

**THE ROLE OF THE ARYL HYDROCARBON RECEPTOR IN INFLAMMATION-
ASSOCIATED COLORECTAL TUMORIGENESIS**

By

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ABSTRACT

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Colorectal cancer (CRC) is the second leading cause of cancer-related mortality in the United States representing a significant social and economic burden. Chronic inflammation and disturbances of the intestinal barrier increase the risk for the development of colorectal cancer. The proper functioning of the intestinal barrier requires the regulation of transcriptional signaling that maintain the balance between intestinal epithelial, local immune cells and intestinal bacteria. Evidence indicates that the aryl hydrocarbon receptor (AHR) is intimately involved in the maintenance of the intestinal barrier by regulating immune cells and modulating inflammation. The AHR is a ligand-activated transcription factor that was identified as an environmental sensor for its role mediating the adaptive metabolism of xenobiotics. In this thesis, we sought to determine the role of the AHR in inflammation-associated colorectal cancer. First, we review the literature related to the role of the AHR in intestinal biology and discuss the AHR-regulation of immune cells and inflammation within the intestinal barrier. Next, we investigated the involvement of the AHR inflammation-associated CRC using both an *Ahr*-deletion mouse model and treatment with an AHR ligand to activate the receptor

signaling. We found that the AHR is protective against the development of inflammation-associated CRC. Next, we examined the segregation of responses to inflammation-associated CRC with the *Ahr* locus. Finally, we provided preliminary data to identify the cell type where the activation of the AHR is required for its protective effect.

DEDICATION

This thesis is dedicated to my lovely family for they unconditional support and love. To my father: Rafael Diaz-Espino, my mother: Luz M. Diaz, my sisters: Jennifer Diaz-Diaz and Diana Diaz-Diaz. To my life partner Carlos Coriano and to my two angels:

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LIST OF ABBREVIATIONS

AHR	Aryl Hydrocarbon Receptor
ILC	Innate lymphoid cells
IEL	Intraepithelial lymphocytes
DC	Dendritic Cells
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
PAHs	Polycyclic aromatic hydrocarbons
PAS	Per-Arnt-Sim
AHH	Aromatic hydrocarbon hydroxylase
NLS	Nuclear localization signal
DRE	Dioxin response elements
P450	Cytochrome P450
AHRR	Aryl Hydrocarbon Receptor Repressor
hAHR	Human Aryl Hydrocarbon Receptor
DV	<i>Ductus Venosus</i>
HAHs	Halogenated aromatic hydrocarbons
CRC	Colorectal Cancer
FAP	Familial Adenomatous Polyposis
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CD	Crohn's disease
DSS	Dextran sodium sulfate
AOM	Azoxymethane
I3C	Indole-3-carbinol
DIM	3,3'-diindolylmethane
ICZ	Indolo [3,2-b] carbazole
FICZ	6-formylindolo[3,2-b]carbazole

LTr-1	2-(indol-3-ylmethyl)-3,3'-diindolylmethane
ILC	Innate lymphoid cells
IEL	Intraepithelial lymphocytes
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF α	Tumor Necrosis Factor- α
IL-6	Interleukin 6
IL-1 β	Interleukin 1 beta
WT	Wild-type
IP	Intraperitoneal
HPLC-UV	High-performance liquid chromatography-ultraviolet

Chapter 1

Literature review:

The aryl hydrocarbon receptor: as a regulator of intestinal biology

Chapter 1

The aryl hydrocarbon receptor: as a regulator of intestinal biology

1.1 Abstract

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcriptional factor that was identified through its role in mediating the adaptive metabolism of xenobiotics such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene, and numerous other polycyclic aromatic hydrocarbons (PAHs). In recent years, numerous studies have shown that AHR is also involved in normal development and physiology. Evidence from multiple laboratories supports the idea that the AHR plays a role in the physiology of barrier organs including the intestinal barrier. In this review, we discuss current knowledge related to the role of the AHR in intestinal biology. First, we provide a summary of the evidence that demonstrates that the AHR is a regulator of intestinal immunity, inflammation, and microbiota. This summary is followed by a discussion of the modulation of the AHR and its signalling as therapy for intestinal diseases and colorectal cancer. Then, we provide a description of the fundamentals of the AHR system including its signal transduction pathways and a description of how the receptor mediates further responses to xenobiotics.

1.2 Introduction

The aryl hydrocarbon receptor (AHR) is an environmentally responsive nuclear receptor that belongs to the Per-Arnt-Sim (PAS) superfamily [1, 2]. The AHR was initially identified through its role in the adaptive metabolism of polycyclic aromatic hydrocarbons (PAHs) and structurally related xenobiotics [3]. In recent years, evidence has accumulated that the AHR plays an essential role in normal animal physiology and development [4-14]. Various experiments focusing on the physiological role of AHR have demonstrated that this receptor is highly expressed in barrier organs such as the skin, the lungs and the intestines [12, 15-17]. The idea that barrier organs represent the first line of defense against external insults is consistent with our initial understandings of AHR biology as a sensor of environmental stimuli. One idea that is emerging is that the AHR may modulate signals important in the regulation of and homing immune cells to barrier epithelia [9, 12, 13, 18, 19]. Several reports describe relationship between AHR and the proper functioning of the intestinal barrier through the modulation of the resident immune cells, inflammatory responses and microbiota interactions [18, 20-35]. Often, this relationship is mediated by the activation of the AHR, by dietary ligands, within the intestinal epithelium [20, 30]. For example, dietary pro ligands, such as indole 3-carbinol (I3C), appear to initiate an AHR signaling cascade that modulates intraepithelial lymphocytes activity, intestinal lymphoid follicle formation and mucosal homeostasis [20, 30]. Taken in sum, these observations indicate that the AHR may be found to be a central player in the etiology and prevention of intestinal pathology.

The purpose of this review is to provide a summary of the current knowledge of the role of AHR signaling in intestinal barrier, specifically as it relates to immune and

inflammatory response coordination and intestinal microbiota modulation. Additionally, we will briefly discuss the role of AHR in intestinal pathologies and how the modulation of the receptor by dietary ligands may ultimately be critical to the prevention and treatment of these diseases.

1.3 The AHR in intestinal health

The intestinal barrier protects the body against external environmental insults while also serving as a selective filter to allow the absorption of nutrients and electrolytes [36, 37]. The intestinal barrier functions, primarily, by maintaining symbiotic interactions with the microbial community and immune cells within the intestinal epithelium [38, 39]. The dynamic crosstalk between these key components of the intestinal barrier represents the foundation of intestinal homeostasis [39]. The intestinal homeostasis is intricate and requires the regulation of transcriptional signaling networks through responses to intercellular communications [40].

One critical regulator of the intestinal barrier is the AHR. The AHR has been shown to be expressed in multiple intestinal cells types and this expression seems to be essential for its regulatory functions [12, 20-23, 41, 42]. For example, the AHR is a critical modulator of the innate lymphoid cells (ILC) and a regulator of intraepithelial lymphocytes (IEL) preservation in the intestines [12, 20, 30]. Both cell populations are important players that control intestinal homeostasis by regulating innate immune responses in addition to maintaining appropriate microbial loads and composition [43, 44]. It has been reported that the AHR is instrumental in the development of intestinal lymphoid organs such as crypto patches through the maintenance of ROR γ t⁺-expressing innate lymphoid cells (ROR γ t⁺ ILC) [45]. Studies using transgenic mice revealed that the postnatal development and expansion of ROR γ t⁺ ILC were impaired in *Ahr* null mice compared with WT mice [10, 46]. The AHR also has been implicated in the regulation of the expression of genes that are involved in the development and

maintenance of RORyt⁺ ILC such as the receptor tyrosine kinase (Kit) and Notch [21, 47, 48]. Although the exact mechanisms underlying AHR regulation of these genes remains unclear, it is known that the AHR is required for maintenance of RORyt⁺ ILC, principally at mucosal sites [48]. In a separate experiment, mice fed a diet lacking AHR ligands showed reduced formation of intestinal lymphoid follicles, RORyt⁺ ILC, and low levels of Kit, while mice fed a diet containing AHR ligands displayed increased levels of both, RORyt⁺ ILC and expression of Kit [21].

These data indicate that the AHR is critical in sustaining adequate and functioning numbers of ILCs over time and that its activation by dietary ligands control the development of RORyt⁺ ILC [10, 30]. The RORyt⁺ ILC are a subset of innate lymphocytes that reside in the lamina propria and are an important source of interleukin (IL)-22 and IL-17 [49, 50]. Importantly, *Ahr* null mice exhibit decreased levels of IL-22 and increased susceptibility to *Citrobacter rodentium* infections [21]. The IL-22 is a cytokine required for the protection against *C.rodentium* infections by the production of antimicrobial peptides (AMPs) [51, 52]. Further experiment demonstrated that the AHR mediate the production of IL-22 by regulating ILC22 [21]. The ILC22 includes a subset of natural killer (NK) cells marker NKp46 (NKp46⁺ ILCs) that require the AHR signal for their development [21].

In addition to ILC, the intestinal barrier harbors a T cell subpopulation known as intraepithelial lymphocytes (IEL). The IEL are an important part of the mucosal host defense against microorganisms and they also participate in epithelial tissue repair [53, 54]. The fact that IELs require AHR activity is indicated by the reports that; 1) *Ahr* null mice exhibit a reduced number of IELs [55], 2) in the absence of AHR ligands, IELs

function is impaired and intestinal epithelial cell turnover are defective [20, 21], and 3) the maintenance of IELs depends critically on T-cell specific AHR expression [23, 46]. Additionally, similar to RORyt⁺ ILC, the number of IEL cells were rescued to normal levels in mice fed a diet containing AHR ligands compared with those fed a diet depleted of AHR ligands [20]. These results demonstrate that the activation of the AHR by dietary compounds has a profound impact on intestinal immune cell function (Figure 1.1)

In addition to IEL and ILCs, it has also been reported that the AHR regulates the differentiation and function of intestinal dendritic cells (DC) and macrophages [11]. Experiments using transgenic mice showed that the expression of the AHR in intestinal mucosal DC and macrophages is required for the development of intestinal epithelial cells [11]. Following exposure to dextran sodium sulfate (DSS), an epithelial damaging agent, mice lacking the AHR in DC displayed increased susceptibility to epithelial injury as defined by decreases in overall body weight and colon length [11]. Similarly, the *Ahr* null mice exhibited higher DSS-induced colitis than the WT mice [20]. Interestingly, our laboratory observed that mice harboring the lower binding affinity allele of the AHR displayed both higher intestinal basal inflammation and increased susceptibility to DSS-induced colitis, characterized by increased levels of pro-inflammatory cytokines and increased incidence of prolapse, compared with mice harboring the high-affinity allele of the AHR. Collectively, these observations indicate that the AHR mediates intestinal inflammation and that the high susceptibility of *Ahr* null mice to intestinal damage may be due to intestinal epithelial barrier malfunction and disrupted homeostasis as result of decrease levels of RORyt⁺ ILC, IL-22 and IEL (Figure 1.2).

The gastrointestinal tract harbors an impressive reservoir of microbes that can both generate AHR ligands that may play a role the maintenance of the intestinal barrier [28, 56, 57]. For example, *Lactobacillus reuteri* produces indole-3-aldehyde (IAld) an indole metabolite that activates AHR and promotes intestinal homeostasis via induction of IL-22 [33]. It has also been shown that the metabolism of tryptophan by *Lactobacillus acidophilus* provides protection against *Candida albicans* infection by AHR-dependent expression of IL-22 [33]. An independent study reported that the commensal probiotic *Lactobacillus bulgaricus* ameliorated DSS-colitis by microbiota-mediated activation of AHR signaling [58]. Thus, this modulation of AHR by gut microbial metabolites is a contributor to the protection of the host against excessive inflammation and infections and for the regulation of intestinal homeostasis (Figure 1.1)

Taken together, these observations support the idea that the AHR is required for the preservation of a healthy intestinal barrier. The AHR-regulation of intestinal homeostasis is a multifactorial process that involves the development, control, and maintenance of immune and epithelial cells and the regulation of host microbiota through the metabolism of dietary ligands. (Figure 1.2). A proper intestinal homeostasis is essential for an adequate immunosurveillance and a healthy intestinal epithelium.

1.4 AHR modulation as therapy for intestinal pathologies

Disturbances of the intestinal barrier can increase the risk for intestinal pathologies including inflammatory bowel disease [59]. Inflammatory Bowel Diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease (CD) are archetypical inflammatory conditions of the intestinal barrier that are perpetuated by the adaptive and innate immune [60]. Ulcerative colitis is characterized by chronic inflammation of the colon and rectum whereas CD encompasses inflammation in any part of the gastrointestinal tract [61]. As a regulator of intestinal inflammation, the AHR has been proposed as a therapeutic target for IBD. Various independent studies in mice using the dietary AHR ligand I3C in DSS-induced colitis have reported both, attenuated colitis phenotypes and protection against the development of colitis [62, 63].

Indole-3-carbinol is a proagonist found in cruciferous vegetables such as broccoli, Brussels sprouts and cabbage [64]. Under the acidic environment of the stomach, I3C undergoes condensation reactions to form several metabolites such as 3,3'-diindolylmethane (DIM) and indolo[3,2-b]carbazole (ICZ) [65-68]. Of these products, ICZ possess the highest affinity for binding to the AHR [69]. In a model of colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS), the treatment with the AHR ligand 6-formylindolo[3,2-b]carbazole (FICZ) decreased the severity of inflammation [63]. In this experiment, the attenuation of colitis was characterized by decreased levels of inflammatory cytokines and induction of IL-22 [63]. In a separate experiment, treatment with the AHR dietary ligand DIM resulted in anti-inflammatory effects on

murine dendritic cells through suppression of the inflammatory cytokines tumor necrosis factor- α (TNF α), IL-1 β , IL-6 and IL-12 [26]. In addition, DIM was shown to diminish DSS-induce colitis by reducing T_H17 cells and increasing the number of T_{reg} cells. Importantly, these effects required the AHR [70]. T_H17 cells have been implicated in the progression of IBD by promoting mucosal inflammation while T_{reg} cells are involved in the maintenance of the epithelial tissue integrity [70].

Dysregulation of the intestinal barrier also increase the risk for colorectal cancer (CRC). Worldwide, CRC is the third most common cancer affecting men and the second most common cancer in women [71]. In the United States, CRC is the second most common cause of cancer-related deaths, representing a significant social and economic burden [60]. Various reports demonstrate that the AHR is a modulator of carcinogenic responses in the colon [62, 63, 72]. One of these reports demonstrates that the deletion of the *Ahr* in *Apc*^{Min/+} mice, a genetic model for colorectal cancer, increased the number of tumors compared with *Apc*^{Min/+} *Ahr*^{+/+} [73]. In addition, treatment with I3C suppressed the tumor development in the cecum and small intestine in *Apc*^{Min/+} mice [73]. Our laboratory recently reported that the AHR is a repressor of colitis-associated CRC [74]. In these experiments, our laboratory employed a chemically-induced model of CRC using treatments with azoxymethane (AOM) as a tumor initiator and DSS as a tumor promoter. Following treatments with the tumor protocol, *Ahr* null mice showed higher colorectal tumor multiplicity than WT mice [74]. The *Ahr* null mice also exhibited increased levels of AOM-induced DNA adducts and DSS-induced colitis than WT mice. Dietary supplementation with I3C decreased the colorectal tumor number as well as the AOM-induced DNA adducts and DSS-induced colitis in WT mice [74]. Importantly, these

observations demonstrated that I3C modulates the induction of tumors by AOM/DSS in an AHR-dependent manner [74].

Studies in mice have shown that some of the AHR dietary ligands have chemopreventive properties and can modulate pathways associated with CRC and other tumors. Several investigations reported that I3C and DIM have protective effects against breast, prostate and ovarian cancer [69]. Curcumin, a food additive, has shown to inhibit the expression and activity of cyclooxygenase 2, leading to a decrease in cell proliferation [75]. The flavonoid chrysin, a compound present in honey, has proven to induce apoptosis in human colorectal cancer cells via the AHR- dependent regulation of TNF- α and β [76]. Together, these findings support the idea that the activation of AHR by natural ligands can modulate inflammatory and anticarcinogenic responses that are critical in the pathogenesis of IBD and CRC.

1.5 Fundamentals of the AHR signaling

The AHR was originally discovered in rodent models that experienced a highly robust and inducible metabolic response to PAHs, such as benzanthracene, benzo(a)pyrene, and 3-methylcholanthrene [77]. Administration of these compounds led to the induction of the synthesis of liver microsomal enzymes, primarily cytochrome P450-dependent monooxygenases, that are capable of metabolic degradation of the xenobiotic compounds that activated the receptor [78, 79]. Collectively, the P450 activity were termed aromatic hydrocarbon hydroxylase (AHH) [77]. Upon further investigations, the adaptive response was revealed to vary significantly across strains of inbred mice [80-82]. For example, C57BL/6J mice were highly sensitive to PAH induction of AHH, whereas DBA mice were comparatively unresponsive [80]. Such differences in PAH responsiveness was soon found to be due to a single autosomal gene. This locus was designated as *Ah*, for aryl hydrocarbon responsiveness [81, 83].

Further studies revealed that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was a more potent inducer of AHH than PAHs [84]. This observation led to the use of radiolabeled TCDD and further identification of the dioxin-binding receptor from the hepatic cytosol of C57BL/6J mice [85]. Competitive binding experiments showed that this site bound dioxin congener with an affinity equivalent to its potency to induce AHH [85, 86]. It was discovered that this binding site segregated with responsive and unresponsive alleles [87]. Together, these observations provided evidence to support the existence of a receptor encoded by the *Ah* locus. This receptor was later named as the AHR. Later, mice with the b allele (*Ahr^b*) were classified as high affinity due to their robust response to the best characterized and most potent AHR agonist 2,3,7,8-

tetrachlorodibenzo-p-dioxin (TCDD) [88]. The d allele (*Ahr^d*) is considered to be the low-affinity version of AHR, as mice harboring this allele are generally 10-fold less responsive to TCDD than mice with the *Ahr^b* [88]. Consequent to the cloning and molecular characterization of the AHR, experiments demonstrated that the decrease in binding affinity in the *Ahr^d* mice is due to a single amino acid substitution (A375V) (Figure 1.4) [88].

The AHR pathways

The term “the AHR adaptive pathway” is used to refer to the AHR-mediated, ligand-induced, XRE-driven up-regulation of xenobiotic metabolism enzymes such as *Cytochrome P450 (Cyp)1a1*, *Cyp1a2*, *Cyp1b1* and the *Glutathione S-transferase 1a* (Figure 1.3) [89, 90]. Many of the AHRE-driven genes mediated by AHR ligand binding are involved in the metabolism of xenobiotic compounds, including PAHs [91, 92]. This observation indicates that the AHR may have evolved to detoxify foreign chemicals [77, 80].

We now know many of the details of AHR signal transduction as it is related to the induction of xenobiotic metabolism [93, 94]. In the absence of a ligand, AHR resides in the cytosol and is bound to the chaperone proteins Hsp90, p23, and Ara9 [95-97]. Upon ligand binding, the AHR changes conformation to expose a putative nuclear export signal, allowing translocation to the nucleus [98-100]. Once in the nucleus, the ligand-bound AHR complex dimerizes with its binding partner, ARNT [101]. The AHR-ARNT dimer recognizes a specific sequence in the DNA (5'-TNGCGTG-3'), known as

an Ah responsive elements (AHRE) [94]. The AhR-ARNT-AHRE complex serves as a transcription factor that further recruits other transcriptional machinery to the target gene promoter [102, 103]. Some of the most recognized genes include the cytochrome P450s and glucuronosyltransferases. These enzymes are involved in phase I and phase II metabolism of xenobiotic compounds and are considered canonical AHR-driven genes [104-106].

Toxicological Pathways

The role of AHR in toxicology is complex. First, it is clear that the AHR can play a significant role in PAH toxicity as it serves as a regulator of their metabolism. But, when we think of AHR-mediated toxicity, we more commonly think of toxic endpoints that are initiated by the binding of AHR to dioxins and dioxin-like compounds (DLCs) [107-109]. Moreover, we think of AHR mediated endpoints such as chloracne, porphyria and liver cancer [108, 109]. These aspects of AHR-mediated toxicology appear to be the result of AHR-mediated alterations in gene expression that occur when DLCs activate the receptor in a manner that is beyond, or different from, the adaptive response. This toxic pathway is often induced by DLCs by virtue of the fact that either 1) they are high-affinity ligands which are persistent and difficult to metabolize and thus overactivate the AHR, or 2) these compounds induce some unique conformation change in the AHR such that unique gene batteries are upregulated that lead to toxicity. [107-109]. Importantly, knockout murine models of various components of the AHR signaling pathway including AHR, ARNT hypomorphs, and the AHR-associated protein Ara9

render the mice unresponsive to TCDD, indicating the toxic effects of TCDD are mediated through AHR [4, 110, 111].

Developmental Pathways

Studies in *Ahr* null murine models have reported severe developmental defects including a patent *ductus venosus* (DV) or shunt in the liver, reduced liver weight, vascular abnormalities in kidney and eyes, alterations in immune cell populations and reduced fertility [4-6, 55, 112]. Interestingly, many of these developmental endpoints appear to require many of the signalling steps of the adaptive pathway described above. For example, AHR mouse models with an altered nuclear localization sequence or an AHR mouse model with a mutated DNA binding domain also display a patent DV [7, 113, 114]. Similarly, in an ARNT hypomorph murine model, many of the same phenotypes are observed, including the patent *ductus venosus*, indicating that a limited amount of ARNT prevents AHR from functioning in its full capacity during development [111]. Evidence for the existence of an endogenous ligand for the AHR come from the observation that gestational administration of TCDD to AHR hypomorphic mice and ARNT hypomorphic mice at embryonic day 16.5 and 18.5 rescued the patent DV phenotype, indicating heightened activation of AHR during this critical period is sufficient to close the liver shunt [115]. These data indicate that activation of AHR and subsequent canonical signaling is required for normal vasculature development. However, it remains unclear what endogenous ligand activates the AHRE during

development to induce normal development or what the set of AHRE regulated genes are required for normal development to occur.

AHR Ligands

The AHR is a promiscuous receptor that can be activated by a myriad of ligands with different chemical structures and binding affinities. To facilitate the review, we have divided the AHR ligands into three categories, environmental contaminants, dietary compounds and endogenous compounds (Table 1.1). For most readers, the most familiar source of ligands come from anthropogenic sources. The halogenated aromatic hydrocarbons (HAHs) and the PAHs are the best known examples of this class [116, 117]. The HAH tend to have longer environmental half-lives and include the DLCs [116, 118-120], whereas exposure to PAHs may be more significant in human populations due to their ubiquitous presence in cigarette smoke, diesel exhaust and cooking smoke [121-123]. Examples xenobiotic ligands that are anthropogenic environmental contaminants include TCDD, dibenzofurans, biphenyls, benzo(a)pyrene, benzanthracene and 3-methylcholanthrene [85, 116, 124].

More recently, a wide compendium of lower affinity compounds has been recognized as AHR ligands. These chemicals are mainly non-toxic dietary compounds such as chrysin, quercetin, I3C and its condensation products DIM and ICZ (Table 1.1) [125, 126]. These dietary compounds are ubiquitously found in fruits and vegetables and their intake could significantly contribute to our overall human exposure to AHR ligands [126-128]. Interestingly, some of these chemicals have been reported to modify

carcinogenesis through a variety of different mechanisms, which indicate their potential as chemopreventive and therapeutic agents [125, 129, 130].

Several compounds have been proposed as endogenous physiological ligands of the AHR. Well known examples include arachidonic acid metabolites and the tryptophan photooxidation product, 6-formylindolocarbazole (FICZ) [116, 131, 132].

Although these chemicals activate the receptor we do not yet have the appropriate temporal or cell specific data to prove that they are the *bona fide* cognate ligands during development that close the liver shunt [116, 133]. What it is known, is that the AHR mediates toxic, biological and developmental responses and its ability to induce malignant responses appears to depend on various factors such as tissue type, environment, and the character of the agonist to which it has been exposed.

1.6 Conclusion and Future directions

Our understanding of the biology of AHR has changed over the years and a wide number of recent studies have provided evidence for the role of this receptor in different biological processes including intestinal health. Studies in *Ahr*-deficient mice have demonstrated that the AHR is required for intestinal homeostasis and for the proper functioning of the mucosal barrier. The AHR-regulation of intestinal homeostasis encompasses the modulation of immune cells, the control of inflammatory responses and the regulation of host microbiota through the metabolism of dietary ligands. Additionally, several reports demonstrate that the activation of the AHR by dietary ligands can lead to anti-inflammatory and chemopreventive effects within the intestines.

Collectively, these observations support the concept that modulation of the AHR can serve as a therapeutic target for IBD and CRC. However, a clear understanding of the mechanisms underlying these beneficial effects is crucial as numerous reports show that the activation of AHR can also provoke carcinogenic responses. Thus, a few questions must be addressed before therapies with AHR ligands can be recommended. For example, how does the deletion and activation of the AHR lead to decreases and increases in cancer? How is AHR involved in the development of CRC? Can the activation of the AHR be modulated to avoid the dioxin-like toxic responses? The answers to these questions will provide insights into the mechanism by which AHR affects the progression and treatment of IBD and colitis-associated colorectal cancer. The following thesis presents a series of studies aimed to understand the role of the AHR in colitis-associated CRC. In chapter 1, we review the literature related to the role of the AHR in intestinal biology and discuss the AHR-regulation of immune cells and

inflammation within the intestinal barrier. In chapter 2, we investigated the involvement of the AHR inflammation-associated CRC. The chapter 3 aimed to examine the segregation of responses to inflammation-associated CRC with the *Ahr* locus. Finally, the chapter 4 was focused on identifying the cell type where the activation of the AHR is required for its protective effect against CRC.

1.7 Figures and Legends

Figure 1.1: The AHR regulates multiple cells in the intestinal barrier

The AHR signaling regulates the intraepithelial lymphocyte (IEC) and different subsets of innate lymphoid cells (ILC). Both cells are involved in the maintenance of the tissue integrity and regulation of bacterial composition. In addition, ILC are involved in protection against bacterial infections and control of the IL-22 production. Dendritic cells and macrophages are also regulated by AHR and are critical for inflammation responses.

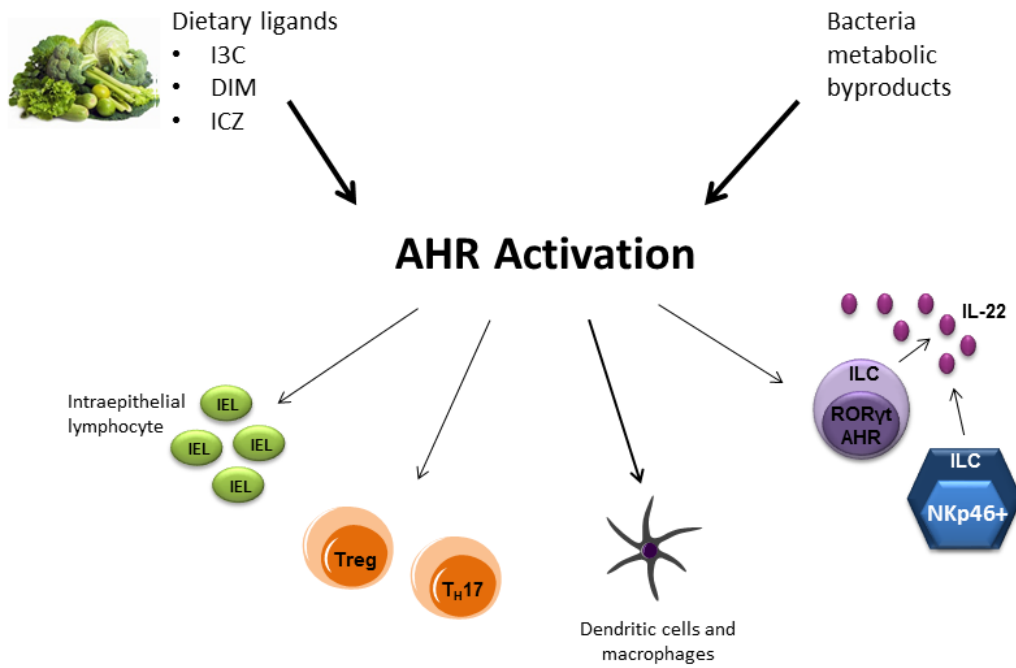
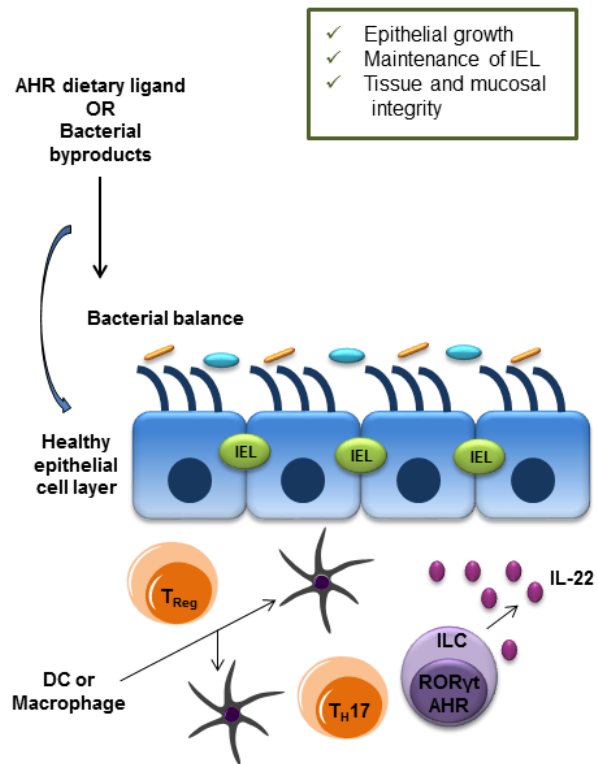


Figure 1.2: AHR-mediated regulation of intestinal homeostasis

Left. Intestinal homeostasis: Ligands of the AHR can be obtained from the diet or bacterial byproducts and are released to the intestines where the AHR signaling regulates the epithelial growth and contributes to the maintenance of the intraepithelial lymphocyte (IEL) and innate lymphoid cell (ILC). This signaling contributes to the tissue and mucosal integrity resulting in reduced inflammation and proper bacterial balance.

Right. The absence of AHR ligands: The lack of AHR signaling in the intestines is associated with reduced epithelial growth, decreased number of IEL and bacterial imbalance. These changes can cause decreased immunosurveillance and increased expression of inflammatory cytokines like IL-1 β , IL-6, and IL-17. In consequence, the risk for infections, chronic inflammation and the development of intestinal pathologies increase.

Intestinal Homeostasis



Absence of AHR ligands

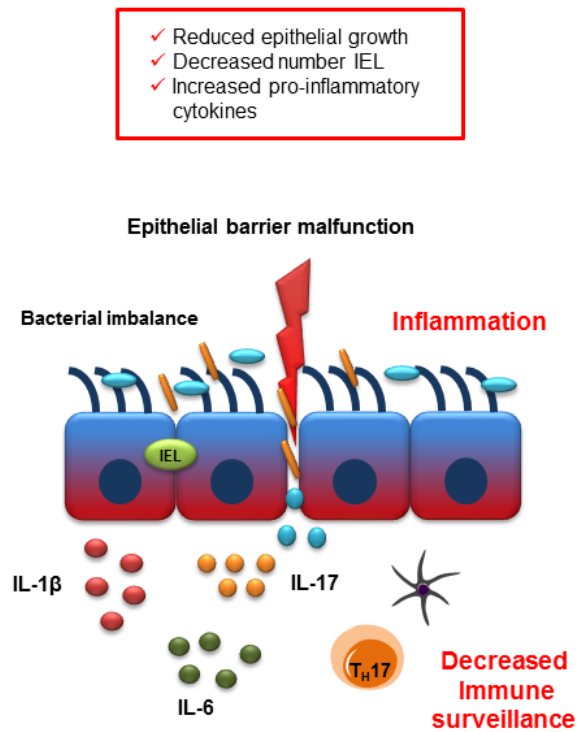


Figure 1.3: AHR signaling pathway

Upon binding its ligand in the cytosol, the AHR translocates to the nucleus where it heterodimerizes with ARNT. The activated AHR-ARNT dimer binds to a specific enhancer sequence, a dioxin responsive element, allowing it to recruit coactivators and basal transcription factors to induce the expression of target genes. Target genes of the AHR include phase-1 xenobiotic metabolizing enzymes such as *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, as well as phase-2 enzymes such as *Glutathione S-transferase 1a*.

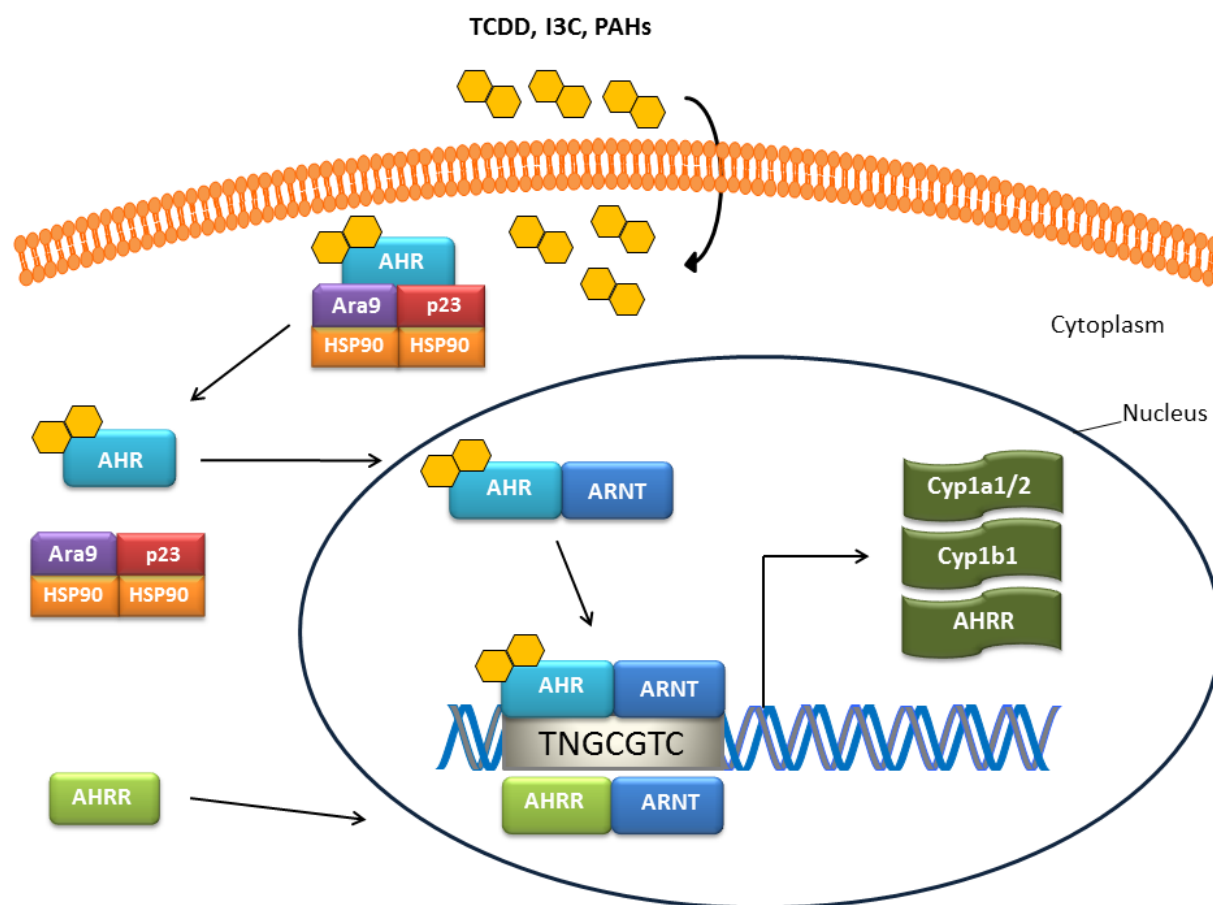


Figure 1.4: AHR domain structure

Schematic presentation of the protein domains found in the AHR^b (dioxin responsive) and AHR^d (dioxin non-responsive) mice. The DNA binding domain is found within the basic helix loop helix (bHLH) region of the AHR. The PAS-A domain facilitates the heterodimerization with ARNT and the chaperone binding, whereas the PAS-B domain contains the ligand binding region. The C-terminal region holds the transactivation domain that is responsible for facilitating transcription after DNA binding. The diagram also shows the change in the amino acid sequence in the ligand binding domain of the AHR^d, which contains an alanine to valine (A375V) substitution.

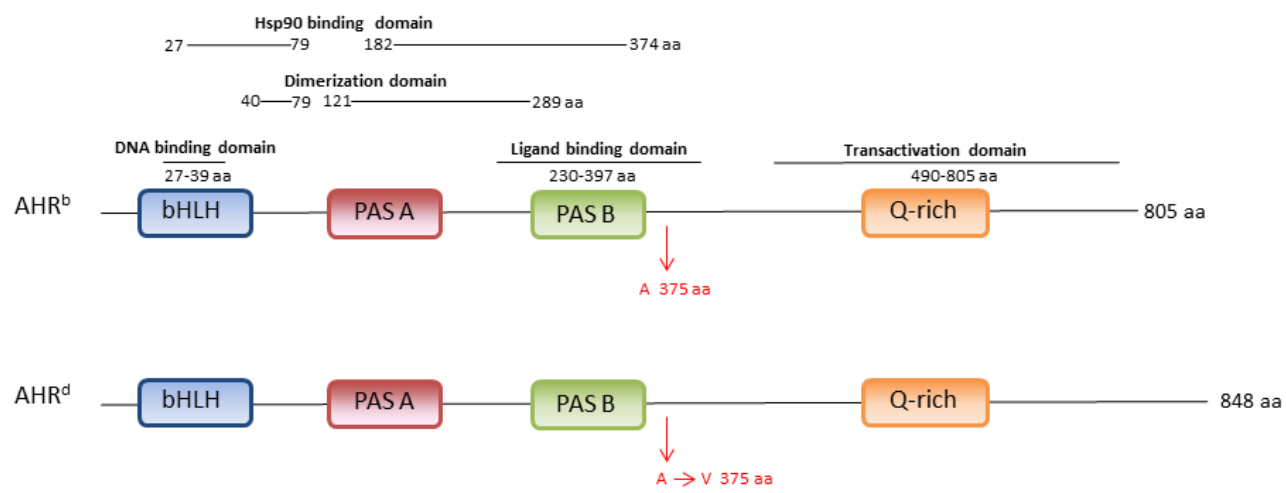


Table 1.1: AHR ligands

Examples of compounds that induce AHR signaling.

Source	Examples	References
Xenobiotics	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	[85, 131, 134]
	3-methylcholanthrene	[135, 136]
	Benzantracene	[137, 138]
	Benzo[a]pyrene	[138, 139]
	β -naphthoflavone	[140, 141]
Dietary	Indole-3-carbinol (I3C)	[65, 66, 142]
	3,3'-diindolylmethane (DIM)	[143, 144]
	Indolo[3,2-b] carbazole (ICZ)	[66, 67]
	2-(indol-3-ylmethyl)-3,3'-diindolylmethane (LTr-1)	[66, 145]
	Chrysin	[76, 128]
	Quercetin	[146, 147]
Endogenous	Arachidonic acid metabolites	[148, 149]
	6-formylindolo[3,2-b]carbazole (FICZ)	[150-152]

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Chapter 2

The aryl hydrocarbon receptor is a repressor of inflammation-associated colorectal tumorigenesis in mouse

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The assessment of the AOM-induced DNA adducts was performed by Silvia Balbo and Romel Dator at the Masonic Cancer Center, University of Minnesota.

Chapter 2

The aryl hydrocarbon receptor is a repressor of inflammation-associated colorectal tumorigenesis in mouse

2.1 Abstract

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the United States. Chronic intestinal inflammation, like Inflammatory Bowel Disease, increases the risk for the development of CRC. The aryl hydrocarbon receptor (AHR), a transcriptional factor and a mediator for xenobiotic metabolism, plays an integral role in intestinal homeostasis by regulating intestinal innate immune cells and inflammation. Therefore, we hypothesize that AHR is involved in the development of colitis-associated CRC. To investigate our hypothesis, we used a mouse model of colitis-associated colorectal tumorigenesis that employs treatment with azoxymethane (AOM) and sodium dextran sulfate (DSS). We examined the role of AHR using both an *Ahr*-deletion mouse model (*Ahr*^{Δ2/Δ2}) and treatment with the AHR pro-agonist indole-3-carbinol (I3C). Tumors, defined as neoplasms, were visually counted and recorded in terms of incidence, multiplicity, and location. Furthermore, we assessed intestinal inflammation by monitoring pro-inflammatory markers using qPCR and measuring colon length. We found that colorectal tumor incidence was increased 32% in *Ahr* null mice and tumor

multiplicity was approximately increased 3-fold compared to WT mice (2.4 v 7, $P < 0.05$). Moreover, colorectal tumor multiplicity was reduced 92% by treatment with I3C in WT mice, while the suppressor effect of I3C was not observed in *Ahr* null mice ($p < 0.05$). These studies have demonstrated that AHR plays a protective role in colitis-associated colorectal tumorigenesis. This conclusion is based on the observations that *Ahr* null mice showed increased number of colorectal tumors and mice treated with I3C exhibited fewer tumors. In addition, this work supports the use of AHR agonists such as I3C as a chemopreventive therapy for IBD-associated CRC in human patients.

2.2 Introduction

Inflammatory Bowel Disease (IBD) is characterized by persistent inflammation of the colon and small intestine, with its two major clinical manifestations being ulcerative colitis (UC) and Crohn's disease (CD) [1]. The UC is described as a state of chronic inflammation of the colon and rectum, whereas CD involves inflammation in the mucosal lining of any part of the gastrointestinal tract [2]. Combined, UC and CD afflict nearly 1.4 million people in the United States [3]. Epidemiological studies have reported that patients with IBD have 6 times higher risk of colorectal cancer (CRC) than the general population, [4] and further, that 10-15% of all deaths in IBD patients are associated with CRC [5].

Although the pathogenesis of IBD-associated CRC is not clearly understood, disturbances of the gut mucosa and dysregulation of intestinal immunity are associated with both the initiation and propagation of the disease [6]. Recent investigations focusing on the aryl hydrocarbon receptor (AHR) have demonstrated its essential role in maintaining intestinal immunity by regulating the homeostasis of innate immune cells [7, 8]. The AHR is a member of the basic helix-loop-helix/Per-Arnt-Sim (PAS) superfamily of transcription factors and has been identified as a mediator of the toxic effects of environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and benzo(a)pyrene [9, 10]. Upon ligand binding, AHR translocates into the nucleus where it forms a heterodimeric complex with the AHR nuclear translocator (ARNT) protein. This nuclear heterodimer recognizes and binds dioxin response elements in the DNA and is followed by subsequent up-regulation of downstream target genes including phase I and

phase II drug metabolizing enzymes (e.g., *Cytochrome P450 (Cyp)1a1*, *Cyp1a2*, *Cyp1b1*, and the *Glutathione S-transferase 1a*.) [9-11].

In addition to its role in xenobiotic metabolism, AHR is thought to be intimately involved in intestinal inflammation and colitis-associated colorectal tumorigenesis. Several studies have shown that *Ahr* null mice exhibit a deficit in intestinal intraepithelial lymphocytes (IELs) and ILC22 cells [12, 13]. Both IEL and ILC22 are essential regulators of intestinal homeostasis and key factors in protection against intestinal infections [14-16]. Additionally, other reports demonstrate that AHR is a regulator for the differentiation of Regulatory T cells (Treg) and T helper (T_H17) cells [17, 18]. These cells have been reported to be critical in the development of autoimmune diseases and inflammation. Importantly, investigators have independently reported that *Ahr*-deficient mice display severe colitis compared with WT mice in a chemically induced experimental model [7, 19]. In these mouse models, dextran sodium sulfate (DSS) induces colitis by generating local inflammation in the colon. This phenotype was ameliorated by treatment with AHR pro-ligands such as indole-3-carbinol (I3C) and 3,3'-diindolylmethane (DIM) [7, 19, 20]. Further, another group showed that treatment with both I3C and DIM suppressed tumor development in the cecum and small intestine in *Apc*^{Min/+} mice, which model the human condition Familial Adenomatous Polyposis (FAP) [21].

Given these observations, we investigated the idea that AHR plays a repressor role in colitis-associated colorectal tumorigenesis. To investigate this hypothesis, we compared the incidence and multiplicity of colorectal tumors between WT and *Ahr* null mice using a chemical-induced, colitis-associated colorectal tumorigenesis model [22].

In this model, azoxymethane (AOM) is used as a colon-specific carcinogen that is administered intraperitoneally and induces tumors by alkylation of DNA [22, 23]. The combined treatment of AOM with Dextran Sodium Sulfate (DSS), a pro-inflammatory chemical, replicates colorectal tumorigenesis promoted by an acute inflammation phase [23]. Therefore, we use this two-stage colorectal tumor initiation-promotion model for the study of inflammation-associated CRC [23-25]. Further, we examined if activation of AHR by the pro-agonist I3C suppresses colorectal tumor incidence and multiplicity in the same model. Collectively, our data show that AHR plays a protective role in colitis-associated colorectal tumorigenesis.

2.3 Methods

Animals. C57BL/6J (Wild-type) and *Ahr* null (*Ahr*^{Δ2/Δ2}) mice were housed in a selective pathogen-free facility on corn cob bedding with chow diet and water ad libitum according to a protocol approved by University of Wisconsin School of Medicine and Public Health, Animal Care and Use Committee. The *Ahr*^{Δ2/Δ2} mouse has been previously described [26, 27]. The null mice in this study have been backcrossed to the C57BL/6J strain for more than 20 generations and are thus >99.9% C57BL/6J background.

Tumor studies. The two stage colorectal tumor initiation-promotion assay, also known as colitis-associated colorectal tumorigenesis model (Fig.1A) consisted of a single dose of 10 mg/kg body weight of azoxymethane (AOM) (concentration: 13.4M; Sigma-Aldrich, St. Louis, MO) by intraperitoneal (IP) injection at 8 weeks of age followed by 1% w/v dextran sodium sulfate (DSS) (Colitis Grade MW: 36,000-50,000; MP Biomedicals, Solon, OH) in drinking water on days 7-12 post-injection [22]. Sixteen weeks later, the mice were sacrificed by CO₂ euthanasia and the large intestine was harvested. The intestine was longitudinally incised along the main axis. The number, location and size of tumors on the luminal surface of the colon and rectum were recorded. For analysis of spontaneous colon and cecal tumors, 52-56 week old mice were evaluated. In these studies, the tumors have been defined as neoplasms.

Diets: To compare the incidence and multiplicity of colorectal tumors between WT and *Ahr* null mice, animals were fed with grain based diet (5001, Test Diet, Richmond, IN). For the dietary treatment with indole-3-carbinol (I3C; Sigma-Aldrich, St. Louis, MO) and

to eliminate the possibility of additional AHR ligands in the diet, mice were fed a purified diet. Specifically, we used either I3C diet (0.1% (w/w) in AIN-76A diet; Test Diet, Richmond, IN) or control diet (AIN-76A; Test Diet, Richmond, IN). For both experiments, mice were fed on the appropriate diet from birth continuously until completion of the colorectal tumorigenesis protocol (Fig.1A). Dams were fed with the diet one week before mating and the progeny was then used for the experiments.

Histology: Segments of colon and rectum from AOM/DSS-treated WT and *Ahr*^{Δ2/Δ2} mice were harvested and fixed in 10% formalin. The formalin fixed tissues were embedded in paraffin, sectioned into 4μm segments and stained with hematoxylin and eosin for histological evaluation.

Assessment of AOM-induced DNA adducts. At eight weeks of age, mice were treated with a single IP injection of 10 mg/kg body weight of AOM (concentration: 13.4M; Sigma-Aldrich, St. Louis, MO). Twelve hours after treatment mice were sacrificed and colon and rectum mucosal cells were collected. DNA was isolated using the Qiagen Gentra Puregene Mouse Tail Kit (Qiagen, Valencia, CA) and adducts quantitation (*O*⁶-methyl-guanine) was performed by capillary liquid chromatography–electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) in selected reaction monitoring (SRM) mode.

Measurement of DNA adducts. *O*⁶-methyl-guanine was purchased from Midwest Research Institute (Kansas City, MO; purity 98%), and [CD₃] *O*⁶-methyl-guanine was

obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). Isotopic purity was greater than 99%. O^6 -methyl-guanine adducts were quantified following a protocol reported in the literature [28]. Briefly, the DNA was dissolved in 1 mL of HCl (0.1 N) and $[CD_3]$ O^6 -methyl-guanine (200 fmol) was added as internal standard. The samples were heated at 90°C for 30 minutes, cooled, and neutralized with 1 N NaOH to pH 7.0. A portion of the hydrolysate (20 μ L) was used for guanine quantification. The remainder of the sample was loaded into a solid-phase extraction cartridge (Strata-X 33 μ m Polymeric Reversed Phase, 30mg/1mL; Phenomenex, Torrance, CA) for sample purification and enrichment. The resulting sample was dissolved in 20 μ L H₂O for LC-ESI-MS/MS analysis.

The LC-ESI-MS/MS analysis was performed on a TSQ Vantage (Thermoelectron, San Jose, CA) triple quadrupole mass spectrometer coupled to NanoLC-Ultra 2D HPLC (Eksigent, Dublin, CA) system. Reversed-phase separation on a Luna C18 column (250mm x 0.5 mm, 100Å, 5 μ m particle size; Phenomenex, Torrance, CA) was performed at a flow rate of 10 μ Lmin⁻¹ using 15 mM of ammonium acetate as solvent A and methanol as solvent B. Subsequently, 1 μ L of the sample was injected into the column. Gradient elution was carried out starting with 2% B for the first 5 minutes and a linear gradient from 5% B to 30% B for 10 minutes and to 75% B for another 2 minutes. The percentage of B was held constant for 2 minutes at 75% and finally a 73% B min⁻¹ to 2% for 1 minute, the column was re-equilibrated for 20 minutes. Adducts were quantified by selected reaction monitoring (SRM) of m/z -166.09 $\rightarrow$ m/z -149.09 for O^6 -methyl-guanine and of the corresponding transition m/z -169.11 $\rightarrow$ m/z -152.12 for $[CD_3]$ O^6 -methyl-guanine. Typical spray voltage was 3.2 kV, sheath gas (N₂)

pressure of 34 arbitrary units, and a capillary temperature of 260°C was used. The SRM analysis was performed using argon (Ar) as collision gas set at 1.3 mTorr, scan width of m/z 0.1, and scan time of 0.3 seconds. Quadrupole resolution was achieved with a Q1 set at m/z 0.7 and Q3 set at m/z 0.7. The analysis was performed in positive ionization mode with a total run time of 40 minutes. Quantitation analysis compares the adduct MS peak area ratio with that of the deuterated standard with a calibration curve.

The amount of guanine in each sample was determined by high-performance liquid chromatography-ultraviolet (HPLC-UV) spectrometric analysis. This was carried out on a Dionex UltiMate 3000 RSLCnano System equipped with a Variable Wavelength Detector set at 254nm (Thermo Scientific, Sunnyvale, CA). Reversed-phase separation on Acclaim PepMap C18 column (300 μm x 15 cm, 100Å, 2 μm particle size; Thermo Scientific, Sunnyvale, CA) was performed at a flow rate of 5 μLmin^{-1} using water as a solvent A and methanol as solvent B. One μL of sample was injected into the column and the temperature was maintained at 40°C using a column oven. Gradient elution was carried out starting with 2% B for the first 3 minutes and a linear gradient from 2% B to 30% B for 22 minutes and to 95% B for another 5 minutes. The percentage of B was held constant for 5 minutes at 95% and finally at 18.6% B min^{-1} to 2% for 5 minutes then the column was re-equilibrated for 10 minutes. Total run time was 50 minutes. A calibration curve was constructed and the amount of guanine was calculated. The amount of O^6 -methyl-guanine was normalized to the total amount of guanine in each sample and expressed as pmol O^6 -methyl-guanine per μmole guanine.

Assessment of DSS-induced colitis. Eight-week-old mice were weighed and treated with 1% w/v dextran sodium sulfate (DSS) (Colitis Grade MW: 36,000-50,000; MP Biomedicals, Solon, OH) in drinking water for 5 days followed by a single oral gavage administration of 1% w/v DSS solution (3 mL) to normalize the time of last exposure. Twenty-four hours after the final DSS administration, mice were weighed, sacrificed and the colon was harvested. Colon length was measured and mucosal tissues were scraped with a surgical blade. The colon mucosal tissues were subsequently used for gene expression analysis.

Gene expression analysis. Total RNA was isolated from mucosa using the Qiagen RNeasy kit (Qiagen, Valencia CA). The isolated RNAs were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA). The mRNA levels were measured with TaqMan Universal PCR Master Mix (Applied Biosystems) and custom-designed probes listed as follows: Assay ID: *IL-1 β* ; *Mm00434228_m1*, *IL-6*; *Mm00446190_m1*, *Tnfa*; *Mm00443260_g1*, *Myc*; *Mm00487804_m1*, *Ahr*; *Mm00478932_m1*, *Cyp1a1*; *Mm00487218_m1*, β -actin; *Mm01205647_g1*, *GAPDH*; 4352339E. The β -actin and GAPDH mRNA levels were measured as internal controls.

Western Blot analysis: Colon and cecum tissue were collected from eight-week-old WT and *Ahr* ^{$\Delta 2/\Delta 2$} mice. Tissues were homogenized in RIPA lysis buffer (Qiagen) containing HaltTM protease inhibitor cocktail (Thermo Scientific, San Jose, CA) and the lysates were sonicated. After centrifugation at 14,000 rpm, the supernatants were

collected. For western blot analysis, 40 µg of proteins was loaded onto NuPAGE protein gels (Life technologies, Carlsbad, CA), electrophoresed and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA). The membrane was incubated with β -catenin (BD transduction laboratories), β -actin or GAPDH antibody (Sigma-Aldrich, St. Louis, MO). To quantify the β -catenin, β -actin and GAPDH protein levels, we measured the western blot signal intensity of the samples through densitometric scanning of the immunostained Immobilon-P membrane (Molecular Imager Gel Doc and Quantity One software, Bio-Rad laboratories, Hercules, CA). The β -catenin protein levels were normalized to β -actin or GAPDH protein levels.

Statistical-Analysis. All statistical analyses were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla California USA), www.graphpad.com. Intergroup comparisons were performed by Fisher exact test or the non-parametric Wilcoxon rank sum test. Differences between groups were considered statistically significant when the p -value was < 0.05 .

2.4 Results

Ahr null mice have an increased susceptibility to colitis-associated colorectal tumorigenesis.

Using a colitis-associated colorectal tumorigenesis model [22] (Fig. 2.1 A) we demonstrated that AHR is protective against colorectal tumor development, as *Ahr* null mice have higher tumor incidence than WT mice ($p < 0.005$) (Fig. 2.1 B). The average colon tumor multiplicity in *Ahr* null mice was nearly three-fold higher than WT mice ($p < 0.0001$) (Fig. 2.1 C). The results of colorectal tumor multiplicity were further classified by gender and colorectal segment (Fig. 2.1 D-E). The increase of colorectal tumor multiplicity in *Ahr* null mice was independent of sex, as the three-fold increase in tumor multiplicity was observed in both male and female mice (male; $p < 0.01$, female; $p < 0.01$) (Fig. 2.1 D). In both WT and *Ahr* null mice, most tumors were located in the distal colon and rectum segments (Fig. 2.1 E). The tumor multiplicities of distal colon and rectum were significantly higher in *Ahr* null mice compared to WT mice (distal colon; $p < 0.01$, rectum; $p < 0.01$), whereas the proximal colon tumor multiplicity was not significantly different between WT and *Ahr* null mice ($p = 0.22$). We also measured the size of colorectal tumors. Interestingly, the average size of colorectal tumors in *Ahr* null mice was approximately 23% smaller than those found in WT mice ($p = 0.03$) (Fig. 2.1 F). Additionally, sections of the colon and rectum were collected for histological analysis. In all evaluated samples, we observed no tissue invasion and the muscularis mucosa was intact (Fig. 2.2), indicating that the tumors formation were adenomas.

To evaluate the spontaneous colorectal tumor development in WT and *Ahr* null mice, we compared the tumor incidence and multiplicity between 52-week old WT mice versus age-matched *Ahr* null mice (Fig. 2.3). In our model, spontaneous colorectal tumor development was extremely low on both lines and we did not detect statistically significant differences in tumor incidence or multiplicity (Fig. 2.3 A-B). Additionally, we assessed the levels of β -catenin protein and *c-Myc* mRNA, which are two genes involved in the molecular mechanism of colorectal cancer [29]. Levels of both β -catenin protein and *c-Myc* mRNA were not significantly increased in any of the two groups of mice (Fig. 2.3 C-D).

***Ahr* null mice have increased AOM-induced DNA damage and DSS-induced inflammation.**

The genotoxic agent azoxymethane AOM employed in this study is a potent colorectal tumor initiator [22]. To compare the AOM-induced colorectal tumor initiation activity between WT and *Ahr* null mice, we measured levels of an established biomarker of DNA adduct formation, O^6 -methyl-guanine [30] (Fig. 2.4). The amount of O^6 -methyl-guanine was undetectable in untreated animals but was increased in AOM-treated WT and *Ahr* null mice (WT: $p < 0.01$; *Ahr* ^{$\Delta 2/\Delta 2$} : $p < 0.01$). The level of O^6 -methyl-guanine was almost two-fold higher in AOM-treated *Ahr* null mice than treatment-matched WT mice ($p < 0.02$).

In our two-stage colorectal tumor protocol, DSS-induced intestinal inflammation plays an important role in colorectal tumor promotion [22, 31]. To investigate the effect

of the *Ahr* deletion on the susceptibility of mice to DSS-induced inflammation, we used reduced colon length as an endpoint to indicate the severity of DSS-induced acute colitis. After oral administration of 1% DSS for 5 days, *Ahr* null mice developed a reduction in colon length compared to untreated *Ahr* null mice ($p < 0.05$). In contrast, colon length was not significantly changed by DSS treatment in WT mice (Fig. 2.5 A). Additionally, we measured mRNA levels of pro-inflammatory markers including the cytokines IL-1 β , TNF α and IL-6 [32], in colonic mucosa of *Ahr* null and WT mice to further assess the extent of acute DSS-induced colitis (Fig. 2.5 B-D). In the colon of *Ahr* null mice, IL-1 β , TNF α and IL-6 mRNA levels were markedly increased by DSS treatment ($p < 0.05$). In WT mice, expression of IL-1 β and IL-6 were not significantly increased by DSS treatment, although there was an increase in TNF α mRNA.

Previous studies have reported that DSS treatment may induce gene expression of *Ahr* and the *Ahr*-driven gene *Cyp1a1*, the highly sensitive and specific surrogate gene for *Ahr* activation, in murine colon mucosa [19]. To investigate whether AOM or DSS treatment stimulated *Ahr* gene expression or AHR activation, we measured the *Ahr* and *Cyp1a1* mRNA levels in the colon of both AOM- or DSS-treated WT mice (Fig. 2.6). Under these conditions, we did not see AOM and DSS-mediated induction of *Ahr*-driven gene expression or enhancement of AHR expression (Fig. 2.6 A-D)

I3C protection from colorectal tumor development is AHR-dependent

To investigate the role of AHR in I3C-mediated protection from colitis-associated colorectal tumors, we first set out to validate that I3C activates AHR in our model. We

confirmed the I3C-induced activation of AHR by demonstrating a significant increase of *Cyp1a1* mRNA in the colon of mice fed with I3C supplementation versus mice fed with the control diet ($p < 0.0001$) (Fig. 2.7). Subsequently, we compared tumor incidence and multiplicity in mice fed with an I3C-supplemented diet to those on a control diet (Fig. 2.8). The colorectal tumor incidence was decreased by 92% in I3C-fed WT mice compared to control diet-fed WT mice ($p < 0.0001$) (Fig. 2.8 A). However, the colorectal tumor incidence was not influenced by I3C treatment in *Ahr* null mice. The colorectal tumor multiplicity in I3C-supplemented WT mice was 33 fold lower than mice on control diet ($p < 0.0001$) (Fig. 2.8 B). In contrast, there was no significant decrease in colorectal tumor multiplicity in *Ahr* null mice when supplemented with dietary I3C ($p = 0.35$).

In addition to high colorectal tumor burden, *Ahr* null mice displayed higher mortality than WT mice when challenged with the AOM/DSS protocol (WT: 4%; *Ahr* ^{$\Delta 2/\Delta 2$} : 57%) (Table 2.1). The mortality was independent of the diet, as 56% and 58% of *Ahr* null mice die when fed with control and I3C-supplemented diet, respectively. Interestingly, 8% of WT mice fed with control the diet died while all WT mice supplemented with I3C survived, however this difference in mortality is not significant ($p = 0.12$).

Influence of I3C on AOM-induced DNA damage and on DSS-induced inflammation

To understand the effect of I3C in the colitis-associated colorectal tumorigenesis model, we measured the levels of DNA adduct formation in AOM-treated WT mice fed with I3C or control diet. In WT mice supplemented with I3C, AOM-induced DNA adducts

were decreased by nearly 38% compared to WT mice on the control diet ($p=0.03$) (Fig. 2.9). In untreated WT mice, DNA adducts formation did not change as a result of I3C-supplementation. Additionally, we evaluated the effect of I3C in DSS-induced inflammation (Fig. 2.10). We observed that the DSS-treated WT mice on I3C diet showed longer colons than WT mice on control diet ($p<0.002$) (Fig. 2.10 A). The mRNA levels of TNF α and IL-1 β were not affected by I3C treatment, though mRNA levels of IL-6 were significantly reduced in WT mice on an I3C-supplemented diet compared to WT mice on control diet ($p=0.03$) (Fig. 2.10 B-D).

2.5 Discussion

Inflammatory bowel disease (IBD) is a well-established risk factor for the development of CRC [33, 34]. While the etiology of IBD is not fully understood, prior investigations demonstrate that dysregulation of intestinal immunity is a significant contributor to its pathophysiology [35, 36]. In the last several years, experiments using *Ahr* deletion mouse models have revealed that AHR is a fundamental player in mediating intestinal inflammation through regulation of both intraepithelial lymphocytes (IELs) and innate lymphoid cells (ILCs) [7, 8]. The relationship between AHR and intestinal immunity and the importance of inflammation in IBD led us to hypothesize that the presence and activation of AHR may impact the development of IBD-associated colorectal cancer. We, therefore, postulated that the absence of AHR would yield an increase in colorectal tumors, while the activation of the receptor by pro-agonists like I3C will protect against tumor development.

Our approach to investigating the role of AHR in IBD-associated colorectal tumor development was to use a chemically-induced colorectal tumorigenesis model that employs treatment with AOM and DSS. In this model, we observed an increased susceptibility to AOM/DSS-induced colorectal tumor development in our *Ahr* null mice (Fig. 2.1) whereas activation of AHR by dietary I3C protected against colorectal tumor development in an AHR-dependent manner (Fig. 2.8). Taken together, these observations demonstrate that AHR protects against IBD-associated colorectal tumor development. Interestingly, the finding that the tumor size was not enlarged in AOM/DSS-treated *Ahr*^{Δ2/Δ2} mice compared AOM/DSS-treated WT mice (Fig. 2.1 F) and

that all tumors were adenomas (Fig. 2.2), suggests that the AHR may be involved in the early stages of colorectal tumorigenesis.

The role of AHR in AOM/DSS-induced colorectal tumors development has not been previously investigated and its mechanism may be multifactorial, including an immunological/inflammatory component and a xenobiotic metabolism component. With regard to xenobiotic metabolism, we have previously reported that *Ahr* null mice display a patent ductus venosus (DV) and therefore have a decreased rate of first-pass metabolism for many foreign compounds, potentially including AOM [37]. The increase O^6 methylation of guanine residues in our null models (Fig. 2.4) suggests that a higher concentration of AOM or its electrophilic metabolite may be reaching this target site. Such an observation is consistent with altered first pass metabolism of AOM, which, as previously mentioned, may be attributed to the hepatic shunt. Additionally, it is reported that the hepatic metabolizing enzymes, such as Cyp2e1, and the β -glucuronidases from intestinal flora are required for the generation of the electrophilic AOM metabolite responsible for alkylating DNA [38-41]. Thus, it is possible that altered intestinal immunity in *Ahr* nulls, possibly through a decrease in the number of resident IELs, is influencing the bacterial loads of intestinal flora. In turn, this could lead to alterations of microbial populations expressing β -glucuronidase (e.g. *Clostridium*, *Peptostreptococcus*, *B. fragilis*, *Staphylococcus*) in the colons of *Ahr* null mice, making them more susceptible to the alkylating and therefore toxic effects of AOM and to the inflammation induced by DSS [42-44]. Collectively, these observations suggest that the high susceptibility of *Ahr* null mice to AOM/DSS may be due to altered xenobiotic

metabolism, altered intestinal microbiota or aberrant response to chemically-induced inflammation.

From these data, we know that most, if not all, of the anticarcinogenic activity of I3C, observed in these studies is mediated through AHR. Indole-3carbinol is a pro-agonist found in the *Brassica* family of vegetables. Following ingestion, I3C undergoes condensation reactions within the acidic gastric environment resulting in several different metabolites including 3,3'-diindolylmethane (DIM) and indolo [3,2-b] carbazole (ICZ), both of which have a high affinity for AHR [45, 46]. In an effort to determine if AHR mediates the protective effects of I3C in our model of IBD-associated CRC, we compared the intestinal tumor burden in WT and *Ahr* null mice following AOM/DSS treatment. Our observation that *Ahr* null mice were not influenced by the treatment of I3C is consistent with the idea that the anticarcinogenic effect of I3C is dependent upon the AHR (Fig. 2.8). Furthermore, we investigated the effect of I3C in AOM-induced DNA adduct formation and in DSS-induced intestinal inflammation. Our data demonstrate that I3C confers protection against electrophilic damage by AOM (Fig. 2.9). The I3C-mediated inflammation is unique in that inhibition of IL-6 alone was observed (Fig. 2.10). Together, these observations suggest that a major component of the I3C-mediated anticarcinogenic effect in colitis-associated colorectal tumorigenesis model is a reflection of its ability to influence AOM bioactivation through first pass metabolism effects or modification of intestinal flora. The importance of the anticarcinogenic effects of I3C as they relate to AHR-mediated intestinal inflammation and subsequent cellular proliferations are yet to be determined.

To date, *Ahr* deletion mice have been generated by three different laboratories [27, 47, 48] and a variety of observations regarding colorectal tumor development as well as the response to chemically-induced colitis have been reported. For example, one group reported that an *Ahr* deletion mouse model displayed significant spontaneous cecal and colon tumor development [21]. They reported that 100% of 11 weeks old *Ahr* null mice (*Ahr*^{Δ1/Δ1}, corresponding to the excision of exon 1 in the *Ahr* gene) bore spontaneous colon or cecal tumors. In our *Ahr* null mouse model (*Ahr*^{Δ2/Δ2}, corresponding to excision of exon 2 in the *Ahr* gene) spontaneous cecal and colon tumors were rare even at 52 weeks of age (Fig. 2.3). Although tumor incidence in *Ahr*^{Δ2/Δ2} was approximately three times higher than in our WT mice, these differences were not significant (Fig. 2.3 A). Additionally, the *Ahr*^{Δ1/Δ1} mice showed abnormal accumulation of β-catenin protein in the small intestine, cecum, and colon. However, the colonic β-catenin levels were not significantly increased in the *Ahr*^{Δ2/Δ2} mice as compared to WT mice. Another difference between our work and current literature is that several reports have demonstrated that treatment with I3C and DIM rescues DSS-induced colitis [7, 19, 20]. In our model, the treatment of I3C minimally influenced the DSS-induced inflammation (Fig. 2.10). These discrepancies may be due to differences in *Ahr* null alleles, mouse genetic background, intestinal microbiota, housing conditions, and protocols used for DSS exposure.

We demonstrated that AHR plays a protective role in IBD-associated colorectal tumorigenesis; the implication of which is that AHR is required for resistance to IBD-associated tumor formation. The clinical importance of this consideration stems from the evolutionarily conserved nature of the AHR such that it is highly conserved among

mammals including humans [9, 10]. Accordingly, genetic or epigenetic gene silencing or environmental inhibition of AHR could represent novel health modifiers for IBD and IBD-associated CRC in humans. In general, our data support the previously described hypothesis that I3C-mediate suppression of AOM/DSS-induced colorectal tumorigenesis. In addition, this is the first study to establish that the protective effect of I3C in the colon is dependent on AHR. This work supports the application of I3C as a chemopreventive agent for IBD-associated CRC and also suggests that the activation of AHR may be used as a chemopreventive strategy against CRC.

2.6 Figures and Legends

Figure 2.1: Susceptibility to AOM/DSS-induced colorectal tumorigenesis was increased in *Ahr* ^{$\Delta 2/\Delta 2$} mice.

(A) Schematic protocol for AOM/DSS-induced colorectal tumorigenesis. At 8 weeks of age, WT mice (n=37) and *Ahr* ^{$\Delta 2/\Delta 2$} (n=20) were treated with a single dose of 10 mg/kg body weight of AOM by IP injection. Seven days after the AOM injection, the mice were given 1% DSS in drinking water for 5 days. Sixteen weeks later, the mice were sacrificed for evaluation of colorectal tumorigenesis. (B) The incidence of colorectal tumors in AOM/DSS-treated WT and *Ahr* ^{$\Delta 2/\Delta 2$} mice. (C-E) AOM/DSS-induced colorectal tumor multiplicity in WT and *Ahr* ^{$\Delta 2/\Delta 2$} mice. (C) Total colorectal tumor multiplicity. (D) Gender-based classification. (E) Segmentation-based classification. (F) The size of colorectal tumors in AOM/DSS-treated WT and *Ahr* ^{$\Delta 2/\Delta 2$} mice. Bars represent mean values. *a*; significantly different relative to WT mice ($p < 0.05$), *b*; significantly different relative to the proximal colon of WT mice ($p < 0.05$), *c*; significantly different relative to the proximal colon of *Ahr* ^{$\Delta 2/\Delta 2$} ($p < 0.05$).

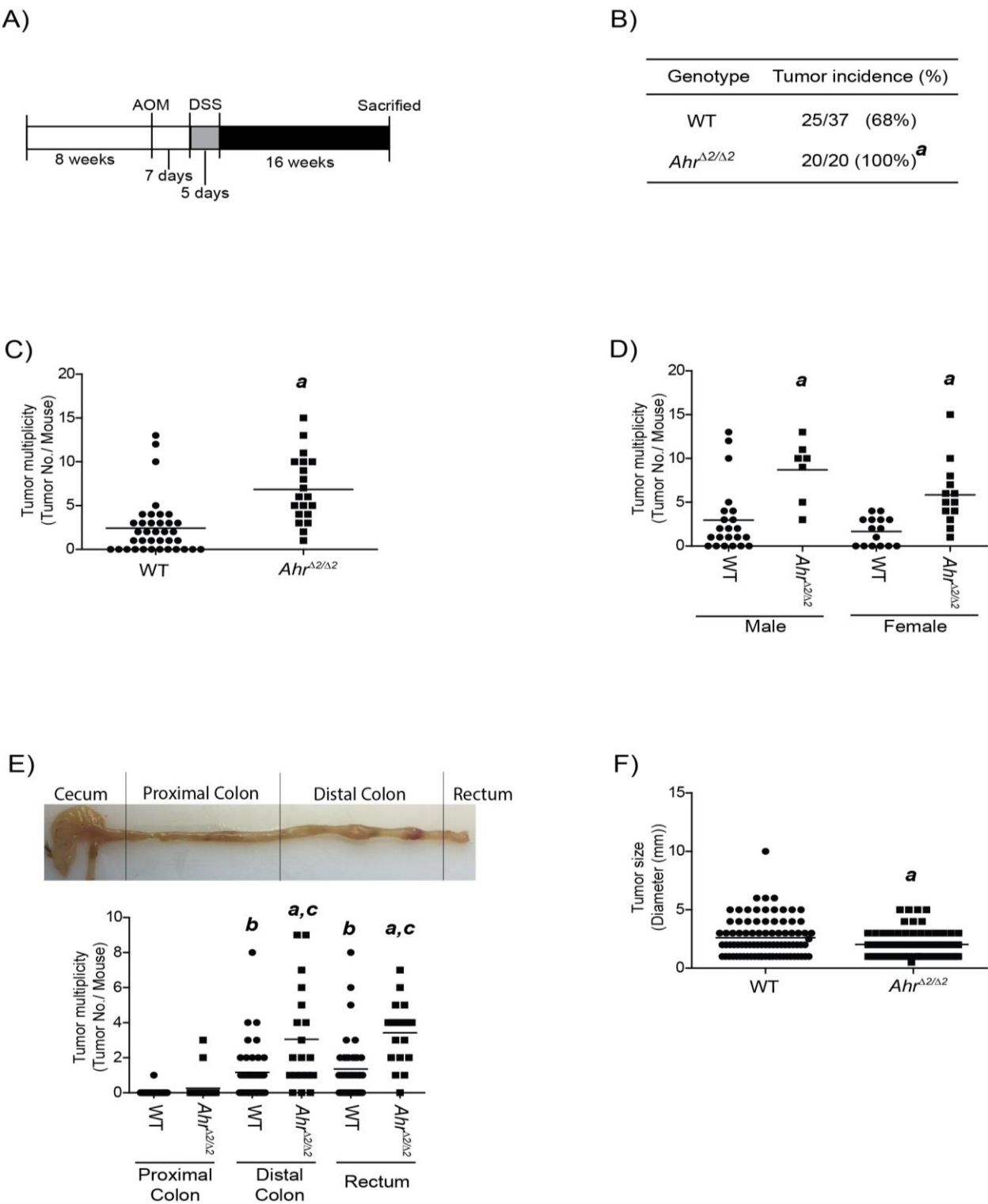
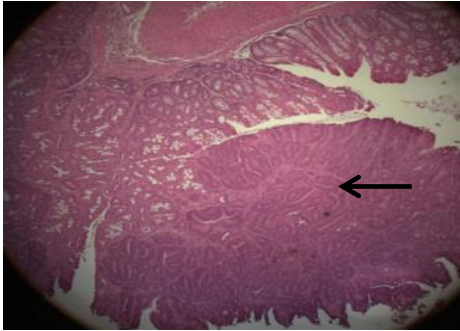


Figure 2.2: AOM/DSS-induced colorectal tumors were classified as adenomas in both WT and *Ahr* ^{$\Delta 2/\Delta 2$} mice.

Histological staining of colon sections from AOM/DSS-treated WT mice (A & B) and *Ahr* ^{$\Delta 2/\Delta 2$} mice (C & D). Mice showed no tissue invasion and an intact muscularis mucosa demonstrating that the tumors were adenoma formation (large black arrows).

Muscularis mucosa (small black arrows).

A) C57BL6



B) C57BL6

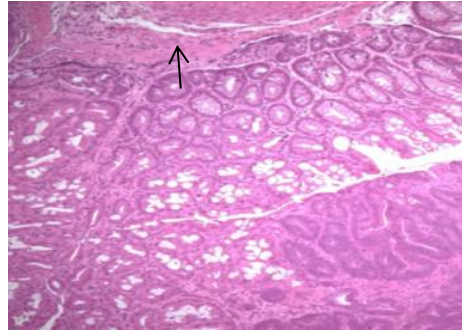
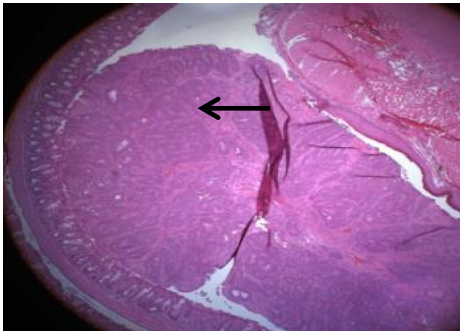
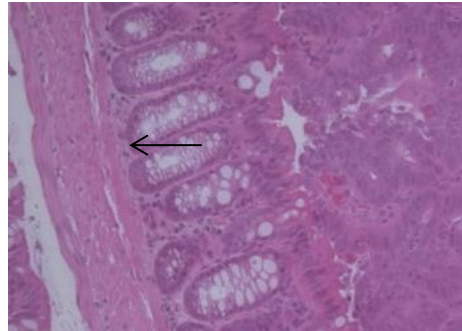
C) $Ahr^{\Delta 2/\Delta 2}$ D) $Ahr^{\Delta 2/\Delta 2}$ 

Figure 2.3: *Ahr* deletion did not significantly influence the spontaneous development of cecal and colon tumors.

(A) The incidence of cecal and colonic tumors in WT and *Ahr*^{Δ2/Δ2} mice (52-56 weeks of age). (B) Colon tumor multiplicity in WT (n=27) and *Ahr*^{Δ2/Δ2} (n=31) mice (52-56 weeks of age). Western blot and quantitative real-time RT-PCR analysis. (C) Relative protein levels of β-catenin in the cecum and colon of WT and *Ahr*^{Δ2/Δ2} mice (n=4). (D) Relative *c-Myc* mRNA levels in the colon of WT and *Ahr*^{Δ2/Δ2} mice. Each group contained more than 4 mice. Error bars represent SEM.

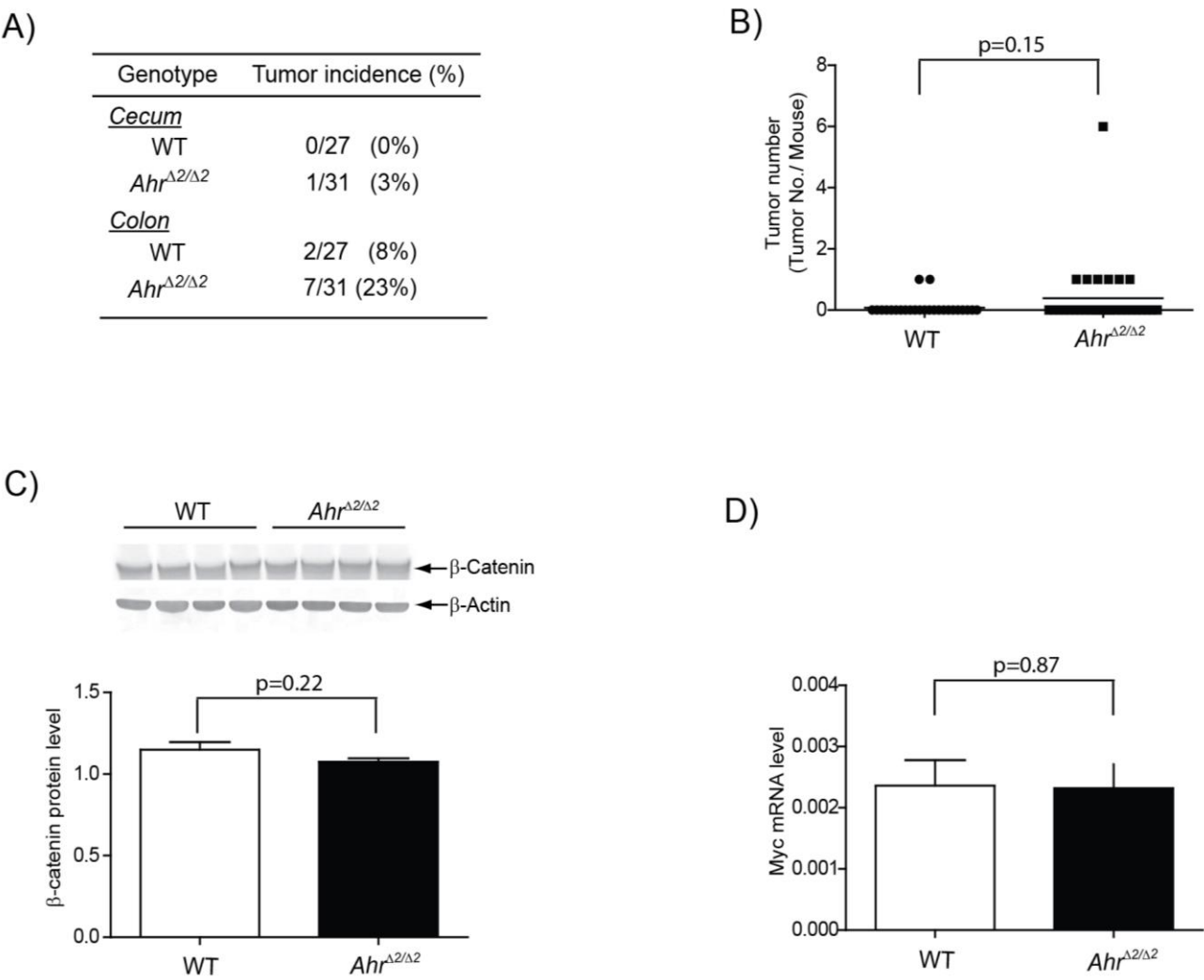


Figure 2.4: $Ahr^{\Delta2/\Delta2}$ mice showed higher levels of AOM-induced DNA adduct than WT mice.

Levels of O^6 -methyl-guanine in colonic mucosa DNA of WT and $Ahr^{\Delta2/\Delta2}$ mice. At 8 weeks of age, WT and $Ahr^{\Delta2/\Delta2}$ mice were administered a single IP injection of PBS or 10 mg/kg body weight of AOM. Twelve hours after the injection, mice were sacrificed. *a*; significantly different relative to the no AOM treated mice ($p < 0.05$), *b*; significantly different compared to the AOM-treated WT mice ($p < 0.05$). Bars represent mean values.

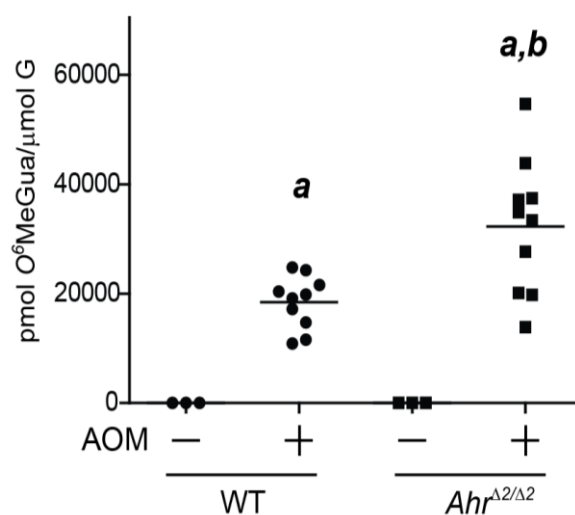


Figure 2.5: *Ahr* ^{$\Delta 2/\Delta 2$} mice displayed higher susceptibility to DSS-induced acute colitis than WT mice.

Male WT and *Ahr* ^{$\Delta 2/\Delta 2$} mice (9 weeks of age) were given 1% DSS in drinking water for 5 days. Twenty-four hours after the treatment, the mice were sacrificed. (A) Colon length. Each group contained more than 13 mice (n=13-23). *a*; significantly different relative to the control *Ahr* ^{$\Delta 2/\Delta 2$} mice ($p < 0.05$), *b*; significantly different relative to DSS-treated WT mice ($p < 0.05$).

Levels of pro-inflammatory cytokine genes were higher in DSS-treated *Ahr* ^{$\Delta 2/\Delta 2$} mice than WT mice.

The IL-1 β (B), TNF α (C) and IL-6 (D) mRNA levels were determined by quantitative real-time RT-PCR and these measured mRNA levels were normalized to β -actin mRNA levels. Each group contained more than 4 mice (n=4-5). *a*; significantly different relative to control *Ahr* ^{$\Delta 2/\Delta 2$} mice ($p < 0.05$), *b*; significantly different relative to DSS-treated WT mice ($p < 0.05$), *c*; significantly different relative to control WT mice ($p < 0.05$), Error bars represent SEM.

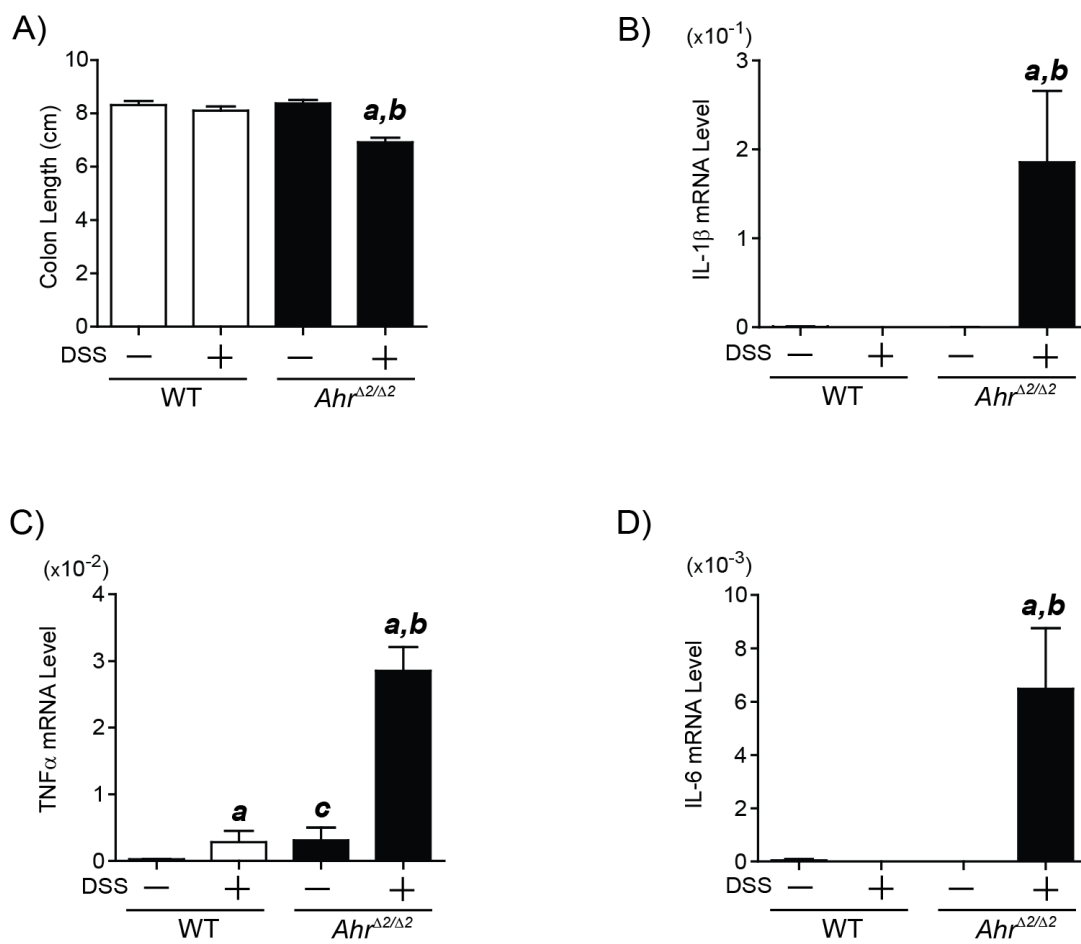


Figure 2.6: Expression of AHR and *Cyp1a1* mRNA in the colon of AOM- or DSS-treated WT mice.

(A-B) At 8 weeks of age, WT mice were administered a single IP injection of PBS or 10mg/kg body weight of azoxymethane (AOM). Twelve hours after the injection, mice were sacrificed. (C-D) At 9 weeks of age, WT mice were given 1% dextran sodium sulfate (DSS) in drinking water for 5 days. Twenty-four hours after the treatment, mice were sacrificed. Total RNAs were isolated from the distal colon mucosa of mice. The AHR (A and C) and *Cyp1a1* (B and D) mRNA levels were determined by quantitative real-time RT-PCR. The measured mRNA levels were normalized to GAPDH mRNA levels. Each group contained more than 3 mice (n=3-5). Error bars represent SEM.

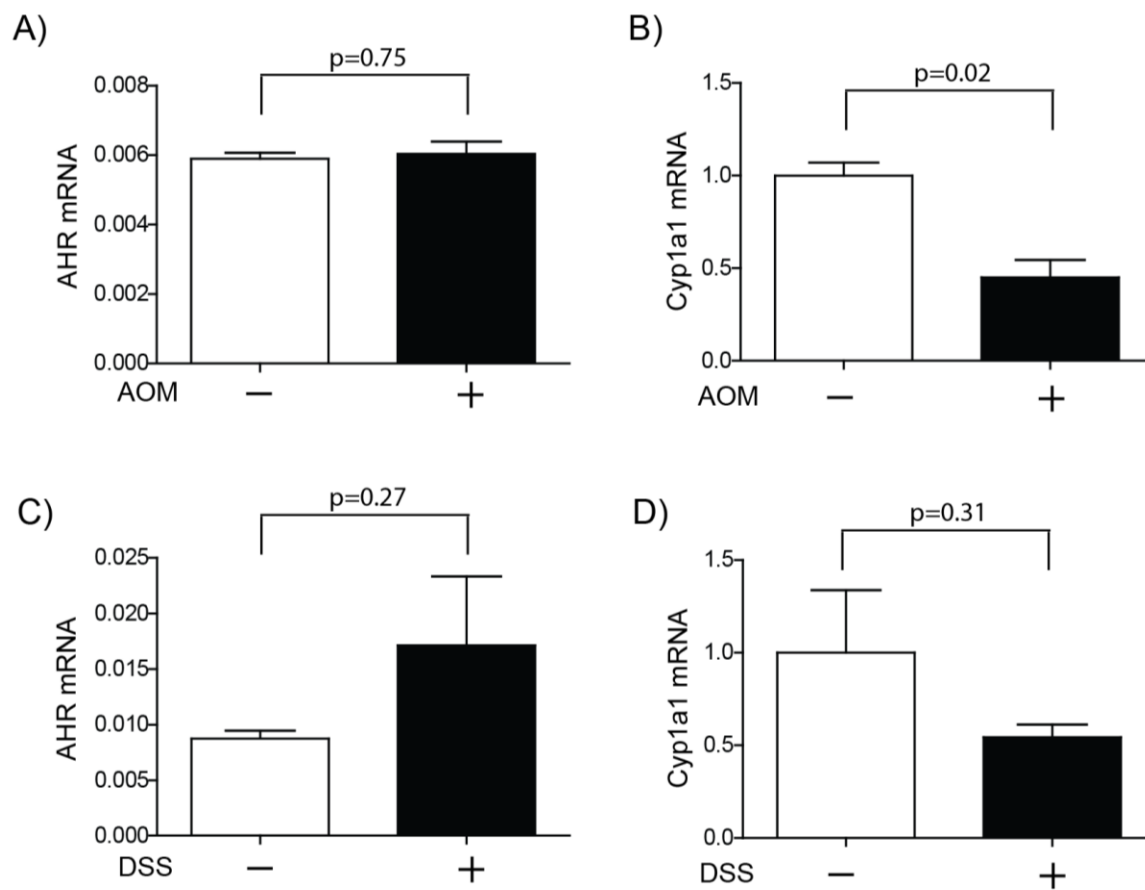


Figure 2.7: I3C significantly induced expression of Cyp1a1 mRNA levels in the colon of I3C-treated WT mice.

Mice were fed with I3C or control diet from birth. At 8 weeks of age, mice were sacrificed and colonic mucosa was scraped. Total RNAs were isolated and Cyp1a1 levels were determined by quantitative real-time RT-PCR. The mRNA levels were normalized to GAPDH mRNA levels. Each group contained 4 mice (n=4). Error bars represent SEM.

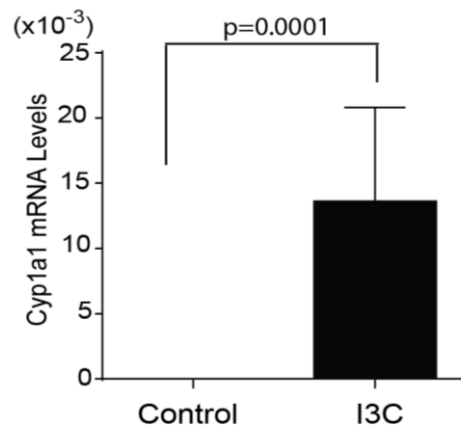


Fig 2.8: I3C suppressed tumor development induced by AOM/DSS in WT mice but not in *Ahr* ^{$\Delta 2/\Delta 2$} .

Mice were fed with I3C or control diet from birth. At 8 weeks of age, WT (Control) (n=24), WT (I3C) (n=29), *Ahr* ^{$\Delta 2/\Delta 2$} (Control) (n=14), *Ahr* ^{$\Delta 2/\Delta 2$} (I3C) (n=11), were treated with a single dose of 10 mg/kg body weight of AOM by IP injection. Seven days after the AOM injection, the mice were given 1% DSS in drinking water for 5 days. Sixteen weeks later, the mice were sacrificed for evaluation of colorectal tumors. (A) The incidence of colorectal tumors in AOM/DSS-treated WT and *Ahr* ^{$\Delta 2/\Delta 2$} mice. (B) AOM/DSS-induced colorectal tumor multiplicity in WT and *Ahr* ^{$\Delta 2/\Delta 2$} mice. *a*; significantly different relative to WT mice treated with control diet ($p < 0.0001$), *b*; significantly different relative to *Ahr* ^{$\Delta 2/\Delta 2$} mice.

Figure 2.9: I3C treatment decreased AOM-induced DNA adducts.

Levels of O^6 -methyl-guanine in colonic DNA of WT mice treated with I3C and control diet (n=5-10). At 8 weeks of age, both groups of WT mice were administered a single IP injection of PBS or 10 mg/kg body weight of AOM. Twelve hours after the injection, mice were sacrificed. Bars represent mean values. *a*; significantly different relative to no AOM treatment group ($p<0.0001$), *b*; significantly different relative to WT mice treated with control diet ($p<0.03$).

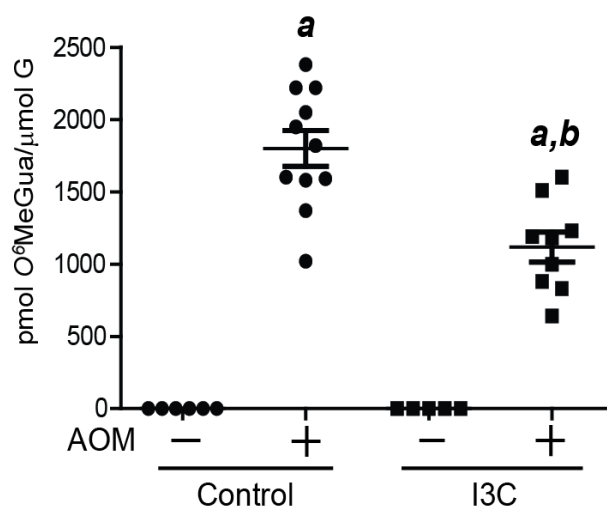
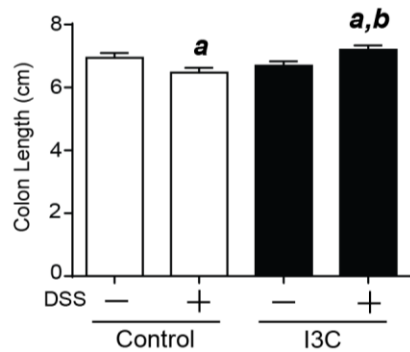


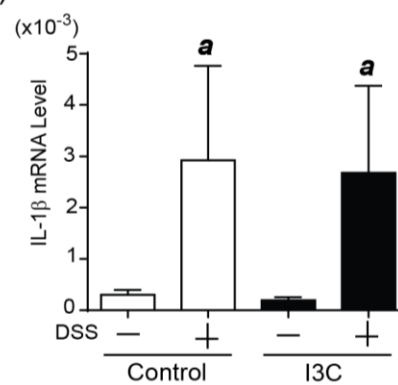
Figure 2.10: I3C partially reduces DSS-induced inflammation.

WT mice (9 weeks of age) treated with I3C and control diet, were given 1% DSS in the drinking water for 5 days. Twenty-four hours after treatment, the mice were sacrificed. Colon length (A). Additionally, total RNAs were isolated from the distal colon mucosa of WT mice treated with or without DSS. The IL-1 β (B), TNF α (C) and IL-6 (D) mRNA levels were determined by quantitative real-time RT-PCR and these measured mRNA levels were normalized to GAPDH mRNA levels. Each group contained more than 10 mice. *a*; significantly different relative to no DSS-treated group ($p < 0.05$), *b*; significantly different relative to WT mice treated with control diet ($p < 0.05$). Error bars represent SEM.

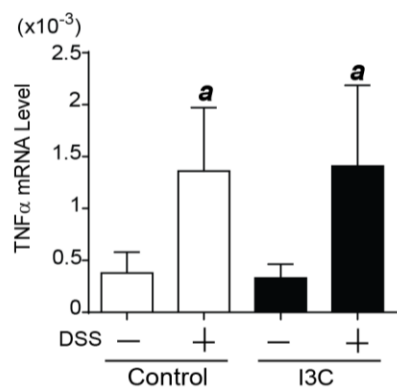
A)



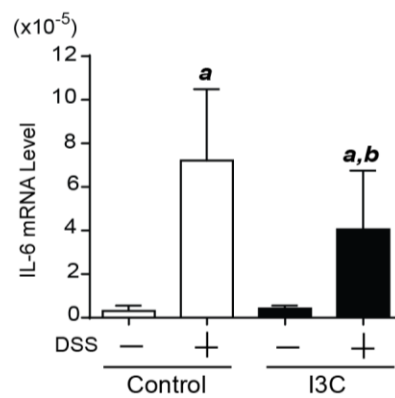
B)



C)



D)



2.7 Tables and Legends

Table 2.1: AOM/DSS-treated $Ahr^{\Delta 2/\Delta 2}$ mice exhibited higher mortality than AOM/DSS-treated WT mice.

At 8 weeks of age, WT (Control) (n=26), WT (I3C) (n=29), $Ahr^{\Delta 2/\Delta 2}$ (Control) (n=32), $Ahr^{\Delta 2/\Delta 2}$ (I3C) (n=26), were treated with a single dose of AOM by IP injection. Seven days after the AOM injection, the mice were given 1% DSS in drinking water for 5 days. Sixteen weeks later, the mice were sacrificed for evaluation of colorectal tumors. The table shows mice that were found dead during the experiment.

Genotype	Diet	Mortality Rate (%)
WT	Control	2/26 (8%)
	I3C	0/29 (0%)
		Total: 2/55 (4%)
$Ahr^{\Delta 2/\Delta 2}$	Control	18/32 (56%)
	I3C	15/26 (58%)
		Total: 33/58 (57%)

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Chapter 3

Segregation of responses to inflammation-associated colorectal tumorigenesis with the Ahr locus

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Chapter 3

Segregation of responses to inflammation-associated colorectal tumorigenesis with the *Ahr* locus

3.1 Abstract

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the metabolism of a wide variety of xenobiotics and regulates a plethora of physiological processes. In mice, two genetic variants of the AHR confers different binding affinities to certain chemicals, where the *Ahr^d* allele has low binding affinity and the *Ahr^b* allele it is known as the high binding affinity. By using mice harboring the *Ahr^b* allele and *Ahr* null mice, our laboratory previously demonstrated that the AHR plays a protective role in inflammation-associated colorectal cancer (CRC). Given that the *Ahr* null mice exhibited a *ductus venosus* and reduced first-pass metabolism, we set out to confirm the protective role of the AHR using the *Ahr^d* mice as a model of low binding affinity allele with normal xenobiotic metabolism. In addition, we wanted to evaluate if the AHR protection was mediated by the ligand binding affinity, thus we examined the segregation of responses to colitis-associated CRC with the *Ahr* locus. To this end, we employed a two-stage colorectal carcinogenesis model using azoxymethane (AOM) as tumor initiator and dextran sodium sulfate (DSS) as a tumor promoter. We found that the colorectal tumor incidence and multiplicity were nearly the same in both *Ahr^b* and *Ahr^d* mice. Unexpectedly, *Ahr^d* mice displayed higher susceptibility to DSS-induced

acute colitis than *Ahr^b* mice. Exposure to I3C-supplemented diet decreased the effects of AOM/DSS in both *Ahr^b* and *Ahr^d* mice. However, the protective effects were diminished in *Ahr^d* mice. Together, our results demonstrate that the binding affinity does not mediate the protective effects of AHR in colitis-associated CRC and confirm that the chemoprotective effects of the I3C are dependent on the AHR.

3.2 Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor that is recognized for its role mediating the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other environmental contaminants [1]. In the absence of a ligand, the AHR exists in the cytosol as a stable complex bound to chaperones such as Hsp90, p23, and Ara9 [2]. After binding its ligand the AHR changes conformation and translocate to the nucleus where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator protein (ARNT) [3, 4]. The active AHR-ARNT complex interacts with a particular sequence in the DNA known as dioxin responsive elements [3]. This interaction induces the expression of numerous Phase I and Phase II xenobiotic metabolizing enzymes such as Cytochrome P450 (*Cyp*) *1a1*, *Cyp1a2*, *Cyp1b1* and the *Glutathione S-transferase 1a* [5, 6]. These xenobiotic-metabolizing enzymes are critical mediators for the detoxification of a wide number of AHR ligands [7].

Early studies exploring the mechanism of induction of the xenobiotic metabolizing enzymes revealed variation in responses to polycyclic aromatic hydrocarbons (PAHs) between mouse strains [8]. Following exposure to 3-methylcholanthrene, the metabolizing enzymes of the C57BL/6 mice were highly inducible, whereas enzymes of the DBA mice were relatively unresponsive [8]. These observations led to the discovery of variant murine *Ahr* alleles that grant low or high binding affinity for different classes of AHR ligands [9]. Mice homozygous for the d allele of the AHR (*Ahr^d*) have the low-affinity form of the receptor as they are ten-fold less responsive to TCDD than mice harboring the b allele (*Ahr^b*, high affinity) [9]. Further sequencing analysis showed that the lower ligand affinity observed in the *Ahr^d* receptor is attributed to a valine

substitution at position 375 of the ligand binding domain [9-11]. In the *Ahr^b*, an alanine residue is found at same position which significantly increases the binding affinity to TCDD [9, 11]. Interestingly, the human AHR (*hAHR*) harbors a valine residue at position 381 of the ligand binding domain and similarly to *Ahr^d*, it exhibits a lower ligand-binding affinity to TCDD compared to the *Ahr^b* [10, 12]. Clearly, genetic variations in the AHR between and within species have an impact on their sensitivity to xenobiotics.

Although the AHR is known for its function as an environmental sensor, emerging data indicate that the AHR is a regulator of various physiological processes including tissue development and homeostasis, immune and inflammatory responses and tumorigenesis [13-19]. Recently, multiples lines of evidence demonstrate that the AHR is a critical regulator of intestinal health [20]. For example, independent studies have reported that the *Ahr*-null mice exhibits an impaired intestinal barrier characterized by exacerbated immune responses, microbial imbalance, and reduced intestinal epithelial cells turnover [21-23]. Therefore, these mice are more susceptible to intestinal bacterial infections, colitis and tumor development [21, 22, 24-27]. Interestingly, *Ahr^b* mice treated with dietary AHR ligands maintained a healthy immune mucosa barrier and were less susceptible to developing intestinal diseases compared with those fed diets lacking AHR ligands [24-26, 28-32]. Recently, our lab demonstrated that the *Ahr*-null mice displayed higher colorectal tumor burden than the *Ahr^b* mice [33]. Additionally, in *Ahr^b* mice, the activation of the AHR *via* dietary supplementation with the AHR ligand indole-3-carbinol (I3C) resulted in significant reduction of colorectal tumors [33]. These observations demonstrated that the AHR protects against the development of colitis-associated colorectal cancer (CRC) in mice [33]. The *Ahr null* mice exhibited a patent

ductus venosus (DV) that allows the portal blood to bypass the liver such that xenobiotics in the blood draining the intestines are not subject to first pass metabolism [14, 15]. Therefore, high susceptibility to colitis-associated CRC observed in the *Ahr null* mice could be a reflection of the liver shunt and reduced metabolism for AOM. Therefore, we wanted to confirm the protective role of the AHR in colitis-associated CRC using the *Ahr^d* mice as a model that does not lead to liver shunting.

Historically, a segregation analysis of responses with the *Ahr* locus has been used to examine the dependence of a particular event on the AHR ligand binding affinity [34]. Thus, we set out to evaluate whether the low affinity binding at the AHR was protective in mice harboring the *Ahr^d* allele, by examining the segregation of responses to colitis-associated CRC with the *Ahr* locus. To this end, we compared the carcinogenic responses between *Ahr^b* and *Ahr^d* mice to a chemically-induced model of colitis-associated CRC. Specifically, we investigated whether the low binding affinity of the AHR affects the response to both, azoxymethane (AOM), a colon carcinogen, and to sodium dextran sulfate (DSS), a colitogenic agent. Finally, we evaluated whether the treatment with indole-3-carbinol (I3C) impacts these responses. Overall, our data demonstrated that the binding affinity does not mediate the protective effects of AHR in colitis-associated CRC, but does influence the chemoprotective effects of I3C.

3.3 Methods

Mice and diets. All experiments were approved by the School of Medicine and Public Health, Animal Care and Use Committee of the University of Wisconsin-Madison.

C57BL/6J mice are homozygous for the high ligand binding affinity allele of the AHR and were used as controls throughout this study and are defined as *Ahr^b*. Congenic mice harboring the low ligand binding affinity allele of the AHR (*Ahr^d*) have been previously described [35]. These mice have been backcrossed >20 times to the C57BL/6J strain making them >99.9% C57BL/6J background. Both *Ahr^b* and *Ahr^d* mice were housed in a selective pathogen-free facility on corn cob bedding. Mice were fed either, the control diet (AIN-76A; Test Diet, Richmond, IN) or a diet supplemented with indole-3-carbinol (I3C; Sigma-Aldrich, St. Louis, MO). Feeding protocol has been previously described [33]. Food and acidified water were provided *ad libitum*.

Tumor studies. A chemically induced model of colitis-associated colorectal cancer [36] was used to cause colorectal tumor formation in both *Ahr^b* and *Ahr^d* mice. The tumor protocol was previously described [33]. Briefly, mice were treated with a single dose of 10 mg/kg body weight of azoxymethane (AOM) (concentration: 13.4M; Sigma-Aldrich, St. Louis, MO) by intraperitoneal (IP) injection at 8 weeks of age followed by 1% w/v dextran sodium sulfate (DSS) (Colitis Grade MW: 36,000-50,000; MP Biomedicals, Solon, OH) in drinking water on days 7-12 post-injection. Mice were sacrificed sixteen weeks after treatment protocol. The colon and rectum were collected, and tumors were visually counted.

Quantification of AOM-induced DNA adducts. At eight weeks of age, both, *Ahr^b* and *Ahr^d* mice were treated with one IP injection of AOM (10 mg/kg body weight; concentration: 13.4M; Sigma-Aldrich, St. Louis, MO). Mice were sacrificed twelve hours after treatment and DNA from colon mucosa cells were isolated as previously described [33]. The DNA was used to quantify the amount of *O*⁶-methyl-guanine, the major mutagenic adduct of AOM [37]. The quantification was performed by capillary liquid chromatography–electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) following a reported protocol [33, 38]. The total *O*⁶-methyl-guanine was normalized to the total amount of guanine present in each sample, and the final amount was reported as pmole *O*⁶-methyl-guanine per μ mole guanine.

Evaluation of DSS-induced colitis. The assessment of DSS-induced colitis was performed at eight weeks of age as previously reported [33]. Mice were sacrificed twenty-four hours after the DSS cycle. Reduced colon length and formation of rectal prolapse were used as indicators of acute colitis [39]. Colon mucosa was collected, and RNA from this tissue was isolated for further gene expression analysis [33].

Gene expression analysis. Total RNA from colon mucosa of both, untreated and DSS-treated mice was prepared for gene expression analysis as previously reported [33]. The mRNA levels of the target genes were measured with TaqMan Universal PCR Master Mix (Applied Biosystems) using custom-designed probes. The following TaqMan probes were used: *Cyp1a1*: Mm00487218_m1, *Cyp1b1*: Mm00487229_m1, *Ahr*: Mm01352370_m1, *IL-1 β* : Mm00434228_m1, *IL-6*: Mm00446190_m1, *TNF α* :

Mm00443260_g1 and GAPDH; 4352339E. The gene expression was reported as relative expression compared with the mRNA levels of GAPDH.

Statistical-Analysis. All statistical analyses were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software (La Jolla California USA), www.graphpad.com. Intergroup comparisons were conducted by Fisher exact test or the non-parametric Wilcoxon rank sum test. Differences between groups were considered statistically significant when the *p*-value was < 0.05 .

3.4 Results

Expression of AHR-regulated genes in Ahr^b and Ahr^d mice.

To validate our mouse model of low or high binding affinity for AHR ligands, we examine the expression of selected AHR-regulated genes in the colon mucosa of Ahr^b and Ahr^d mice fed with I3C (Fig. 3.1 A-C). Initially, we confirmed that both Ahr^b and Ahr^d mice were responsive to I3C treatment by demonstrating a significant increase of *Cyp1A1*, *Cyp1B1*, and *Ahrr* mRNA levels in the colon of mice treated with I3C diet related to mice fed control diet ($p < 0.05$). Then, we confirmed that Ahr^d mice were less responsive to I3C treatment than Ahr^b mice, as their mRNA levels of *Cyp1A1*, *Cyp1B1* and *Ahrr* were significantly lower than the levels observed in Ahr^b mice ($p < 0.05$). Also, we found that in the absence of I3C Ahr^b mice exhibited higher mRNA levels of *Ahrr* than Ahr^d ($p = 0.03$).

Ahr^b and Ahr^d mice are equally susceptible to AOM/DSS-induced colorectal tumorigenesis.

Previous studies from our laboratory demonstrated that Ahr^b mice were less susceptible to colitis-associated CRC than *Ahr*-null mice [33]. Therefore, we wanted to investigate whether the low binding affinity allele of AHR impacts mice susceptibility to colitis-associated CRC. Using a two-stage colorectal tumor model, we demonstrated that Ahr^b and Ahr^d mice are equally susceptible to colitis-associated colorectal tumorigenesis (Fig. 3.2). After the treatment with AOM and DSS protocol, there was no

significant difference in colorectal tumor incidence, multiplicity or tumor size between *Ahr^b* and *Ahr^d* mice treated with the control diet ($p>0.05$) (Fig. 3.2 A-C).

Further, we examined the colorectal tumor incidence and multiplicity in *Ahr^b*, and *Ahr^d* mice fed a diet supplemented with I3C (Fig. 3.2 A and B). Consistent with our previous findings, the colorectal tumor incidence and multiplicity of *Ahr^b* mice fed I3C diet was significantly decreased compared with *Ahr^b* mice fed the control diet ($p<0.0001$). Interestingly, we also observed a significant reduction in the colorectal tumor incidence and multiplicity of I3C-fed *Ahr^d* mice ($p=0.0008$). However, the reduction in colorectal tumor burden in I3C-fed *Ahr^b* mice was greater than the reduction observed in I3C-fed *Ahr^d* mice; tumor development in *Ahr^b* was decreased by 90% versus 54.5% detected in *Ahr^d* ($p=0.03$).

I3C treatment decreased AOM-induced DNA adducts in both *Ahr^b* and *Ahr^d* mice.

The tumor initiation stage of our model of colitis-associated CRC consists of a single dose of AOM. The AOM is a potent colon carcinogen that induces the formation of *O*⁶-methyl-guanine DNA adducts [40]. To examine the susceptibility of mice harboring the low binding affinity allele of the AHR to AOM, we quantified the levels of *O*⁶-methyl-guanine in the colon mucosa of *Ahr^b* and *Ahr^d* mice treated with AOM and fed the control diet (Fig. 3.3). Both *Ahr^b* and *Ahr^d* mice exhibited formation of *O*⁶-methyl-guanine adducts. The *Ahr^d* mice showed apparently lower amounts of adducts relative to *Ahr^b* mice; however, this difference was not significant ($p=0.11$). Subsequently, we evaluated the effects of I3C on AOM-induced DNA adducts by measuring the levels *O*⁶-methyl-

guanine in mice fed I3C-supplemented diet. As previously reported in chapter 2 figure 2.9, levels of DNA adducts were decreased in I3C-fed *Ahr^b* mice compared with control-fed *Ahr^b* mice. Interestingly, the treatment of I3C also reduced the levels of DNA adducts in *Ahr^d* mice ($p=0.047$).

Ahr^d mice exhibit a higher inflammatory state than Ahr^b mice.

The involvement of inflammation in colorectal carcinogenesis is irrefutable as individuals with chronic intestinal inflammation are at increased risk of developing CRC [41, 42]. To better understand the effects of AOM and DSS in our mouse model, we set out to characterize the inflammatory state of the *Ahr^b* and *Ahr^d* mice. We measured mRNA levels of the pro-inflammatory cytokines IL-1 β , TNF α and IL-6 in the colon mucosa of mice fed the control diet (Fig. 3.4). Levels of all three pro-inflammatory cytokines were markedly higher in *Ahr^d* mice than *Ahr^b* mice, and the difference in mRNA levels of TNF α was significant ($p=0.0002$) (Fig. 3.4 B).

Ahr^d mice have increased susceptibility to DSS-induced acute colitis.

To investigate the susceptibility of our congenic *Ahr^d* mice to DSS-induced colitis, we evaluated markers of acute inflammation in the colon mucosa derived from *Ahr^b* and *Ahr^d* mice sacrificed twelve hours after a cycle of DSS. Specifically, we measured reduced colon length and quantified levels of inflammatory cytokines (Fig. 3.5). There was no significant difference in the colon length between *Ahr^b* and *Ahr^d* mice treated

with the control diet ($p=0.22$) (Fig. 3.5 A). Quantification of the levels of pro-inflammatory cytokines revealed that *Ahr^d* mice were more susceptible to DSS- induced colitis. The mRNA levels of IL-1 β and TNF α were significantly increased in *Ahr^d* mice relative to *Ahr^b* mice (IL-1 β : $p=0.049$; TNF α : $p=0.036$) (Fig. 3.5 B-C). Although the mRNA levels of IL-6 were also increased in *Ahr^d* mice relative to *Ahr^b* mice, the difference was not significant ($p=0.09$) (Fig. 3.5 D). Additionally, we assessed the development of rectal prolapse as it is an indicator of the severity of the DSS-induced colitis [43] (Fig. 3.5 E). Interestingly, 27% of the *Ahr^d* mice developed rectal prolapse while none of the *Ahr^b* mice showed prolapse.

As previously reported, we observed that I3C influenced the DSS-induced colitis in *Ahr^b* mice [33]. I3C-fed *Ahr^b* mice displayed longer colons and a significant reduction in mRNA levels of IL-1 β relative to control fed mice (colon length: $p=0.0063$; IL-1 β : $p=0.009$) (Fig. 3.5 A-B). However, there was no significant reduction in the mRNA levels of TNF α and IL-6 in I3C-treated *Ahr^b* mice (TNF α : $p=0.09$; IL-6: $p=0.38$) (Fig. 3.5 C-D). Therefore, we wanted to investigate the effect of I3C in DSS-treated *Ahr^d* mice. Examination of inflammatory markers proved that the dietary supplementation with I3C also reduced the DSS-induced colitis in *Ahr^d* mice. The mRNA levels of IL-1 β and TNF α were significantly decreased by treatment with I3C (IL-1 β : $p=0.048$; TNF α : $p=0.032$). A longer colon and reduced levels of IL-6 was observed in I3C-fed *Ahr^d* mice. However, these differences were not significant (colon length: $p=0.06$; IL-6: $p=0.35$). Surprisingly, the treatment of I3C eliminated the formation of rectal prolapse.

3.5 Discussion

In past years, numerous investigations have provided evidence that the AHR is a critical regulator of intestinal health by maintaining the intestinal immune homeostasis [21, 23, 44]. Notably, the AHR signaling is required for the regulation of intestinal immune cells, inflammatory responses and ligand-dependent modulation of intestinal microbiota [44-47]. Compelling data also show that the AHR contributes to the susceptibility of mice to intestinal inflammatory diseases [24, 25, 48]. In this regard, we recently demonstrated that the AHR signal acts as a repressor of the development of colitis-associated colorectal cancer (CRC) [33]. Importantly, this conclusion was derived from both, a mouse model lacking a functional AHR and *Ahr^b* mice harboring the high ligand binding affinity allele of the AHR. The present study had two purposes: first, to confirm the protective role of the AHR in colitis-associated CRC using the *Ahr^d* mice as a model of low binding affinity allele with normal xenobiotic metabolism, and second, to examine the segregation of responses to colitis-associated CRC with the *Ahr* locus.

Our strategy to study colorectal tumorigenesis in mice with divergent alleles of the AHR was straightforward; we used congenic mice harboring the high or low ligand binding affinity allele of the AHR, *Ahr^b* and *Ahr^d*, respectively. We confirmed the low-ligand binding affinity allele in the *Ahr^d* mice by showing that they were significantly less responsive to treatment with I3C than *Ahr^b* mice (Fig. 3.1). Then, we implemented an AOM/DSS colitis-associated CRC model to show that the binding affinity does not mediate the protective effects of AHR in colitis-associated colorectal cancer but does influence the protective effects of I3C. These conclusions are based on the following

observations. First, there was no significant difference in the colorectal tumor incidence and multiplicity between the *Ahr^b* and *Ahr^d*, indicating that the two mouse strains are equally susceptible to AOM/DSS-induced colorectal tumors. Second, the *Ahr^b* mice were more responsive to the chemopreventive effects of I3C, as the reduction in the colorectal tumors was higher in *Ahr^b* than in *Ahr^d* (Fig. 3.2). These data support our previous observation that the anticarcinogenic effects of I3C against colitis-associated tumorigenesis are dependent on the AHR and are also influenced by the binding affinity to the receptor.

In addition, these results indicate that the *Ahr^d* mice is a useful model for the low-affinity AHR protein in studying the effects of carcinogens and chemopreventive agents. As mentioned before, the *Ahr*-null mice exhibit a liver shunt and their responses to carcinogens may be altered due to decreased metabolism. Because the *Ahr^d* mice exhibit normal livers and blood filtration, the xenobiotics undergo phase I metabolism. This represents a valuable tool for studying the role of the AHR in carcinogenesis by circumventing the metabolic defects observed in *Ahr*-null mice.

Our two-stage tumorigenesis model encompassed treatments with both AOM and DSS. Regarding the AOM, both *Ahr^b* and *Ahr^d* mice developed AOM-induced DNA adducts (Fig. 3.3). Furthermore, we evaluated the effect of I3C on AOM-induced DNA adducts. Both *Ahr^b* and *Ahr^d* mice showed a reduction in the formation of DNA adducts when supplemented with I3C. These observations confirm our previous data on the ability of I3C to reduce the AOM metabolism. In addition, these data indicate that the low-affinity AHR may still efficiently respond to treatment with AHR ligands to prevent the effects of carcinogens.

The DSS is a colitogenic drug that induces direct damage to the mucosal barrier and promotes the development of colorectal tumors [49]. To better comprehend the effects of DSS-induced colitis in the *Ahr^d* mice, we initially examined their inflammatory state by comparing levels of pro-inflammatory markers with those in *Ahr^b* mice. A significant increase in the pro-inflammatory markers demonstrates that *Ahr^d* mice have a higher colonic inflammation than *Ahr^b* mice (Fig. 3.4). Further, we showed that the *Ahr^d* mice were more susceptible to DSS-induced colitis than *Ahr^b* mice as increased levels of pro-inflammatory cytokines, as well as the development of rectal prolapse, a sign of advanced colitis, were observed (Fig. 3.5). Taken together, these data suggest that the low-affinity AHR leads to a higher inflammatory state and in consequence increased inflammatory responses induced by DSS.

The role of the AHR in intestinal inflammation has been recognized as *Ahr* null mice exhibited reduced levels of intestinal immune cells and impaired mucosal barrier [21, 44]. Based on these observations, it is plausible that the low-affinity AHR may be causing abnormalities in the mucosal epithelial barrier and intestinal immune cells. In turn, this could lead to a chronic inflammatory state and perhaps increase the susceptibility to tumor development in a longer response time. Further studies are needed to characterize the susceptibility of *Ahr^d* mice to short and long term protocols of colitis-associated CRC.

We also analyzed the effects of I3C in both DSS-treated *Ahr^b* and *Ahr^d* mice (Fig.3.5). As expected, the treatment of I3C moderately decreased the DSS- induced inflammation in *Ahr^b* mice. Interestingly, I3C-fed *Ahr^d* mice exhibited protection against

DSS-induced colitis. Indeed, levels of levels of IL-1 β and TNF α , as well as rectal prolapse were decreased by the treatment of I3C.

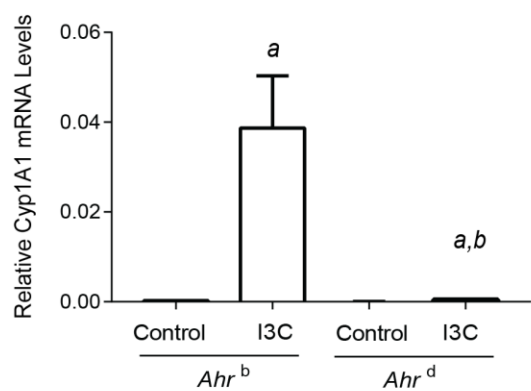
The work reported herein provides essential information that contributes to the characterization of the low-affinity AHR mice and their response to colitis-associated CRC. First, we demonstrated that under our protocol of sixteen weeks, *Ahr^b* and *Ahr^d* mice are equally susceptible to AOM/DSS-induced colorectal tumor. Second, we found that the *Ahr^d* were less responsive to treatment with I3C, but its response was sufficient to provide protection against both AOM and DSS. The clinical implication of these observations arises from the similarities between the mouse *Ahr^d* and human AHR, and suggests that I3C may have protective effects in human patients as well. Third, we showed that the *Ahr^d* mice exhibited higher intestinal inflammatory state than mice carrying the high-affinity AHR suggesting that this mouse model could be a useful tool to study the pathogenesis of human intestinal diseases provoked by heightened states of mucosal inflammation.

3.6 Figures and Legends

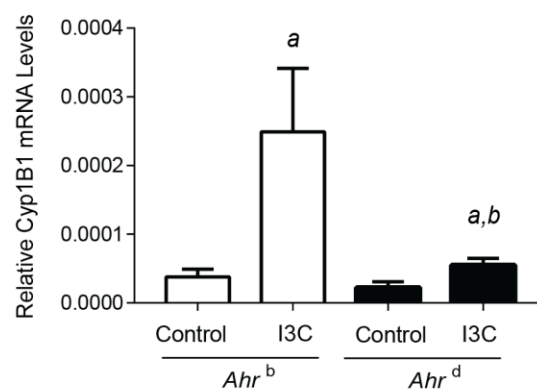
Figure 3.1: Expression of AHR-associated genes in the colon of *Ahr^b* and *Ahr^d* mice.

Mice were fed either control or I3C supplemented diet. At eight weeks of age, mice were sacrificed, and the colon mucosa was scraped and used for RNA isolation. The total RNAs was used to measure the expression of AHR-associated genes. The mRNA levels of (A) *Cyp1A1*, (B) *Cyp1B1* and (C) *Ahrr* were determined by quantitative real-time RT-PCR. The quantification of mRNA levels was normalized to GAPDH levels. Each group contained 6 mice (n=6). Error bars represent SEM. *a*; significantly different compared to control diet ($p<0.05$), *b*; significantly different relative to *Ahr^b* mice fed with I3C ($p<0.05$), *c*; significantly different relative to *Ahr^b*-control diet mice ($p<0.05$).

A)



B)



C)

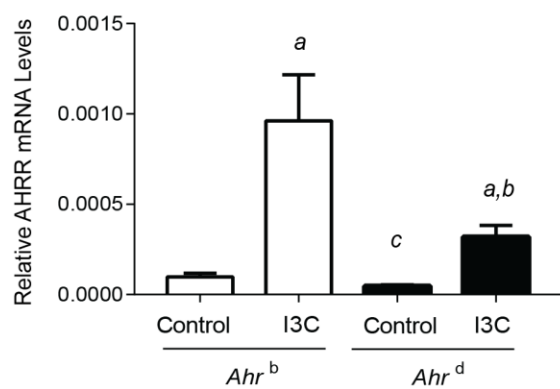


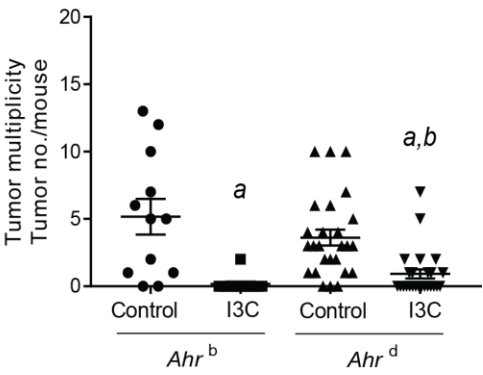
Figure 3.2: *Ahr^d* and *Ahr^b* mice are equally susceptible to AOM/DSS-induced colorectal cancer.

Ahr^b and *Ahr^d* mice were fed with either control or I3C supplemented diet and treated with a single IP injection of AOM (*Ahr^b* control diet (n=12); *Ahr^b* I3C diet (n=12)) | (*Ahr^d* control diet (n=26); *Ahr^d* I3C diet (n=25)). Seven days after the AOM treatment, mice were given a single cycle of 1% DSS in drinking water for five days. Mice were sacrificed sixteen weeks after treatment for colorectal tumor quantification. (A) The incidence of colorectal tumors in *Ahr^b* and *Ahr^d* mice. (B) Colorectal tumor multiplicity in *Ahr^b* and *Ahr^d* mice. (C) Tumor size in *Ahr^b* and *Ahr^d* mice. *a*; significantly different compared to mice fed with control diet ($p < 0.05$), *b*; significantly different compared to *Ahr^b* mice fed with I3C diet ($p < 0.05$).

A)

Genotype	Diet	Tumor incidence (%)
<i>Ahr^b</i>	Control	10/12 (83 %)
	I3C	1/12 (8%) ^a
<i>Ahr^d</i>	Control	23/26 (88%)
	I3C	10/25 (40%) ^{a,b}

B)



C)

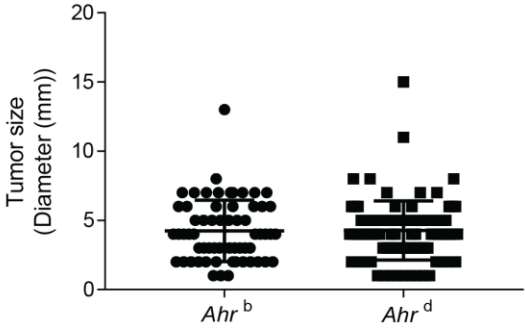


Figure 3.3: I3C treatment decreased AOM-induced DNA adducts in both *Ahr^b* and *Ahr^d* mice.

Levels of O^6 -methyl-guanine. *Ahr^b* and *Ahr^d* mice were treated with control or I3C diet. When mice reached eight weeks of age, they were treated with an IP injection of AOM. Mice were sacrificed twenty-four hours after treatment and DNA were isolated from colon mucosa. The DNA was used to measure the levels of O^6 -methyl-guanine. Each group contained 6 mice (n=6). Mice were treated with a single dose of AOM and sacrificed twelve hours after the injection. a; significantly different compared to mice fed with control diet (p<0.05).

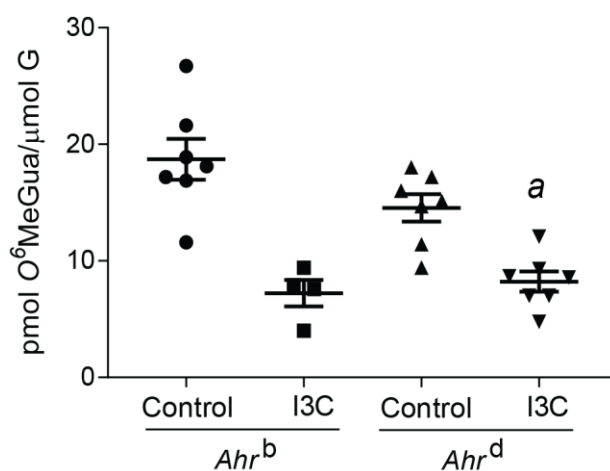
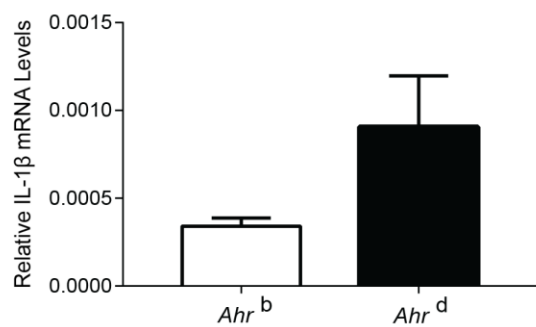


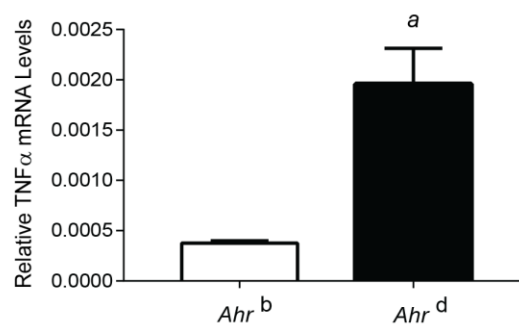
Figure 3.4: *Ahr^d* mice exhibited higher basal inflammatory state in the colon than *Ahr^b* mice.

Levels of pro-inflammatory cytokines in colon mucosa of *Ahr^b* and *Ahr^d*. The IL-1 β (B), TNF α (C) and IL-6 (D) mRNA levels were examined by quantitative real-time RT-PCR. The results were normalized to the mRNA levels of GAPDH. Each group has 6 mice (n=6). a; significantly different relative to control *Ahr^b* mice (p<0.05). Error bars represent SEM.

A)



B)



C)

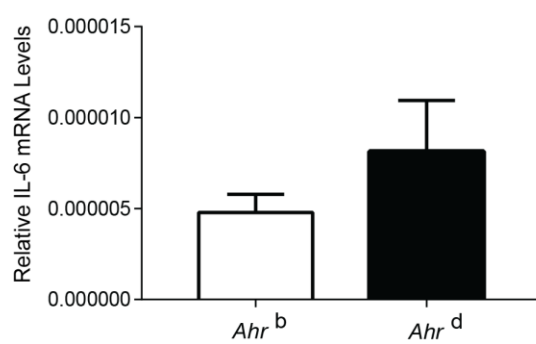
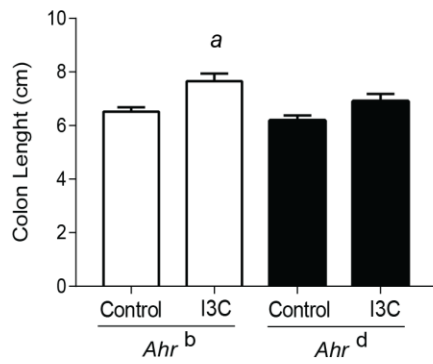


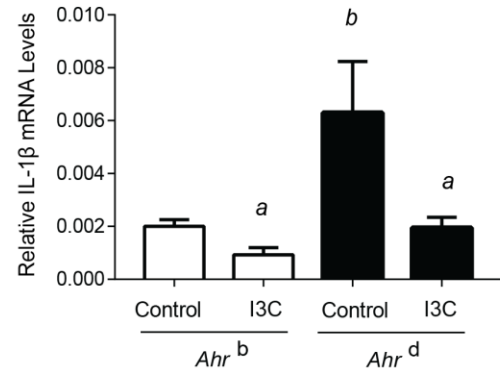
Figure 3.5: *Ahr^d* mice showed higher susceptibility to DSS-induced acute colitis than *Ahr^b* mice.

Markers of DSS-induced acute colitis. The *Ahr^b* and *Ahr^d* mice were treated with either control or I3C diet followed by a cycle of 1% DSS in drinking water for five days. Mice were sacrificed twelve hours after treatment and markers of acute colitis were recorded. (A) Colon length; Levels of pro-inflammatory cytokines (B) 1IL-1 β , (C) TNF α and (D) IL-6; (E) Rectal prolapse. The mRNA levels of the pro-inflammatory cytokines were determined by quantitative real-time RT-PCR and normalized to GAPDH levels. Each group contained 6 mice (n=6). *a*; significantly different relative to mice fed with control diet ($p < 0.05$), *b*; significantly different compared to *Ahr^b* mice treated with control diet ($p < 0.05$). Error bars represent SEM.

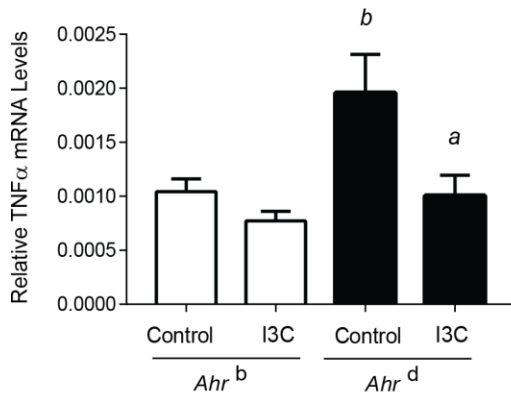
A)



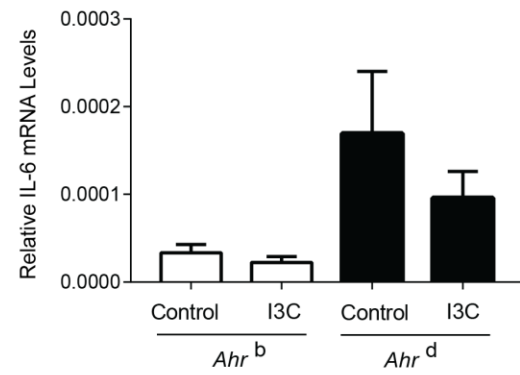
B)



C)



D)



E)

Genotype	Diet	Rectal Prolapse
<i>Ahr^b</i>	Control	0/12 (0%)
	I3C	0/12 (0%)
<i>Ahr^d</i>	Control	7/26 (27%) ^{a,b}
	I3C	0/25 (0%)

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Chapter 4

Preliminary studies to identify the cell autonomous role of the aryl hydrocarbon receptor in inflammation-associated colorectal cancer.

The assessment of the AOM-induced DNA adducts was performed by Silvia Balbo and Romel Dator at the Masonic Cancer Center, University of Minnesota.

Chapter 4

Preliminary studies to identify the cell autonomous role of the aryl hydrocarbon receptor in inflammation-associated colorectal cancer.

4.1 Abstract

The aryl hydrocarbon receptor (AHR) is a transcription factor that belongs to the Per-Arnt-SIM (PAS) superfamily. It has become clear that the AHR is involved in the maintenance of the intestinal immune system by unknown mechanisms. Using global *Ahr* null mice, our laboratory previously demonstrated that the AHR plays a protective role in colitis-associated colorectal cancer (CRC). In this series of experiments, we set out to identify the cell population in which AHR signaling was essential for this biology. We hypothesized that AHR signaling in the intestinal epithelia or resident immune cells was responsible for its protective effects against colitis-associated CRC. To test our hypothesis, we generated mice conditionally null for *Ahr* in a cell-specific manner using Cre-Lox technology. Employing our two-stage model of CRC, we examined the role of AHR signaling in intestinal epithelial cells, macrophages, endothelial and T cells in mediating the protective effects against colitis-associated CRC. Evaluation of colorectal tumor multiplicity revealed that the loss of AHR in the endothelial cell compartment had the most significant influence on the colorectal tumor burden in mice. Further studies are needed to confirm this observation including the assessment of DSS-induced colitis in mice conditionally null for *Ahr* in the endothelial cells.

4.2 Introduction

The aryl hydrocarbon receptor is a transcription factor and a member of the Per-Arnt-Sim (PAS) superfamily. The signal AHR transduction pathway is regulated by xenobiotic ligands including, pollutants, pharmaceuticals, and dietary constituents [1-4]. Upon ligand-binding, a conformation change in the AHR is provoked and this is followed by receptor translocation to the nucleus and heterodimerization with the AHR nuclear translocator (ARNT). The activated AHR/ARNT dimer drives the transcription of various xenobiotic metabolizing genes such as *CYP1A1*, *1A2* and *1B1* and the *Glutathione S-transferase 1a* [5-7].

The AHR was first identified through its role in mediating the adaptive metabolism of xenobiotics, including polycyclic aromatic hydrocarbons (PAH's). Recently, it has been suggested that the AHR is important in various biological processes including the maintenance of the intestinal immune barrier [8-11]. Investigations using mutant mouse models have demonstrated that the AHR is required for the proper functioning of the intestinal mucosa barrier through its regulation of intraepithelial lymphocytes and innate lymphoid cells [10, 12-14].

Our laboratory previously demonstrated that the AHR plays a protective role in colitis-associated colorectal cancer (CRC) [15]. This conclusion was based, in part, on the observation that after treatment with an initiation-promotion colorectal tumor protocol, the *Ahr*-null mice exhibit higher colorectal tumor burden than WT mice [15]. Importantly, these experiments were done in *Ahr* global nulls, where the AHR has been deleted in all cells and cell types of the mouse. Therefore, to better understand the protective role of the AHR in colitis-associated CRC, we set out to determine the identity

of the cells where AHR signaling is important for this outcome. Specifically, we asked if AHR resident in intestinal epithelial cells, macrophages, endothelial, or T cells are involved in the CRC phenotype.

Given the suggested role of the AHR in the maintenance of intraepithelial lymphocytes of the intestine and its role in modulating intestinal inflammation, we hypothesized that the protective effects of the AHR against colitis-associated CRC could be attributed to its signal in the intestinal epithelial and/or resident T cells. To test our hypothesis, we employed a strain of mice in which AHR was expressed in a conditional fashion using a Cre recombinase transgene that excised the receptor gene in intestinal epithelial cells, macrophages, endothelial cells, or T cells. We then exposed the mice to a model of colitis-associated CRC (azoxymethane, AOM, as a tumor initiator and sodium dextran sulfate, DSS, as a tumor promoter) and examined colorectal tumor multiplicity. Our data indicate that the loss of the AHR in the endothelial cells, but not in resident immune cells or epithelial, may increase the susceptibility to colitis-associated CRC.

4.3 Methods

Mice and diets. The transgenic mice were previously described: *Ahr* allele (*Ahr*^{fx/fx}) [16], *Cre*^{Villin} [17], *Cre*^{LysM} [18], *Cre*^{CDH5} [19] and *Cre*^{LCK} [20]. To generate a mouse conditionally null for *Ahr* in a specific cell type, we crossed our *Ahr*^{fx/fx} to animals expressing the Cre recombinase transgene driven by tissue-specific promoters as presented in Figure 4.1 (*Cre*^{Villin}: epithelial cells of the small and large intestine, *Cre*^{LysM}: macrophages, *Cre*^{CDH5}: endothelial cells and *Cre*^{LCK}: T cells). In each group of mice, the *Ahr*^{fx/fx} littermates were used as a control. All mice were housed in a selective pathogen-free facility on corn cob bedding and fed with chow diet (5001, Test Diet, Richmond, IN). Food and acidified water were provided *ad libitum*. All experiments and animal care were approved by the School of Medicine and Public Health, Animal Care and Use Committee of the University of Wisconsin-Madison.

Tumor studies. A colitis-associated colorectal cancer model that employed treatments with azoxymethane (AOM) and sodium dextran sulfate (DSS) was used to induce colorectal tumor in mice [21]. Our tumor protocol has been previously described [15]. Briefly, mice were treated with a single IP injection of AOM followed by a single cycle of 1% DSS in drinking water for five days. Mice were sacrificed sixteen weeks after treatment protocol. The colon and rectum were collected, and tumors were visually counted.

Assessment of Ductus Venosus. The global *Ahr null* mouse develops a patent *ductus venosus* (DV) in the liver. Therefore, for this study, the DV patency was evaluated by perfusion of the liver with trypan blue as previously reported [22]. Briefly, mice were sacrificed by CO₂ euthanasia, and their abdominal cavity was opened to expose the liver. The portal vein was cannulated with a 22G needle, and the liver was flushed with PBS, followed by 1% trypan blue using gravity flow. An incision of the vena cava was made to allow outflow. Perfused liver that remained pink (bypassed by the dye) was considered positive for DV.

Quantification of AOM-induced DNA adducts. Mice were treated with a single IP injection of AOM (10 mg/kg body weight; concentration: 13.4M; Sigma-Aldrich, St. Louis, MO) and sacrificed twelve hours after treatment. The DNA from colon mucosa cells were isolated as previously described [15] and was used to quantify the amount of O⁶-methyl-guanine, the principal mutagenic adduct of AOM [23]. The quantification of O⁶-methyl-guanine was performed by capillary liquid chromatography–electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) following a reported protocol [15, 24]. The total O⁶-methyl-guanine was normalized to the total amount of guanine present in each sample, and the final amount was reported as pmole O⁶-methyl-guanine per μ mole guanine.

Statistical-Analyses. The statistical analyses were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software (La Jolla California USA), www.graphpad.com. Intergroup comparisons were conducted by the nonparametric

Wilcoxon rank sum test. Differences between groups were considered statistically significant when the p -value was < 0.05 .

4.4 Results

Ahr^{fx/fx}Cre^{CDH5} mice displayed higher AOM/DSS-induced colorectal tumors

To evaluate whether the deletion of the AHR in intestinal epithelial cells, macrophages, endothelial cells or T cells influence the susceptibility to inflammation-associated colorectal cancer, we exposed the mice to an AOM/DSS protocol. Colorectal tumor incidence did not differ in all conditional mouse strains compared with their respective control mice (*Ahr^{fx/fx}* vs *Ahr^{fx/fx}Cre^{villin}*, *Ahr^{fx/fx}* vs *Ahr^{fx/fx}Cre^{LysM}*, *Ahr^{fx/fx}* vs *Ahr^{fx/fx}Cre^{CDH5}* and *Ahr^{fx/fx}* vs *Ahr^{fx/fx}Cre^{LCK}*) (Fig. 4.2). Interestingly, the colorectal tumor multiplicity in *Ahr^{fx/fx}Cre^{CDH5}* mice was significantly increased compared with treated littermates *Ahr^{fx/fx}* ($p=0.048$). There was no significant difference in the tumor burden of *Ahr^{fx/fx}Cre^{villin}*, *Ahr^{fx/fx}Cre^{LysM}* and *Ahr^{fx/fx}Cre^{LCK}*, when compared with their respective control matched mice.

Ahr^{fx/fx}Cre^{CDH5} mice exhibited patent ductus venosus

Our laboratory has shown previously that deletion of the AHR in endothelial cells causes patent ductus venosus, similar to that observed in global *Ahr*-null mice [22]. To evaluate whether the deletion of AHR affects the liver vasculature in any of our conditional mouse models, we assessed the DV status by liver perfusion (Fig. 4.3). Patent ductus venosus was confirmed in 100% of the *Ahr^{fx/fx}Cre^{CDH5}* mice whereas *Ahr^{fx/fx}Cre^{villin}*, *Ahr^{fx/fx}Cre^{LysM}* and *Ahr^{fx/fx}Cre^{LCK}* mice exhibited normal liver.

AOM-induced DNA adducts

In our model of colitis-associated CRC, the AOM-induced DNA adducts represent the initiation stage of the tumorigenesis process. We set out to determine the effects of AOM by quantifying the amount of O^6 -methyl-guanine in the colon mucosa of our three conditional mouse models (Fig. 4.4). Importantly, given the observations that $Ahr^{fx/fx}Cre^{CDH5}$ mice displayed higher tumor burden and exhibited a DV, which might influence first pass metabolism of AOM, we wanted to examine the possibility that the deletion of the AHR in the endothelial cells also provokes an alteration in AOM-induced DNA adducts. However, the amount of O^6 -methyl-guanine was not significantly different in any of the $Ahr^{fx/fx}Cre^{villin}$, $Ahr^{fx/fx}Cre^{CDH5}$ and $Ahr^{fx/fx}Cre^{LysM}$ mice compared with their respective matched controls.

4.5 Discussion

In chapter 2, we show that the *Ahr*-null mice are more susceptible to colitis-associated colorectal CRC than WT mice. This conclusion was based on the fact that *Ahr* nulls exhibited higher colorectal tumor burden and increased levels of both AOM-induced DNA adducts and DSS-induced inflammation. These observations demonstrated that the AHR plays a protective role against colitis-associated colorectal cancer through multiple mechanisms [15]. Here, we set out to identify the cell type where AHR signaling is required for this protective effect. We chose to focus on four candidate cell types that we predicted might be involved in AHR-mediated regulation of the intestinal mucosa barrier; The intestinal epithelial cells, The macrophages, The endothelial cells and The T cells [13, 25-30]. We postulated that AHR signaling in one or more of these cell types is responsible for the AHR's protective effects against colitis-associated CRC. To test this idea, we generated mice conditionally null for *Ahr* in these four cells types and evaluated their contribution to the susceptibility to AOM/DSS-induced colorectal tumorigenesis.

Following treatment with AOM and DSS, we evaluated the colorectal tumor multiplicity in mice with *Ahr*^{fx/fx}*Cre*^{villin} (loss of AHR in intestine epithelial cells), *Ahr*^{fx/fx}*Cre*^{LysM} (loss of AHR in macrophages), *Ahr*^{fx/fx}*Cre*^{CDH5} (loss of AHR in endothelial cells) and *Ahr*^{fx/fx}*Cre*^{LCK} (loss of AHR in T cells) (Fig. 4.2). We found that the loss of AHR in the endothelial cells significantly increased the colorectal tumor burden as *Ahr*^{fx/fx}*Cre*^{CDH5} mice displayed higher tumor multiplicity than the control mice. These data indicate that the AHR signaling in the endothelial cells may contribute to the AHR protective effects against colitis-associated colorectal cancer.

Although these data were significant, we noticed that the average of the tumor burden in the control mice group was below the average of tumors observed in the control groups of $Ahr^{fx/fx}Cre^{villin}$, $Ahr^{fx/fx}Cre^{LysM}$ and $Ahr^{fx/fx}Cre^{LCK}$ which were run in parallel. Thus, we would like to perform a multiple comparison statistical test to evaluate the veracity of our data. Also, we would like to repeat this experiment to confirm that the deletion of the AHR in the endothelia is critical to our phenotype. Another reason to repeat these studies was that deletion of the AHR in $Ahr^{fx/fx}Cre^{villin}$ and $Ahr^{fx/fx}Cre^{LCK}$ mice led to small, but statistically insignificant decreases in colorectal tumor multiplicity. One important consideration for future experiments is that the expression of the *villin* transgene occurs in the villus and crypt epithelial cells of the small and large intestines, and is not unique to the colon [17]. Since this model is not specific to the colonic epithelial cells, the reduction in colorectal tumor burden observed in $Ahr^{fx/fx}Cre^{villin}$ mice cannot be attributed only to the colonic epithelial cells. A conditional *Ahr*-null mice model specific to the proliferative compartment of the colon should be used to thoroughly examine the effect of the AHR signaling in colon epithelial cells in AOM/DSS-associated CRC.

Regarding the deletion of the AHR in the macrophages, we found that tumor multiplicity was nearly the same between $Ahr^{fx/fx}Cre^{LysM}$ and the control mice. At the first sight, this observation suggests that the macrophages are not essential to our phenotype. However, it has been reported that the Cre expression occurs in only approximately 50% of the macrophages [31]. Therefore, our observation is not conclusive and further examination is needed to characterize the role of the macrophages in the protective role of the AHR.

Our lab has previously reported that the *Ahr*-null mice exhibit a patent *ductus venosus* (DV) resulting in a portosystemic shunt, and therefore they display decreased first pass biotransformation of various xenobiotics [22, 32]. Thus, it is possible that the high colorectal tumor burden observed *Ahr*-null mice may be a reflection of their reduced metabolism of AOM causing increased levels of AOM-induced DNA adducts. To investigate this possibility in our conditional *Ahr*-null mice and to determine if the DV was contributing to the susceptibility to tumor development, we examined both the DV status and the AOM-induced DNA adducts in *Ahr^{fx/fx}Cre^{villin}*, *Ahr^{fx/fx}Cre^{LysM}*, *Ahr^{fx/fx}Cre^{CDH5}* and *Ahr^{fx/fx}Cre^{LCK}* mice. Liver perfusion studies demonstrate that *Ahr^{fx/fx}Cre^{CDH5}* do have a patent *ductus venosus* whereas *Ahr^{fx/fx}Cre^{Villin}*, *Ahr^{fx/fx}Cre^{LysM}* and *Ahr^{fx/fx}Cre^{LCK}* mice exhibited normal liver perfusion (Fig. 4.2). Further, we evaluated the susceptibility to AOM-induced DNA adducts (Fig. 4.3). There was no significant difference in the levels of *O*⁶-methyl-guanine between the *Ahr^{fx/fx}Cre^{CDH5}*, *Ahr^{fx/fx}Cre^{Villin}* and *Ahr^{fx/fx}Cre^{LysM}* mice when compared with their respective matched control mice. This observation suggests that the deletion of the AHR in the endothelial cells, intestinal epithelial cells and macrophages is not involved in the high susceptibility to AOM-induced DNA adducts observed in *Ahr*-null mice. Unfortunately, we were unable to evaluate the AOM-induced DNA adduct in *Ahr^{fx/fx}Cre^{LCK}* mice. Nevertheless, since the tumor burden in these mice was not significantly different we would not expect an increased level of AOM-induced DNA adducts.

Importantly, these data indicate that the DV phenotype of the AHR endothelial cell deletion model is not a contributor to the susceptibility to AOM-induced DNA adducts in these mice. This conclusion is based on the observation that the

Ahr^{fx/fx}*Cre*^{CDH5} mice exhibited a DV but the levels of *O*⁶-methyl-guanine were not significantly increased when compared with control mice.

Since our model of colitis-associated CRC contains a tumor promotion stage based on the treatment of DSS, future experiments will be focused on evaluating the DSS-induced colitis in our mice models. The examination of the susceptibility to inflammation in the *Ahr*^{fx/fx}*Cre*^{CDH5} mice could add valuable data that would allow us to confirm our previous observation that the loss of the AHR signaling in the endothelial cells increases the susceptibility to colitis-associated CRC, possibly by increasing the susceptibility to inflammation.

Regarding the responses of *Ahr*^{fx/fx}*Cre*^{villin} mice to DSS-induced inflammation, it has been reported that the deletion of the AHR in the intestinal epithelial cells (*Ahr*^{fx/fx}*Cre*^{villin}) aggravated the DSS-induced colitis [33]. Interestingly, following an AOM/DSS-induced tumor protocol, our *Ahr*^{fx/fx}*Cre*^{villin} mice exhibited no significant difference in the tumor burden. Thus, future evaluation of the DSS-induced colitis is needed to examine the role of the AHR signaling in intestinal epithelia in our model of colitis-associated colorectal cancer. Since this mouse model is not unique to the colon epithelial cells, a conditional *Ahr*-null mouse model specific to the proliferative compartment of the colon should be included to thoroughly examine the effect of the AHR signaling in colon epithelial cells in AOM/DSS-associated CRC.

Multiple observations have been reported regarding the response of *Ahr*^{fx/fx}*Cre*^{LCK} mice to different protocols of DSS-induced colitis. Following administration of 1% DSS, these mice displayed reduced colitis than the control mice [33]. In a separated experiment, where the colitis was induced by treatment with 2% DSS, *Ahr*^{fx/fx}*Cre*^{LCK}

exhibited similar responses to colitis than the control mice [34]. We observed no significant difference in the tumor burden of the $Ahr^{fx/fx}Cre^{LCK}$, although we need to assess the AOM-induced DNA adducts in these mice, perhaps its responses to the colitis-associated CRC is related to the reduced inflammation described in these reports. Future experiments will include the assessment of AOM and DSS-induced colitis to further characterize the role of the T cells in our CRC phenotype.

It has been reported that the loss of the AHR receptor in the macrophages ($Ahr^{fx/fx}Cre^{LysM}$) does not influence the DSS-induced colitis [33]. This observation is comparable to our tumor multiplicity data as the tumor burden in $Ahr^{fx/fx}Cre^{LysM}$ was similar to the $Ahr^{fx/fx}$ control mice. To confirm the involvement of the macrophages in the protective role of the AHR, we will next examine the effects of DSS-induced colitis in $Ahr^{fx/fx}Cre^{LysM}$ mice.

The work reported herein provides preliminary data for determining the cell autonomous role of the AHR in inflammation-associated CRC. Further experiments are needed to thoroughly evaluate the role of the AHR signaling in The intestinal epithelial cells, The macrophages, The endothelial cells and The T cells in mediating the protective effects against colitis-associated CRC. Future experiments will be a focus on repeating the tumor studies and evaluating the DSS-induced colitis in our mice models.

4.6 Figures and Legends

Figure 4.1: Mice breeding scheme

To generate a mouse conditionally null for *Ahr* in a specific cell type, we crossed our homozygous *Ahr*^{fx/fx} to animals expressing the Cre recombinase transgene driven by tissue-specific promoters (*Cre*^{Villin}: epithelial cells of the small and large intestine, *Cre*^{LysM}: macrophages and *Cre*^{CDH5}: endothelial cells, *Cre*^{LCK}: T cells). The *Ahr*^{fx/fx}*Cre*^{-/-} were used as control mice.

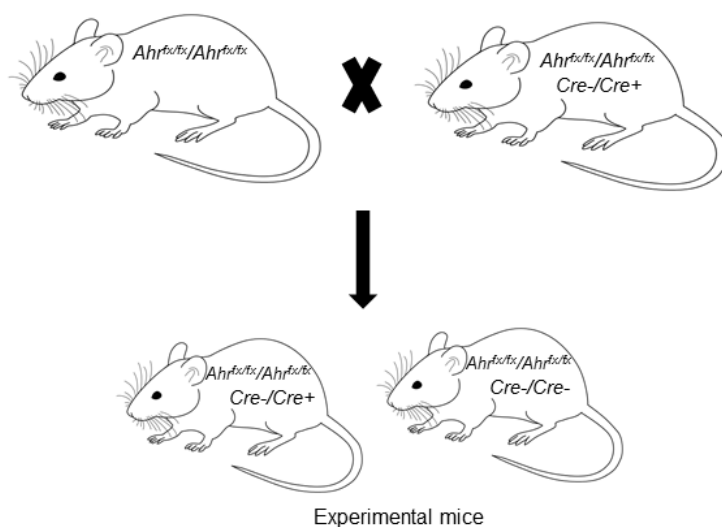


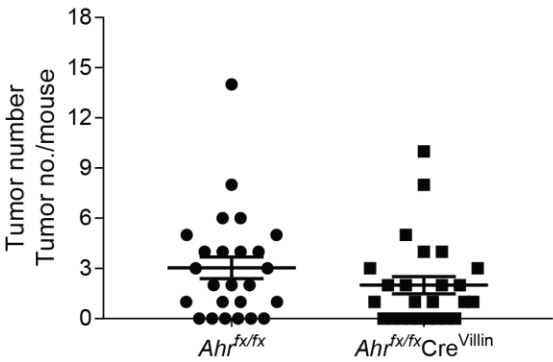
Figure 4.2: $Ahr^{fx/fx}Cre^{CDH5}$ mice displayed higher AOM/DSS-induced colorectal tumorigenesis

AOM/DSS-induced colorectal tumorigenesis. At eight weeks of age, mice were treated with the tumor induction protocol. Mice were sacrificed sixteen weeks after treatment, and the colon and rectum were harvested for the evaluation of tumors. Tumor incidence and multiplicity in: $Ahr^{fx/fx}Cre^{villin}$ (A and B) (n=25); $Ahr^{fx/fx}Cre^{LYSM}$ (C and D) (n=22-27) and $Ahr^{fx/fx}Cre^{CDH5}$ (E and F) (n=23). $Ahr^{fx/fx}Cre^{LCK}$ (G and H) (n=32). a; significantly different compared to $Ahr^{fx/fx}$ control mice ($p < 0.05$). Error bars are represented as SEM.

A)

Genotype	Tumor incidence (%)
<i>Ahr</i> ^{fx/fx}	19/25 (76%)
<i>Ahr</i> ^{fx/fx} Cre ^{Villin}	16/25 (64%)

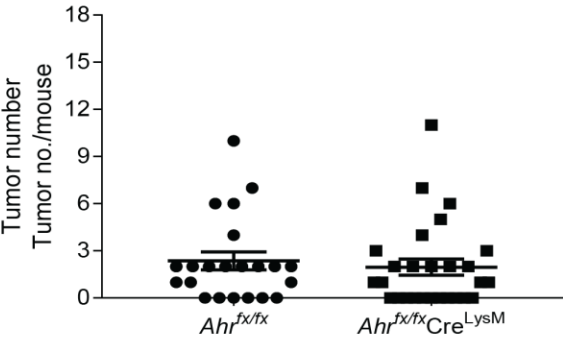
B)



C)

Genotype	Tumor incidence (%)
<i>Ahr</i> ^{fx/fx}	16/22 (73%)
<i>Ahr</i> ^{fx/fx} Cre ^{LysM}	16/27 (60%)

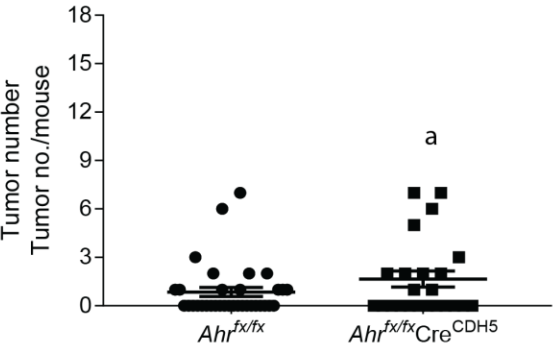
D)



E)

Genotype	Tumor incidence (%)
<i>Ahr</i> ^{fx/fx}	5/23 (22%)
<i>Ahr</i> ^{fx/fx} Cre ^{CDH5}	11/23 (48%)

F)



G)

Genotype	Tumor incidence (%)
<i>Ahr</i> ^{fx/fx}	20/32 (63%)
<i>Ahr</i> ^{fx/fx} Cre ^{LCK}	20/32 (63%)

H)

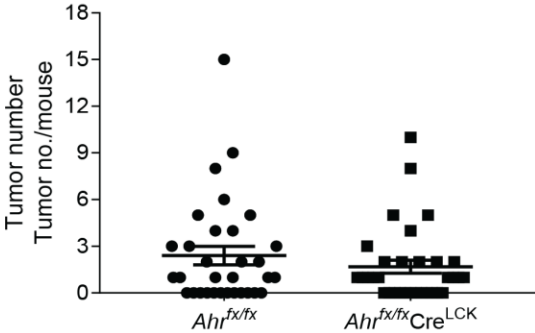


Figure 4.3: Assessment of patent *ductus venosus*

Patent *ductus venosus* status was evaluated by liver perfusion. A) *Ductus venosus* status in, $Ahr^{fx/fx}Cre^{villin}$, $Ahr^{fx/fx}Cre^{LysM}$ and $Ahr^{fx/fx}Cre^{CDH5}$. *Ductus venosus* status in $Ahr^{-/-}$ and $Ahr^{fx/fx}$ has been previously reported [16, 22]. B) Representation of perfused livers. *Left*: Normal liver; full coloration of the liver indicates no DV as closed portal branched retain the tryptophan blue. *Right*: Patent *ductus venosus*; the tryptophan blue enter to the portal vein and bypass the liver through the DV into the inferior vena cava. LLW: liver lobe weights.

A)

Genotype	% of patent DV	Normal LLW	Matched Control	n
<i>Ahr</i> ^{-/-}	100 *	YES	YES	-
<i>Ahr</i> ^{fx/fx}	0 **	YES	YES	-
<i>Ahr</i> ^{fx/fx} <i>Cre</i> ^{Villin}	0	YES	YES	0/14
<i>Ahr</i> ^{fx/fx} <i>Cre</i> ^{LyzM}	0	YES	YES	0/19
<i>Ahr</i> ^{fx/fx} <i>Cre</i> ^{CDH5}	100	NO	YES	10/10
<i>Ahr</i> ^{fx/fx} <i>Cre</i> ^{LCK}	0	YES	YES	0/14

* Lavis *et. al.* (2000)** Walisser *et. al* (2005)

B)



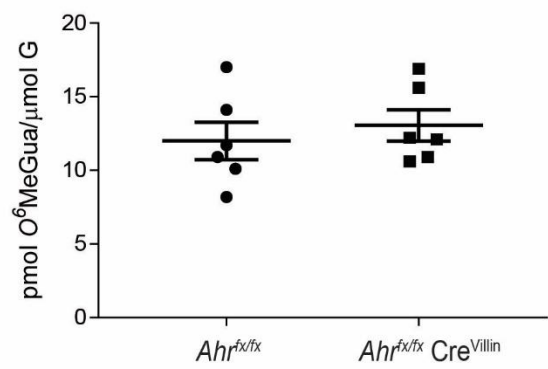
Normal liver

Liver with
patent DV

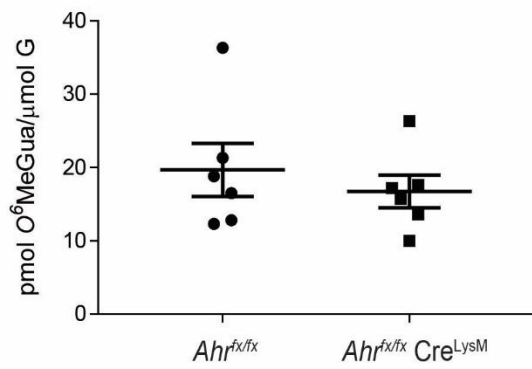
Figure 4.4: AOM-induced DNA adducts

Evaluation of AOM-induced DNA adducts. At eight weeks of age, $Ahr^{fx/fx}Cre^{villin}$, $Ahr^{fx/fx}Cre^{LysM}$ and $Ahr^{fx/fx}Cre^{CDH5}$ mice were treated with a single injection of AOM. Twelve hours after the injection, mice were sacrificed, and DNA from colon mucosa was isolated for further quantification of the levels of O^6 -methyl-guanine. Error bars are represented as SEM.

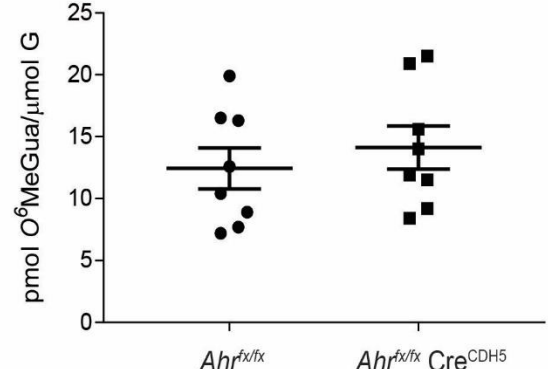
A)



B)



C)



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