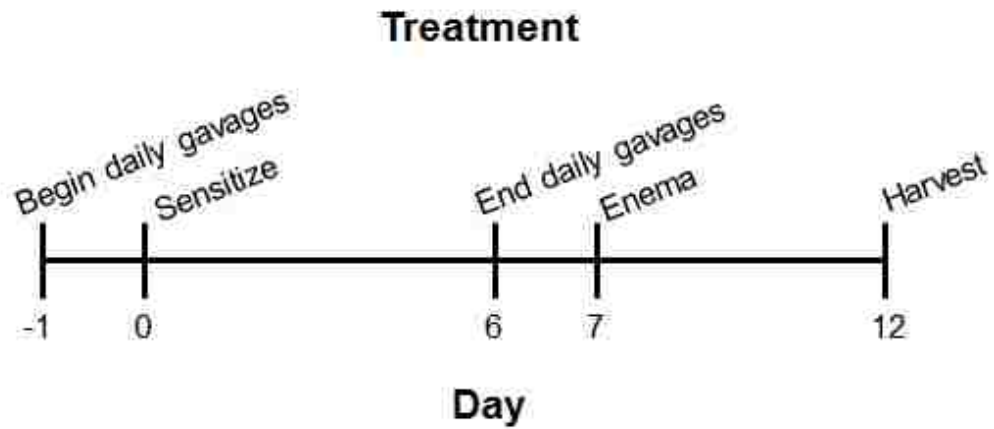
Figure 3.1. **Experimental design.** This schematic represents the experimental design utilized for the studies in which animals were gavaged daily with 100 mg/kg I3C, peripherally sensitized with 5% TNBS, intrarectally injected with 2.5 mg TNBS, and subsequently harvested on day 12 for evaluation.

Colon processing and histology. Colons were excised from each mouse and washed with PBS to remove debris. For histological analysis, a 1 cm section from the distal colon was fixed in 2% paraformaldehyde, processed in the Shandon Citadel 2000 Automated Tissue Processor (Thermo Fisher Scientific, Waltham, MA) with vacuum unit, embedded using the Shandon Histocentre 2 Embedding unit, sectioned at 7 μm using the Thermo Shandon Finesse 325 microtome, and stained with hematoxylin and eosin (H&E) in the Shandon Veristain 24-4 Slide Stainer. Colons were assessed microscopically by a blinded individual to determine the severity of inflammation based on an established scoring table (Benson and Shepherd, 2011a). Slides were imaged using the Nikon E800 Microscope with Cambridge Research Instrumentation, Inc. and Nuance camera software version 1.62 at 10X magnification. The remaining colon was divided into two sections for cell isolation, as described in *Supplemental Material and Methods,* and homogenization in lysis buffer from which the supernatants were analyzed for cytokine production, as described below.

Cytokine assays. Supernatants from homogenized colon tissue were examined for the levels of IL-1β, IL-6, IL-10, IL-12, IL-17, and IFN-γ via enzyme-linked immunosorbent assay (ELISA). Samples were analyzed according to the manufacturer's instructions using mouse cytokine-specific BD OptEIA ELISA kits (BD Biosciences, San Diego, CA) or R&D ELISA kits (Minneapolis, MN).

qRT-PCR. RNA was isolated from colon tissue with Trizol reagent (Invitrogen, Carlsbad, CA) and subjected to RNA clean-up using the Total RNA Kit with optional DNase

treatment (Omega Bio-Tek, Norcross, GA) according to the manufacturers' instructions. First strand cDNA synthesis was performed using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Resulting cDNA was subjected to qRT-PCR using commercially obtained primers (SABiosciences, Frederick, MD or Integrated DNA Technology, Coralville, IA) including aryl hydrocarbon receptor (*AHR*), aldehyde dehydrogenase family 1, subfamily A1 (*ALDH1A1*), aldehyde dehydrogenase family 1, subfamily A2 (*ALDH1A2*), estrogen receptor alpha (*ESR1*), estrogen receptor beta (*ESR2*), indoleamine-2,3-dioxygenase 1 (*IDO1*), indoleamine-2,3-dioxygenase 2 (*IDO2*), interleukin 6 (*IL6*), interleukin 10 (*IL10*), interleukin 17A (*IL17A*), interleukin 17F (*IL17F*), interleukin 22 (*IL22*), transforming growth factor, beta 1 (*TGFB1*), transforming growth factor, beta 2 (*TGFB2*), and transforming growth factor, beta 3 (*TGFB*3). Reactions were performed with PerfeCTa SYBR Green Supermix (Quanta Biosciences) on an Agilent Technologies Stratagene Mx3005P® QPCR System (Santa Clara, CA).

Flow cytometry. Lamina propria mononuclear cells from colonic tissue and cells from the mesenteric lymph nodes (MLNs) were isolated and evaluated for expression of accessory molecules by flow cytometry, as previously described (Benson and Shepherd, 2011). Briefly, after cells were washed with PAB (1% bovine serum albumin and 0.1% sodium azide in PBS), they were blocked with Fc block (BioLegend, San Diego, CA) to eliminate non-specific staining. Subsequently, cells were stained for 10 min using optimal concentrations of fluorochrome-conjugated monoclonal antibodies. Antibodies used in these experiments included CD4-PE (GK1.5), CD25-PerCP/Cy5.5 (PC61), CD11c-APC (HL3), MHC2-FITC (M5/114.15.2), CD103-Pacific Blue (2E7), and their

corresponding isotype controls, all of which were purchased from BioLegend or BD Biosciences (San Jose, CA). Two hundred thousand events were collected using a BD FACSAria flow cytometer and analyzed using FACSDiva (Version 6.1.2, BD Biosciences, San Jose, CA) and FlowJo (Version 8.7.1, TreeStar, Inc., Ashland, OR) software programs.

Statistics. All statistical analyses were performed using GraphPad Prism 4.0a for Macintosh (GraphPad Software, San Diego, CA). Student's *t*-test was used to compare vehicle-treated groups and I3C-treated groups while 2-way analysis of variance (ANOVA) with Bonferroni's post-hoc test was used to compare multiple groups. Values $p \leq 0.05$ were determined to be significant.

RESULTS

Severity of TNBS-induced colitis is reduced with I3C treatment in females but not males. I3C is a prevalent dietary compound with anti-inflammatory properties so its potential to decrease inflammation in a murine model of colitis was evaluated. This compound has been used extensively in clinical trials with no serious long-term adverse effects reported. Therefore, the dose used in this study is comparable to a dose in humans taking I3C supplements (Minich *et al*., 2007). In order to assess the potential for I3C to ameliorate colitis, key indicators that mark the onset of Crohn's disease, including significant body weight loss, loose stools, and dehydration, were evaluated first. Female mice treated with I3C did not lose as much weight and recovered more rapidly than vehicle-treated mice (Figure 3.2A). The clinical severity, as determined by the overall

Figure 3.2
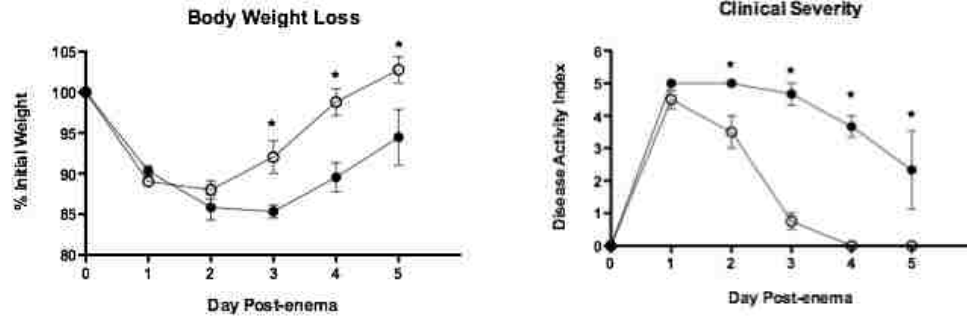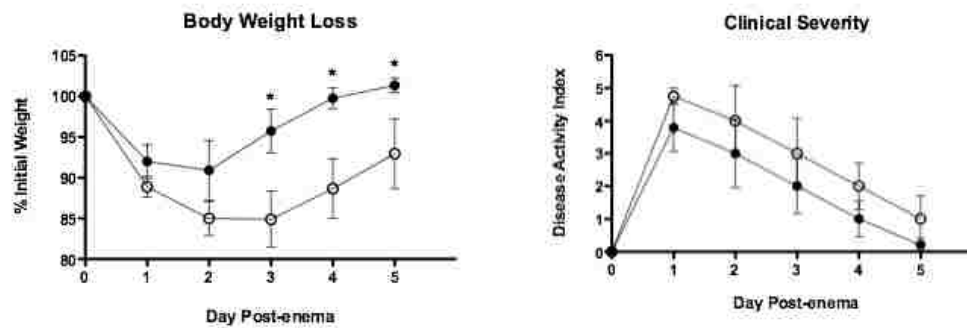
Figure 3.2. I3C ameliorates colitis in female, but not male, mice exposed to TNBS. Mice were monitored daily after disease induction for body weight loss and other clinical signs including body condition, dehydration state, and stool consistency. Clinical severity was determined by combining the symptom scores for a total possible severity score of 9. Closed circles represent vehicle-treated mice, and open circles represent I3C-treated mice. Results are shown as mean ± SEM (n=5) and representative of three separate experiments. *indicates significance of p≤0.05.

body condition, stool consistency, and dehydration state, was also significantly less severe in female I3C-treated mice. Conversely, when compared to vehicle-treated mice, male mice treated with I3C lost weight, regained that weight more slowly, and experienced a trend towards more severe clinical signs (Figure 3.2B).

Inflammation and tissue damage in the colon decreases following treatment with I3C in females. Since intestinal inflammation associated with Crohn's disease is transmural in nature, inflammatory cell infiltration and tissue lesions were evaluated in the colon. In line with the clinical signs, female I3C-treated mice colon inflammation decreased from 4.0 in vehicle-treated mice to 1.4 with I3C treatment (Figure 3.3A). Conversely, the damage score increased from 4.0 to 5.3 in male vehicle- and I3C-treated mice, respectively (Figure 3.3B). This increase, however, was not statistically significant.

I3C exerts differential effects on cytokine production in colonic tissue of TNBS-exposed mice. Colitis is associated with significant production of pro-inflammatory cytokines. To better understand how I3C may alter the onset of Crohn's disease, cytokine production in colonic tissue was measured. In I3C-treated female mice, IFN-γ levels decreased from 337 pg/ml to 174 pg/ml (Figure 3.4). IL-10 increased from 591 pg/ml to 680 pg/ml, but this was not statistically significant. It should be noted that IL-10 was significantly increased in one of the three experiments while just a trend towards increased IL-10 was observed in the other experiments. I3C did not alter the production of IL-1β, IL-6, IL-12, or IL-17 in female mice. In I3C-treated male mice, IL-1β and IL-6 were both increased from 332 pg/ml to 817 pg/ml and from 66 pg/ml to 154 pg/ml,

Figure 3.3
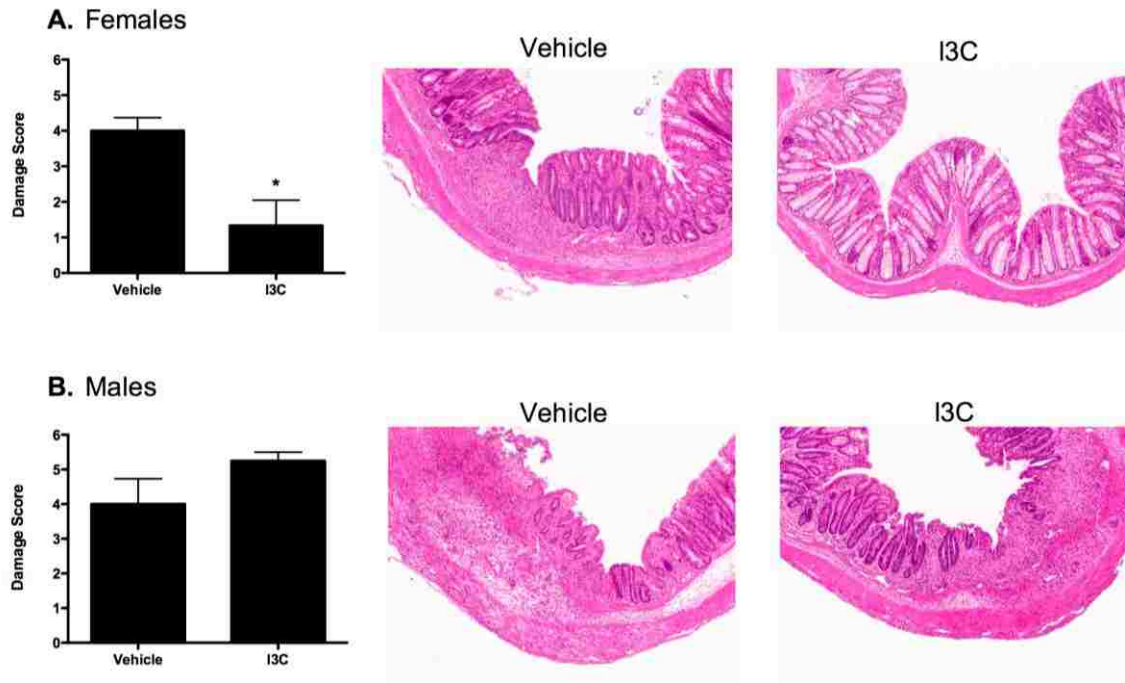
Figure 3.3. Sex-specific effects of I3C on colon damage. Colon tissue was removed on day 5 post-enema and evaluated histologically to determine tissue damage. The final damage score was based on the severity inflammatory cell infiltration and mucosal lesions, both of which were scored on a scale of 0-3. Representative images are displayed to the right of the graph. Results are shown as mean $\pm$ SEM (n=5) and representative of three separate experiments. *indicates significance of $p \leq 0.05$.

Figure 3.4
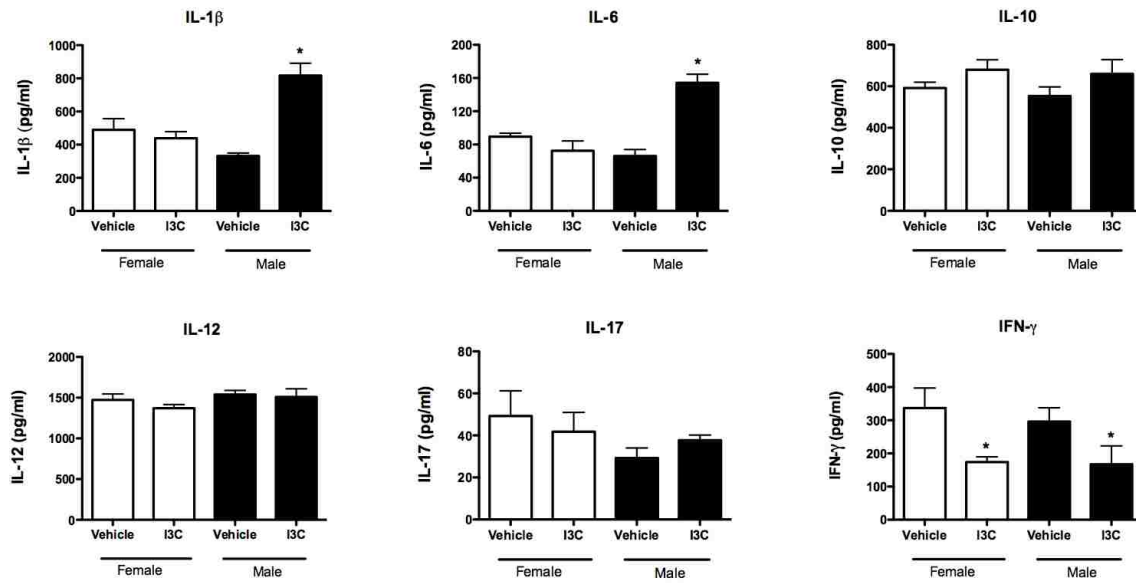
Figure 3.4. I3C induces production of anti-inflammatory and pro-inflammatory mediators in colonic tissue of TNBS-treated mice. Colon tissue was harvested five days after TNBS enema delivery, homogenized, and assessed for cytokine production. Results are shown as mean ± SEM (n=5) and representative of three separate experiments. *indicates significance of p≤0.05.

respectively. IFN-γ levels decreased from 296 pg/ml to 167 pg/ml. As with female mice, I3C did not alter the production of IL-12 or IL-17.

Differential regulation of gene expression by I3C in TNBS-induced colitis. Since exposure to other dietary chemicals leads to the induction of regulatory genes that correlates to decreased colitis, gene expression was evaluated in vehicle- and I3C-treated mice five days after the TNBS enema (Table 3.1). Interestingly, I3C-treated female mice downregulated *IL17A* expression by 3.5 fold while male mice treated with I3C upregulated *IL17A* expression by 8.1 fold. A similar trend was observed with *IL6* expression with decreased levels in females and increased levels in males treated with I3C. There was also a trend of increased expression of *ALDHA1* and *TGFB* families of genes in females and decreased levels in male mice. More specifically, in female I3C-treated mice, there was a 2.3 fold decrease in *ALDH1A2* levels and a 2.2 fold decrease in *TGFB1* transcript levels. Conversely, in male I3C-treated mice, *TGFB3* expression was increased by 6.6 fold while increases in other *TGFB* family members and the *ALDH1A* genes were not statistically significant. Notably, *IL10* expression was decreased by 2.4 fold in male mice but not significantly altered in female mice. Expression levels of *AHR*, *ESR1*, and *ESR2* were unchanged in both female and male mice five days post-enema.

Alterations in gut cell populations were not observed five days after TNBS enema. T cells and dendritic cells (DCs) are particularly important for maintaining homeostasis in the intestinal immune system. Therefore, cells were isolated from the colon tissue and the mesenteric lymph nodes (MLNs) and evaluated for phenotypic changes that could

Table 3.1. Differential regulation of gene expression by I3C in TNBS-induced colitis[a]

Gene	Female Fold change	Female p-value	Male Fold change	Male p-value
AHR	-1.1	0.4	4.3	0.2
ALDH1A1	-1.2	0.4	1.2	0.4
ALDH1A2	-1.5	0.2	1.7	0.3
ESR1	1.1	0.4	2.7	0.3
ESR2	1.1	0.4	4.3	0.2
IDO1	1.2	0.4	-1.3	0.3
IDO2	1.4	0.3	1.3	0.4
IL6	-2.4	0.08	4.1	0.2
IL10	-2.2	0.2	-2.4*	0.02
IL17A	-3.5*	0.05	8.1*	0.05
IL17F	-2.3	0.06	-1.2	0.5
IL22	-1.7	0.3	1.3	0.4
TGFB1	-2.2*	0.03	2.7	0.2
TGFB2	-1.6	0.06	1.8	0.2
TGFB3	-1.1	0.4	6.6*	0.05

[a]Colon tissue was harvested, homogenized in Trizol, and subjected to qRT-PCR to assess alterations in gene transcription. Fold change is relevant to vehicle-treated mice and normalized to β-actin. Data are representative of a single experiment with n=5 mice per treatment group. *indicates significance of $p \leq 0.05$.

Table 3.2. I3C does not significantly alter critical immune cells in colon[a]

A. Colon (Percent Population)

Immune cell markers	Female		Male	
	Vehicle	I3C	Vehicle	I3C
CD4	2.4 ± 0.4	2.8 ± 0.6	3.1 ± 0.2	4.9 ± 1.5
CD25	77.9 ± 7.6	70.4 ± 5.5	67.5 ± 5.1	67.1 ± 15.4
CD11c	2.7 ± 0.5	3.4 ± 0.8	3.9 ± 0.2	4.1 ± 0.6
MHC2	35.1 ± 10.3	40.1 ± 6.8	48.7 ± 6.2	36.8 ± 13.9
CD103	73.1 ± 6.0	68.9 ± 4.6	64.2 ± 3.4	65.3 ± 11.2

B. Colon (Mean Fluorescence Intensity)

Immune cell markers	Female		Male	
	Vehicle	I3C	Vehicle	I3C
CD4	536 ± 51	492 ± 18	512 ± 21	520 ± 21
CD25	426 ± 4	433 ±15	443 ± 25	417 ± 26
CD11c	570 ± 73	620 ± 78	651 ± 21	559 ± 102
MHC2	3039 ± 159	3072 ± 153	2780 ± 167	3166 ± 405
CD103	340 ± 38	290 ± 14	368 ± 26	326 ± 28

C. MLNs (Percent Population)

Immune cell markers	Female		Male	
	Vehicle	I3C	Vehicle	I3C
CD4	18.3 ± 0.9	21.8 ± 1.4	26.1 ± 3.1	20.2 ± 4.4
CD25	8.1 ± 0.7	6.9 ± 0.4	6.1 ± 0.6	8.2 ± 1.6
CD11c	1.4 ± 0.1	1.9 ± 0.2	1.6 ± 0.3	1.3 ± 0.3
MHC2	82.5 ± 1.6	81.1 ± 2.3	81.5 ± 1.5	83.5 ± 0.7
CD103	18.3 ± 2.6	14.2 ± 1.0	11.7 ± 1.0	17.4 ± 4.9

D. MLNs (Mean Fluorescence Intensity)

Immune cell markers	Female		Male	
	Vehicle	I3C	Vehicle	I3C
CD4	1115 ± 21	1183 ± 24	1176 ± 37	1254 ± 59
CD25	296 ± 23	472 ± 17	465 ±14	488 ± 23
CD11c	1208 ± 70	1114 ± 77	916 ± 62	1042 ±160
MHC2	5440 ± 132	5196 ± 273	5050 ±153	5009 ± 257
CD103	2094 ± 235	2230 ± 363	1819 ± 308	1637 ± 713

[a]Leukocytes were isolated from colonic tissue and mesenteric lymph nodes (MLNs) five days after enema administration. For T cells, CD25+ cells were gated out of the CD4+ population, and for dendritic cells MHC2+ and CD103+ cells were gated out of the CD11c+ population. Results are representative of three independent experiments (n=5).

indicate the presence of regulatory cells following exposure to I3C. Five days after administration of the TNBS enema, no significant changes in T cells or DCs were observed (Table 3.2).

DISCUSSION

The cruciferous vegetable constituent I3C is a potent anti-cancer agent, but its effects on inflammatory responses, especially in the gut, are not well documented. Since I3C and its primary metabolite DIM recently have been shown to produce anti-inflammatory and immunoregulatory effects *in vitro* (Benson and Shepherd, 2011b; Chang *et al*., 2011; Cho *et al*., 2008; Tsai *et al*., 2010), these compounds have the potential to elicit protective effects in diseases characterized by chronic inflammation. The current conventional medicines for Crohn's disease, a chronic inflammatory disease of the gastrointestinal tract, can be accompanied by severe adverse effects so many patients have turned towards natural and complementary therapies as part of their treatment regimens. Consequently, we evaluated the potential of I3C to decrease the severity of Crohn's disease using a murine model of colitis.

To characterize the effects of I3C on colitis, we evaluated the hallmark signs of disease onset: significant body weight loss, clinical severity, damage to colonic tissue, and inflammatory mediator production. I3C suppressed the body weight loss and the severity of clinical signs, but this effect was surprisingly only observed in female mice while male mice displayed a trend to a more severe disease phenotype. Furthermore, inflammatory cell infiltration and tissue lesions were decreased in the colons of female, but not male, mice. The acid condensation product DIM has been reported to ameliorate

the DSS model of colitis in terms of clinical signs and histological scoring of the tissue. In this report, however, Balb/c mice of an unspecified sex were used in the experiments (Kim *et al.*, 2009). Using the prototypical AhR ligand TCDD, our laboratory previously reported that TCDD dampened disease severity in TNBS-induced colitis (Benson and Shepherd, 2011a). In those studies we used male and female mice, and both sexes benefited from TCDD administration.

Colitis induction is characterized by increased production of inflammatory mediators. Thus, an important aspect of potential therapeutic would be to decrease the production of these damaging mediators. Interestingly the profile of cytokines produced in the gut was quite surprising. As expected, the exacerbated disease state in males was associated with increased production of pro-inflammatory cytokines following I3C treatment. In female mice, only IFN-γ production was reduced. With DIM treatment in DSS-induced colitis, decreased levels of IL-6, IFN-γ, TNF-α, PGE-2, and NO were observed (Kim *et al.*, 2009). Additionally, there was a trend towards decreased IL-10 production at highest dose evaluated (20 mg/kg). The authors concluded that decreased inflammation was likely due to decreased NF-κB binding activity in colon tissue.

In contrast to I3C, following TCDD treatment of both female and male mice we observed decreases in the pro-inflammatory cytokines and no change in IL-10 (Benson and Shepherd, 2011a). Furthermore, TCDD treatment resulted in significant increases in the expression of *AHRR*, *CYP1A1*, and *IDO* in the colons of mice five days after enema delivery. I3C primarily affected *ALDH1A2, IL17A*, *IL10* and the *TGFB* family members. Importantly, I3C did not have lasting effects on *AHRR* or *CYP1A1* gene expression (data not shown), which suggests that the AhR is not the sole or primary factor responsible for

the suppression of TNBS-induced colitis in females. These differences suggest that different mechanisms of action are responsible for mediating the effects of the dietary AhR ligands TCDD and I3C. Since I3C possesses anti-estrogenic properties, the estrogen receptor (ER) may also play a role, especially since the AhR and the ER have been demonstrated to physically interact (Matthews *et al*., 2005; Ohtake *et al*., 2003; Wormke *et al*., 2003). Although we did not observe changes in ER expression five days after colitis induction, additional studies assessing the role of the ER in mediating the effects of I3C in TNBS colitis would be worthwhile.

Even though there was not an overwhelming decrease in pro-inflammatory cytokines in female mice, I3C may be acting on several important immune cells in the gut to ameliorate colitis. Dendritic cells (DCs) play important role in maintaining intestinal immune homeostasis. In a recent report from our laboratory, we demonstrated that bone marrow-derived DCs grown in the presence of I3C and cultured with antigen-specific naïve CD4+ T cells generated Foxp3+ regulatory T cells (Tregs) (Benson and Shepherd, 2011b). Therefore, it is possible that DCs increased Tregs in the gut consequently leading to the decreased gut inflammation observed in I3C-exposed mice. This would be consistent with what we observed in TNBS-treated mice exposed to TCDD (Benson and Shepherd, 2011a). Although we did not observe any significant alterations in cell populations on day five post-enema, it is possible that the functions of DCs and T cells in the mesenteric lymph nodes and colon tissue were altered earlier in the inflammatory response. Thus, there may be direct and/or indirect effects of I3C on T cells in the gut, and this possibility must be further evaluated.

Taken together, our results demonstrate the novel finding that I3C imparts sex-specific effects in the TNBS colitis model. Since these findings could have significant implications for Crohn's disease patients consuming increased amounts of cruciferous vegetables and/or I3C dietary supplements, additional studies are warranted to define the specific mechanisms responsible for the divergent effects on colonic inflammation induced by I3C.

ACKNOWLEDGMENTS

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CHAPTER 4

Mice lacking the aryl hydrocarbon receptor are less sensitive to colonic inflammation induced by TNBS

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ABSTRACT

The aryl hydrocarbon receptor (AhR) has been identified as a potential therapeutic target for chronic inflammatory diseases, as activation of the AhR by various ligands can suppress disease severity. In the process of confirming the role of the receptor in mediating the effects in murine colitis, several investigators demonstrated that AhR knockout (KO) mice were more sensitive to inflammatory stimuli and consequently developed a more severe disease phenotype. Similarly we set out to further evaluate the role of the receptor in TNBS-induced colitis in our laboratory. In contrast to published results, our AhR KO mice exposed to TNBS developed a less severe disease state, as demonstrated by decreased weight loss, decreased colonic inflammation, and decreased expression of pro-inflammatory cytokine genes. However, by using the AhR antagonist CH223191 in AhR wild-type (WT) exposed to TNBS, we generated the anticipated results in that CH223191-treated mice developed colitis and also displayed increased colonic inflammatory mediator production as well as increased expression of inflammatory genes, which are indicative of a more severe disease state. Taken together, our results demonstrated that AhR KO mice in this animal facility are less sensitive to intestinal inflammation induced by TNBS. Since there was no definitive explanation for the decreased responsiveness to TNBS in AhR KO mice, additional studies are necessary to better understand the gut mucosal immune responses in these mice.

KEY WORDS

Aryl hydrocarbon receptor (AhR), inflammation, colitis

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is critical in modulating immune responses and other physiological processes, as evident by the potentially adverse effects exerted by its prototypical ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Importantly, these effects induced by TCDD are not usually observed in AhR knockout (KO) mice thereby emphasizing the role of this receptor in the documented pathologies. The AhR also plays important roles in normal cellular and developmental processes, as abnormalities in AhR KO mice have been observed including hypertrophy and fibrosis of cardiac and hepatic tissues, lymphocyte depletion in the spleen, skin lesion, and rectal prolapses in aging AhR KO mice (Schmidt *et al.*, 1996; Fernandez-Salguero *et al.*, 1997). Notably, the AhR is critical in regulating inflammatory responses. AhR KO mice are more susceptible to inflammatory stimuli, such as lipopolysaccharide, as increased production of inflammatory mediators and increased mortality due to sepsis have been documented (Kimura *et al.*, 2009; Sekine *et al.*, 2009).

Since the AhR has critical roles in immune and inflammatory responsiveness, numerous studies in recent years have targeted the AhR via TCDD and other ligands for the treatment of chronic inflammatory disorders. While performing our studies using the TNBS colitis model, reports emerged regarding the role of the AhR in the dextran sodium sulfate (DSS) colitis model, a chemically induced colitis model that primarily triggers a Th2-mediated immune response in the gut. AhR activation by TCDD was also protective in these studies, as decreased disease severity and decreased production of inflammatory mediators was decreased (Takamura *et al.*, 2010). The role of the receptor in this model was also investigated independent of ligand treatment. AhR KO mice exposed to DSS

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developed a more severe colitis phenotype that resulted in the death of several mice (Arsenescu *et al.*, 2011; Furumatsu *et al.*, 2011).

Our laboratory has previously demonstrated the beneficial effects of the AhR ligands TCDD and I3C against 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis (Benson and Shepherd, 2011). In line with other investigators, we wanted to use AhR KO mice to definitively demonstrate that the receptor is indeed involved in mediating the effects of various ligands that dampened disease severity. Therefore, in this study we first aimed to reproduce the results generated in AhR KO mice independent of ligand treatment and hypothesized that TNBS-exposed AhR KO mice should experience a more severe disease phenotype compared to AhR wild-type (WT) mice. Our results surprisingly revealed that AhR KO mice were less sensitive to the intestinal inflammation induced by TNBS. When AhR WT mice were exposed to an AhR antagonist prior to TNBS, however, colitis was successfully induced. Thus, distinctly different outcomes were observed in our animal facility in mice lacking receptor versus blocking receptor activity in wild-type mice.

MATERIALS AND METHODS

Laboratory animals. Six to eight week old AhR WT and AhR KO female mice (both on the C57Bl/6 background) were bred and maintained in the animal research facility at the University of Montana. C57Bl/6 AhR WT mice were originally obtained from The Jackson Laboratories (Bar Harbor, ME) and bred in-house. C57Bl/6 AhR KO (B6.AhR[tm1Bra]) mice were obtained from Dr. Paige Lawrence (University of Rochester Medical College, Rochester, NY) and bred as previously described (Schmidt and

Bradfield, 1996). All mice were housed under specific pathogen-free conditions and maintained on 12 h dark/light cycles. Throughout each experiment animals were individually caged, and standard laboratory food and water were provided *ad libitum*. All protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee and adhered to the current NIH guidelines for animal usage.

Chemicals. 2,4,6-trinitrobenzenesulfonic acid (TNBS) was obtained from Sigma-Aldrich and dissolved in 4:1 acetone:olive oil for pre-sensitization or 40% ethanol (EtOH) for enema administration. The acetone/olive oil solution was used for the vehicle in the antagonist experiment. The AhR antagonist CH223191 (Sigma-Aldrich) was dissolved in DMSO (final concentration 0.07%) and further diluted in PBS for i.p. delivery.

Colitis induction. Colitis was induced with TNBS, as previously described (Benson and Shepherd, 2011). As shown in Figure 4.1, pre-sensitization was conducted on day 0, as previously described (Benson and Shepherd, 2011). For experiments utilizing an AhR antagonist, 10 μg CH223191 in PBS was delivered via i.p. injection on day 6. The dose as well as the delivery and timing were based on the study conducted by Monteleone and colleagues (2011). Body weight loss and clinical signs were monitored daily. Briefly, the severity of each clinical sign was scored on a scale 0-3 and combined to determine the total severity score. On day 12, mice were euthanized via CO_2 asphyxiation followed by cervical dislocation.

Figure 4.1
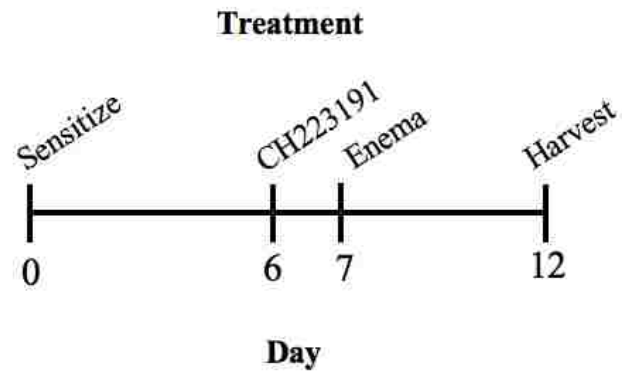
Figure 4.1. **Experimental design.** AhR WT and KO mice were peripherally sensitized with TNBS and subsequently received an enema containing TNBS to induce colitis on day 7. In experiments utilizing the AhR antagonist in AhR WT mice, CH223191 was delivered via i.p. injection on day 6. Tissues were harvested 5 days after disease induction for evaluation.

Colon processing and histology. Colons were excised from each mouse and washed with PBS to remove debris. For histological analysis, a 1 cm section from the distal colon was fixed in 2% paraformaldehyde, processed in the Shandon Citadel 2000 Automated Tissue Processor (Thermo Fisher Scientific, Waltham, MA) with vacuum unit, embedded using the Shandon Histocentre 2 Embedding unit, sectioned at 7 μm using the Thermo Shandon Finesse 325 microtome, and stained with hematoxylin and eosin (H&E) in the Shandon Veristain 24-4 Slide Stainer. Colons were assessed microscopically by a blinded individual to determine the severity of inflammation based on the scale developed by our laboratory (Benson and Shepherd, 2011). Briefly, the extent of inflammatory cell infiltration and tissue lesions was scored on a scale 0-3 and combined to determine a total damage score. Slides were imaged using the Nikon E800 Microscope with Cambridge Research Instrumentation, Inc. and Nuance camera software version 1.62 at 10X magnification. Another section of the colon was homogenized in lysis buffer from which the supernatants were analyzed for cytokine production using commercially available ELISA kits. The last section of the colon was homogenized in Trizol for gene expression analysis.

Cytokine assays. Supernatants from homogenized colon tissue were examined for the levels of IL-6, IL-10, IL-12, IL-17, IL-22, IFN-γ, and TNF-α via enzyme-linked immunosorbent assay (ELISA). Samples were analyzed according to the manufacturer's instructions using mouse cytokine-specific BD OptEIA ELISA kits (BD Biosciences, San Diego, CA) or R&D ELISA kits (Minneapolis, MN).

qRT-PCR. RNA was isolated from colon tissue with Trizol reagent (Invitrogen, Carlsbad, CA) followed by RNA clean-up using the Total RNA Kit with optional DNase treatment (Omega Bio-Tek, Norcross, GA) according to the manufacturers' instructions. First strand cDNA synthesis was performed using 500 ng RNA and qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Resulting cDNA was subjected to qRT-PCR using commercially obtained primers (SABiosciences, Frederick, MD) including aryl hydrocarbon receptor (*AHR*), cytochrome P450 A1 (*CYP1A1*), interferon gamma (*IFNG*), interleukin 6 (*IL6*), *IL10, IL12, IL17A, IL22*, and tumor necrosis factor alpha (*TNFA*). Reactions were performed with PerfeCTa SYBR Green Supermix (Quanta Biosciences) on an Agilent Technologies Stratagene Mx3005P® QPCR System (Santa Clara, CA). Resulting data were normalized to β-actin, and fold changes were calculated using the ΔΔCt method, which compares threshold values of the samples of interest for a particular gene relative to a housekeeping gene.

Statistics. All statistical analyses were performed using GraphPad Prism 4.0a for Macintosh (GraphPad Software, San Diego, CA). Student's *t*-test was used to compare WT versus KO groups while 2-way analysis of variance (ANOVA) with Bonferroni's post-hoc test was used to compare multiple groups. Values p≤0.05 were determined to be significant.

RESULTS

TNBS colitis is less severe in AhR KO mice. In order to investigate the role of the AhR in colitis, the clinical signs of colitis were first monitored in AhR WT and KO mice that were not treated with an AhR ligand. As shown in Figure 4.2, both WT mice and KO mice rapidly lost more than 10% of their initial body weights following TNBS exposure. Surprisingly, the AhR KO mice were less sensitive than AhR WT mice, as they lost less body weight (Figure 4.2A) and recovered more rapidly from clinical signs (Figure 4.2B). Furthermore, the degree of inflammatory cell infiltration and tissue lesions was also lower in KO mice compared to WT mice, as the average damage score was 4.7 in AhR WT mice and 2.5 in AhR KO mice (Figure 4.2C).

Inflammatory and protective mediator production in mice experiencing colitis. To further evaluate how AhR KO mice respond to TNBS-induced colitis, the production of cytokines critical to potentiating colonic inflammation were assessed. In AhR KO mice, IL-10 production increased from 1096 pg/ml in AhR WT mice to 1252 pg/ml, and IL-12 production decreased from 1628 pg/ml in WT mice to 1342 pg/ml in AhR KO mice (Figure 4.3). No significant changes were observed in IL-6, IL-17, IL-22, IFN-γ, or TNF-α levels in the colon.

Figure 4.2

A.

B.

C.
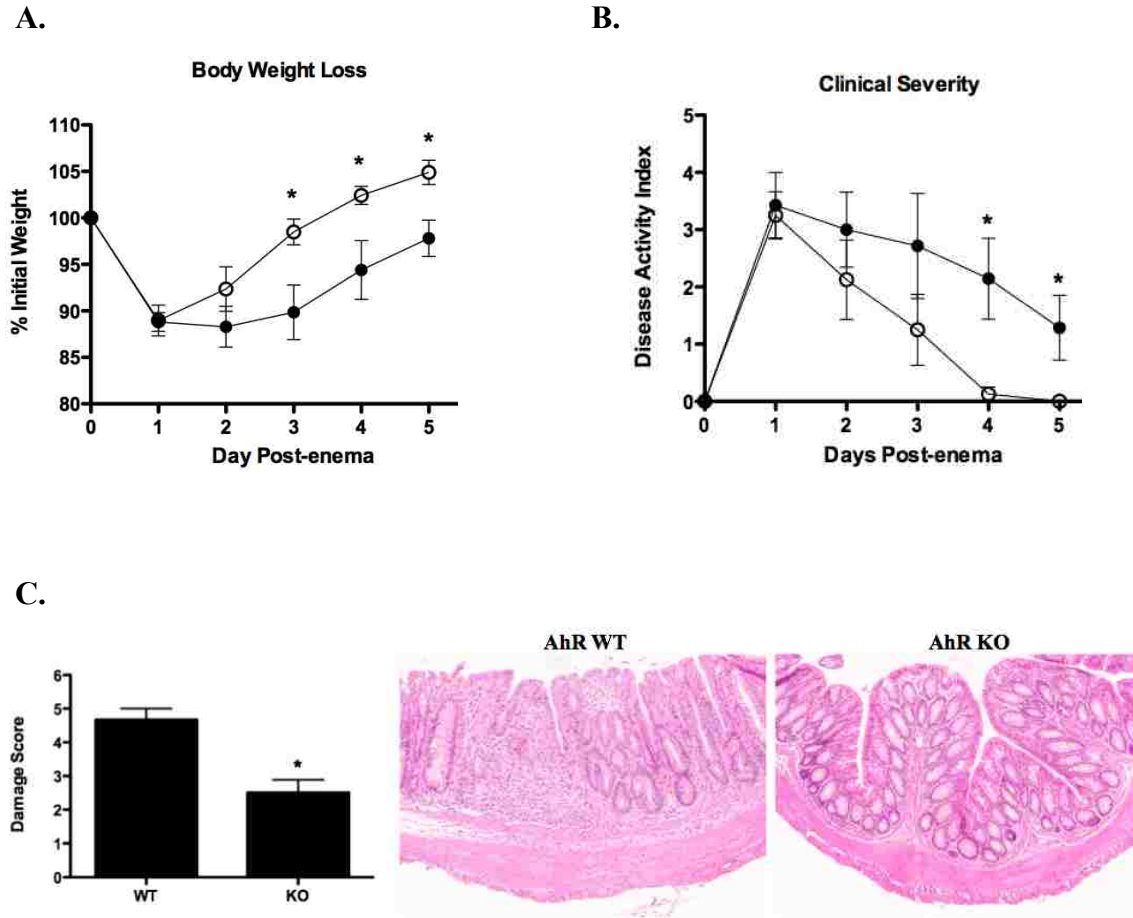
Figure 4.2. TNBS colitis is less severe in AhR KO mice. AhR WT mice (•) and AhR KO mice (°) were monitored daily after disease induction for body weight loss (A) and other clinical symptoms including body condition, dehydration state, and stool consistency (B). Damage in colonic tissue was also evaluated for inflammatory cell infiltration and tissue lesions (C). Results are shown as mean ± SEM (n=5) and representative of three separate experiments. *indicates significances of p≤0.05.

Figure 4.3
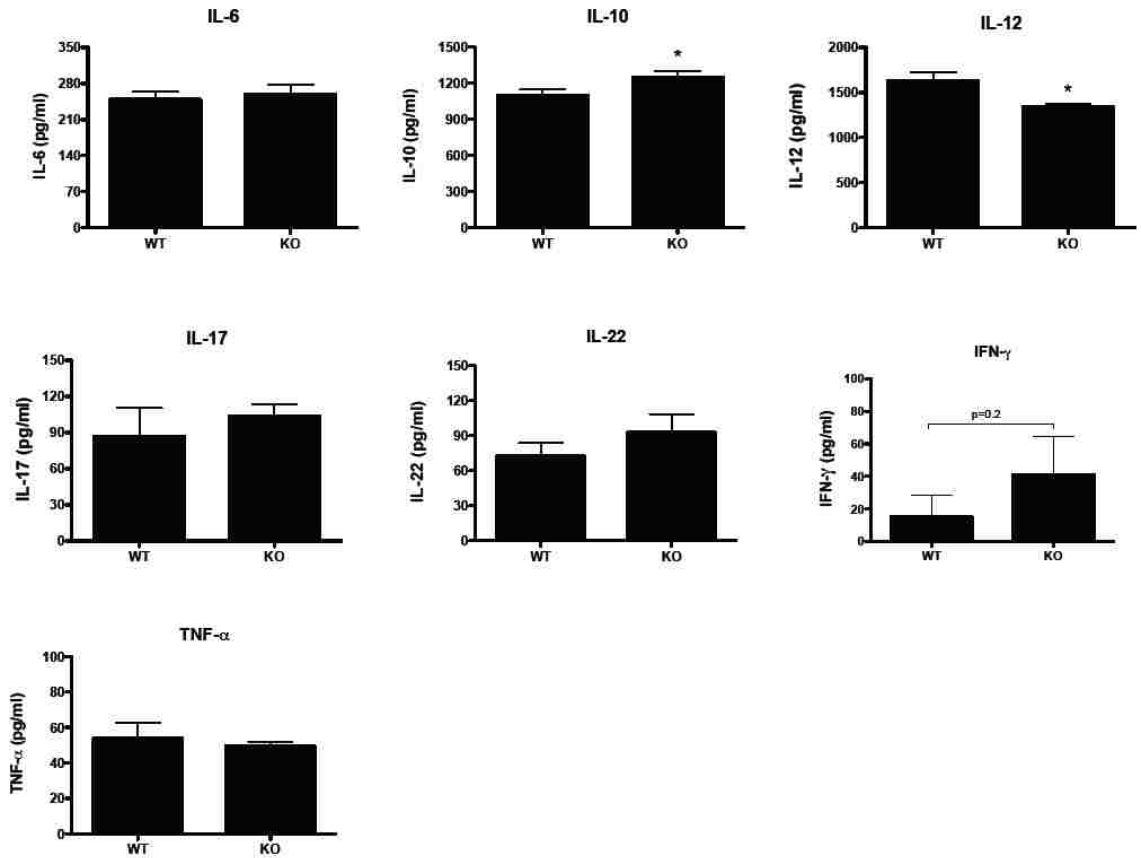
Figure 4.3. Inflammatory and protective mediator production during colitis. Colonic tissues from AhR WT and KO mice were homogenized, and cytokine levels in the supernatants were measured by ELISAs. Results are shown as mean ± SEM (n=5) and representative of three separate experiments. *indicates significances of p≤0.05.

Altered gene expression in AhR KO mice exposed to TNBS. Modifications in colonic gene expression were evaluated next to better understand the unexpected results in TNBS-exposed AhR KO mice. In contrast to the relatively unchanged protein expression, several genes were down-regulated in TNBS-exposed AhR KO mice compared to AhR WT mice (Table 4.1). Notably, decreased mRNA expression was observed in *AHR* by 154-fold*, IFNG* by 46.7-fold, *IL6* by 10.9-fold, *IL10* by 5.9-fold, *IL12* by 8.7-fold, *IL17A* by 13.9-fold, and *TNFA* by 12.0-fold. No significant changes were observed in *CYP1A1 or IL22* expression.

AhR WT mice exposed to the AhR antagonist CH223191 develop colitis. Recently it was demonstrated that the AhR antagonist CH223191 generated results similar to studies using AhR KO mice (i.e. more severe disease phenotype) in mice exposed to TNBS (Monteleone *et al.*, 2011). Since we did not observe a more severe outcome in conventional AhR KO mice treated with TNBS, this AhR antagonist was used to block AhR activity in wild-type mice during TNBS-induced colitis. As expected, mice exposed to the EtOH control only (with no TNBS enema and with/without CH223191) did not develop disease, as demonstrated by no decrease in body weight or increase in severity of clinical signs (Figure 4.4A). While both groups of mice exposed to TNBS developed colitis, the difference between CH223191 and its PBS vehicle was not statistically significant. Histological evaluation revealed significant inflammatory cell infiltration and tissue lesions following TNBS exposure (Figure 4.4B). However, CH223191-treated mice experienced a trend towards increased colonic damage, as indicated with a score of 5.0 with CH223191 treatment compared to the score of 4.38 with vehicle treatment.

Table 4.1. Gene expression is decreased in the gut of AhR KO mice during colitis

Gene	Fold change	p-value
AHR	-154*	0.02
CYP1A1	-1.7	0.4
IFNG	-46.7*	0.007
IL6	-10.9*	0.006
IL10	-5.9*	0.008
IL12	-8.7*	0.03
IL17A	-13.9*	0.02
IL22	-1.6	0.3
TNFA	-12.0*	0.03

Colon tissue was harvested on day 5 post-enema, homogenized in Trizol, and subsequently subjected to qRT-PCR to assess changes in gene expression. Fold change is relevant to AhR WT mice and normalized to β-actin. Data are representative of one experiment (n=5). *indicates significances of p≤0.05.

Figure 4.4

A.
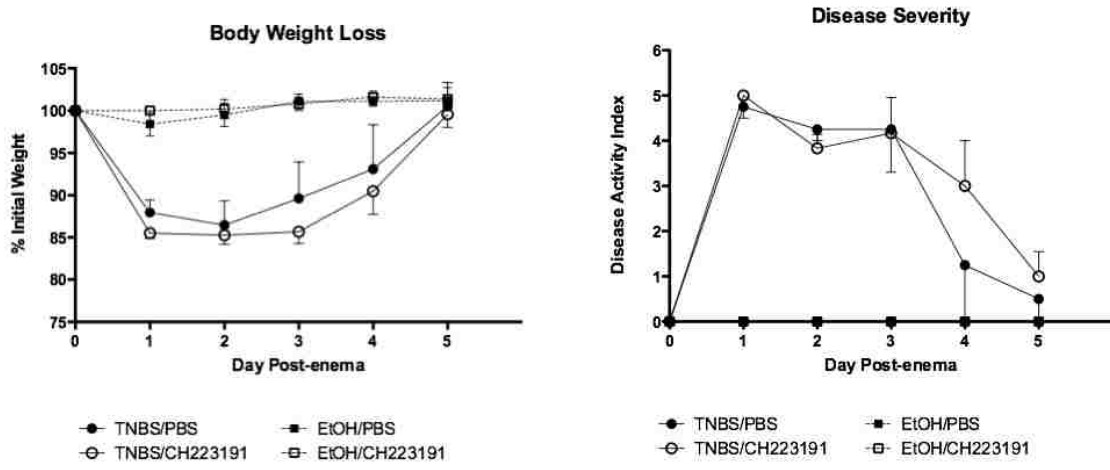
B.
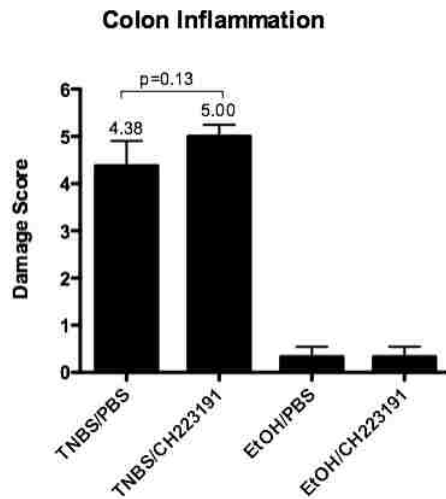
Figure 4.4.   TNBS colitis is induced in CH223191-treated WT mice.   Mice were monitored daily after disease induction for body weight loss and other clinical symptoms including body condition, dehydration state, and stool consistency (A).   Damage in colonic tissue was also evaluated for inflammatory cell infiltration and tissue lesions (B). Results are representative of one experiment (n=3-6). *indicates significances of p≤0.05.

CH223191 treatment alters inflammatory mediator and gene expression profiles.
Treatment with the AhR antagonist CH223191 significantly altered cytokine production
in the colon of TNBS- and vehicle-exposed mice (Figure 4.5). In TNBS-exposed mice,
CH223191 increased IL-6 from 411 to 1462 pg/ml, IL-10 from 287 to 340 pg/ml, IL-17
from 158 to 196 pg/ml, and IFN-γ from 117 to 176 pg/ml. Conversely, IL-12 and TNF-α
production was significantly decreased. In absence of the TNBS inflammatory stimulus,
mice exposed to CH223191 exhibited increased IL-10 production from 319 to 410 pg/ml
and increased IL-22 production from 99 to 142 pg/ml.

CH223191 also induced several changes in colonic gene expression on day 5
post-enema (Table 4.2). In TNBS-exposed mice, CH223191 significantly increased the
expression of several inflammatory mediators including *IFNG* by 12.5-fold, *IL6* by 3.2-
fold, *IL10* by 9.9-fold, *IL12* by 9.5-fold, and *IL17A* by 6.2-fold. Although CH223191
had no effect on *AHR* gene expression, *CYP1A1* expression was not increased, which is a
hallmark indicator of AhR activation by an agonist. In line with other investigators, *AHR*
expression was significantly upregulated by 636-fold in TNBS-exposed mice compared
to the ethanol vehicle control (data not shown). Finally, in the absence of TNBS,
CH223191 did not induce changes in colonic gene expression at the time of harvest.

Figure 4.5
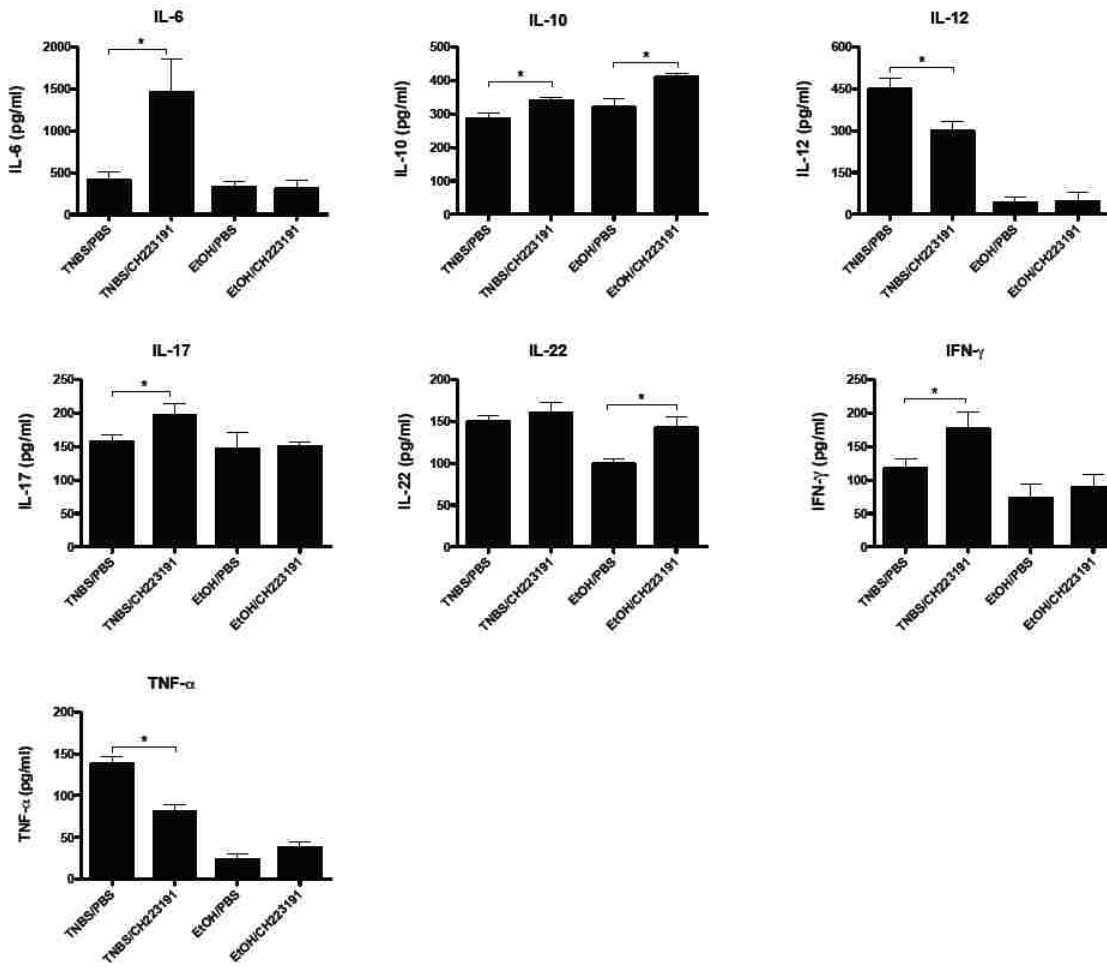
Figure 4.5. CH223191 alters cytokine production in AhR WT mice during colitis.
Colonic tissues were collected on day 5 post-TNBS enema, homogenized, and cytokine
levels in the supernatants measured by ELISA. Results are shown as mean ± SEM (n=5)
and representative of one experiment (n=3-6). *indicates significances of p≤0.05.

Table 4.2. Gene expression is altered in the gut of CH223191-treated mice

Gene	TNBS		EtOH	
	Fold change	p-value	Fold change	p-value
AHR	2.3	0.2	-1.3	0.4
CYP1A1	1.2	0.4	-1.7	0.3
IFNG	12.5*	0.02	-1.5	0.3
IL6	3.2*	0.05	1.5	0.3
IL10	9.9*	0.04	-1.0	0.5
IL12	9.5*	0.02	1.3	0.4
IL17A	6.2*	0.04	-1.1	0.5
IL22	1.0	0.5	-1.2	0.4
TNFA	1.2	0.4	1.4	0.3

Colon tissue was harvested on day 5 post-enema, homogenized in Trizol, and subsequently subjected to qRT-PCR to assess changes in gene expression. Fold change is relevant to vehicle-treated mice and normalized to β-actin. Data are representative of one experiment (n=3-6). *indicates significances of $p \leq 0.05$.

DISCUSSION

Since immune suppression is a classic response following activation of the AhR, this signaling pathway has been investigated as a new therapeutic target for chronic inflammatory diseases, such as Crohn's disease and multiple sclerosis. In fact, numerous studies have been published recently regarding the beneficial effects of AhR activation by TCDD to reduce disease severity (Quintana *et al.*, 2008; Kerkvliet *et al.*, 2009; Takamura *et al.*, 2010; Benson and Shepherd, 2011). Recently several laboratories have focused their studies on the role of the AhR in intestinal inflammation. As such, AhR KO mice have been used to confirm the role of the AhR in mediating the effects of various ligands. In addition to demonstrating that the AhR was responsible for mediating the protective effects against colitis, it has also been shown that AhR KO mice were more sensitive to inflammation (Arsenescu *et al.*, 2011). Similarly, in this study we also aimed to definitively demonstrate the role of the AhR by using conventional AhR KO mice in the TNBS-induced colitis model.

We first evaluated the role of the AhR in TNBS-induced colitis independent of AhR ligand treatment in AhR WT and KO mice. Surprisingly, AhR KO mice appeared to be less sensitive to disease severity and colonic inflammation. Furthermore, there was a significant decrease in the expression of several pro-inflammatory genes in the colon including *IFNG, IL6, IL12, IL17A,* and *TNFA*. Thus, decreased inflammatory mediator production may be partially responsible for reduced inflammation in the gut of our AhR KO mice. This finding is in complete contrast to a previously published report that was conducted in WT and AhR KO mice using DSS-induced colitis, as it was revealed that AhR KO mice exposed to DSS died within 7 days of the beginning of treatment whereas

the AhR WT mice developed colitis as expected (Arsenescu *et al.*, 2011). Furumatsu and colleagues also confirmed increased mortality in AhR KO mice exposed to DSS and consequently suggested that the endogenous ligands that regulate homeostasis in AhR WT mice cannot do so in AhR KO mice thereby resulting in exacerbated DSS-induced colitis (Furumatsu *et al.*, 2011). Although this hypothesis is plausible for their results, it is not consistent with the ameliorated disease state we observed in AhR KO mice treated with TNBS to induce colitis.

Instead of using AhR KO mice, several laboratories use chemical antagonists to block AhR receptor activity to specifically evaluate the role of the AhR in mediating various physiologic responses. One such antagonist is CH223191, which was first identified as an antagonist by Kim and colleagues because this compound blocked TCDD-induced gene transcription responsible for mediating the toxic effects of TCDD (Kim *et al.*, 2006). However, CH223191 is a ligand-selective antagonist as it inhibits some AhR agonists, such as TCDD and related halogenated aromatic hydrocarbons, but not others, such as polycyclic aromatic hydrocarbons, flavonoids, and indirubin (Zhao *et al.*, 2010). Moreover, it is currently unknown if this antagonist blocks the binding of endogenous ligands. Nevertheless, using an antagonist such as CH223191 can be advantageous over AhR KO mice since it is used in WT mice that develop normally and do not have other physiological defects that have been reported in conventional AhR KO mice.

Accordingly, the AhR antagonist CH223191 was administered to AhR WT mice prior to inducing colitis with TNBS (Monteleone *et al.*, 2011). In this study, the authors observed increased disease severity and inflammation in mice co-treated with TNBS and

CH223191, which is consistent with the observations in AhR KO mice exposed to DSS. Since our results in the TNBS-induced colitis model using AhR KO mice were not consistent with previously published reports using either TNBS or DSS, we performed an additional study using CH223191 in an experimental approach (i.e. dose and regimen) similar to Monteleone and colleagues (2011). We found that administration of CH223191 to AhR WT mice resulted in the induction of colitis by TNBS. This outcome was distinctly different than what we previously observed with AhR KO mice in which colitis disease severity was attenuated in AhR WT mice. Interestingly, although the differences were not statistically significant, there was also a trend towards increased colonic damage and more severe clinical signs in mice exposed to CH223191.

In addition to the effects on disease severity observed in TNBS-treated mice, CH223191 also significantly altered the production of several pro-inflammatory cytokines. There was increased production of IL-6, IL-17, and IFN-γ, which is supportive of the anticipated exacerbated disease phenotype. Interestingly, CH223191 decreased IL-12 and TNF-α production, which was not observed by Monteleone and colleagues (2011). The expression of several inflammatory genes was also increased in CH223191-treated mice with the TNBS inflammatory stimulus, which is suggestive of a more severe disease phenotype. However, we did not detect a decrease in *AHR* gene expression induced by CH223191 at the time of harvest, an effect that was observed by Monteleone and colleagues (2011). It is possible that AhR protein levels decreased in colonic tissue even though gene expression was unchanged; however, this possibility has yet to be evaluated. Nevertheless, the trend toward an anticipated increased disease

severity and increased inflammation was observed in our AhR WT mice exposed to CH223191.

While we could not replicate the published results of colitis in AhR KO mice, we were able to generate results that were more consistent with published observations using an AhR antagonist in AhR WT mice. Thus, several factors may be affecting the immune responsiveness in the gut of our AhR KO mice. First of all, it must be noted that there are multiple conventional AhR KO mice commercially available on the C57Bl/6 background. One strain was generated by the Gonzalez laboratory by targeting exon 1 for deletion (Fernandez-Salguero *et al.*, 1997) whereas another strain was generated by the Bradfield laboratory by targeting exon 2 for deletion (Schmidt *et al.*, 1996). Although several similar pathologies are observed in both AhR KO mice, there are numerous discrepancies between the two null alleles (reviewed in (Lahvis and Bradfield, 1998). This may be due to different targeting strategies that can alter the function of neighboring genes, different genetic backgrounds used to generate AhR KO mice, and/or the variability of housing environments. In our studies we used the mice generated from the Bradfield laboratory; however, other conventional AhR KO mice were used in other studies. Furumatsu and colleagues used another null mouse generated by the Fujii-Kuriyama laboratory (Shimizu *et al.*, 2000), while Arsenescu and colleagues (2011) did not specify which AhR KO mice strain they obtained from Jackson Laboratories. Therefore, it is very possible that the differences in the AhR null alleles contribute to the inconsistencies observed in our studies compared to the current literature.

Additionally, we observed sex-specific effects on colitis severity when dietary AhR ligand indole-3-carbinol was administered to mice. Although not previously

documented, male and female AhR KO mice in our facility could respond differently to absence of the AhR. In our studies, we used female mice, as at the time of the study we had a greater availability of female AhR KO mice. Arsenescu and colleagues (2011) used male AhR KO mice while Furumatsu and colleagues (2011) did not specify the sex of mice utilized. Thus, a combination of sex differences and difference in null alleles could be contributing to the decreased disease severity observed in our studies.

Finally, it is also possible that the microbiome and/or various environmental conditions (i.e. bedding, food, etc…) in this facility have significantly impacted the inflammatory responsiveness of AhR KO mice making them less susceptible to TNBS-induced colitis. Accordingly, AhR KO fecal samples were sent to Charles River for a PCR Rodent Infectious Agent (PRIA) panel analysis to assess the presence of parasites, viruses, bacteria, and fungi that may be affecting gut mucosal immune responses (data not shown). Results revealed that our AhR KO mice tested positive for mouse norovirus (MNV), *Pasturella pneumotropica-Heyl and Helicobacter hepaticus*. MNV and *P. pneumotropica* are very common in many animal facilities, and MNV has been present in our animal facility for several years. AhR KO mice are known to have *Helicobacter* present in the gut, which is a contributing factor to rectal prolapses in mice greater than 9 months (Schmidt *et al.*, 1996; Fernandez-Salguero *et al.*, 1997), an effect that we have observed over the last several years in our AhR KO colony. MNV has been attributed to worsened intestinal inflammation in a bacteria-induced model of colitis (Lencioni *et al.*, 2008). Although some pathogens are present in the gut of AhR KO mice, these pathogens are not necessarily supportive of making these mice less sensitive to colitis induction by TNBS. However, there could be a shift in commensal bacteria in this

facility that alters immune responsiveness to TNBS, which has yet to be investigated. Furthermore, preliminary microarray and proteomics analyses (Appendix 1) conducted on naïve AhR KO mice did not provide a definitive answer to explain the discrepancies, but the data suggested that decreased NF-kB signaling and decreased apoptosis in the AhR KO gut may contribute to the decreased disease severity in AhR KO mice. Future studies evaluating the importance of these signaling pathways in the gut of AhR KO mice in this facility may help elucidate the mechanisms responsible for their decreased sensitivity to TNBS.

Collectively, our results demonstrate that AhR KO mice in the University of Montana Laboratory Animal Facility are less sensitive to intestinal inflammation induced by TNBS whereas AhR WT mice treated with an antagonist develop colitis as expected. By achieving the documented trend towards increased disease severity using the AhR antagonist in AhR WT mice, this strongly suggests that a complex set of factors is drastically impacting the ability of these mice to respond to the TNBS inflammatory stimulus. Additional studies are warranted to better understand the gut mucosal immune responses in our AhR KO mice.

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CHAPTER 5

Dietary ligands of the aryl hydrocarbon receptor induce anti-inflammatory and immunoregulatory effects on murine dendritic cells

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ABSTRACT

Activation of the aryl hydrocarbon receptor (AhR) in immune cells, such as dendritic cells (DCs), can lead to suppressed immune responses. Although AhR activation is most recognized for mediating the effects of its prototypical ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), many compounds existing in dietary sources can also bind the AhR. Since the immunomodulatory effects of indole-3-carbinol (I3C) and indirubin-3'-oxime (IO) have yet to be investigated in DCs, we evaluated the potential immunomodulatory effects of these compounds on murine DCs. We hypothesized that I3C and IO suppress immune and inflammatory responses in DCs. We found that both I3C and IO decreased the expression of CD11c, CD40, and CD54 while they increased expression of MHC2 and CD80. Following LPS-activation, I3C and IO suppressed the production of pro-inflammatory mediators including TNF-α, IL-1β, IL-6, IL-12, and nitric oxide but increased IL-10 levels. These effects of I3C and IO were partially mediated by the AhR. Additionally, immunoregulatory genes, such as *ALDH1A*, *IDO* and *TGFB*, were upregulated following treatment with I3C or IO. Both I3C and IO decreased basal levels of NF-κB p65 but only I3C suppressed the LPS-induced activity of RelB. Finally, when cultured with naïve T cells, BMDCs treated with the dietary AhR ligands increased the frequency of Foxp3+ Tregs in an antigen-specific manner. Taken together, these results indicate that I3C and IO exhibit immunosuppressive and anti-inflammatory effects on DCs. Since I3C and IO are significantly less toxic than TCDD, these natural products may ultimately become useful therapeutics for the treatment of autoimmune and inflammatory diseases.

INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that bridge the innate and adaptive branches of the immune system. As such, these cells are critical to mounting successful immune and inflammatory responses against antigens as well as promoting tolerance. Following antigen uptake in the periphery, DCs mature and migrate to lymph nodes where they present processed antigen to naïve T cells. Many factors contribute to the ensuing adaptive immune response. One of these involves activation of the aryl hydrocarbon receptor (AhR), the cytosolic ligand-activated transcription factor responsible for the toxic and immunomodulatory effects of its prototypical ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The AhR is expressed in immune cells and is involved in many important physiological processes, as AhR deficient mice have numerous defects including slowed early growth, anal prolapses, and hepatic abnormalities (Schmidt *et al.*, 1996). Importantly, AhR activation by TCDD in DCs results in immune suppression via induction of indoleamine-2,3-dioxygenase (IDO) and subsequent generation of regulatory T cells (Tregs) (Vogel *et al.*, 2008; Mezrich *et al.*, 2010; Nguyen *et al.*, 2010; Simones and Shepherd, 2011).

Besides environmental contaminants, additional compounds present in the diet have the potential to bind and activate the AhR. One of these dietary constituents is indole-3-carbinol (I3C), which is found in cruciferous vegetables, and another is indirubin, which is a component in the traditional Chinese medicine Danggui Longhui Wan. However, neither of these compounds binds the AhR as potently as TCDD. Both I3C and indirubin have well-documented anti-cancer properties, as they both work in part by inhibiting cyclin dependent kinases that leads to cell cycle arrest in various cell lines.

More specifically, I3C has been evaluated for the treatment of both breast and prostate cancer (Weng *et al.,* 2008) while indirubin has been used traditionally for the treatment of chronic myelocytic leukemia (Eisenbrand *et al*., 2004).

Despite these known effects on cancerous cells, the effects of I3C and indirubin on immune cells have not yet been investigated in depth. I3C suppresses the production of pro-inflammatory cytokines in macrophages (Chen *et al.*, 2003; Tsai *et al.*, 2010; Chang *et al.*, 2011) while indirubin has been demonstrated to suppress these mediators in splenocytes and microglial cells (Kunikata *et al.*, 2000; Jung *et al.*, 2011). Therefore, studies evaluating the effects of these dietary AhR ligands on additional immune cell populations, such as DCs that constitutively express the AhR, are warranted since these compounds are present in the diet or readily consumed supplements and have great potential as complementary therapies in chronic inflammatory diseases.

In this study we aimed to define the specific immunomodulatory effects of I3C and indirubin on murine bone marrow-derived DCs (BMDCs). We hypothesized that these natural AhR ligands alter DC maturation such that they suppress immune and inflammatory responses in DCs. To test this hypothesis, we conducted an array of *in vitro* experiments to assess changes in BMDC fate and function following exposure to I3C and indirubin. The results obtained in this study suggest that both I3C and indirubin generate immunosuppressive effects on DCs that may promote a regulatory environment, which may be useful to suppress chronic inflammatory diseases and/or autoimmunity *in vivo*.

MATERIALS AND METHODS

Chemicals and reagents. Indole-3-carbinol (I3C) and indirubin-3'-oxime (IO), a commercially available indirubin derivative that is also an AhR agonist (Guengerich *et al.*, 2004), were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO (Sigma-Aldrich). Both compounds were >99% pure when purchased, and the purities of I3C and IO were also confirmed in house prior to use via NMR and HPLC, respectively (data not shown).   For initial concentration-response studies, I3C was used at concentrations of 25, 50, 100, and 200 μM and IO was used at 0.01, 0.1, 1.0, and 5.0 μM. For all other experiments, I3C and IO were used at final concentrations of 50 μM and 1 μM, respectively. The final concentration of solvent used in cell culture was below 0.1% and did not induce DC cytotoxicity.

Mice. Six- to 8-week old male $AhR^{+/+}$, $AhR^{-/-}$, and OTII $Foxp3^{egfp}$ mice (all on a C57Bl/6 background) were bred and maintained in the animal research facilities at the University of Montana. C57Bl/6 $AhR^{+/+}$ were originally obtained from The Jackson Laboratories (Bar Harbor, ME) and bred in-house. C57Bl/6 $AhR^{-/-}$ ($B6.AhR^{tm1Bra}$) mice were generously provided by Dr. B.P. Lawrence (University of Rochester Medical College, Rochester, NY) and bred as previously described (Schmidt and Bradfield, 1996). OTII $Foxp3^{egfp}$ mice were kindly provided by Dr. Randolph J. Noelle (Dartmouth Medical School, Lebanon, NH), who originally obtained these mice from Dr. A. Rudensky (University of Washington School of Medicine, Seattle, WA), and bred as previously described (Wang *et al.*, 2008). To verify identity of mutant mice prior to use in experiments, $AhR^{-/-}$ mice and OT II $Foxp3^{egfp}$ mice were genotyped via PCR and

phenotyped via flow cytometry, respectively. The genotype of AhR$^{+/+}$ and AhR$^{-/-}$ mice is based on the presence of AhR^b or AhR^d alleles. The AhR$^{+/+}$ mice possess the AhR^b allele (AhR$^{b/b}$), which is 300bp, whereas AhR$^{-/-}$ mice possess the d allele (AhR$^{d/d}$), which is 260bp. AhR$^{+/-}$ mice possess both the b and d alleles thus resulting in both the 300bp and 260bp products. Mice were housed under specific pathogen-free conditions and maintained on 12 h dark/light cycles. Standard laboratory food and water were provided *ad libitum*. All protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee and adhered to the current National Institutes of Health (NIH) guidelines for animal usage.

Bone marrow-derived dendritic cells (BMDCs). BMDCs were prepared as previously described (Bankoti et al., 2010). Briefly, bone marrow cells were collected by flushing murine femurs and tibias with complete media (cRPMI) comprised of RPMI (GibcoBRL, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT), 50 μM mercaptoethanol, 20 mM HEPES, 10 mM sodium pyruvate, and 50 μg/ml gentamicin (GibcoBRL, Grand Island, NY). Subsequent gradient centrifugation using the Lympholyte$^®$-M reagent (Cedarlane Laboratories Limited, Ontario, Canada) removed red blood cells. The hematopoietic cells were cultured at a density of 1x10^6 cells/ml in tissue culture flasks or 6-well plates in cRPMI supplemented with 30 ng/ml murine GM-CSF (PeproTech, Rocky Hill, NJ) and treated with AhR ligands or vehicle control. Cells were grown for 7 d at 37°C and 5% CO_2. Media, growth factor, and AhR ligand treatment were refreshed on days 3 and 5. On day 7, non-adherent cells representing immature DCs were harvested and purified using anti-CD11c (N418) beads (Miltenyi Biotec,

Auburn, CA) and the Miltenyi AutoMACS per the manufacturer's instructions. BMDC purity was verified via flow cytometry, and cells subsequently cultured were >90% CD11c+. Cells were ≥95% viable as determined by Trypan blue exclusion.

Flow cytometry. Accessory molecule expression on isolated cells was determined by flow cytometry, as previously described (Shepherd *et al.*, 2001). Briefly, cells were washed with PAB (1% bovine serum albumin and 0.1% sodium azide in PBS). Fc block (BioLegend, San Diego, CA) was used to eliminate non-specific staining. Optimal concentrations of fluorochrome-conjugated monoclonal antibodies were used to stain cells for an additional 10 min on ice. Antibodies used in these experiments included CD11c-APC (HL3), MHC2-PECy7 (M5/114.15.2), CD86-AlexaFluor700 (GL-1), CD80-PE (16-10A1), CD54-Pacific Blue (YN1/1.7.4), CD40-FITC (3/23), CD4-APC (RM4-5), CD25-PerCPCy5.5 (PC61), and their corresponding isotype controls, all of which were purchased from BioLegend (San Diego, CA) or BD Biosciences (San Jose, CA). One to five hundred thousand events were collected using a BD FACSAria flow cytometer and analyzed using FACSDiva (Version 6.1.2, BD Biosciences, San Jose, CA) and FlowJo (Version 8.7.1, TreeStar, Inc., Ashland, OR) software programs.

Cell activation and cytokine assays. Purified vehicle- or AhR ligand-treated BMDCs were cultured in 6-well plates at a density of $1x10^6$ cells/ml and stimulated with 1 μg/ml lipopolysaccharide [LPS, *Escherichia coli* (055:B5), Sigma-Aldrich] for 24 h. Cells were harvested for immunophenotypic and qRT-PCR analyses while supernatants were collected and evaluated for cytokine production using enzyme linked immunosorbent

assays (ELISAs). Levels of IL-1β, IL-2, IL-6, IL-10, IL-12, IFN-γ and TNF-α were measured using BD ELISA kits (BD Biosciences, San Jose, CA) per the manufacturer's instructions. Levels of nitric oxide (NO) were measured using the Griess Reagent System (Promega, Madison, WI) per the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR). RNA was isolated from BMDCs with Trizol reagent (Invitrogen, Carlsbad, CA) followed by RNA clean-up using the Total RNA Kit with optional DNase treatment (Omega Bio-Tek, Norcross, GA) according to the manufacturers' instructions. First strand cDNA synthesis was performed using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Resulting cDNA was subjected to qRT-PCR using commercially obtained primers (SABiosciences, Frederick, MD) including aldehyde dehydrogenase 1 family, member A1 (*ALDH1A1)*, aldehyde dehydrogenase 1 family, member A2 (*ALDH1A2)*, cytochrome P450 A1 (*CYP1A1*), indoleamine-2,3-dioxygenase 1 (*IDO1*), indoleamine-2,3-dioxygenase 2 (*IDO2*), interleukin 6 (*IL6*), interleukin 10 (*IL10*), interleukin 27 (*IL27*), latent binding protein 3 (*LTBP*), transforming growth factor, beta 1 (*TGFB1*), transforming growth factor, beta 2 (*TGFB2*), and transforming growth factor, beta 3 (*TGFB3*). Reactions were performed with PerfeCTa SYBR Green Supermix (Quanta Biosciences) on an Agilent Technologies Stratagene Mx3005P® QPCR System (Santa Clara, CA). Resulting data were normalized to β-actin, and fold changes were calculated using the ΔΔCt method, which compares threshold values of the samples of interest for a particular gene relative to a housekeeping gene.

NF-kB activity. DMSO-, I3C-, and IO-treated BMDCs were prepared and purified, as described above. Purified BMDCs ($2x10^6$ cells/well) were stimulated with LPS (1 µg/ml) for 45 min in 6-well plates. Cells were harvested, and nuclear protein extracts were prepared using the Active Motif Nuclear Lysis kit (Active Motif, Carlsbad, CA). Protein concentrations were measured using the Bradford reagent (Bio-Rad Laboratories, Hercules, CA), and nuclear protein (3 µg/well) was subsequently used in the Active Motif TransAM colorimetric assay to evaluate the DNA binding activity of NF-κB p65 and RelB per the manufacturer's instructions.

DC:T cell co-cultures. BMDCs were grown in the presence of the DMSO vehicle, I3C, or IO for 7 d and subsequently purified, as described above. BMDCs were cultured with 1 µg/ml $OVA_{323-339}$ peptide (Mimotopes, Clayton, Victoria, Australia) for 2 h and washed twice prior to culturing with T cells. Spleens as well as the popliteal and brachial lymph nodes of OTII Foxp3^{egfp} mice were harvested, and CD4+ T cells were purified to >75% using a naïve CD4 T cell isolation kit (Miltenyi Biotec, Auburn, CA) and the Miltenyi AutoMACS per the manufacturer's instructions. Cells were ≥95% viable as determined by Trypan blue exclusion. DCs and T cells were co-cultured for 3 d at a ratio of 1:5 (DC:T cells) in 96-well plates. On day 3, cells were harvested, evaluated via flow cytometry for the frequency of CD4+Foxp3+ T cells, and supernatants were collected for subsequent evaluation of cytokine production, as described above.

Statistical analyses. Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Dunnett's post-hoc test while data sets with 2 groups were analyzed by student's t-test. Values of $p < 0.05$ were considered significant.

RESULTS

Concentration-dependent effects of I3C and indirubin-3'oxime (IO) on the viability and proliferation of BMDCs. Since the effects of I3C and IO on DCs have not yet been evaluated and both compounds can trigger cell cycle arrest, the concentration-dependent effects on proliferation and viability of these dietary AhR ligands was initially examined in GM-CSF-derived BMDCs. Concentrations of I3C were based on physiologically relevant concentrations (reviewed in Howells *et al.*, 2007) while IO concentrations were based on other published *in vitro* studies (reviewed in Eisenbrand *et al.*, 2004), as the bioavailability of IO has not yet been determined to our knowledge. Neither compound affected the viability of the non-adherent, immature DCs at any concentration tested; however, all concentrations of I3C examined decreased DC cell numbers, the highest concentrations of I3C (100 μM and 200 μM) decreased cell proliferation by as much as 60% and 97%, respectively, as indicated by decreased cell numbers (Figure 1). The highest concentration of IO (5 μM) also significantly decreased DC cell numbers. Therefore, based on the results obtained in this initial experiment demonstrating no cytotoxicity and slight anti-proliferative effects, concentrations of 50 μM I3C and 1 μM IO were selected for subsequent experiments.

Figure 5.1
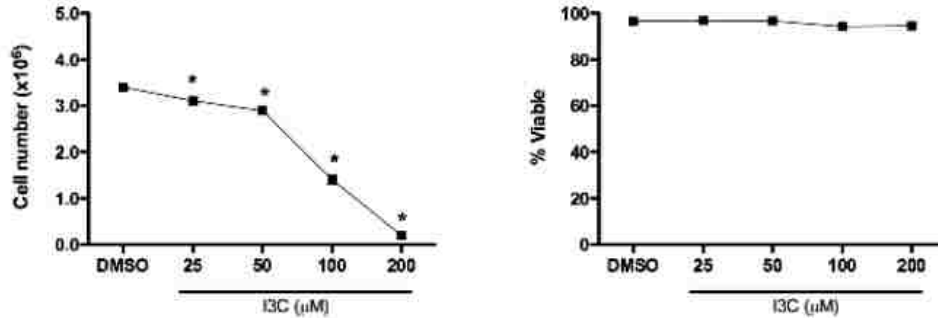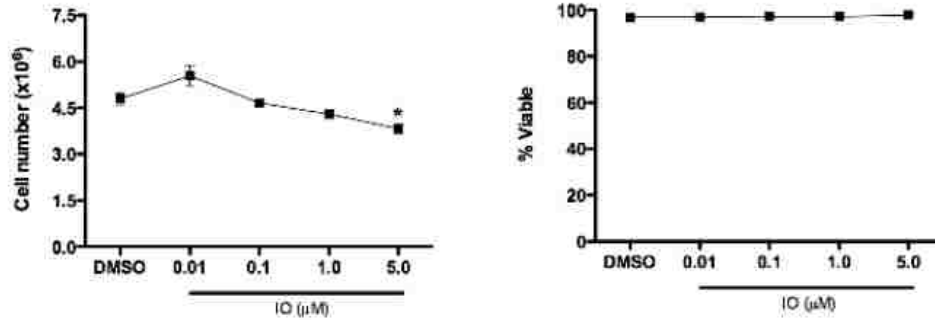
Figure 5.1. **Concentration-dependent effects of I3C and IO on BMDC proliferation and viability.** BMDCs were grown in the presence of the 0.1% DMSO vehicle control or various concentrations of I3C (A) and IO (B). After 7 d, non-adherent cells were stained with Trypan blue to determine cell numbers and viability. Results are representative of 3 separate experiments with n=3. *indicates significance of $p \leq 0.05$.

Immunophenotypic changes induced by I3C and IO. After determining non-cytotoxic concentrations of I3C and IO, the effects of these compounds on BMDC immunophenotype were evaluated via flow cytometry (Figure 2). Both compounds significantly decreased the relative expression of CD11c, CD40, and CD54 on BMDCs while the expression of MHC class 2 (MHC2), MHC2^{hi} and CD80 was increased. Differential effects were observed on CD86 expression, as I3C increased the expression of this co-stimulatory molecule while IO decreased its expression.

I3C and IO alter the LPS-induced changes in surface marker expression and cytokine production. DCs play critical roles in mounting appropriate immune responses to various pathogens and inflammatory insults; therefore, the effects of LPS stimulation on BMDCs treated with I3C or IO were examined. Alterations in cell surface molecule expression were assessed initially (Figure 3). Both compounds inhibited the LPS-induced upregulation of CD11c and CD40 while enhancing the LPS-induced increase in the expression of MHC2 and CD80. I3C upregulated CD86 and CD54; however, IO slightly decreased the LPS-induced expression of both CD86 and CD54.

The effects of I3C and IO on pro-inflammatory cytokine production following LPS activation in BMDCs were also assessed. As anticipated, 1 μg/ml LPS induced the production of several pro-inflammatory cytokines including IL-1β, IL-6, IL-12, and TNF-α. BMDCs exposed to I3C or IO and subsequently stimulated with LPS generally produced lower levels of pro-inflammatory cytokines (Figure 4). I3C and IO decreased the LPS-induced production of IL-1β from 1506 pg/ml to 1040 pg/ml and 941 pg/ml, respectively. This effect was also observed with the LPS-induced IL-6 production in

Figure 5.2
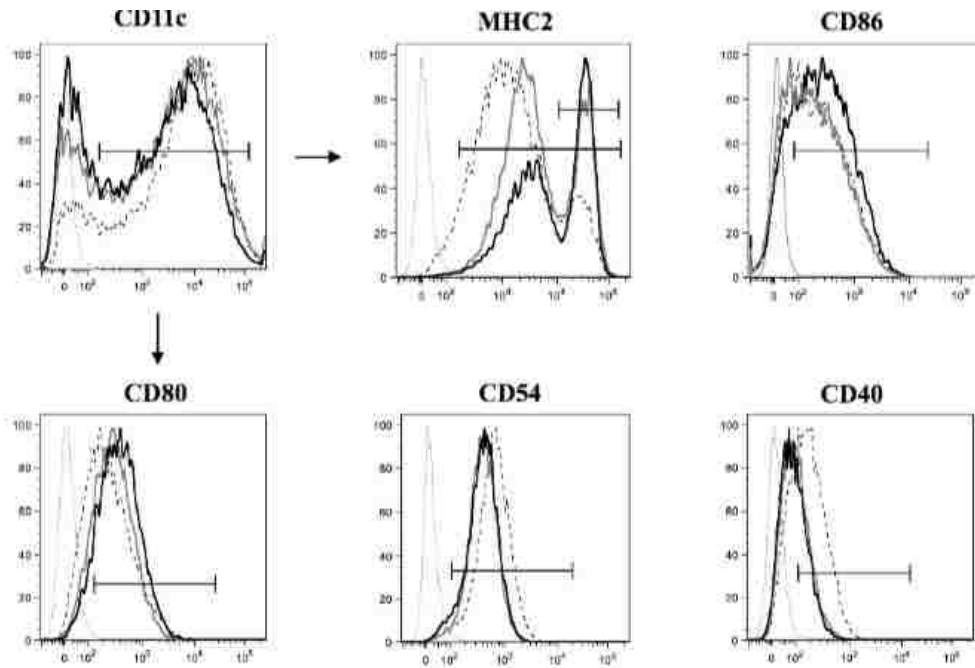
Surface marker	DMSO	I3C	IO
CD11c	18406 ± 655	12333 ± 775*	13919 ± 313*
MHC2	7176 ± 131	18453 ± 655*	16245 ± 843*
MHC2hi	33640 ± 350	36800 ± 691*	36496 ± 837*
CD86	386 ± 5	437 ± 10*	346 ± 8*
CD80	404 ± 5	521 ± 15*	445 ± 11*
CD54	749 ± 8	474 ± 8*	462 ± 6*
CD40	210 ± 4	106 ± 2*	130 ± 4*

Figure 5.2. Alterations in BMDC phenotype following AhR ligand treatment.
BMDCs were grown in the presence of the DMSO vehicle control, 50 μM I3C, or 1 μM IO for 7 d and subsequently evaluated for their relative expression (MFI) of surface markers via flow cytometry (numerical values are listed in the corresponding table below the histograms). Thin gray lines indicate isotype control, dotted black line indicate DMSO-treated cells, thick black lines indicate I3C-treated cells, and thick gray lines indicate IO-treated cells. Arrows indicate MHC2/MHC2^{hi}, CD86, CD80, CD54, and CD40 expression on CD11c+ cells. Results are representative of 3 separate experiments with n=3. *indicates significance of p≤0.05.

Figure 5.3
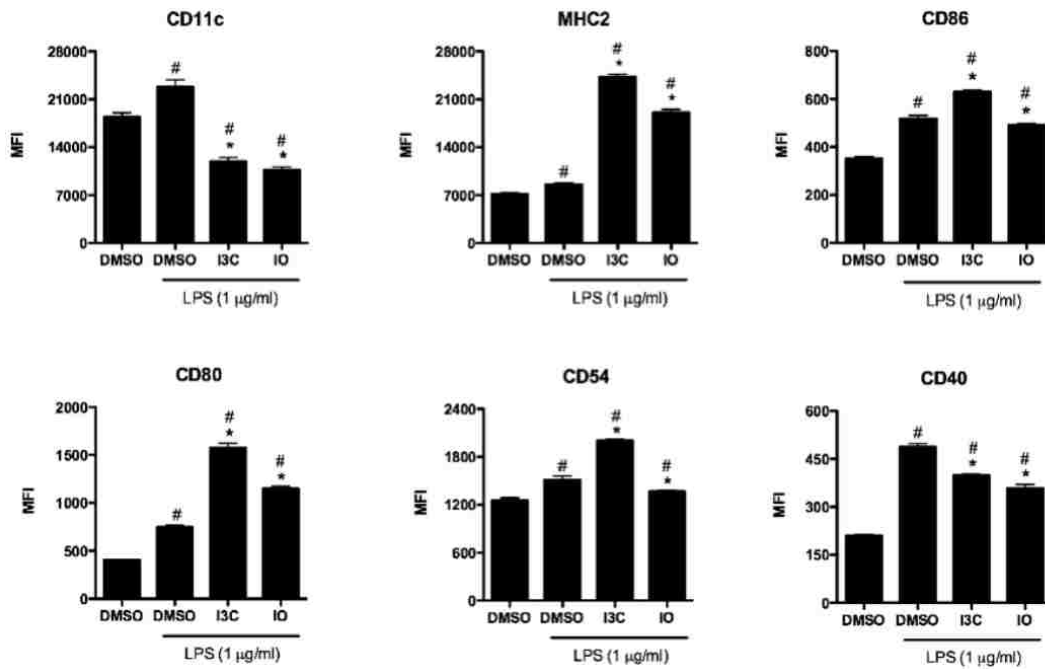
Figure 5.3. Dietary AhR ligands alter LPS-induced changes in surface phenotype of BMDCs. BMDCs were grown in the presence of the DMSO vehicle control, 50 μM I3C, or 1 μM IO for 7 d and purified. The immature BMDCs were subsequently treated with 1 μg/ml LPS for 24 h, and the immunophenotype was evaluated by flow cytometry. Results are representative of 3 separate experiments with n=3. #indicates significance of $p \leq 0.05$ compared to unstimulated DMSO control, *indicates significance of $p \leq 0.05$ compared to LPS-stimulated DMSO control.

Figure 5.4
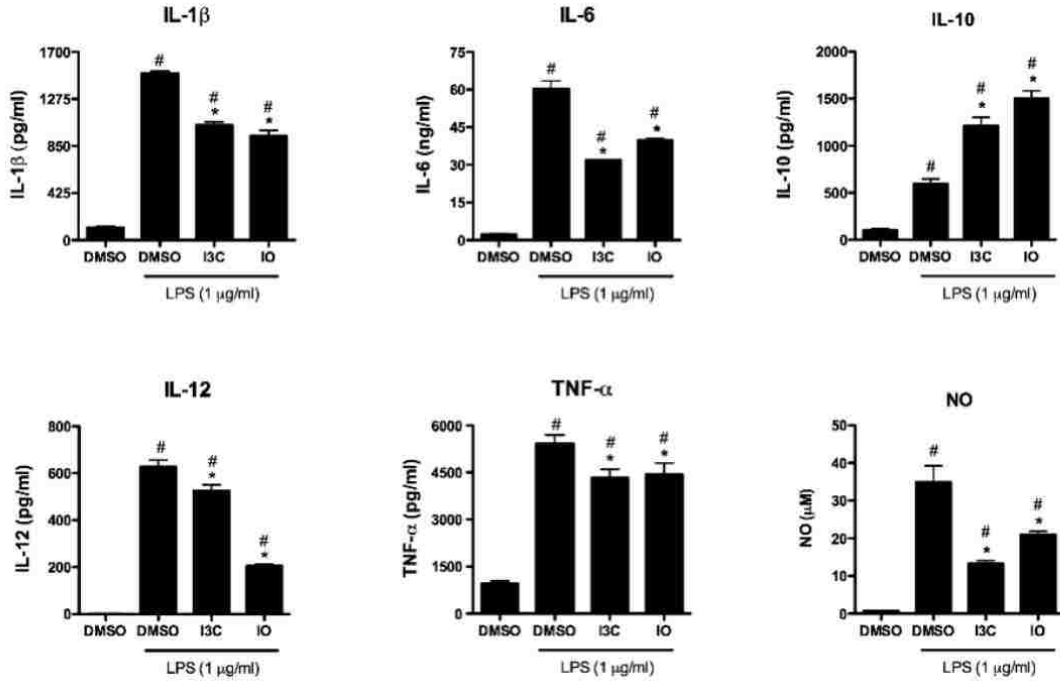
Figure 5.4. Suppression of LPS-induced pro-inflammatory cytokine production by I3C- and IO-treated BMDCs. BMDCs were grown in the presence of the DMSO vehicle control, 50 μM I3C, or 1 μM IO for 7 d and purified. The immature BMDCs were subsequently treated with 1 μg/ml LPS for 24 h, and protein levels of cytokines present in the supernatants were measured by ELISA. Results are representative of 3 separate experiments with n=3. #indicates significance of $p \leq 0.05$ compared to unstimulated DMSO control, *indicates significance of $p \leq 0.05$ compared to LPS-stimulated DMSO control.

which I3C and IO decreased IL-6 levels from 60 ng/ml to 32 ng/ml and 40 ng/ml, respectively. IL-12 production decreased from 628 pg/ml to 525 pg/ml with I3C and 204 pg/ml with IO. This effect was also observed with TNF-α, as this cytokine was decreased from 5415 pg/ml to 4338 pg/ml with I3C and to 4427 pg/ml with IO. Lastly, the LPS-induced nitric oxide (NO) production was suppressed from 35 μM to 13 μM with I3C and to 21 μM with IO. Conversely, I3C and IO increased the production of the anti-inflammatory cytokine IL-10 after LPS stimulation from 598 pg/ml to 1211 pg/ml and 1501 pg/ml, respectively. Taken together, these results suggest that I3C and IO significantly suppress the inflammatory responsiveness of LPS-stimulated BMDCs by inhibiting inflammatory mediator production and promoting anti-inflammatory mediator production.

I3C and IO induce immunoregulatory genes in BMDCs. AhR activation in DCs results in the induction of regulatory genes, an effect that correlates with a DC-induced expansion of CD4+Foxp3+ Tregs (Bankoti *et al.*, 2010; Quintana *et al.*, 2010; Simones and Shepherd, 2011). Thus, the expression of several key immunoregulatory genes was assessed in BMDCs treated with I3C or IO to further evaluate the immunosuppressive potential of these dietary AhR ligands (Table 1). Without stimulation, BMDCs grown in the presence of I3C upregulated *ALDH1A1* by 3.6 fold, *CYP1A1* by 5.2 fold, *IDO1* by 6.7 fold, *IDO2* by 5.7 fold, *TGFB2* by 2.4 fold, and *TGFB3* by 5.8 fold (Table 1A). Conversely, *ALDH1A2* and *IL27* decreased by 2.3 fold and 4.1 fold, respectively. Following LPS stimulation, *IDO1*, *IDO2, IL6, TGFB2*, and *TGFB3* was upregulated while *IL10, IL27,* and *TGFB1* decreased in I3C-treated BMDCs. Similar trends were

observed in IO-treated BMDCs with and without LPS stimulation (Table 1B). In unstimulated cells, IO upregulated *ALDH1A1* by 2.6 fold, *CYP1A1* by 4.7 fold, *IDO1* by 3.1 fold, *IDO2* by 3.0 fold, *TGFB2* by 3.5 fold, and *TGFB3* by 4.0 fold. In LPS-activated cells, *IDO1, IDO2, TGFB2*, and *TGFB3* were upregulated and *ALDH1A2, IL6*, and *IL27* were downregulated. Overall, in an inflammatory environment, I3C- and IO-treated BMDCs increase their expression of key immunoregulatory genes and thus may possess the potential to suppress immune responses.

AhR-dependence of I3C and IO on BMDCs. To investigate whether the observed effects of I3C were dependent on the AhR, BMDCs from AhR$^{-/-}$ mice were generated in the presence of I3C or IO. Surface marker expression of unstimulated and LPS-stimulated cells (Tables 2A and 2B) as well as cytokine production (Table 2C) was assessed. When AhR-deficient BMDCs were grown in the presence of I3C, changes in the expression of CD11c and CD54 were not observed relative to the vehicle control thereby indicating that the effects on these molecules were dependent on the AhR. The immunomodulatory effects of IO appeared to be more dependent on the AhR, as changes in CD11c, MHC2, CD80, and CD54 were not observed in unstimulated AhR$^{-/-}$ BMDCs. LPS-activated cells revealed that the expected alterations in the expression of CD11c on I3C-treated BMDCs, and CD11c, MHC2, CD80 and CD54 on IO-treated BMDCs were not observed in cells lacking the AhR. Additionally, suppression of many of the LPS-induced pro-inflammatory cytokines examined was observed in AhR-deficient BMDCs treated with I3C (IL-1β, IL-6, TNF-α and NO) and IO (IL-6, IL-12 and TNF-α).

Table 5.1. Dietary AhR ligands induce regulatory gene expression in unstimulated and LPS-stimulated BMDCs[a]

A. Indole-3-carbinol (I3C)

Gene	Unstimulated		LPS	
	Fold change	p-value	Fold change	p-value
ALDH1A1	3.6*	0.008	-1.1	0.420
ALDH1A2	-2.3*	<0.001	-3.4*	<0.001
CYP1A1	5.2*	0.008	1.0	0.475
IDO1	6.7*	0.001	13.5*	<0.001
IDO2	5.7*	<0.001	5.1*	<0.001
IL6	1.3	0.328	1.5*	0.008
IL10	-1.6	0.144	-1.6*	0.040
IL27	-4.1*	<0.001	-2.6*	<0.001
LTBP	-1.2	0.324	1.2	0.139
TGFB1	1.1	0.373	-1.3*	0.032
TGFB2	2.4*	<0.001	2.5*	0.001
TGFB3	5.8*	0.005	1.8*	0.034

B. Indirubin-3'-oxime (IO)

Gene	Unstimulated		LPS	
	Fold change	p-value	Fold change	p-value
ALDH1A1	2.6*	0.040	1.4	0.140
ALDH1A2	-1.2*	0.019	-1.4*	0.016
CYP1A1	4.7*	0.009	-1.4	0.262
IDO1	3.1*	0.004	6.6*	0.001
IDO2	3.0*	<0.001	4.2*	<0.001
IL6	1.2	0.367	-1.3*	0.016
IL10	-1.4	0.051	-1.2	0.262
IL27	-3.4*	<0.001	-3.1*	0.004
LTBP	1.4	0.407	1.6*	0.011
TGFB1	1.1	0.244	1.1	0.362
TGFB2	3.5*	0.001	3.2*	0.001
TGFB3	4.0*	0.027	1.7*	0.037

[a]Purified BMDCs grown in the presence of vehicle, 50 µM I3C or 1 µM IO for 7 d were activated with 1 µg/ml LPS for 24 h to evaluate changes in gene transcription by qRT-PCR. Fold change is relevant to vehicle-treated cells and normalized to β-actin. Results are representative of 3 separate experiments. *indicates significance of $p \leq 0.05$ compared to the vehicle control.

Table 5.2. Immune modulation by I3C and IO is not entirely AhR-mediated in BMDCs[a]

A. Immunophenotype of unstimulated cells

Surface marker	AhR^{+/+}			AhR^{-/-}		
	DMSO	I3C	IO	DMSO	I3C	IO
CD11c	3391 ± 142	2603 ± 326*	2219 ± 312*	4611 ± 548	4822 ± 107	3992 ± 280
MHC2	2833 ± 81	8056 ± 400*	6432 ± 255*	1970 ± 100	2912 ± 125*	2067 ± 100
CD86	1299 ± 15	1493 ± 3*	1270 ± 10	1279 ± 5	950 ± 27*	1180 ± 31*
CD80	1176 ± 23	1901 ± 8*	1412 ± 8*	839 ± 14	1129 ± 14*	822 ± 13
CD54	548 ± 7	507 ± 11*	435 ± 6*	649 ± 6	630 ± 12	633 ± 10
CD40	334 ± 72	290 ± 11*	291 ± 5*	409 ± 18	308 ± 16*	358 ± 6*

B. Immunophenotype of LPS-stimulated cells

Surface marker	AhR^{+/+}			AhR^{-/-}		
	DMSO	I3C	IO	DMSO	I3C	IO
CD11c	1950 ± 74	1702 ± 55*	1770 ± 96	2665 ± 74	2645 ± 98	2198 ± 174
MHC2	3182 ± 37	8189 ± 61*	6514 ± 170*	1817 ± 11	3386 ± 143*	1901 ± 71
CD86	1433 ± 9	1787 ± 21*	1370 ± 8*	1665 ± 5	1304 ± 8*	1523 ± 25*
CD80	1219 ± 29	3257 ± 92*	1874 ± 30*	981 ± 15	1544 ± 52*	998 ± 39
CD54	510 ± 6	545 ± 3*	439 ± 2*	583 ± 5	650 ± 12*	560 ± 17
CD40	452 ± 11	417 ± 12*	373 ± 21*	589 ± 12	497 ± 9*	499 ± 18*

C. Cytokine production following LPS stimulation

Cytokine	AhR^{+/+}			AhR^{-/-}		
	DMSO	I3C	IO	DMSO	I3C	IO
IL-1β (pg/ml)	1056 ± 53	813 ± 31*	992 ± 42*	1212 ± 56	862 ± 69*	1053 ± 52
IL-6 (ng/ml)	47 ± 2	21 ± 1*	24 ± 1*	47 ± 0.3	31 ± 1.6*	36 ± 2.6*
IL-10 (pg/ml)	598 ± 49	1211 ± 92*	1501 ± 83*	417 ± 67	127 ± 10*	543 ± 67
IL-12 (pg/ml)	1463 ± 159	984 ± 25*	427 ± 30*	1921 ± 140	2018 ± 93	1323 ± 88*
TNF-α (pg/ml)	5347 ± 181	5121 ± 135*	3962 ± 283*	7471 ± 603	5972 ± 280*	5040 ± 194*
NO (μM)	9.3 ± 0.3	2.4 ± 0.5*	7.5 ± 0.8*	28 ± 1	16 ± 2*	30 ± 2

[a]AhR^{+/+} and AhR^{-/-} BMDCs were grown in the presence of vehicle, 50 μM I3C or 1 μM IO and subsequently immunophenotyped (A) prior to or following activation (B) with LPS (1 μg/ml) for 24 h. Cytokine production was also assessed (C). Mean fluorescence intensity (MFI) and protein concentrations values are shown as mean ± standard error. Data are representative of 2 independent experiments with n=3. *indicates significance of p≤0.05 compared to DMSO control of respective cell type.

Conversely, the effects of I3C on IL-12 and the effects of IO on IL-1β, IL-10 and NO were demonstrated to be dependent on the AhR. Interestingly, IL-10 production was decreased in LPS-stimulated, AhR$^{-/-}$ BMDCs treated with I3C in contrast to the effects observed in AhR$^{+/+}$ BMDCs. Finally, the induction of several immunoregulatory genes also appeared to be independent of the AhR, as significant upregulation of *ALDH1A1, ALDH1A2, IDO1*, and TGFB2 was observed in I3C and IO-treated AhR-deficient BMDCs (data not shown). Thus, both I3C and IO exert effects on BMDCs by a mechanism that is partially, but not entirely, dependent on the AhR.

I3C and IO differentially alter NF-kB signaling in BMDCs. Because the immunomodulatory effects of I3C and IO on BMDCs were not entirely dependent on the AhR, NF-kB binding activity was evaluated since both I3C and IO can disrupt NF-kB signaling (Figure 5). In unstimulated cells, both I3C and IO reduced the activity of nuclear NF-kB p65; however, similar effects were not observed in LPS-activated DCs. Conversely, there was a trend for increased basal levels of RelB following IO but not I3C treatment, whereas I3C, but not IO, significantly decreased the levels of RelB following LPS activation.

I3C- and IO-treated BMDCs promote the generation of antigen-specific Tregs. AhR-activated immunoregulatory DCs can induce the generation of CD4+Foxp3+ Tregs (Quintana *et al.*, 2010; Simones and Shepherd, 2011); therefore, the potential for dietary AhR ligand-treated BMDCs to induce OVA$_{323-339}$-specific Tregs was investigated. Culturing naïve CD4+ OTII T cells for 3 days in the presence of I3C- or IO-treated,

OVA$_{323\text{-}339}$-loaded BMDCs significantly increased the frequency of CD4+Foxp3+ T cells (Figure 6A). When compared to vehicle-treated BMDCs, I3C treatment increased the frequency of OTII Tregs by approximately 2.4-fold while IO treatment increased the frequency by almost 2-fold. Cytokine production was also measured in the supernatants from these cultures (Figure 6B). OTII T cells cultured with IO-treated BMDCs significantly increased IL-2 production from 112 pg/ml to 6505 pg/ml. I3C- and IO-treated BMDCs decreased the production of IFN-γ in the co-cultures by approximately 33% and 45%, respectively. IL-10 production was also decreased by both the I3C- and IO-treated BMDCs when cultured with naïve OTII T cells.

Figure 5.5
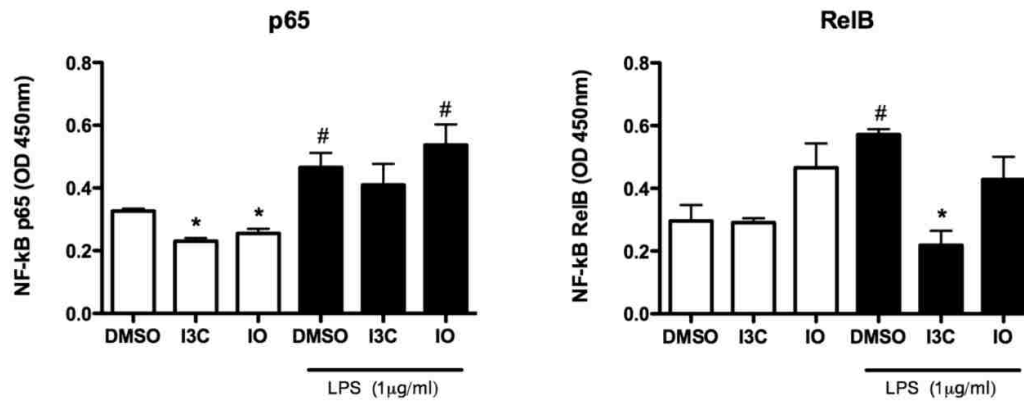
Figure 5.5. I3C and IO differentially alter NF-kB signaling. Purified I3C- and IO-treated BMDCs were stimulated with 1 µg/ml LPS for 45 min, and nuclear protein extracts were subsequently prepared for evaluation of the binding activity of NF-kB p65 (A) and RelB (B). Results are representative of 1 experiment with n=3. #indicates significance of p≤0.05 compared to unstimulated vehicle control and *indicates significance of p≤0.05 compared to vehicle control of similarly activated samples.

Figure 5.6

A. Treg frequency
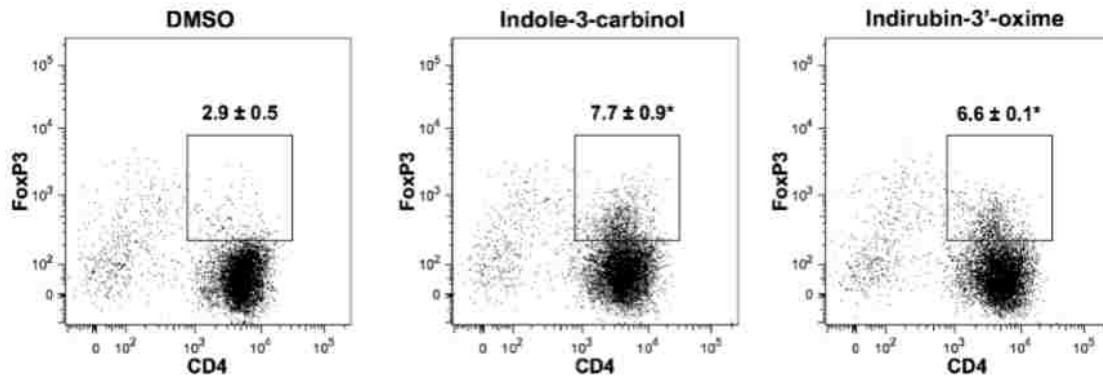
B. Cytokine production
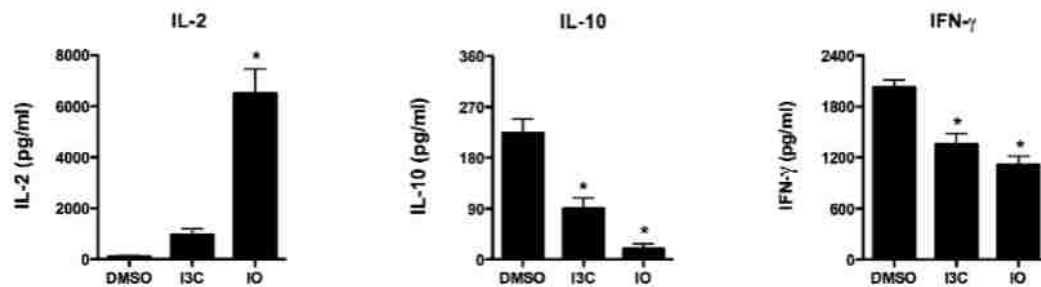
Figure 5.6. DCs treated with dietary AhR ligands increase the frequency of CD4+Foxp3+ Tregs and alter cytokine production. AhR ligand-treated BMDCs and naïve CD4+ OTII T cells were prepared as described in the *Materials and Methods*. (A) The percent of CD4+Foxp3+ T cells is shown in the histogram. (B) Cytokine production of IL-2, IL-10 and IFN-γ was also measured from the culture supernatants as described in the *Materials and Methods*. Results are representative of 2 separate experiments with n=3. *indicates significance of p≤0.05.

DISCUSSION

Modulating the fate and function of DCs has important therapeutic implications in the treatment of a large variety of immune-mediated diseases. The AhR signaling pathway can play an important role in shaping the fate and function of DCs in immune and inflammatory responses. Given that activation of the AhR generates immunoregulatory cells, including DCs and Tregs, it is essential to evaluate the effects of non-toxic AhR ligands, including agonists and antagonists, on critical cell populations in the immune system. I3C and indirubin are particularly interesting dietary AhR ligands since they both possess potent anti-cancer properties, but currently our knowledge of their effects on immune cells is limited. Therefore, in this study we investigated the potential of I3C and indirubin to promote immunosuppressive and anti-inflammatory effects via the AhR.

Following AhR activation in DCs, several changes in cell surface molecule expression, cytokine production, and regulatory gene induction contribute to suppressive and tolerogenic immune responses. The exogenous, prototypical ligand TCDD and the endogenous ligand 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) both decrease the expression of CD11c and upregulation of MHC2 and CD86 in murine BMDCs (Bankoti *et al.*, 2010). Alternatively, the small molecule VAF347 decreases CD86 and HLA-DR in human DCs (Ettmayer *et al.*, 2006) while the environmental contaminant benzo(a)pyrene (BaP) induces few changes in the expression of CD11c, MHC2, and CD86 on murine DCs (Hwang *et al.*, 2007). Consistent with the results from TCDD- and ITE-treated DCs, we demonstrated that I3C and IO decreased the expression of CD11c, CD40, and CD54 while increasing the expression of MHC2

and CD80. Decreased CD11c expression on DCs following exposure to I3C and IO suggests altered differentiation of these professional antigen-presenting cells. Furthermore, since CD40 and CD54 are critical immune molecules involved in the interactions of T cells with DCs, decreased expression of these markers on DCs suggests that their ability to successfully activate effector T cells may be defective. Although both I3C and IO are AhR agonists (Miller, 1997; Guengerich *et al.*, 2004), changes in the immunophenotype of BMDCs were not entirely dependent on the AhR as observed when using BMDCs from mice lacking the AhR. Similarly, it has been noted that the effects of BaP are not entirely AhR-dependent (Hwang *et al.*, 2007). This is distinctly different than the effects of TCDD, which are entirely dependent on the AhR (Bankoti *et al.*, 2010). Overall, the changes in surface marker expression on DCs treated with I3C and IO could be contributing to the generation of immune suppression, in part via AhR activation.

Cytokines are soluble mediators that play a critical role in influencing immune responses. Following activation by bacterial stimuli, such as LPS, DCs secrete many pro-inflammatory cytokines that can promote inflammatory responses. Consequently, we determined the effects I3C and IO on the LPS-induced production of inflammatory mediators. In general, we found that I3C and IO suppressed the secretion of many pro-inflammatory cytokines thereby indicating anti-inflammatory activity. Interestingly, TCDD was recently shown to increase the LPS-induced production of IL-6 and TNF-α (Bankoti *et al.*, 2010) whereas other AhR ligands including BaP, ITE, and VAF347 decrease the production of pro-inflammatory cytokines (Ettmayer *et al.*, 2006; Lawrence *et al.*, 2008; Quintana *et al.*, 2010). Thus, I3C and IO elicit cytokine patterns much more

similar to readily metabolized AhR ligands than TCDD, an extremely stable and persistent AhR agonist.

To further explore the immunosuppressive and anti-inflammatory properties of dietary AhR ligands, gene transcription was evaluated in unstimulated and LPS-activated BMDCs. Both I3C and IO upregulated *IDO* and *TGFB* genes, which have important implications in the generation of regulatory immune cells. Several investigators have demonstrated that AhR activation by TCDD in DCs induces regulatory genes that promote the expansion of Tregs (Mellor *et al.*, 2004; Tas *et al.*, 2007; Bankoti *et al.*, 2010; Simones and Shepherd, 2011). Our laboratory previously reported that TCDD induced *IDO1, IDO2,* and *TGFB3* in both unstimulated and LPS-stimulated BMDCs. Prior to activation, TCDD upregulated *TGFB3* while *TGFB1* and *TGFB2* were upregulated following LPS activation (Bankoti *et al.*, 2010). Importantly, the induction of IDO expression by DCs has been associated with the non-canonical NF-κB pathway (Tas *et al.*, 2007; Vogel *et al.*, 2008). Aldehyde dehydrogenase is associated with retinoic acid metabolism, which has been linked to regulatory DCs that drive Treg expansion. In our studies, *ALDH1A1* was upregulated by both compounds while *ALDH1A2* did not seem to have an important role in producing DCs of a regulatory phenotype. This effect was also observed with the endogenous ligand ITE, as *ALDH1A1* was induced in DCs possessing regulatory functions (Quintana *et al.*, 2010). Overall, upregulation of these immunoregulatory genes by I3C and IO suggest that these compounds have the potential to generate a regulatory environment that promotes the induction of regulatory T cells.

Because DCs acquired an immunoregulatory phenotype following treatment with I3C or IO, we evaluated their ability to generate Tregs from naïve CD4+ T cells in an

antigen-specific manner. In this study, I3C- and IO-treated BMDCs significantly increased the frequency of CD4+Foxp3+ T cells *in vitro*. These findings are consistent with a study that demonstrated that indirubin increased CD4+CD25+Foxp3+ T cells *in vivo* (Zhang *et al.*, 2007). However, in this study it was not determined if indirubin acted directly on the T cells or indirectly via other immune cells such as the DCs. To date, no studies have reported direct and/or indirect effects of I3C on Foxp3+ Treg induction. TCDD, VAF347, and ITE have all been shown to increase CD4+CD25+Foxp3+ Tregs (Hauben *et al.*, 2008; Quintana *et al.*, 2010; Simones and Shepherd, 2011). ITE was recently demonstrated to induce Tregs both directly via effects on T cells and indirectly by altering DC function. Moreover, these effects in the DCs were found to be dependent on the generation of retinoic acid, as Foxp3+ Treg generation was blocked following the addition of a retinoic acid inhibitor (LE135) to the DCs (Hauben *et al.*, 2008; Quintana *et al.*, 2010; Simones and Shepherd, 2011). On the other hand, a recent study from our laboratory demonstrated that DCs treated with TCDD increased the frequency of OVA-specific, CD4+Foxp3+ Tregs in an IDO-dependent manner (Simones and Shepherd, 2011). Thus, based on the imunoregulatory profiles of DCs exposed to different AhR ligands, it is possible that Treg induction can occur through several mechanisms including retinoic acid, kynurenines, and/or TGF-β.

Both I3C and indirubin have traditionally been used in anti-cancer and anti-inflammatory applications. To date, neither I3C nor IO has been specifically evaluated for their potential anti-inflammatory effects on DCs, and information regarding the effects of these compounds on other immune cells is also limited. I3C has been assessed *in vitro* using the murine macrophage cell line RAW264.7 and primary murine

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macrophages. These studies revealed that I3C decreased the LPS-induced production of several inflammatory mediators, such as nitric oxide (NO) and TNF-α, which has been associated with decreased translocation of NF-κB into the nucleus (Chen *et al.*, 2003; Tsai *et al.*, 2010; Chang *et al.*, 2011). Our laboratory has recently demonstrated that I3C ameliorates disease severity and inflammation associated with a murine model of colitis (manuscript in preparation), which is consistent with our previously published work regarding the anti-inflammatory and immunosuppressive effects of TCDD on colitis (Benson and Shepherd, 2011). The anti-inflammatory effects of indirubin have been considerably less studied. Kunikata and colleagues reported that indirubin inhibited IFN-γ production from human myelomonocytic HBL-38 cells, IL-6 production by murine splenocytes, and ear swelling in a murine model of delayed-type hypersensitivity (Kunikata *et al.*, 2000). Most recently, IO inhibited the LPS-induced production of NO, TNF-α, IL-1β, and PGE-2 by rat brain microglia cells via inhibition of NF-κB activation (Jung *et al.*, 2011).

Since suppression of NF-κB signaling has been associated with decreased inflammatory mediator production by both I3C and IO, it is likely that these compounds also interfere with NF-κB signaling to contribute to the anti-inflammatory responses we observed in BMDCs. The AhR can interact with NF-kB signaling components (Ruby *et al.*, 2005; Vogel *et al.*, 2007a; Vogel *et al.*, 2007b). Therefore, it is conceivable that I3C and IO may interact with the AhR and/or NF-κB in DCs to reduce their inflammatory responsiveness. In this study, we demonstrated that I3C and IO decrease the binding activity of NF-κB p65 found in the nucleus of the BMDCs, but only I3C significantly decreased RelB activity following LPS activation. Therefore, it is possible that altered

NF-κB signaling contributes, at least in part, to the suppression of LPS-induced inflammatory cytokine production in DCs. Overall, our data suggest that both the AhR and NF-κB signaling pathways may contribute towards the immunoregulatory and anti-inflammatory effects of I3C and IO in DCs. Additional studies utilizing DRE and NF-κB luciferase reporter assays conducted in DCs may help delineate the contribution of these pathways, as most studies on these compounds to date have been conducted in cancer cells. For example, it was shown that 60 μM I3C decreased relative luciferase activity in breast cancer cells with regards to NF-κB activity (Rahman KM *et al*., 2004) while the DRE-driven luciferase of indigoids has been documented in a derivative of human hepatoma HepG2 cells (Guengerich *et al*., 2004).

Given that the effects observed in this study were not entirely dependent on either the AhR or NF-κB, it is possible that other factors contributed to the observed effects, as IO has been reported to inhibit Src kinase activity and subsequently the phosphorylation of STAT3, which has been implicated as an important factor in Th17 cell differentiation (Nam *et al*., 2005; Kimura *et al*., 2007). AhR activation leads to the generation of Tregs and IO may decrease Th17 differentiation so these factors together may contribute to the observed immunosuppressive environment.

It should be noted that in the acidic environment of the stomach, I3C forms the acid condensation product diindolylmethane (DIM). Since I3C is rapidly metabolized into DIM following oral consumption, the use of I3C in cell culture experiments has been criticized, as it has been shown that DIM is primarily responsible for the anti-cancer and anti-proliferative effects *in vivo*. However, a recent study demonstrated that following the addition of I3C to cultured cells, DIM spontaneously forms and accounts for much of the

documented effects *in vitro* (Bradlow and Zeligs, 2010). Accordingly, we expect that DIM also formed in our cell cultures and thereby is at least partially responsible for the observed effects on murine BMDCs.

Collectively, we have demonstrated that the dietary AhR ligands I3C and IO exert anti-inflammatory and immunoregulatory effects on DCs *in vitro*. Although both of these compounds bind the AhR, the effects we observed on cell surface marker expression, cytokine production, and gene transcription were not entirely dependent on the AhR. Moreover, altered NF-kB signaling pathway may be contributing to the anti-inflammatory effects of both I3C and IO. Since no severe adverse reactions have been reported in humans consuming I3C or indirubin (clinical trials reviewed in Eisenbrand *et al.*, 2004; Minich and Bland, 2007), these compounds may ultimately be useful complementary therapeutics for treating inflammatory disorders by altering DC fate and function, consequently creating an immunosuppressive environment. Importantly, our results also suggest that the generation of regulatory DCs and T cells following exposure to I3C and indirubin may significantly reduce the utility of these natural products as anti-cancer reagents. It is plausible that different metabolism occurs *in vivo* versus *in vitro* leading to a differential effect in animals compared to cell culture. It is also possible that the anti-proliferative effects of I3C/DIM on tumor cells combined with additional anti-inflammatory and regulatory effects on the immune system may limit damage generated by the tumor cells permitting a more robust immune response. Therefore, focused in vivo studies to investigate these possibilities are warranted based on previously published studies and our results presented herein.

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CHAPTER 6: CONCLUSIONS

Crohn's disease is a multifactorial gastrointestinal disorder of unknown etiology that affects millions of people worldwide. The goal of current conventional therapeutics is to induce and maintain remission by controlling the inflammation that causes symptoms. This is accomplished with five basic categories of treatments (aminosalicylates, corticosteroids, immunomodulators, antibiotics, and biologics) that are used in combination to treat patients, as corticosteroids rapidly reduce inflammation while immune modulators help maintain remission, for example. These medications, however, must be tailored to each patient and are also accompanied by severe adverse effects, especially with extended use. Thus, there is a need to develop new therapeutics that more effectively and more safely control and possibly prevent intestinal inflammation.

The aryl hydrocarbon receptor (AhR) has emerged recently as an attractive therapeutic target for several disease states including those that involve chronic inflammation. The AhR is perhaps most recognized for mediating the toxic effects of its prototypical ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) via events that are both dependent and independent of dioxin response elements (DREs). A notable effect of AhR activation by TCDD is the generation of Foxp3+ regulatory T cells (Tregs), which potently suppress inflammation. Due to its severe adverse effects, TCDD would not be a realistic therapeutic option for human diseases. Therefore, there is a need to identify AhR ligands that selectively block DRE-driven transcription associated with TCDD toxicity but still repress acute phase genes. These selective AhR modulators ultimately have the

potential to be used in combination with current therapies for inflammatory diseases and hormone-dependent cancers since they do not exert the toxic effects associated with TCDD (McDougal *et al.*, 2001; Okino *et al.*, 2009; Murray *et al.*, 2010; Murray *et al.*, 2011; Smith *et al.*, 2011).

Since AhR activation by various ligands can generate immunoregulatory and anti-inflammatory effects and little is currently known about the role of the AhR in gut immune responses, we explored the role of AhR activation in the mucosal immune system and the potential for natural ligands to ameliorate intestinal inflammation without eliciting toxic effects. Our central hypothesis was that that *AhR agonists found in the diet have the potential to dampen inflammation associated with Crohn's disease*. We utilized a murine model of colitis to evaluate the role of the receptor in the gut and also conducted *in vitro* experiments to further investigate the mechanisms of dietary AhR ligands on important immune cells present the gut. The following sections highlight the primary conclusions from this project and future directions worth pursuing to extend the research.

Dietary AhR ligands modulate intestinal inflammatory responsiveness

The first goal of this project was to test the hypothesis that AhR activation by the prototypical AhR ligand TCDD would reduce the inflammation generated in a murine model of Crohn's disease by promoting an immunosuppressive environment in the gut. We demonstrated for the first time that TCDD suppressed 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced intestinal inflammation, as indicated by decreased disease severity, decreased inflammatory mediator production, and increased Foxp3+ Tregs in colon tissue

(Benson and Shepherd, 2011a). Thus, the amelioration of colitis likely occurred via the Treg generation following AhR activation.

Since TCDD is not a viable treatment option for Crohn's disease patients, it is necessary to identify other AhR agonists lacking the toxic effects associated with TCDD. Indole-3-carbinol (I3C) is a natural AhR agonist present in cruciferous vegetables that possesses the potential to dampen inflammation (Takada *et al.*, 2005; Kim *et al.*, 2009; Tsai *et al.*, 2010; Chang *et al.*, 2011). We found that I3C exerted sex-specific effects on disease severity by dampening inflammation in female, but not male, mice (Benson and Shepherd, 2011c). Due to the potential crosstalk with several signaling pathways, complex interactions of the AhR with the estrogen receptor and/or nuclear factor-kappa B (NF-kB) signaling pathways are likely responsible for this effect (Ohtake *et al.*, 2003; Wormke *et al.*, 2003; Matthews *et al.*, 2005).

Collectively, these *in vivo* studies demonstrated the importance of AhR activation in the gut by exerting anti-inflammatory and immunoregulatory effects. Several investigators have also reported an increased frequency of Tregs following AhR activation, which consequently suppressed autoimmune diseases induced in mice including multiple sclerosis (Quintana *et al.*, 2010), diabetes (Kerkvliet *et al.*, 2009), and uveoretinitis (Zhang *et al.*, 2010). With respect to colitis, several ligands have been shown to suppress intestinal inflammation following AhR activation. These compounds that activate AhR signaling include the I3C metabolite diindolylmethane (DIM) (Kim *et al.*, 2009), 6-formylindolo[3,2-b]carbazole (FICZ) (Monteleone *et al.*, 2011), and *Lactobacillus bulgaricus* OLL1181 (Takamura *et al.*, 2011). To our knowledge, however, I3C is the first documentation of an AhR ligand exerting sex-specific effects on

intestinal inflammation. This suggests that effects of ligands may vary based on the context of inflammation in certain tissues. Moreover, I3C is a commercially available supplement so it could have significant implications for self-medicating patients who want to relieve symptoms not suppressed by conventional medicine or to relieve the symptoms of the medicine.

Regulatory cells present in the gut are induced by natural AhR ligands

The second primary goal of this project was to investigate the mechanism(s) by which dietary AhR ligands exert their effects on important immune cells found in the gut. Dendritic cells (DCs) constitutively express the AhR and are a critical cell population in the gut that maintains tolerance by promoting the expansion of Tregs. We found that I3C, as well as the traditional Chinese medicine component indirubin, exerted anti-inflammatory effects via the reduction of lipopolysaccharide (LPS)-induced inflammatory cytokines in bone marrow-derived DCs (BMDCs). I3C and indirubin increased the expression of several regulatory genes including indoleamine-2,3-dioxygenase (*IDO*), transforming growth factor beta (*TGFB*), and aldehyde dehydrongenase (*ALDH1A)*. The ligand-treated BMDCs also induced Foxp3+ Tregs when co-cultured with antigen-specific naïve CD4+ T cells (Benson and Shepherd, 2011b). Collectively, the results obtained demonstrate the anti-inflammatory and immunoregulatory effects of I3C and indirubin.

Several mediators, especially IDO, TGF-β, and retinoic acid, are critical for maintaining a regulatory environment to prevent inappropriate responses to food particles and commensal bacteria in the gut. In line with our *in vitro* findings, it was recently

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demonstrated that immunoregulatory DCs suppressed TNBS-induced colitis, which was partially dependent on IDO (Hoshino *et al.*, 2011). In fact, IDO inhibition exacerbates TNBS-induced colitis (Gurtner *et al.*, 2003). Previous investigations in our laboratory revealed that TCDD generates regulatory DCs capable of inducing Foxp3+ Tregs in an IDO-dependent manner (Simones and Shepherd, 2011). Thus, it is possible that I3C or indirubin administered to mice would induce regulatory cells capable of suppressing disease severity in the gut. Furthermore, the results obtained in DCs could also be extended to intestinal epithelial cells (IECs), which are AhR-expressing antigen presenting cells that produce protective mediators necessary to prevent inappropriate immune responses to food particles and commensal bacteria. Importantly, IECs also condition DCs to have a regulatory phenotype via TGF-β and retinoic acid (Iliev *et al.*, 2009). Taken together, natural AhR ligands induce a regulatory phenotype in DCs without exerting overt cytotoxicity, which is crucial to effectively dampening inflammation, and thus may also be effective treatment options for those afflicted with inflammatory bowel diseases.

Future directions

Collectively, the data generated in this project demonstrate that the AhR plays an important role in mediating gut immune responsiveness. Dietary AhR ligands possess the potential to impart regulatory and anti-inflammatory functions by acting on several cell populations including DCs, intestinal epithelial cells, and lymphocytes. To expand this research, several additional studies would better define the role of the AhR in intestinal immune tissue. Since the studies in this project utilized an acute model of colitis, it is

necessary to confirm the observed effects of AhR ligands in a chronic model of colitis, which is in many ways more representative of human Crohn's disease. Another important avenue to pursue is the evaluation of the effects of AhR ligands in combination with conventional treatment (i.e. corticosteroids) that can rapidly reduce inflammation. Finally, one of the most fascinating topics in current research involves trying to understand how the gut microbiome interacts with the immune system. Thus, it would be useful to evaluate how AhR activation by various ligands in the gut mucosa specifically alters gut bacteria populations and their functions.

In general, the most recent therapeutic approaches under development for treating inflammatory bowel diseases are aimed at improving mucosal barrier integrity via stem cells, restoring homeostasis of the gut bacteria via probiotics, and identifying vehicles that deliver potent therapeutics directly to the intestines (Plevy and Targan, 2011; Scholmerich, 2011). Selectively modulating AhR activity is a novel approach to suppress disease severity, and our studies provide insight into natural AhR ligands that can dampen intestinal inflammation by promoting an immunosuppressive environment. Clearly, the AhR is a promising novel therapeutic target that warrants further investigation for its potential to improve the function of gut mucosal immune responses.

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APPENDIX 1

Colonic gene and protein expression of aryl hydrocarbon receptor deficient mice

OVERVIEW

Since the aryl hydrocarbon receptor (AhR) knockout (KO) mice in the University of Montana laboratory animal facility were unexpectedly less sensitive to colitis, preliminary studies were conducted to evaluate gene and protein expression in the colons of naïve AhR KO mice compared to naïve AhR wild-type (WT) mice. Since T cells play a critical role in mounting inappropriate immune responses in colitis, and inflammation causes the clinical signs of the disease, these two pathways were specifically evaluated in the gene expression analyses. Moreover, proteomics analyses are quite extensive so changes in proteins involved in these pathways were of particular interest.

METHODS

Laboratory animals. Six to eight week old AhR WT and AhR KO mice (both on the C57Bl/6 background) were bred and maintained in the animal research facility at the University of Montana. C57Bl/6 AhR WT mice were originally obtained from The Jackson Laboratories (Bar Harbor, ME) and bred in-house. C57Bl/6 AhR KO (B6.AhR[tm1Bra]) mice were obtained from Dr. Paige Lawrence (University of Rochester Medical College, Rochester, NY) and bred as previously described (Schmidt *et al*., 1996). All mice were housed under specific pathogen-free conditions and maintained on 12 h dark/light cycles.

SuperArray. Three AhR WT samples and three AhR KO samples were pooled for gene expression analysis. RNA and cDNA was prepared as described above. Resulting cDNA was subjected to the RT2 Profiler PCR Array Systems for T helper Cell Differentiation and NF-kB Signaling Pathway per the manufacturer's instructions (SA Biosciences, Frederick, MD). Reactions were performed with PerfeCTa SYBR Green Supermix (Quanta Biosciences) on an Agilent Technologies Stratagene Mx3005P® QPCR System (Santa Clara, CA). Resulting data were analyzed using SA Bioscience's Web-Based PCR Array Data Analysis tool.

Proteomics. The colonic mucosa was collected as previously described (Calpe-Berdiel *et al.*, 2007). Briefly, colons were removed, washed with ice cold PBS, and opened longitudinally such that the mucosal layer could be lightly scraped. Approximately 5 mg of mucosal tissue was assessed for protein expression, as described by Lai and colleagues (Lai *et al.*, 2011). Briefly, mucosa was solubilized, trypsinized, and fractionated via HPLC. MS was used to identify and quantify peptides. Resulting data were analyzed using Ingenuity Systems Pathway Analysis software (Ingenuity Systems Inc., Redwood City, CA).

RESULTS

Genes involved with Th cell differentiation and NF-kB signaling were upregulated in AhR KO mice. Components of Th cell differentiation and NF-kB signaling were investigated by commercially available array kits. Numerous genes involved in T helper cell differentiation were upregulated including *IL-4, IL-13, IFN-γ,* and *Tbx21* while only *Ccr6* was downregulated in AhR KO mice (Table 1A). No clear bias towards any T helper cell subset (ie Th1, Th2, Th17 or regulatory T cells) was observed. Moreover, many genes involved in the NF-kB signaling pathway were also upregulated while decreases in gene expression were not observed in AhR KO mice (Table 1B). Overall, gene expression data suggested no clear skewing of T helper cell subsets and also implied that KO mice may have enhanced NF-kB signaling thereby making them more susceptible to inflammation, which is still inconsistent with the results observed in the TNBS model.

AhR KO mice have decreased expression of proteins involved in NF-kB signaling, apoptosis, and antigen presentation. A total of 2374 proteins were identified in AhR WT and KO mice. Interestingly, more proteins were downregulated than upregulated, as shown in Tables 2 and 3. Of the proteins exhibiting at least a -1.5 fold change, there were several related to immune and inflammatory responsiveness in the gut including Card10, Mapk14 (p38-α), Bax, Fadd, and Ctsb. There were also several immune and inflammatory genes upregulated including Saa1, Fkpb5, Atrn, and Ctsl; however increased expression of these proteins are not consistent with the decreased inflammation observed in AhR KO mice following TNBS administration. Networking analyses performed with Ingenuity Systems software revealed that the top network with the

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protein expression fold changes at least +/- 1.5 is cell death, cellular movement, and gene expression (results not shown). There were 20 focus proteins in this network including Bax, Brp44L, Card10, Ctbp2, Ctsb, Eif2s2, Fadd, Hcfc1, and Pik3r6 among several others.

Table 1. Gene expression in the colon of AhR KO mice

 A. T helper cell differentiation

Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
Cacna1f	3.24	*Ifng*	16.40	*Il2ra*	4.10
Ccl7	2.42	*Il12b*	5.26	*Il4*	2.98
Ccr3	3.22	*Il12rb2*	7.39	*Il5*	3.85
Ccr6	-2.25	*Il13*	4.04	*Il9*	7.19
Csf2	4.04	*Il18r1*	2.31	*Myb*	2.11
Fasl	27.40	*Il18rap*	9.49	*Nr4a3*	2.31
Gata3	4.52	*Il1r1l*	3.13	*Stat1*	2.26
Gata4	4.13	*Il2*	16.52	*Stat4*	4.58
Havcr2	2.04	*Il21*	19.51	*Tbx21*	2.18
Icos	3.55				

 B. NF-kB signaling pathway

Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
Atf2	2.55	*Fos2*	2.68	*Ripk2*	2.68
Casp1	2.75	*Htr2b*	2.99	*Stat1*	2.31
Casp8	2.60	*Ifng*	12.04	*Tlr6*	2.35
Ccl2	2.45	*Il10*	5.46	*Tlr7*	4.63
Csf2	7.62	*Il1a*	5.50	*Tlr8*	3.48
Csf3	5.58	*Il1b*	4.26	*Tnf*	2.10
Egr1	2.17	*Il6*	24.59	*Tnfsf14*	4.68
Fasl	12.47	*Nlrp12*	13.09		

Colon tissue from AhR WT and KO mice was homogenized in Trizol and subjected to qRT-PCR to assess changes in gene expression. Genes with fold changes +/- 2.0 relative to WT mice are shown in the table. Data are representative of one experiment in which tissue from three mice were pooled for each group.

Table 2. Downregulated protein expression in AhR KO mice

Protein		Fold Change	p-value
Card10	Caspase recruitment domain-containing protein 10	-4.5	0.1
Eml5	Echinoderm microtubule-associated protein-like 5	-2.9	0.2
Bphl	Valacyclovir hydrolase	-2.7	0.05
Mptx	Mucosal pentraxin	-2.4	0.2
Plbd1	Phospholipase B-like 1	-2.3	0.08
Rab5b	RAB5B, member RAS oncogene family	-2.2	0.3
Hmbs	Porphobilinogen deaminase, Isoform 2	-2.1	0.09
Ctbp2	C-terminal binding protein 2	-2.1	0.2
Ccdc109a	Coiled-coil domain-containing protein 109A	-2.1	0.1
Srrm1	Serine/arginine repetitive matrix protein 1, Isoform 2	-2.0	0.01
Eif2s2	Eukaryotic translation initiation factor 2, subunit 2 (beta)	-2.0	0.04
Dhrs11	Dehydrogenase/reductase SDR family member 11	-2.0	0.04
Bax	Apoptosis regulator BAX	-2.0	0.01
Col6a3	Collagen alpha-3(VI) chain	-1.9	0.3
Ttll13	Tubulin polyglutamylase TTLL13	-1.9	0.01
Sec13	Protein SEC13 homolog	-1.9	0.08
Sema6a	Semaphorin-6A, Isoform 2	-1.9	0.05
Hdhd2	Haloacid dehalogenase-like hydrolase domain-containing protein 2	-1.9	0.05
Msh2	DNA mismatch repair protein Msh2	-1.9	0.03
Clpp	Putative ATP-dependent Clp protease proteolytic subunit, mitochondrial	-1.8	0.06
Npepl1	Aminopeptidase NPEPL1	-1.8	0.1
Wwp2	NEDD4-like E3 ubiquitin-protein ligase WWP2	-1.8	0.2
Tmem43	Transmembrane protein 43	-1.7	0.2
Rbm3	Rbm3 protein	-1.7	0.2
Pik3r6	Phosphoinositide 3-kinase regulatory subunit 6, Isoform 2	-1.7	0.09
Cbara1	Calcium uptake protein 1, mitochondrial	-1.7	0.09
Rpl7A	Ribosomal protein L7A	-1.6	0.4
S100a11	Protein S100-A11	-1.6	0.3
Cask	Peripheral plasma membrane protein CASK	-1.6	0.5
Pcdh1	Protocadherin 1	-1.6	0.1
Sacm1l	Phosphatidylinositide phosphatase SAC1	-1.6	0.4
Mtnd4	NADH-ubiquinone oxidoreductase chain 4	-1.6	0.2
Ndufb7	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	-1.6	0.2
Parp3	NAD+ ADP-ribosyltransferase 3 PARP-3	-1.6	0.3
Ttc39a	Tetratricopeptide repeat protein 39A, Isoform 2	-1.6	0.06
Phactr4	Phosphatase and actin regulator 4, Isoform 2	-1.6	0.3
Hcfc1	Host cell factor 1	-1.6	0.06
Gm10093	Histone deacetylase	-1.6	0.1
Gcn1l1	GCN1 general control of amino-acid synthesis 1-like 1	-1.6	0.03

Ndufaf1	Complex I intermediate-associated protein 30, mitochondrial	-1.6	0.5
Brp44l	Brain protein 44-like	-1.6	0.3
Nars	Asparaginyl-tRNA synthetase, cytoplasmic	-1.6	0.2
Gm12597	Alpha-interferon	-1.6	0.3
Smpdl3a	Acid sphingomyelinase-like phosphodiesterase 3a	-1.6	0.3
Acss2	Acetyl-coenzyme A synthetase, cytoplasmic	-1.6	0,2
Tmem14c	Transmembrane protein 14C	-1.5	0.4
Slc35c2	Slc35c2 protein	-1.5	0.3
Shank1	SH3 and multiple ankyrin repeat domains protein 1	-1.5	0.2
Rab11fip1	Rab11 family-interacting protein 1	-1.5	0.02
Fadd	Protein FADD	-1.5	0.1
Pfdn5	Prefoldin subunit 5	-1.5	0.2
Mtx2	Metaxin 2	-1.5	0.2
Mapk14	Mitogen-activated protein kinase 14, Isoform 3	-1.5	0.2
Ppp1r12a	Protein phosphatase 1 regulatory subunit 12A, Isoform 2	-1.5	0.1
Picalm	Phosphatidylinositol-binding clathrin assembly protein, Isoform 2	-1.5	0.1
Carkd	Carbohydrate kinase domain-containing protein, Isoform 2	-1.5	0.3
Ppip5k1	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 1	-1.5	0.06
Ccdc47	Coiled-coil domain containing 47	-1.5	0.2
Coq3	Coenzyme Q3 homolog methyltransferase	-1.5	0.3
Ctsb	Cathepsin B	-1.5	0.2
Rpl31	60S ribosomal protein L31	-1.5	0.04

Colonic mucosa from naïve AhR WT and KO mice were analyzed for protein expression, as described in *Materials and Methods*. Fold changes of $\geq$ -1.5 compared to WT mice are listed in the table. Results are representative of one experiment with n=3.

Table 3. Upregulated protein expression in AhR KO mice

Protein		Fold Change	p-value
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	1.5	0.3
Saa1	Serum amyloid A protein	1.5	0.4
Reep5	Receptor expression-enhancing protein 5	1.5	0.3
Rab27b	RAB27b, member RAS oncogene family	1.5	0.2
Ndufa7	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	1.5	0.3
Obscn	Obscurin, Isoform 3	1.5	0.3
Fkbp5	FK506 binding protein 5	1.5	0.3
Col5a3	Collagen type V alpha 3 chain	1.5	0.3
Cc2d2a	Coiled-coil and C2 domain-containing protein 2A	1.5	0.3
Clns1a	Chloride channel, nucleotide-sensitive, 1A	1.5	0.3
Cisd1	CDGSH iron-sulfur domain-containing protein 1	1.5	0.5
Atrn	Attractin	1.5	0.04
Rpl28	60S ribosomal protein L28	1.5	0.2
Prkaa1	5'-AMP-activated protein kinase catalytic subunit alpha-1	1.5	0.1
Psmd9	26S proteasome non-ATPase regulatory subunit 9	1.5	0.3
Snrpd2	Small nuclear ribonucleoprotein Sm D2	1.5	0.1
Kcnc3	Potassium voltage-gated channel subfamily C member 3	1.5	0.2
Ube2d2	Ubiquitin carrier protein	1.5	0.3
Rangap1	Ran GTPase-activating protein 1	1.5	0.2
Snrpd2	Small nuclear ribonucleoprotein Sm D2	1.6	0.1
Mlec	Malectin	1.6	0.2
Cmas	N-acylneuraminate cytidylyltransferase, Isoform 2	1.6	0.2
Hdgf	Hepatoma-derived growth factor	1.6	0.1
Ctsl	Cathepsin L	1.6	0.006
Ist1	IST1 homolog	1.6	0.2
Nme1	Nucleoside diphosphate kinase (Fragment)	1.7	0.2
Sh3bgrl3	SH3 domain-binding glutamic acid-rich-like protein 3	1.8	0.04
Mt1	Metallothionein-1	1.8	0.2
Pgm3	Phosphoacetylglucosamine mutase	2.1	0.06
Rps11	40S ribosomal protein S11	2.5	0.1

Colonic mucosa from naïve AhR WT and KO mice were analyzed for protein expression, as described in *Materials and Methods*. Fold changes of $\geq +1.5$ compared to WT mice are listed in the table. Results are representative of one experiment with n=3.

DISCUSSION

Since our results in both AhR WT and KO mice were quite different than the published data, we further investigated the gut of naïve AhR KO mice for factors that may contribute the unexpected immune response in TNBS colitis. Thus, we screened gene expression of T helper cell differentiation and NF-kB signaling components. Gene expression data did not indicate an increase in a specific T helper cell subset and actually indicated heightened NF-kB signaling response. Again, these results are consistent with the literature in that an exacerbated disease state would be expected in AhR KO mice during colitis, which is not what we observed in TNBS-treated colitic mice.

Proteomic analyses, however, revealed decreased expression of many proteins involved in NF-kB signaling, apoptosis, and antigen presentation. The proteins involved in these physiologic pathways have important roles in the intestinal immune system, so their decreased expression may at least partially account for the decreased disease severity observed in AhR KO mice treated with TNBS. Dampening NF-kB signaling via Card10 and Mapk14 can decrease inflammation. Since increased gene expression of NF-kB signaling components was observed, this could be indicative of a compensatory mechanism of increasing gene expression due to decreased response to stimuli. Decreased apoptosis via Bax and Fadd has been reported to have a protective effect in intestinal epithelial cells (IECs), as increased apoptosis in IECs affects barrier integrity thereby exacerbating colitis. In Crohn's disease patients, apoptotic rates of IECs are increased from 2% to 5%, which enables luminal antigens to enter mucosal tissue (Schulzke *et al.*, 2006). Moreover, anti-TNF therapy decreases apoptosis and restores epithelial barrier integrity (Zeissig *et al.*, 2004). Since the TNBS model of colitis

revolves around TNBS binding colonic proteins prior to recognition by the immune system, decreased antigen presentation would dampen the ensuing immune insult typically caused by TNBS. However, it must be emphasized that protein expression was evaluated in whole mucosa and not specific cell populations. Thus, the results may not accurately reflect what is occurring in each cell population that can alter immune responsiveness in the gut.

The data presented in Appendix 1 is preliminary so future studies could be conducted to confirm these results. It is also expected that by extending these analyses into TNBS-treated AhR WT and AhR KO mice we may be better able to understand how gene and protein expression is changed in the context of this potent inflammatory stimulus. Ultimately this data could help delineate specific pathways responsible for the decreased inflammation observed in our AhR KO mice.

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