THE ROLE OF THE ARYL HYDROCARBON RECEPTOR IN
MODULATING SKIN AND INTESTINAL EPITHELIAL PHYSIOLOGY

A Dissertation in
Biochemistry, Microbiology, and Molecular Biology

by
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ABSTRACT

Barrier tissues such as the skin and intestine are important for the first line of defense against injury and exposure to potentially harmful toxicants or pathogens from the external environment. The cellular response in these tissues to stimuli often begins with altered levels of gene expression. Ligand-activated transcription factors such as the aryl hydrocarbon receptor (AHR) are important mediators of this response. Due to the complex nature of AHR activation and its often context-specific role in health and disease, the use of relevant in vitro and in vivo models is necessary to fully elucidate its specific roles in a particular tissue. This dissertation describes the characterization of an AHR-regulated gene in the skin which is induced in response to AHR activation and inflammatory stimuli, the development of a mouse model for studying AHR activation during ultraviolet light exposure, and the development of a mouse model for use in determining the role of AHR expression in intestinal epithelial cells. The results of these studies confirm both the complex nature of AHR activation in different contexts and the importance of its expression in barrier tissues.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa(s)</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>AHH</td>
<td>aryl hydrocarbon hydroxylase</td>
</tr>
<tr>
<td>AHR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>BaP</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>BNF</td>
<td>β-Naphthoflavone</td>
</tr>
<tr>
<td>CCL20</td>
<td>Chemokine (C-C motif) ligand 20</td>
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<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CH223191</td>
<td>2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide</td>
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<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1</td>
</tr>
<tr>
<td>CXCL2</td>
<td>chemokine (C-X-C motif) ligand 2</td>
</tr>
<tr>
<td>CXCL5</td>
<td>C-X-C motif chemokine 5</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>cytochrome P450, family 1, subfamily A, polypeptide 1</td>
</tr>
<tr>
<td>DIM</td>
<td>3, 3’-diindolylmethane</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DRE</td>
<td>dioxin response element</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sodium sulfate</td>
</tr>
<tr>
<td>FICZ</td>
<td>6-Formylindolo(3,2-b)carbazole</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GNF351</td>
<td>N-(2-(1H-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9H-purin-6-amine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>HAH</td>
<td>halogenated aromatic hydrocarbon</td>
</tr>
<tr>
<td>HSP90</td>
<td>90-kDa heat shock protein</td>
</tr>
<tr>
<td>I3C</td>
<td>indole-3-carbinol</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICZ</td>
<td>indolo[3,2-b]carbazole</td>
</tr>
<tr>
<td>IEC(s)</td>
<td>intestinal epithelial cell(s)</td>
</tr>
<tr>
<td>IL1B</td>
<td>interleukin 1 beta</td>
</tr>
<tr>
<td>IL6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>mAHR</td>
<td>mouse aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PTGS2</td>
<td>prostaglandin-endoperoxide synthase 2</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAhRM</td>
<td>selective aryl hydrocarbon receptor modulator</td>
</tr>
<tr>
<td>SGA360</td>
<td>1-allyl-3-(3,4-dimethoxyphenyl)-7-(trifluoromethyl)-1H-indazole</td>
</tr>
<tr>
<td>S100A9</td>
<td>S100 calcium binding protein A9</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachloro-dibenzo-p-dioxin</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>3-MC</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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</table>
ACKNOWLEDGEMENTS

One of my favorite quotes, and one that I think is especially apt in regards to scientific inquiry, comes from Sir Isaac Newton: “If I have seen further, it is by standing upon the shoulders of giants.” Though there is some debate concerning whether Newton wrote this statement in a letter to his fellow scientist, Robert Hooke, with a certain degree of sarcasm, at this particular moment, I use it in all earnestness. I could not be where I am today without the wisdom and encouragement of so many people. First of all, I would like to thank my advisor, Dr. Gary Perdew. His enthusiasm and endless optimism about the potential of scientific research has been an inspiration. I would also like to thank my committee members, Drs. Frisque, Paulson, Peters, and Dudley for their support throughout this process. Thank you to my fellow lab members, especially Dr. Iain Murray, Dr. Tejas Lahoti, Dr. Brett DiNatale, Nate Girer, Troy Hubbard, Elif Muku, Dr. Ann Kusnadi, and Kelly Wagner. A special thanks to Dr. Adam Glick and members of his lab including Dr. Anand Ravindran, Dr. Nick Blazanin, Jeongin Son, and Michael Podolsky for their help and knowledge concerning skin research. A debt of thanks is due to Stephanie Bora of Dr. Cantorna’s lab for helping me to gavage my mice and teaching me how to conduct the *C. rodentium* experiment. A great deal of thanks belongs to my fellow classmates in the BMMB program, especially my good friend Kristen Browder. A great amount of thanks is due to my hardworking undergraduate mentees, Angela Alnemri, Shana Santarelli, and especially Jake Boyer. I would also like to acknowledge all of the wonderful people “downstairs” in the mouse facility who do a fantastic job of taking care of our animals, and especially Mark Borkowski, who has been such a pleasure to work with.
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CHAPTER 1: INTRODUCTION

1.1 THE ARYL HYDROCARBON RECEPTOR

1.1.1 The aryl hydrocarbon receptor as an environmental sensor

The aryl hydrocarbon receptor (AHR) (also known as the Ah receptor) is a ligand-activated transcription factor capable of eliciting physiological changes in response to environmental cues through its ability to regulate a diverse array of genes through direct transcriptional regulation and through indirect protein-protein interactions with other transcription factors. Upon its initial discovery and characterization, it was studied primarily as a mediator of toxicity, as its characteristic target genes included members of the cytochrome P450 (CYP) family of enzymes. More recent studies involving the AHR have shifted focus to the physiological role of the receptor within different tissues, cell types, and disease states, in addition to continued interest in its toxicological effects. One important step in this effort was the generation of in vivo mouse models, including the AHR knock out ($Ahr^{-/-}$) mouse, followed by the development of additional models including cell-specific knock outs, constitutively active, and “humanized” mouse models. Increased expression of AHR at barrier tissues such as the lungs, skin and intestine [1] implicate it as an important mediator in modulating cellular responses to environmental stimuli. This review will provide a summary of basic AHR biology, including the AHR signaling pathway, target genes, and ligands, followed by a description of AHR-relevant mouse models, and concluding with a summary of the physiology of the skin and intestinal tract and an overview of the current knowledge of the role of AHR in these tissues.
1.1.2 The AHR signaling pathway and target genes

The prototypical AHR signaling pathway is the most clearly defined mode of AHR activation. AHR, in the absence of bound ligand, is located in the cytoplasm bound to a chaperone core complex, which consists of two units of heat shock protein 90 (HSP90) [2], one unit of X-associated protein 2 (XAP2) [3], and one unit of p23 [4]. HSP90 is required for the stability and ligand-binding ability of AHR in the cytoplasm upon initial complex formation [5, 6]. Upon activation via ligand-binding, the AHR translocates to the nucleus, dissociates from its chaperone complex [7], and binds to its heterodimeric partner, the aryl hydrocarbon receptor nuclear translocator (ARNT) [8]. The AHR/ARNT complex then recognizes and binds to the core sequence, 5′-T/GCGTG-3′ [9], known as the dioxin response element (DRE), xenobiotic response element (XRE), or aryl hydrocarbon response element (AHRE). Through the interaction of the AHR/ARNT complex with functional DRE sequences, expression of genes involved in a wide array of cellular functions is influenced. Persistent presence of ligand causes AHR protein degradation, during which the AHR is exported from the nucleus and degraded by the cytoplasmic proteasome [10], with the transcription activation domain, and the DNA-binding domain of both AHR and ARNT playing a role in their degradation [11].
Figure 1.1 The AHR signaling pathway.
The essential steps of the AHR signaling pathway are outlined. The first step involves ligand binding to the AHR while in complex with its chaperone proteins in the cytoplasm. Upon ligand binding, the AHR and chaperone complex translocates into the nucleus, upon which the complex dissociates from AHR. AHR binds to its heterodimeric partner, ARNT (aryl hydrocarbon receptor nuclear translocator), followed by the AHR/ARNT complex binding to dioxin response elements (DREs) in the promoter of inducible genes, leading to gene transcription. See text for the appropriate references.

The first target genes characterized for the AHR include those encoding enzymes involved in drug metabolism. Prototypical examples of these genes include those encoding cytochrome P450 (CYP), family 1, subfamily a, polypeptide 1 (CYP1A1) [12], CYP1A2 [13], and CYP1B1 [14]. CYP enzymes are involved in Phase I metabolism, and therefore in the clearance of potentially toxic substances from the body. Due to this function, they are also conversely linked to increased toxicity through the generation of
harmful intermediates. For example, benzo(a)pyrene (BaP), through the AHR signaling pathway, leads to the induction of CYP1A1 expression. The CYP1A1 enzyme can subsequently metabolize BaP to the mutagenic intermediate, the trans-7,8-diol 9,10-epoxide of BaP [15]. AHR also induces expression of certain genes encoding proteins involved in Phase II drug metabolism, including NAD(P)H:oxidoreductase (NQO1) [16], several glutathione S-transferases [17] and UDP-glucuronosyltransferases [18]. Due to the role of AHR in the regulation of these enzymes, the AHR activation pathway is often associated with a toxic response [9].

The AHR has been implicated in a variety of cellular processes in addition to its roles in drug metabolism. These processes include, but are not limited to, cell cycle control [19], cellular proliferation [20], inflammation [21, 22], growth regulation [23], differentiation [24], and Th17 cell regulation [25]. Additionally, AHR can regulate its own activity through induction of the aryl hydrocarbon receptor repressor (AHRR), itself an AHR-regulated gene [26].

1.1.3 Classification and structure of the AHR

The AHR belongs to the basic helix-loop-helix family of transcription factors. Within that family, it is a member of the subgroup termed the Period (PER)/aryl hydrocarbon receptor nuclear translocator (ARNT)/single-minded (SIM, after the Drosophila protein) (PAS) domain proteins, which are characterized by two PAS regions (PAS-A and PAS-B). The PAS domains confer the ability of these transcription factors to sense signals and influence physiologic responses. The functional domains of the mouse AHR (mAHR) were characterized through deletion analysis. The basic (b) region is required for the interaction between AHR and DRE sequences (i.e., DNA binding), while
the helix-loop-helix (HLH) motif is involved in the heterodimerization between AHR and ARNT. The PAS domain is also critical for AHR/ARNT dimerization and subsequent binding of AHR/ARNT to its cognate response element. Additional functions of the PAS regions include binding of AHR to HSP90, XAP2, and ligand in the cytoplasm. The carboxyl terminus of the AHR contains three transactivation (TA) domains, including acidic, glutamine-rich, and serine/threonine-rich domains (Figure 1.2) [27-30].

Figure 1.2: A representation of the mouse AHR functional domains.
Figure was adapted from Fukunaga, et. al. [27], and Murray, et. al. [29]. b, basic; HLH, helix-loop-helix; PAS, PER-ARNT-SIM; XAP2, X-associated protein 2; HSP90, heat shock protein 90. Figure not drawn to scale.

1.1.4. Classification of AHR ligands

AHR ligands are numerous, structurally diverse, and derived from a variety of sources, including environmental pollutants, microbial products, dietary chemicals, plant polyphenols, photoproducts, and pharmaceuticals, and vary in their relative affinity for AHR. These ligands can further be broadly classified as agonists, antagonists, or selective aryl hydrocarbon receptor modulators (SAhRMs). Figure 1.3 depicts the chemical
structures of representative AHR ligands, and a brief description of each class follows below.

**Figure 1.3: Chemical structures of AHR ligands.**
Representative structures of different classes of AHR ligands are depicted. TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; BaP, benzo(a)pyrene; BNF, beta-naphthoflavone; FICZ, 6-formylindolo[3,2-b]carbazole; ICZ, indolo[3,2-b]carbazole; GNF351, N-(2-(1H-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9H-purin-6-amine; CH223191, 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolyazo-phenyl)-amide; SGA360, 1-allyl-3-(3,4-dimethoxyphenyl)-7-(trifluoromethyl)-1H-indazole.
1.1.5 Agonists

Agonists are typically defined as ligands which activate the AHR through the canonical signaling pathway, leading to nuclear translocation of the receptor and induction of target genes through DRE-mediated activation. These chemicals may originate from exogenous sources or may be produced endogenously. They encompass several different categories of ligand, and are briefly described below.

Xenobiotics

AHR function was originally described through its ability to be activated by a variety of planar, hydrophobic environmental pollutants. These included halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), halogenated dibenzofurans, and diphenyls and polycyclic aromatic hydrocarbons (PAHs), such as 3-methylcholanthrene (3-MC), BaP, 7,12-dimethylbenz[a]anthracene (DMBA) and relatively non-toxic flavonoids, such as beta-naphthoflavone (BNF). Though PAHs are toxic compounds, they are metabolized more readily than the HAHs such as TCDD. TCDD is the most potent HAH agonist known for AHR. Its low rate of metabolism (half life in humans is ~10 years) leads to its biological persistence and the development of toxicity. Many toxic effects are associated with TCDD exposure, including but not limited to, lethality, wasting syndrome, teratogenesis, endocrine disruption, chloracne, and toxicities of the hepatic, immune, cardiovascular, and reproductive systems [29, 31].

Endogenous ligands

Another category of AHR ligands include those produced endogenously that result in biological consequences through AHR activation. Examples of these agonists include tryptophan metabolites such as kynurenic acid [32], kynurenine [33], indoxyl 3-sulfate [34], and 6-formylindolo[3,2-b]carbazole (FICZ) [35]. An additional class of
endogenous ligands for the AHR are lipids and their metabolites, including lipoxin A4 [36], low-density lipoproteins that are modified by shear stress [37], and leukotriene A4 metabolites [38].

**Dietary ligands**

Dietary AHR ligands have been identified and characterized from vegetables and fruits. Cruciferous vegetables including broccoli and Brussels sprouts contain glucobrassicin, which can be enzymatically cleaved to indole-3-carbinol (I3C) and indole-3-acetonitrile (I3ACN). The acid environment of the stomach leads to the condensation of I3C and I3ACN, leading to the production of additional AHR ligands, including 3,3’diindolylmethane (DIM), 2-(indol-3-ylmethyl)-3,3’-diindolylmethane, and indolo[3,2-b]carbazole (ICZ). All of these ligands have varying degrees of affinity for the AHR, though ICZ has the highest affinity [39]. Additional diet-derived ligands of AHR include certain flavonoids, such as quercetin and kaempferol [40].

**Microbial-derived ligands**

Ligands for AHR that are produced by microbes have also been characterized, especially in barrier tissues, which are highly populated with microbiota. In human skin scales from patients with diseases connected to the yeast *Malassezia*, several AHR ligands were identified, including indirubin, FICZ, ICZ, malassezin, tryptanthrin, and pityriacitrin [41]. Intestinal microbes also produce metabolites that are AHR ligands, including indole, indole-3-acetate, and tryptamine [42, 43]. As additional studies of the interactions between the microbiota and host are conducted, more microbial ligands of AHR are likely to be identified.
1.1.6 Antagonists

The development or discovery of antagonists for AHR-mediated activity arose from the need to inhibit its toxic effects, as well as a means of studying its biological functions. Pharmacologic compounds were developed to inhibit AHR activity, and naturally occurring chemicals were characterized as exhibiting AHR-repressive properties. Specific examples of AHR antagonists include the xenobiotics GNF351 [44], StemRegenin 1 (SR1) [45], CH223191 [46], alpha-naphthoflavone (αNF) [47], 6,2’,4’-trimethoxyflavone (TMF) [48], 3’-methoxy-4’-nitroflavone (MNF) [49], and the natural compound resveratrol [50]. These AHR antagonists vary in their affinity for AHR, and in their species- and cell-type specificities, as well as ligand-selective inhibitions. For example, CH223191 was found to inhibit TCDD and other HAH-induced AHR-dependent reporter gene expression, while failing to block AHR-dependent expression when increased by PAHs, flavonoids, or indirubin [51]. In the same study, the authors reported that TMF and MNF also exhibited preferential inhibition of TCDD when compared to BNF. Likewise, GNF351 has been shown to exhibit selective inhibition of AHR activity in vivo limited to the ileum and colon, due to its poor absorption and its metabolism by CYP enzymes after oral gavage [52]. Additionally, αNF is able to antagonize the effects of TCDD, but is an agonist itself at micromolar concentrations [47]. The appropriate use of AHR antagonists for inhibiting AHR-mediated activity therefore needs to be evaluated carefully to be sure the most useful antagonist is being used for a particular application.
1.1.7 Selective aryl hydrocarbon receptor modulators

Due to the overt toxicity exhibited by potent agonists, it has become desirable to develop selective compounds for the AHR. These chemicals allow the AHR to mediate beneficial biological processes, while avoiding the potentially toxic effects, allowing the AHR to be pursued as a more viable drug target. The term “SAhRM,” (selective aryl hydrocarbon receptor modulator) refers to two distinct classes of ligand in the literature. SAhRMs were initially defined by the work of Dr. Stephen Safe and refer to a class of AHR ligands which, much in the manner of selective estrogen receptor modulators (SERMs), exhibit specific agonistic or antagonistic properties dependent on the species, tissue, or cell type [53]. Examples of SAhRMs encompassing this definition include Diindolylmethane (DIM) [54] and 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) [55], which both exhibit anti-estrogenic effects in an AHR-dependent manner without mediating induction of hepatic Cyp1a1 expression, a typical consequence of toxic AHR agonists such as TCDD.

An additional definition of SAhRMs was later established by the work of Dr. Gary Perdew. After observing that the repression of the acute-phase response gene serum amyloid A (Saa), is accomplished in an AHR-dependent, but DNA-binding independent manner, it was hypothesized that selective modulators could exist that could repress specific inflammatory gene expression while avoiding a potentially toxic response [56]. Subsequent studies provided evidence for this concept, in that the SERM, Way-169916, was also capable of binding to AHR and repressing SAA1 expression in an AHR-dependent manner, while failing to induce DRE-dependent CYP1A1 expression [57]. Chemical modification of Way-169916 led to the development of SAhRMs with specific
affinity for the AHR that exhibit the ability to repress acute phase gene expression independent of DNA binding, including the compound SGA360 [58]. The development and characterization of derivatives of SGA360 with greater affinity for the AHR, as well as the determination of the mechanism of SAhRM-mediated effects, is the subject of current research.

1.1.8 Species-specificity in ligand binding

Multiple studies have shown that affinity for a particular AHR ligand often differs between species. For example, while TCDD is an agonist for both mouse and human AHR, the mouse AHR binds TCDD with an affinity~10 fold higher than human AHR, and thus exhibits greater toxic effects upon exposure [59]. In contrast, certain “naturally” derived AHR agonists, such as indole [42] and indirubin [59] exhibit greater affinity for the human AHR. This difference in binding affinity makes in vivo comparative studies between mouse and human difficult to interpret. The development of appropriate in vivo models for the study of AHR-mediated effects on physiology is important for determining the relevance of AHR activation under particular circumstances.

1.2 THE USE OF IN VIVO MODELS FOR THE STUDY OF AHR

Many experimental in vivo models have been used and developed to elucidate the physiological role of the AHR. These models in the mouse include the use of laboratory strains expressing alleles of AHR with different ligand-binding affinities, complete ablation of AHR through the development of the Ahr−/− mouse, the knockout out of the AHR in specific cell types or tissues, models with mutations affecting specific aspects of the AHR signaling pathway, constitutively active AHR (CA-AHR) models (complete or
cell-type specific), and “humanized” AHR mice. Below is summarized how mouse models have been used to clarify the physiological function of AHR \textit{in vivo}.

1.2.1 \textit{Ahr} alleles in laboratory mouse strains exhibit differences in ligand binding affinity

While most mouse models used to study the AHR are generated with a particular hypothesis in mind, the existence among laboratory mouse strains of alleles encoding an AHR that exhibit differences in ligand-binding affinities have been useful for studying \textit{in vivo} ligand-mediated effects. The observation that different inbred laboratory mouse strains varied in their response to aromatic hydrocarbons as measured by the induction of monooxygenase activity led to the hypothesis that a genetic mutation in an inducible receptor could be the cause of this difference in affinity [60]. Two laboratory mouse strains, C57BL/6J and AKR/J, exhibited differences in the inflammatory response upon exposure to the AHR agonist DMBA. The C57BL/6J mice were highly reactive to DMBA and AKR/J mice were minimally reactive, as evidenced by the development of severe skin ulceration in all C57BL/6J mice tested and no ulceration in the AKR/J mice [61]. Administration of 3-MC induced hepatic AHH activity in C57BL/6J mice (4.8-fold), while failing to induce in DBA/2J mice (0.85-fold), classifying the C57BL/6J mice as “inducible”, while the DBA/2J were classified as “noninducible.” Analysis using genetic crosses and backcrosses between these two strains revealed the susceptibility to be dependent on a single autosomal dominant gene [62]. The “inducible” or “responsive” allele was termed \textit{Ah}^b (named for C57BL/6J mice), while the “noninducible” or “nonresponsive” allele was called \textit{Ah}^d (named for DBA/2J mice, but also found in AKR/J mice) [63].
A total of four distinct Ah alleles have been characterized in common laboratory mouse strains [63]. Some of the traits that distinguish the four alleles from each other are summarized in Table 1.1, based on the findings of Poland and colleagues in two studies [63, 64]. The three Ah\(^b\) alleles have high affinity for aromatic hydrocarbons and are therefore responsive, while the Ah\(^d\) allele exhibits minimal affinity and responsiveness for aromatic hydrocarbons. Mutagenesis studies demonstrated that the difference in ligand binding capacity for aromatic hydrocarbons between the Ah\(^b\) and Ah\(^d\) alleles is due to the substitution of a valine in the Ah\(^d\) allele for the alanine found in all three of the Ah\(^b\) alleles [63]. The Ah\(^b-1\) allele encodes the AHR protein of the smallest size (95 kDa), and is likely the most stable, due to its high thermostability compared to the other forms of the Ah\(^b\) allele [64]. An additional indication of the stability of the Ah\(^b-1\) allele is that it does not require sodium molybdate for stability, while it is required in buffer during the isolation of the Ah\(^d\) allele. The Ah\(^b-2\) and Ah\(^d\) alleles encode proteins of the same size, and in fact are different in only three amino acid substitutions. Interestingly, Poland also noted the presence of a 130 kDa AHR variant in the deer mouse, *Peromyscus maniculatus*, though this mouse does not share much similarity with *Mus* strains [64], demonstrating that additional variations of the Ah allele may exist in wild populations of mice.
Table 1.1: Summary of the experimental parameters used to distinguish different forms of the \( Ah \) allele in \( Mus \) strains of laboratory mice.

Experimental parameters used in two studies by Poland and colleagues [63, 64]. \( t_{1/2} \) indicates the measurement of the half-life of specific ligand binding capacity at 35°C. n.d., not determined by the authors.

The difference in \( Ahr \) allelic status in laboratory mouse strains has been associated with physiological consequences. Variation was observed in the inflammatory response and tumor susceptibility in mice phenotypically selected for maximum or minimum acute inflammatory reaction (AIR), known as AIRmax and AIRmin mice, respectively. This response is induced by the subcutaneous injection of polyacrylamide beads, allowing for the study of the endogenous factors controlling the local acute inflammation, independent of antigen [65]. This response was originally established independent of any consideration of AHR, but studies subsequently linked \( Ah \) allelic status to this phenotype. Repeated epicutaneous applications over the course of five days of the tumor inducer and AHR agonist DMBA led to cutaneous inflammation characterized by acute epidermal proliferation, inflammatory cell infiltration, increased expression of \( Il1b, Tnf, Il6, Tgfb1 \), and \( Cyp1b1 \), and eventual elevated skin and lung tumor incidence in the AIRmin mice. Genetic analysis revealed that the more susceptible...
AIRmin mice were homozygous for the 375A polymorphism, identifying them as \( Ah^{b.f} \) allele mice, while the less susceptible AIRmax mice carried the 375V mutation, indicating the \( Ah^{d} \) allele [66]. An additional, earlier study found that higher levels of expression of the high-affinity \( Ah \) allele led to increased fertility, longevity, and fitness [67]. The differences in binding affinities for ligand, especially between the AHR\(^b\) and AHR\(^d\) receptor forms, make mice that express these receptors useful tools for determining \textit{in vivo} relevance of ligand-mediated effects, though the physiological consequences of these interactions is most likely highly complex.

1.2.2 The AHR knockout mouse

Three \( Ahr \)-null (\( Ahr^{-/-} \)) mouse models (or knockout (KO)) were independently generated in the middle to late 1990’s in the laboratories of Dr. Frank Gonzalez [68] (annotated as \( Ahr^{-/-} \) (1)) for the simplicity of the following discussion), Dr. Christopher Bradfield [69] \( Ahr^{-/-} \) (2)), and Dr. Fujii-Kuriyama [70] \( Ahr^{-/-} \) (3)) using homologous recombination. Certain similarities were observed in all three generated strains. No lethality was observed \textit{in utero} due to the disruption of \( Ahr \) expression, as normal Mendelian distributions were observed during heterozygous \( (Ahr^{+/+}, Ahr^{+/-}, \text{and } Ahr^{-/-}) \) matings \( (1:2:1 \text{ for } Ahr^{+/+}, Ahr^{+/-}, \text{and } Ahr^{-/-} \text{ mice, respectively}) \). \( Ahr^{-/-} \) mice from all three strains exhibited a slower growth rate within the first four weeks of life. CYP1A1 and CYP1A2 gene expression or enzymatic activity were not induced in response to aromatic hydrocarbons (TCDD in \( Ahr^{-/-} \) (1) and \( Ahr^{-/-} \) (2) mice, and 3-methylcholanthrene (3MC) in \( Ahr^{-/-} \) (3)), confirming the requirement of AHR for the mechanism of action of these pollutants.

However, notable differences were also observed between the strains. The \( Ahr^{-/-} \) (2) and \( Ahr^{-/-} \) (3) strains were viable and fertile with no observed increase in postnatal
death, while the $A hr^{-/-}$ (1) mice exhibited 40-50% mortality within 4 days of birth, though the survivors reached maturity and were fertile [68]. The $A hr^{-/-}$ (1) and $A hr^{-/-}$ (2) strains exhibited liver abnormalities, but the observations differed in regards to phenotype and severity. Both strains had smaller livers, with a 50% reduction in size in 4 week old animals of the $A hr^{-/-}$ (1) strains, and a 25% reduction in size in the $A hr^{-/-}$ (2) strain. The $A hr^{-/-}$ (1) mice exhibited portal tract fibrosis, mild to moderate cholangitis, eosinophilia of the periportal hepatocytes, centrilobular hypercellularity, and glycogen depletion [68]. The $A hr^{-/-}$ (2) strain also exhibited portal fibrosis, but microvesicular fatty metamorphosis of hepatocytes, prolonged extramodullary hematopoiesis, and portal hypercellularity was also evident in mice three weeks of age or younger [69]. Liver damage, including portal fibrosis was not as notable in the $A hr^{-/-}$ (3) mice. The initial paper characterizing the $A hr^{-/-}$ (3) mice focused on determining if AHR is required for the teratogenicity of TCDD, which was confirmed to be the case, as $A hr^{-/-}$ (3) fetuses did not develop cleft palate or hydronephrosis after TCDD exposure of the pregnant dams, while their wild-type ($A hr^{+/+}$) and heterozygote counterparts did exhibit symptoms of TCDD-induced teratogenesis [70]. Additional studies confirmed that AHR expression is necessary for the teratogenic effects of TCDD [71].

Studies subsequent to the initial characterization reports for the $A hr^{-/-}$ mice described additional effects and abnormalities that are entirely dependent on AHR expression, including ligand-mediated effects as well as development defects. The $A hr^{-/-}$ mouse model demonstrated that toxic endpoints due to TCDD exposure are mediated entirely through the AHR, including immune suppression [72], thymic atrophy, and liver lesion development [73]. Developmental defects were also observed, such as the failure
of the ductus venosus to close in the neonatal mouse [74], which was later found to be dependent on AHR expression in the endothelial/hematopoietic cells [75]. Additionally, reproductive success was impaired in Ahr−/− females, as evidenced by the mice experiencing difficulty maintaining pregnancies, increased mortality during pregnancy and lactation, and challenges with rearing offspring to weaning age [76]. The development of the Ahr−/− mouse strains was an essential step toward characterizing the function of AHR in normal physiology apart from its role in xenobiotic metabolism.

1.2.3 AHR signaling pathway mutations

Another method of determining the specific functions of AHR in vivo is to inhibit particular aspects of its signaling pathway. Two mouse models have been generated by the laboratory of Dr. Christopher Bradfield in which the AHR signaling pathway is disrupted. One strain was generated in which the AHR nuclear localization/DNA binding domain is disrupted [77], as well as a second strain in which the AHR is capable of chaperone interaction, ligand binding, nuclear translocation, and heterodimerization with ARNT, but is not capable of binding DREs [78]. Both of these strains exhibit resistance to TCDD-mediated toxicity and abnormal liver development as observed in Ahr−/− mice, demonstrating the requirement for DRE-mediated activity for these processes. These mutants and the future development of other mutants of the AHR signaling pathway could provide useful tools for dissecting the various aspects of AHR-mediated activity under different physiological conditions.

1.2.4 Constitutively active models

Mouse models have been generated in which a constitutively active form of the AHR (CA-AHR) is expressed. These models were developed in order to study the
consequences of sustained activation of the AHR *in vivo*. CA-AHR mice have shorter life spans and develop glandular stomach tumors [79], have an increased population of mature bone marrow-derived B cells and a decreased population of CD5-expressing B cells (B1) in the peritoneum [80], and an increased incidence of hepatocarcinogenesis after initiation with *N*-nitrosodiethylamine [81]. Additional models have been developed in which AHR is only constitutively active in certain cell populations. CA-AHR expression only in the keratinocytes led to the postnatal formation of severe skin lesions resembling atopic dermatitis in the mice [82]. Specific expression of CA-AHR in T cells led to a decreased number of thymocytes, but an increase in the percentage of CD8+ thymocytes, possibly indicating skewed differentiation in these mice. These transgenic mice also had a suppressed response to immunization, as exhibited by the decreased numbers of T cells and B cells in the spleen [83]. These models help to clarify the physiological effects of overactive AHR *in vivo* but may not be physiologically relevant.

**1.2.5 Mouse models expressing the human form of the AHR**

“Humanized” mice, in which the human form of a receptor in either the whole animal or a specific tissue is expressed, have been developed as experimental models. These models are developed to partially overcome the challenge imposed by species-specific differences in ligand affinity for several different xenobiotic receptors, including the AHR [84]. A knock-in mouse expressing the human AHR (hAHR) was generated to determine the relative sensitivity of the human AHR to TCDD-mediated effects, although the level of AHR protein expression appears to be low, complicating the interpretation of the results obtained. With this in mind, the hAHR mice did not induce levels of *Cyp1a1* and *Cyp1a2* to as high of a degree as C57BL/6J mice in response to TCDD. Additionally,
fetuses exposed prenatally to TCDD did not exhibit developmental abnormalities as severe as C57BL/6J mice [85]. The use of a model in which hAHR is expressed only in the hepatocytes demonstrated that hAHR binds the ligands indirubin and quercetin with higher affinity than C57BL/6J mice using competitive ligand binding assays [86]. Using this same hepatocyte-specific hAHR mouse, it was determined that mouse and human AHR differentially induce genes in response to TCDD, as it was found that only ~18% of gene levels were similarly elevated and ~49% of gene levels were similarly repressed by both receptors [87]. Humanized models are useful for determining relevance of the activation of the AHR by particular ligands while still utilizing in vivo models.

1.3 THE PHYSIOLOGICAL ROLE OF AHR IN THE SKIN AND INTESTINE

As a sensor of chemical signals, the importance of AHR in barrier organ systems exposed directly to the outside environment (i.e., the skin, intestine, lung) has gained increased interest as a research focus. This portion of the review will focus on the role of AHR in the physiology of the skin and intestine, as well as its role in the development of pathologies of those tissues. The focus will be on what has been learned from mouse models about the physiological roles of the receptor and how pharmacological invention leads to understanding the role of AHR and how its activity can be manipulated by ligands to promote a healthy environment in these systems.

1.3.1 Structure and composition of the skin

The skin comprises the first barrier against external injury and stimuli and has been evolutionarily adapted for defense against detrimental external threats, including pathogens, radiation, extreme temperature changes, and mechanical injury. It is composed of two layers, the topmost epidermis and the underlying dermis, which are
connected by a basement membrane. The epidermis is further composed of four distinct layers, with the stratum basale next to the dermis, followed by the stratum spinosum, stratum granulosum, and the stratum corneum (also called the cornified layer), comprising the uppermost layer of the skin [88]. Keratinocytes are the primary cell type of the epidermis, representing about 85% of the epidermal cells [89]. Melanocytes are also present in the epidermis, and are responsible for the production of melanin, located in melanosomes. Melanin is the protein responsible for pigmentation in the skin, hair, and eye and is involved in the skin tanning process. It consists of polymers formed by the conversion of L-tyrosine to L-DOPA, a process catalyzed by tyrosinase, the rate-limiting enzyme of melanogenesis. Melanin provides protection for the keratinocytes from DNA damage due to UV exposure through its ability to absorb UV radiation, and melanocytes transfer the melanosomes through dendrites to the cytoplasm of the keratinocytes and position them over the nucleus of the cells [90]. The dermis is composed of extracellular matrix including collagen and elastic tissue, as well as stromal cells including dermal fibroblasts, fibrocytes, cells of the blood and lymph vessels, and nerves [88, 91]. Both layers also contain resident immune cells. The epidermis contains Langerhans cells, which are the resident antigen-presenting dendritic cells, dendritic epidermal T cells (specific to mice), and CD8+ cytotoxic T cells. The dermis contains more variety in its resident immune cell population, which includes dendritic cell (DC) subsets (dermal DCs and plasmacytoid DCs), T cell subsets (CD4+ T helper 1 (T_H1), T_H2 and T_H17 cells, γδ T cells and natural killer T cells), macrophages and mast cells [91].
1.3.2 UV-mediated skin inflammation and carcinogenesis

Though most human beings are exposed to ultraviolet (UV) radiation almost daily through sunlight, chronic exposure can lead to detrimental effects. The UV spectrum is composed of three different types: UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280nm). UVC has the highest energy, and the maximum absorption spectrum for DNA (at 260 nm) lies within its wavelength range, and it therefore exhibits the greatest potential to cause harm to the skin. However, most of it is inhibited from reaching the surface of the Earth because of its absorption by the ozone layer, and it is therefore not physiologically relevant in the study of the effects of UV exposure on humans. UVA and UVB penetrate the atmosphere, reach Earth’s surface, and are absorbed by human skin, and therefore have the most relevance to human biology. UVA has the lowest energy of the three types, but is able to penetrate the skin to the greatest depth. It is weakly carcinogenic, and is the primary cause of photoaging. While both UVA and UVB are involved in the formation of freckles, wrinkles, and skin cancer, UVB is the primary cause of photocarcinogenesis because it contains higher energy levels than UVA and is the largest component of UV irradiation penetrating the dermis [92, 93]. The acute phenotypic effects of UVB exposure include sunburn, erythema, tanning, immunosuppression and the formation of apoptotic sunburn cells. UVB irradiation also leads to the induction of the inflammatory response through increased production of cytokines, chemokines, and other signaling molecules [92].

Chronic UV exposure can lead to cancer, including melanoma. UVB is a complete carcinogen, because exposure to it is sufficient to induce cancer without an initiator or promoter [94]. Another factor contributing to the carcinogenic potential of UV is its immunosuppressive properties. Evidence for this phenomenon was observed when
tumors in mice repeatedly exposed to UV were transplanted into normal recipients. The normal recipients (mice not exposed to UV) were able to resist tumor growth, and recipient mice with a compromised immune response were susceptible to continued growth of the transplanted tumors [95].

1.3.3 AHR and skin

Multiple studies have demonstrated that the positive or negative biological consequences of AHR activation in the skin are highly dependent on the presence or absence of a pathological condition, the specific ligand causing AHR activation or inhibition, and other contributing factors. There is a fine line in regards to AHR activation between healthy skin and a disease phenotype. General observations concerning AHR function in the skin are also complicated by the responses of various cell types found in the skin, including keratinocytes, sebocytes, melanocytes, and skin immune cells, all of which have been shown to express AHR [96].

1.3.4 Agonist-mediated activation of AHR in the skin: detrimental or beneficial?

The first negative consequences linked to AHR activation in the skin arose from the observation of the effects due to overexposure to polycyclic pollutants. The classic hallmark of TCDD overexposure in humans is the development of chloracne, which presents with symptoms such as epidermal hyperplasia, loss of sebaceous glands, and the presence of epithelial cysts [1]. Evidence that persistent activation of the AHR in the skin leads to dermal pathologies was demonstrated in a study using mice which express a constitutively active form of AHR in the keratinocytes. These mice developed postnatal inflammatory skin lesions resembling atopic dermatitis [82]. Excessive topical exposure to PAHs can lead to skin cancer, the development of which has been shown to be AHR-
dependent, as it was observed that weekly topical applications of BaP over the course of 25 weeks in \( Ahr^{+/+} \) and \( Ahr^{-/-} \) mice lead to the development of tumors, which were completely absent in \( Ahr^{-/-} \) mice [97].

In contrast to these observations, in which it could be argued that ligand-activated AHR is detrimental in the skin, examples of the possible therapeutic potential of AHR agonists have been characterized in the literature. One example is coal tar, which, despite containing PAHs, has been shown to be an effective remedy for the management of atopic dermatitis symptoms. The beneficial effects of coal tar therapy have been linked to the AHR, as it was observed that exposure of human keratinocytes to coal tar resulted in the activation of AHR, and the AHR-dependent induction of the epidermal barrier genes filaggrin (\( FLG \)) and hornerin (\( HRNR \)) [98]. Additionally, the endogenous AHR agonist FICZ was able to decrease adverse symptoms in mice topically treated with Imiquimod (IMQ), which induces psoriasis-like skin inflammation [99].

Though, as discussed above, \( Ahr^{-/-} \) mice were shown to be resistant to PAH-induced skin carcinogenesis, the complete loss of AHR leads to other detrimental skin effects, suggesting an endogenous role for the AHR in the maintenance of skin homeostasis in the absence of overt xenobiotic exposure. Scaly skin lesions were observed in 53% of \( Ahr^{-/-} \) mice which were not evident in age-matched \( Ahr^{+/+} \) mice [100]. \( Ahr^{-/-} \) mice exhibit impaired barrier function in the skin, as demonstrated by increased transepidermal water loss. This defect of the barrier was due at least partially to the absence of AHR in the keratinocytes, as mice with an AHR-specific knockout in the keratinocytes exhibited transepidermal water loss, but AHR-specific knockout in Langerhans cells did not. Additionally, the skin microbiota of \( Ahr^{-/-} \) mice was found to
contain more variety than wild-type counterparts, possibly indicating less stability of the microflora in the skin of these mice [101]. Ahr<sup>-/-</sup> mice treated with IMQ exhibited exacerbated symptoms, including elevated inflammatory gene expression and increased epidermal and stratum corneum thickness when compared to Ahr<sup>+/+</sup> mice treated with IMQ. It was determined using bone marrow chimeras that non-hematopoietic derived cells (i.e., keratinocytes) were primarily responsible for the increased inflammatory reaction to Imiquimod observed in Ahr<sup>-/-</sup> mice [99].

Another factor by which the AHR can contribute to skin health is through activation by ligands produced by skin microflora. FICZ was detected in skin scale extracts from patients suffering inflammatory skin diseases associated with the yeast genus Malassezia, which, while a commensal skin microorganism, can become pathogenic under particular, as yet poorly defined, conditions. Other high affinity AHR agonists were identified in these patient-derived extracts, including indirubin, indolo[3,2-b]carbazole (ICZ), tryptanthrin, malassezin, and pityriacitrin [41]. Due to the many different factors that can influence AHR in the skin under physiological conditions, the beneficial or negative effects of AHR ligand-mediated activation in the skin are complex and context-dependent.

1.3.5 Effects of UV exposure on AHR activation

Another way through which the AHR influences skin physiology is through its ability to mediate the effects of UV exposure. A possible link between UV and AHR was noted due to the observation that UV exposure induces AHH activity in neonatal rat skin [102], and that CYP1A1 and CYP1B1 are induced in a time- and dose-dependent manner in the human epidermis after UVB exposure [103]. One potential explanation is that
photoproducts are formed endogenously upon exposure to UV light that act as agonists for the AHR. This possibility was demonstrated to be one link between AHR and UV upon the characterization of the endogenous AHR ligand, 6-Formylindolo(3,2-b)carbazole (FICZ). FICZ is a high-affinity agonist of the AHR, which has been demonstrated to be produced upon tryptophan exposure to UV light [104]. Human keratinocytes exposed to UV in the presence of tryptophan were shown to express higher levels of CYP1A1 when compared to keratinocytes exposed to UV only, and the induction of CYP1A1 by FICZ or UV was shown to be AHR-dependent [105]. FICZ was found to be produced in human keratinocytes after exposure to UVB, albeit under non-physiological conditions (i.e., high UVB dose, tryptophan starvation followed by incubation with $^{13}\text{C}_1 1\text{5N}_2$tryptophan one hour prior to UVB exposure). The authors also determined that UVB exposure induces AHR translocation to the nucleus, followed by induction of $CYP1A1$ [106].

Loss or inhibition of AHR in the skin can also have potentially protective effects in regard to UV exposure, as presence or activation of AHR may also have negative consequences after UV exposure. $Ahr^{-/-}$ mice failed to express mRNA levels of the pro-inflammatory and pro-carcinogenic mediator cyclooxygenase-2 (COX-2) (or prostaglandin-endoperoxide synthase 2 ($Ptgs2$)) [107] after exposure to UVB, though it was induced in $Ahr^{+/+}$ mice [106]. AHR was anti-apoptotic in human keratinocytes exposed to UVB, which could be deleterious if cells with DNA damage are able to persist. This effect operated through an AHR-dependent mechanism involving the cell cycle regulators E2F1 and checkpoint kinase 1 (CHK1), and $Ahr^{-/-}$ mice on an SKH1 background exhibited decreased E2F1 and CHK1 protein levels [108]. AHR was also
found to mediate local UV-mediated immunosuppression in a model of contact hypersensitivity (CHS). In this model, repeated UV exposure prevents sensitization for a hapten, such as 2,4-dinitrofluorobenzene (DNFB). The AHR antagonist MNF increased sensitivity to DNFB after exposure to UV, and Ahr\(^{-/-}\) mice were similarly responsive to DNFB after UV exposure, demonstrating a role for AHR in this process [109].

Another potential role of the AHR in the mediation of the response of the skin to UV exposure is the development of pigmentation. Both mouse [110] and human [111] melanocytes express AHR. Pigmentation in response to UV was shown to be at least partially AHR-dependent, as Ahr\(^{-/-}\) mice did not tan to the same extent as their wild-type counterparts, as demonstrated by decreased melanin content and tyrosinase activity after UVB exposure. The decreased tanning response exhibited by Ahr\(^{-/-}\) mice was due to decreased AHR activity in the melanocytes because mice with Ahr knocked out only in the keratinocytes displayed a normal tanning response [110]. AHR activation due to TCDD exposure led to an increase in the expression of the melanogenesis genes TYR (tyrosinase) and TYRP2 (dopachrome tautomerase), increased tyrosinase activity, and elevated melanin content in normal human melanocytes [111].

Overall, it seems that the ideal level of AHR activation in the skin is highly context specific. Persistent activation, as exemplified by the CA-AhR model in keratinocytes, and the development of chloracne upon TCDD exposure is most certainly detrimental, while the total absence or inhibition is also not beneficial for proper skin health maintenance.
1.3.6 The intestine: the epithelial barrier, gut microbiota, and inflammatory bowel diseases

The mammalian digestive track is composed of the small intestine, which is responsible for 95% of the absorption of nutrients, and the large intestine or colon, which is primarily involved in the absorption of water. The epithelium of both the small and large intestine is composed of a single cell layer organized into crypts, as well as villi in the small intestine. Both of these structural components increase the surface area and therefore the digestive and absorptive potential of the intestinal epithelium. This layer provides separation from the intestinal lumen, located on the apical side of the intestinal epithelial cells (IECs), which is composed of the gut microbiota, mucus layer, and digested material, from the lamina propria on the basolateral side of the IECs, where the effector immune cells reside. The intestinal epithelium is therefore an essential component of the barrier function of the gut. Stem cells located at the base of the crypts are responsible for replenishing the IECs, which have a turnover rate of every four to five days. The intestinal epithelium is composed of several different cell types, including enterocytes, Goblet cells, Paneth cells, enteroendocrine cells, and follicle-associated epithelium and microfold (M) cells. Enterocytes are the most abundant cell type of the intestinal epithelium, and are responsible for the absorption of nutrients and water. Goblet cells secrete mucins which create the mucus barrier between the luminal contents and the intestinal epithelium. Paneth cells, found only in the small intestinal crypts, produce antimicrobial peptides and provide protection for the stem cells. Enteroendocrine cells secrete hormones, and M cells, located above the Peyer’s patches in the small intestine, sample the luminal contents [112-114].
The microbial community of the intestine has a profound influence on the health or disease status of the gastrointestinal (GI) tract. Though body surfaces exposed to the environment are also inhabited with microbes, the intestine harbors the highest percentage, with the colon accounting for over 70% of the microbial population of the body. Directly after birth, the human intestine begins to be populated with bacteria, with the largest increases in numbers and diversity occurring during the first year of life. After the first year until the end of life, the microbial numbers stabilize, but the composition continues to evolve. The composition of the microbiota in an individual is influenced by a variety of factors, including maternal contribution, diet, exposure to the environment, and antimicrobial treatments. Commensal microbial populations contribute to the essential, beneficial functions of the gut, including the regulation of digestion, innate and adaptive immune cells, and the metabolism of nutrients. However, altered commensal populations are also linked to the development of intestine-related pathologies, including inflammatory bowel disease (IBD), obesity, type 2 diabetes, and colon cancer [115, 116].

An inappropriate, chronic inflammatory response to the gut microbiota is associated with the development of IBD, which includes Crohn’s disease (CD) and ulcerative colitis (UC). CD can be localized anywhere throughout the GI tract, though it is most common in the terminal ileum and perianal regions, while UC occurs only in the colon. Genetic susceptibility is also a contributing factor to disease development, and disruption of the intestinal barrier is a hallmark of these conditions [117, 118].

1.3.7 The role of AHR in the maintenance of intestinal health
Defining the role of the AHR in the GI tract has become an increasingly popular area of research, due to its ability to be regulated by a variety of endogenous, dietary, and
microbial ligands. *In vivo* mouse models have been useful for elucidating the role of AHR in intestinal function under both homeostatic and disease conditions. A description of the *in vivo* models of intestinal disease used in these studies followed by an overview of the current literature involving AHR function in the gut is summarized below.

### 1.3.8 Chemically-induced colitis models in the laboratory mouse

To mimic the pro-inflammatory state found in the intestinal tract of those suffering from IBD, chemically-induced colitis models are used in rodent studies. These models include dextran sodium sulfate (DSS)-, 2,4,6-trinitro benzene sulfonic acid (TNBS)-, and oxazolone-induced colitis. DSS colitis is induced through supplementation of the drinking water of rodents. Continual ingestion leads to acute colitis in the animal, characterized by weight loss, bloody diarrhea, ulcers, and immune cell infiltration, likely due to disruption of the intestinal barrier through damage to the epithelial cells of the basal crypts [119]. This model is useful for the study of the role of the innate immune system in the development of acute colitis, as C.B17 severe combined immunodeficient mice, which lack T and B cells, were still susceptible to the effects of DSS [120]. TNBS colitis is induced via intrarectal administration of TNBS in ethanol. This method induces colitis through the disruption of the mucosal barrier by ethanol, and by the use of TNBS as a hapten. This causes the colonic proteins, including those of the host and/or microbiota, to become immunogenic, leading to the elicitation of an immune response. Similar to the TNBS-induced method, oxazolone is a hapten which is administered intrarectally in ethanol leading to weight loss, diarrhea, and a reduction in goblet cells as well as high mortality rates [119]. The choice of the appropriate model is dependent on the particular aspects of intestinal physiology that are being investigated.
1.3.9 AHR expression and activation is protective in chemically-induced colitis models

Many recent independent studies have demonstrated the protective role of AHR in experimental colitis models as well as the therapeutic potential of AHR agonists in IBD. Evidence for the importance of AHR in the maintenance of intestinal health was provided by the observation that DSS was 100% lethal to Ahr−/− mice by day 7 of exposure, whereas 80% of Ahr+/+ mice and 100% of Ahr+/− mice survived [121]. Additional studies confirmed that Ahr−/− mice experience more severe colitis symptoms than mice expressing the AHR [122, 123]. Arsenescu and colleagues originally proposed that AHR antagonists may be viable drugs for the treatment of IBD, in part because Ahr+/− mice displayed the healthiest phenotype under DSS colitis, though they acknowledged that AHR activity modulation through diet could also be a remedy [121]. In contrast to their proposal, many studies have shown the positive impact of AHR agonists on disease development in experimental colitis models. Oral pre-treatment with TCDD one week prior to the induction of DSS colitis led to an increase in body weight, a decreased inflammation score, increased colon length, and decreased inflammatory gene expression when compared to mice exposed to DSS alone. This preventative effect may be at least partially due to a TCDD-dependent elevation of prostaglandin E2 (PGE2) levels, which are protective in the gut, since exposure to indomethacin, a nonselective cyclooxygenase 1 and cyclooxygenase 2 inhibitor, suppressed the ability of TCDD to decrease the severity of colitis symptoms [124]. Prior exposure to TCDD also alleviated the symptoms of TNBS colitis, as evidenced by decreased colonic inflammation and damage, less severe weight loss, and a reduction of pro-inflammatory cytokines. It was also noted that the decreased severity of colitis in this study could be due to enhanced Foxp3+ regulatory
T cell (T<sub>reg</sub>) presence in the colon of TCDD treated mice as assessed by immunohistochemistry, but this observation conflicted with the authors’ flow cytometry analysis of the colon tissue, in which they noted a decrease in Foxp3+ T<sub>reg</sub> cells in TCDD exposed mice [125]. A previous study had demonstrated that TCDD could induce the expansion of Foxp3+ T<sub>reg</sub> cells <em>in vivo</em> and that this TCDD-dependent expansion could decrease the severity of experimental autoimmune encephalomyelitis (EAE), an <em>in vivo</em> model of multiple sclerosis. This effect was determined to be ligand specific, as FICZ treatment increased the severity of EAE in the mice [126]. The ability of TCDD to decrease colitis severity through induction of T<sub>reg</sub> cells was demonstrated in another study utilizing the DSS colitis model [127].

Investigations into the protective function of agonist-activated AHR continue to further clarify the mechanisms by which the receptor decreases the severity of experimental colitis. Many of these studies use non-toxic or naturally-occurring agonists and investigate the role of AHR in mediating the immune response in the amelioration of colitis-induced disease symptoms. The non-toxic AHR agonist BNF decreased the severity of DSS colitis, demonstrating that other agonists besides TCDD could be effective in relieving colitis symptoms [122]. The tryptophan metabolite FICZ improved symptoms in both DSS- and TNBS-induced colitis models, while treatment with the antagonist CH223191 increased the severity of colitis. The protection afforded by FICZ was due to decreased pro-inflammatory cytokine expression as well as increased IL-22 expression [128], which is protective in the intestinal tract [129] and is regulated by AHR [130]. A tryptophan-containing diet decreased severity of DSS colitis in wild-type mice but did not improve conditions in <em>Ahr</em><sup>−/−</sup> mice [131]. Additionally, kynurenine improved
colitis with an associated increase in IL-10 receptor [132]. The indole-3-carbinol condensation product and AHR agonist DIM led to improvement of oxazolone-induced colitis, potentially through a mechanism involving the promotion of regulatory T cell numbers and a decrease in Th2/Th17 cell numbers [133]. Additionally, indigo improved DSS-induced colitis symptoms, but this effect was lost in Ahr−/− mice treated with DSS [134].

Research into the effects of probiotics on the maintenance of intestinal health and their effects on disease development and progression are currently a pursuit of many laboratories. Since bacterially-derived ligands of AHR have been identified, new emphasis on the role of AHR activation by microbial products is an emerging area of study. Exposure of mice to a lyophilized heat-killed solution of the probiotic lactic acid bacterial strain Lactobacillus bulgaricus OL1181 was shown to increase Cyp1a1 in the colon, as well as improve DSS colitis-mediated symptoms [135]. The same laboratory later found another probiotic strain, Propionibacterium freudenreichii ET-3, derived from Swiss-type cheese, that produced 1,4-dihydroxy-2-naphthoic acid (DHNA), which was capable of activating the AHR and decreasing DSS colitis severity, in part by increasing levels of the antimicrobial proteins RegIIIβ and RegIIIγ [136]. These studies consistently show the beneficial functions of agonist-activated AHR for the alleviation of inflammatory intestinal disease, and that ligands derived from dietary and beneficial microbial sources can aid in disease remediation.

1.3.10 Intestinal immune cell subtypes and microbial- and diet-derived AHR ligands

As noted in the results summarized above, studies investigating the role of dietary activation of AHR and the involvement of the microbiota have begun to clarify its role in
the intestinal tract, particularly in regards to intestinal resident immune cell populations. Intestinal intraepithelial lymphocytes (IELs) are a population of intestinal immune cells which are influenced by AHR activation. IELs are a specialized group of T cells divided into two groups: γδ and αβ T cell receptor expressing IELs. The αβ subset is further composed of CD8αβ and CD8αα T cells. IELs populate the intestine prenatally, but after birth their development and function is influenced by the gut microbes. IELs communicate with epithelial cells and participate in mucosal host defense through their role in maintaining epithelial homeostasis and facilitating repair [137]. The presence of AHR was determined to be required for the maintenance of IELs in the skin and intestine, but not for their development and proliferation. Ahr−/− mice reconstituted with wild-type IELs prior to DSS exposure were able to recover from the effects of colitis unlike the Ahr−/− mice which were not reconstituted, demonstrating the protective function of IELs. Additionally, the authors found that an AHR ligand-sufficient diet could improve colitis-induced symptoms compared to mice on an AHR ligand-deficient diet. Loss of AHR also led to an increased bacterial burden in the mice. The authors conclude that the activation of AHR through dietary ligands is necessary to maintain populations of IELs and a healthy intestine [123]. Additional studies demonstrated that the beneficial effects of FICZ in DSS colitis include the decrease of pro-inflammatory factors such as epithelial-derived IL7, CD8αβ+, CD4+, and CD8+ IEL subpopulations, and an increase in protective TCRγδ+ IELs [138]. These results were supported by a study in which FICZ again improved DSS colitis-induced symptoms and subsequently prevented a reduction in CD8αα+TCRαβ+ IELs which was concurrent with increased expression of IL-15 receptor
and AHR. FICZ also induced an increase in levels of IL-10 and decreased IFN-γ in CD8αα⁺TCRαβ⁺ IELs during colitis [139].

A growing body of work is further defining the role of AHR in the function of innate lymphoid cells (ILCs), particularly the retinoic receptor-related orphan receptor (ROR)γt-expressing ILCs. ILCs are necessary for the development of lymphoid organs both before and after birth in the intestine, including lymph nodes and Peyer’s patches (prenatal) and cryptopatches and isolated lymphoid follicles (postnatal). Their function is controlled by environmental cues, and they produce both IL-22 and IL-17 in response to signals from commensal microbes [137]. AHR is required for the postnatal maintenance and expansion of intestinal RORγt-expressing ILCs, but not for their development, and for the formation of intestinal lymphoid follicles. These processes are dependent on AHR transcriptional activation via dietary ligands including the phytochemicals found in standard rodent chow or by indole-3-carbinol (I3C). Additionally, AHR-mediated expansion of RORγt⁺ ILCs confers protection against intestinal pathogenic attaching and effacing bacterial infections in a *Citrobacter rodentium* model [140]. An independent study confirmed the importance of AHR for the maintenance and function of RORγt⁺ ILCs. This study further found that lack of AHR led to a higher incidence of apoptosis and a decreased production of IL-22 in the ILCs. *C. rodentium* infection was lethal to all *Ahr⁻/⁻* mice by day 10 post-infection, but ectopic expression of IL-22 led to survival of the mice [141]. The same research group further expanded on these findings. Due to the reduction in IL-22-producing ILCs, both a commensal microbe (segmented filamentous bacteria) and Th17 cell numbers were increased in *Ahr⁻/⁻* mice [142]. However, the results of a third study showed that ILC22 cell numbers were not impaired by a lack of dietary or
microbial ligands for AHR, though $Ahr^{−/−}$ mice had decreased amounts of intestinal ILC22 cells which led to an impaired response to bacterial infection in the gut [143]. An additional study reports the importance of microbiota-derived tryptophan metabolites in the induction of $Il22$ expression by AHR, which contributed to the formation of a microbial community that is varied but also provides protection from fungal overgrowth [144]. These studies point to a complex role for dietary or microbial-activated AHR in the maintenance of intestinal homeostasis.

1.3.11 AHR and in vivo models of intestinal carcinogenesis

Consistent with the numerous results demonstrating a protective role for AHR in inflammatory bowel disease models, intestinal carcinogenesis studies overall support a positive role for AHR activation in decreasing incidence or severity of intestinal cancer. Spontaneous colonic tumors in the cecum close to the ileocecal junction develop in $Ahr^{−/−}$ mice, which means that AHR could have tumor suppressor capabilities. To explore the potential mechanism of how AHR can inhibit tumor formation, a mouse model of human hereditary familial adenomatous polyposis, a condition which leads to the formation of colonic tumors, was used to explore the therapeutic potential of AHR ligands. The model is known as $Apc^{Min/+}$ (Adenomatous polyposis coli, multiple intestinal neoplasia/+), in which mice develop small intestinal adenomatous polyps due to a mutation in $Apc$. In this model, AHR agonists including I3C and DIM led to a decrease in the number of small intestinal polyps, which involves AHR E3 ubiquitin ligase activity, and $β$-catenin degradation [145]. The formation of intestinal tumors in the $Ahr^{−/−}$ mice is at least partially dependent on a pro-inflammatory state, because tumors were reduced in germ-free $Ahr^{−/−}$ mice, and double knock-out $Ahr^{−/−} ASC^{−/−}$ mice, even in the presence of $β$-catenin
accumulation. ASC−/− mice lack the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), a component of the inflammasome involved in the activation of caspase-1 and therefore increased production of IL-1β in response to cellular signals such as those produced in response to injury or bacteria. In support of this, inhibition of caspase-1 led to a reduction in tumors in Ahr−/− mice [146]. Ahr−/− mice were again found to be more susceptible to intestinal tumor formation than their wild-type counterparts in a colitis-associated colonic tumorigenesis model, in which mice are exposed to azoxymethane followed by a DSS treatment. In wild-type (Ahr+/+) mice, I3C was able to reduce tumor numbers in this model, while the protective effect of I3C did not alter tumorigenesis in Ahr−/− mice [147]. Though these studies suggest a preventative role for AHR in intestinal carcinogenesis, sustained activation of AHR in a constitutively-active model led to the formation of glandular stomach tumors in the mice [79].

1.3.12 Expression of AHR in human IBD samples

Whether the insights gained from mouse models about the protective role of AHR activation in the gut translates to viable therapies for humans remains to be more fully investigated. Two studies report conflicting results concerning levels of AHR in IBD patients compared to healthy controls. The first study found that CYP1A1 expression was increased in colonic biopsy samples from patients with CD compared to samples from healthy patients. Analysis via immunohistochemistry of AHR protein levels in the colon samples demonstrated that AHR was mostly expressed in the epithelial layer of healthy patients, while tissues from the patients with CD exhibited increased AHR-expressing cells in the lamina propria [121]. In the second study, Ahr expression and protein levels
were decreased in inflamed colonic mucosal samples of patients with UC and CD, compared to control samples taken from volunteers with healthy colons. Additionally, in a comparison between uninflamed and inflamed tissues from patients with UC or CD, Ahr expression was reduced in the inflamed samples [128]. Additional work will need to be conducted to determine if the results from in vivo mouse models of intestinal disease and the beneficial effects of AHR activation in these models translate to viable therapeutics for human disease.

1.4 HYPOTHESIS AND SPECIFIC AIMS

As the current literature demonstrates, the in vivo function of AHR is varied and complex, particularly in barrier tissues such as the skin and intestine. The overall hypothesis for the work presented in this dissertation is that the AHR is an important mediator of the molecular response of the epithelium, particularly the skin and intestine and that activation and/or inhibition of AHR function has profound biological consequences. The specific aims presented to address this hypothesis include:


2. Develop a hairless mouse model expressing the high affinity Ahb-I allele and the low affinity Ahd allele to identify potential AHR-mediated effects in the skin under inflammatory conditions.

3. Develop a mouse model with a specific deletion of AHR in the intestinal epithelium to determine if the protective effects mediated by AHR in the gut are dependent on its expression in that particular compartment.
The results and mouse models presented in this dissertation help to further build the body of knowledge about the function of the AHR in barrier tissues. Future applications of the mouse models described here will further help to clarify the role of AHR in these tissues.
1.5 BIBLIOGRAPHY


CHAPTER 2: EXPRESSION OF THE NEUTROPHIL CHEMOATTRACTANT GENE (C-X-C MOTIF) LIGAND 5 IS REGULATED BY THE ARYL HYDROCARBON RECEPTOR IN MOUSE KERATINOCYTES

2.1 ABSTRACT

Chemokines are components of the skin microenvironment which enable immune cell chemotaxis. Identification of pathways regulating the expression of specific chemokines may lead to the development of novel therapeutics. Transcription factors, including the AHR, are important mediators of chemokine regulation. Here, we demonstrate that the AHR regulates expression of chemokine (C-X-C motif) ligand 5 (Cxcl5), a neutrophil-specific chemoattractant, in primary mouse keratinocytes. AHR-mediated regulation of Cxcl5 is due to direct transcriptional activity upon treatment with AHR agonists such as TCDD. Additionally, AHR mediates synergistic induction of Cxcl5 upon exposure to an agonist and the inflammatory cytokine IL1B. This synergy is confined primarily to keratinocytes, as dermal fibroblasts did not achieve the same level of synergistic induction. AHR-specific antagonists were able to reduce basal and induced levels of Cxcl5, demonstrating the potential for pharmacological intervention. Exposure of mice to UV light followed by treatment with the AHR agonist FICZ significantly induced Cxcl5 expression in skin compared to UV alone, and this response was absent in Ahr−/− mice. These results establish AHR as an important mediator of Cxcl5, with implications for the treatment of inflammatory skin diseases.
2.2 INTRODUCTION

The skin represents the primary barrier to the environment and external insult, and the dermal molecular response to injury is critical in mitigating potential threats. Chemokines are one of many factors produced to aid in this protection. These low molecular weight cytokines form a gradient from the site of injury or infection to the bloodstream by which immune cells can traffic to damaged or infected areas. The relative abundance of these inflammatory mediators is regulated through changes in the amount produced by resident immune cells and local epithelial cells [1]. However, production of chemokines can lead to an overabundance of immune cells, resulting in inflammation and tissue damage [2]. Keratinocytes, the most abundant cell type of the epidermis, contribute to the formation of the immune microenvironment through the production of chemokines and other mediators [3].

Chemokines are classified according to the arrangement of cysteine residues at the N-terminus. One of these subclasses is characterized by the C-X-C motif, and further divided into Glu-Leu-Arg (ELR)$^+$ or ELR$^-$ CXC chemokines. The ELR$^+$ CXC chemokines induce neutrophil chemotaxis through specific interaction with the G-protein coupled receptors CXCR2 and CXCR1, and in the mouse include CXCL1, CXCL2, CXCL5 [4]. Though the chemokines of this family perform a similar function, recent studies have identified non-redundant functions within the CXC family. CXCL5 impairs chemokine scavenging through binding to the Duffy Antigen Receptor for Chemokines (DARC), causing an increase in plasma levels of CXCL1 and CXCL2 which in turn leads to an impairment of the chemokine gradient, decreased neutrophil chemotaxis to the
lungs, and a subsequent increase in *E.coli* numbers and heightened mortality in a murine pneumonia model [5].

CXCL5, also known as LPS-induced CXC chemokine (LIX) in mice, is produced by a variety of cells including the epithelial cells of barrier tissues, including alveolar epithelial type II cells [6], enterocytes [7], and keratinocytes [8]. It has also been linked to a variety of dermal pathologies. In human and rat skin, CXCL5 is the principal mediator of UV-induced pain [9]. Elevated *Cxcl5* expression is observed in mouse skin following topical exposure to 12-0-Tetradecanoylphorbol-13-acetate (TPA) [10]. *Cxcl5* expression is increased in keratinocytes after IL17 exposure, linking it to the pathogenesis of psoriasis [11]. Though NFκB is one factor identified as a possible regulator of *Cxcl5* transcription [12], other factors that regulate *Cxcl5* expression in the skin in response to environmental signals have yet to be identified.

One potential regulator of *Cxcl5* expression is the AHR, as recent studies implicate it as an important factor in the control of immune signaling. As a ligand-activated transcription factor, it has been implicated in regulating the inflammatory response in barrier tissues, including the intestine and skin [13]. AHR target genes include those involved in a myriad of cellular processes, including inflammatory signaling. Previous studies from our laboratory identified inflammation-related genes that are regulated synergistically in an AHR-dependent manner in the presence of additional inflammatory stimuli, including interleukin 6 (*IL6*) [14] in human tumor cell lines and chemokine (C-C motif) ligand 20 (*Ccl20*) in primary mouse peritoneal macrophages [15]. In this study, we have determined that *Cxcl5* is regulated in a similar AHR-dependent manner in primary mouse keratinocytes with *in vivo* relevance in a UVB exposure model.
Our results further define how environmental factors may influence the inflammatory microenvironment in the skin under pro-inflammatory conditions.
2.3 MATERIALS AND METHODS

Animals and husbandry
All mouse lines were bred in-house after acquisition. C57BL/6J mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME). Ahr\textsuperscript{−/−} mice (B6.129-Ahr\textsuperscript{tm1Bra}/J) were a gift from Dr. Christopher Bradfield (University of Wisconsin, Madison, WI.) Animals were housed in specific pathogen-free conditions under a 12 h light/dark cycle with \textit{ad libitum} access to standard chow and water in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University.

Chemicals and reagents
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was a kind gift from Dr. Stephen Safe (Texas A&M University). Formylindolo(3,2-b)carbazole (FICZ) was purchased from Enzo Life Sciences (Farmingdale, NY), Beta-naphthoflavone (BNF) was purchased from INDOFINE Chemical Company, Inc. (Hillsborough, NJ), and indolo[3,2-b]carbazole (ICZ) was purchased from Matrix Scientific (Columbia, SC). N-(2-(1H-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9H-purin-6-amine (GNF351) was provided by the Genomics Institute of the Novartis Research Foundation (San Diego, CA). 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide (CH223191) was purchased from ChemBridge Corp. (San Diego, CA), and 1-Allyl-3-(3,4-dimethoxyphenyl)-7-(trifluoromethyl)-1H-indazole (SGA360) was synthesized as described previously [16]. Recombinant mouse IL1B was purchased from PeproTech (Rocky Hill, NJ).
Plasmids
The oligonucleotides, 5’ - CGATGCGTGCATGCATGCATGCATGCGTGC - 3’ and 5’ - CCACACGA
CGCACACGCACGCACGCACGCACGCACGCACGCACGCACGCATGCGTGCGTGCGTGCGTGCGTGTGGGTAC-3’ were annealed and inserted into the pGL3 promoter to generate a pGL3pro-mCxl5 plasmid (promoter sequence containing DREs). The control plasmid pcDNA3 was obtained from Invitrogen, and pSV40βgal was purchased from Promega.

Mouse Cxcl5 promoter analysis
Promoter analysis of mouse Cxcl5 was conducted using the FASTA sequence (NCBI) to examine the region 1 to 2 kb upstream of the transcription start site. This sequence was analyzed for potential DREs using the JASPAR CORE database.

Cxcl5 reporter assay
Hepa1 cells were seeded in 6-well plates and transfected for 4 h with the Genlantis GenePORTER 3000 Transfection Reagent (San Diego, CA) and various plasmids as described by the manufacturer, after which the cells were placed in complete medium. Following overnight incubation, the cells were treated for 6 h, then lysed in a buffer containing 25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM CDTA, 10% glycerol, and 1% Triton X-100, and combined with Luciferase Assay Substrate (Promega, Madison, WI). Luciferase activity was recorded using a Turner TD-20e luminometer and normalized to β-galactosidase activity.

Electrophoretic mobility shift assay (EMSA)
Gel shift assays were conducted as described previously [17]. Briefly, mouse AHR and mouse ARNT proteins were in vitro translated using T7 rabbit reticulocyte lysate
(Promega). The proteins were combined and incubated with HEDG buffer (25 mM HEPES, 1 mM EDTA, 10% glycerol, pH 7.5), the indicated treatments, and 1x10^6 cpm of ^32^P-labeled DRE probe (mouse Cxcl5 probes used: Forward Probe: 5’ – ATGCATGCGTGCA
TGCATGCGTGCGTCGTGCGTACGTGCGTGCGTGACGTGCGTG – 3’, Reverse Probe: 5’ – CACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGCACGTGCA
TGCACGCAT – 3’. The DRE present in the proximal region of Cyp1a1 was used as a positive control. Lysate translated from empty plasmid was substituted for ARNT for the negative control. Lysate was resolved on a 6% non-denaturing DNA retardation gel (Invitrogen), fixed in a solution of 10% methanol, 10% glacial acetic acid, 10% glycerol, and 70% water for 20 min., vacuum dried and analyzed via autoradiography. Competition EMSA using 10X non-radioactive probe and ^32^P-labeled oligonucleotides was conducted in the presence or absence of 10X non-radioactive oligonucleotides by mass.

**Isolation and culture of primary keratinocytes and dermal fibroblasts**
Primary keratinocytes were isolated from neonatal C57BL/6J (WT, Ahr^+/+), Ahr^+/−, or Ahr^-/- pups and cultured as previously described [18], [19]. Briefly, neonatal pups (0-2 days old) were euthanized using CO2 asphyxiation on ice for 45 minutes. The pups were washed for 10 minutes with betadine solution, followed by rinsing with 70% ethanol. In a laminar flow hood, the skin was removed from each pup. The limbs above the knee and the tail were excised. An incision was made from the hole left by the removal of the tail along the spine to the nose. The skin was carefully peeled away and then removed in one piece. The skin was placed dermis-side down on a plate on ice until all skins were collected. The skins were floated on 0.25% trypsin (Corning, 25-050-C1 via Mediatech, Inc. (Manassas, VA)), dermis side down at 4°C overnight. The next day, the dermal and
epidermal layers were separated, and the epidermal layers were collected in a glass dish with “High Ca\(^{2+}\)” media (EMEM (Lonza, 06-174G), 8% chelexed fetal bovine serum, 0.2% P/S, supplemented with 1.4 mM Ca\(^{2+}\)). The epidermal layers were chopped finely with scissors, followed by pipetting with a sterile, 10-mL pipette. The media containing the epidermal pieces was filtered through a sterile 100 µm nylon filter. The cells were pelleted for 5 minutes at 800 rpm in an Eppendorf 5702 Centrifuge, using an A-4-38 rotor and the pellet was washed with High Ca\(^{2+}\) media and pelleted again. The pellet was resuspended in 0.2 mM Ca\(^{2+}\) media, seeded, and grown at 36°C, 7% CO\(_2\). The following day, the media on the cells was changed to media containing 0.05 mM Ca\(^{2+}\). The cells were used for experiments two days following the media change.

Primary dermal fibroblasts were isolated from the dermal layer following keratinocyte isolation. Briefly, dermal fibroblasts were isolated using media supplemented with Collagenase I. The dermal layers were combined, diced, and incubated in a shaker at 37°C for 30 min, followed by the addition of 200 U/dermis of DNAse in PBS (20,000 U/mL) and briefly swirled. DMEM, 10% FBS, and 1% P/S were added to the cells after the addition of the DNAse solution. Cells were kept at room temperature for 10 minutes with no additional shaking. The dermal solution was filtered into a new tube through a 100 µm filter, and centrifuged at 1100 rpm for 5 minutes. The pellet was resuspended in the DMEM media. The cells were centrifuged at 400 rpm for 5 minutes. The supernatant was collected and centrifuged again for 5 minutes at 400 rpm, after which the supernatant was again collected. Dermal fibroblasts were seeded and cultured at 37°C with 5% CO\(_2\).
RNA isolation and quantitative reverse transcription PCR
Total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO), and was then converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was conducted using PerfeCTa SYBR Green Supermix for iQ (Quanta Biosciences, Beverly, MA) on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Primers used for real-time PCR are listed in Table 1. Ribosomal protein, large, P0 (Rplp0) was used to normalize gene expression.

<table>
<thead>
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<th>Gene</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5' → 3')</th>
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Table 2.1: Mouse primer sequences used for qRT-PCR.

Mouse LIX enzyme-linked immunosorbent assay (ELISA)
Quantification of secreted LIX (CXCL5) in mouse primary keratinocyte culture media was determined using the Quantikine® ELISA for Mouse LIX kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Western blot analysis
Cytosolic protein was isolated from primary keratinocytes and primary dermal fibroblasts using a lysis buffer containing MENG buffer (20 mM MOPS, 2 mM EDTA, 0.02% sodium azide, 10% Glycerol, pH 7.4), 1% IGEPAL, and cOmplete Mini protease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany). The supernatant was collected after ultracentrifugation at 42000 rpm for 30 minutes and stored in liquid
nitrogen until further use. Samples were resolved by 8% tricine-SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Rpt1 antibody was used to probe for AHR and was generated as previously described [20], and β-actin antibody was purchased from Santz Cruz Biotechnology, Inc. (Dallas, Texas). Biotin-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) and $^{125}$I-streptavidin were used for detection and radioactivity was visualized using BioMax film. $^{125}$I-streptavidin was prepared as described previously [21]. ImageJ software was used for Western blot quantification.

**UV irradiation and treatment**

Age-matched female C57BL/6J or *Ahr*<sup>−/−</sup> mice were intraperitoneally injected with avertin prior to irradiation with a dose of 360 mJ/cm<sup>2</sup> UVB from UV bulbs (American Ultraviolet Light Co.) covered with cellulose triacetate (Kodak) to allow only UV wavelengths between 280 and 320 nm. Output of the UV bulbs was measured using a UVX radiometer (UVP, Upland, CA). After UV exposure, the left ears of the mice were treated with DMSO (vehicle) in acetone, while the right ears received 100 ng of FICZ in acetone. Mice received two further applications of the treatments at 8 and 16 h post-UV exposure, and were sacrificed by CO<sub>2</sub> asphyxiation 24 h after the initial exposure. The ears were collected, snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

**Statistical analyses**

GraphPad Prism 5 software was used to conduct all statistical analyses. Data were analyzed using one-way ANOVA followed by Tukey’s multiple comparison test, unless
otherwise indicated in figure legends. Data represent mean ± S.E.M., with $p$-value ≤ 0.05 (*), $p$-value ≤ 0.01 (**), $p$-value ≤ 0.001 (***).
2.4 RESULTS

The mouse Cxcl5 promoter contains multiple responsive DREs

*In silico* analysis of the mouse Cxcl5 promoter revealed a sequence of nine overlapping putative DREs between 1 to 2 kb upstream of the transcription start site, as well as a putative RelA binding element (Figure 2.1a). To determine if the DREs are responsive to AHR-mediated transcription, mouse hepatoma Hepa1 cells were transfected with a luciferase-driven reporter linked to a plasmid containing the mouse Cxcl5 DRE sequence. Treatment with the AHR ligands TCDD and indolocarbazole (ICZ) significantly induced luciferase-driven reporter activity compared to vehicle treatment (Figure 2.1b), demonstrating the functionality of the DREs.

AHR-ARNT complexes bind putative DRE sequences upstream of Cxcl5 in vitro

An EMSA using *in vitro*-translated AHR and ARNT was conducted to provide evidence that the induction of luciferase activity observed in Figure 2.1b is due to direct binding of the AHR/ARNT complex to the mouse Cxcl5 DRE sequence (Figure 2.1c). Binding of the AHR to either the Cyp1a1 or Cxcl5 DRE sequence occurred only in the presence of ARNT, and is enhanced in the presence of TCDD or FICZ. In the presence of 10X non-radioactive probe, this binding is not evident (Figure 2.1d). These data demonstrate that the AHR/ARNT heterodimer is capable of directly binding to DREs contained in the promoter of mouse Cxcl5 *in vitro* and that the AHR-mediated increase of Cxcl5 expression is most likely due to direct AHR/ARNT-DNA binding.
Figure 2.1: Cxcl5 is an AHR-responsive gene.

(a) Scheme of the mouse Cxcl5 promoter, depicting the sequence of nine overlapping DREs and a potential RelA binding site. (b) Hepa1 cells were transfected with a plasmid expressing the mouse Cxcl5 DRE linked to a luciferase reporter construct and treated with vehicle, TCDD, or ICZ for 6 h. (c) An EMSA was performed with in vitro translated mouse AHR and ARNT. TCDD or FICZ was added to the translated proteins and 1x10^6 cpm of P<sup>32</sup>-labeled DRE probe. The putative DRE present in the proximal region of Cyp1a1 was used as a positive control. (d) P<sup>32</sup>-labeled oligonucleotide was used in presence or absence of 10X non-radioactive oligonucleotide by mass. The arrow indicates binding of the AHR/ARNT/DRE complex. Letters (i.e., “a”, “b”, “c”) denote compared columns, with the significance indicated by number of asterisks as noted in Materials and Methods.
TCDD treatment results in the induction of Cxcl5 expression in mouse keratinocytes

To examine the response of primary keratinocytes to TCDD, cells isolated from Ahr+/+ mice were exposed to 2 nM TCDD and gene expression of Cxcl5 was measured using qRT-PCR. TCDD results in a direct increase of Cxcl5 transcript levels, as Cxcl5 mRNA levels were increased 20-fold in response to TCDD, and the prototypical AHR response gene Cyp1a1 was induced >300-fold (Figure 2.2a). The AHR antagonist GNF351 significantly reduced TCDD-mediated induction of both genes (Figure 2.2a). To determine if this response is specific to Cxcl5, the expression of the ELR+CXC chemokines Cxcl1 and Cxcl2 was analyzed. Only Cxcl5 was induced upon 2 nM TCDD exposure when compared to Cxcl1 and Cxcl2 (Figure 2.2a). These data support the concept that the DREs upstream of the mouse Cxcl5 gene are functional and responsive to AHR-mediated transcriptional activation and AHR-mediated regulation is specific to Cxcl5.

AHR agonists mediate synergistic induction of Cxcl5 in primary keratinocytes in the presence of IL1B

Since TCDD alone is capable of inducing a robust induction of Cxcl5 expression in primary mouse keratinocytes (Figure 2.2a), we wanted to determine if TCDD in combination with a relevant inflammatory cytokine would induce a synergistic increase in Cxcl5 expression. IL1B induces secretion of CXCL5 in an in vivo model of peritonitis [22], and was therefore selected for this experiment. Primary keratinocytes were exposed to TCDD, FICZ or BNF for 1 h followed by IL1B treatment for an additional 4 h. Each of the agonists and IL1B alone significantly increased Cxcl5 expression, while co-treatment of agonists and IL1B induced a synergistic induction of Cxcl5, with an approximately 6-fold (TCDD), 8-fold (FICZ) and 4-fold (BNF) induction compared to
IL1B treatment only (Figure 2.2b, left panel). Consistent with previous studies, AHR agonists significantly induced Cyp1a1 expression in the keratinocytes (Figure 2.2b, right panel). These data demonstrate that the synergistic induction of Cxcl5 in the presence of IL1B is AHR-mediated and is not restricted to TCDD.

To further demonstrate the AHR dependence of synergistic induction of Cxcl5, primary keratinocytes from Ahr<sup>+</sup>/ and Ahr<sup>-/-</sup> were treated with 2 nM TCDD followed by treatment with IL1B. TCDD in combination with IL1B induces a 5-fold induction of Cxcl5 expression in the Ahr<sup>+</sup>/ keratinocytes compared to IL1B alone, but fails to induce synergy in the Ahr<sup>-/-</sup> cells (Figure 2.2c). IL1B treatment alone induced Cxcl5 expression to the same degree in Ahr<sup>+</sup>/ and Ahr<sup>-/-</sup> keratinocytes and Cyp1a1 induction was observed only in the Ahr<sup>+</sup>/ keratinocytes. These data demonstrate that the presence of activated AHR is crucial for the synergistic induction of Cxcl5 expression in keratinocytes, but the AHR is not required for IL1B-mediated induction of Cxcl5.
Figure 2.2: AHR agonists induce Cxcl5 expression in mouse primary keratinocytes and mediate synergistic induction of Cxcl5 in the presence of IL1B.

(a) Ahr\textsuperscript{+/+} keratinocytes were pre-treated with vehicle or GNF351 for 8 h, followed by treatment with vehicle or TCDD for 4 h. (b) Ahr\textsuperscript{+/-} keratinocytes were treated with vehicle, TCDD, FICZ, or BNF for 1 h, followed by PBS or IL1B for 4 h. Statistical significance observed for Cxcl5 with the treatments IL1B, TCDD, FICZ, and BNF only compared to vehicle was assessed when those treatments were compared to each other in the absence of agonist and IL1B combination treatment values. (c) Ahr\textsuperscript{+/-} or Ahr\textsuperscript{-/-} keratinocytes were pre-treated for 1 h with vehicle or TCDD followed by PBS or IL1B for 4 h. Expression levels were determined by qRT-PCR. Letters (i.e., “a”, “b”, “c”) denote compared columns within each individual graph, with the significance indicated by number of asterisks as noted in Materials and Methods.
Keratinocytes are the primary mediators of AHR-dependent synergistic Cxcl5 induction

The consequences of AHR activation are highly context-specific and dependent on factors such as pathological state, tissue, and cellular environment. To determine if AHR-dependent synergy observed in primary keratinocytes is cell-type specific, primary keratinocytes and primary dermal fibroblasts were treated with TCDD followed by IL1B. TCDD with IL1B significantly induces Cxcl5 expression in dermal fibroblasts compared to IL1B alone, but the extent of the induction does not reach the levels observed in keratinocytes (1.3-fold induction (Figure 2.3a, right panel) compared to 6.8-fold induction, respectively (Figure 2.3a, left panel). The difference in induction in these two skin-derived cell types may be due to the 2-3-fold higher levels of AHR protein in keratinocytes compared to dermal fibroblasts (Figure 2.3b). These data demonstrate that the AHR-mediated synergistic induction of Cxcl5 is primarily restricted to keratinocytes.

Figure 2.3: Keratinocytes are the primary mediators of AHR-dependent synergistic Cxcl5 expression.

(a) Primary keratinocytes or primary dermal fibroblasts from Ahr+/+ mice were treated for 1 h with vehicle or TCDD followed by treatment with PBS or IL1B for 4 h. Expression of Cxcl5 was determined by qRT-PCR. Letters (i.e., “a”, “b”) denote compared columns within each individual graph, with the significance indicated by number of asterisks as noted in Materials and Methods. (b) Western blot depicting relative levels of AHR protein in primary keratinocytes or primary dermal fibroblasts from Ahr+/+ mice. Quantification was conducted using ImageJ software and each value was normalized to β-actin.
**Cxcl5 expression is repressed by AHR inhibitors**

We investigated whether pharmacologic inhibition of AHR is able to reduce Cxcl5 production due to the ability of AHR agonists to induce its expression. Previous studies showed that these AHR inhibitors were not toxic to primary mouse keratinocytes [23]. To determine if pharmacological inhibition of AHR activation is effective in decreasing Cxcl5 levels, primary mouse keratinocytes were treated with the AHR antagonists GNF351 or CH223191, followed by IL1B for a 5 h time frame. Treatment with GNF351 and CH223191 decreased levels of IL1B-induced Cxcl5 2.0- and 1.8-fold, respectively, compared to IL1B treatment alone (Figure 2.4a, left panel). Basal levels of Cxcl5 expression were also significantly decreased by treatment with GNF351 or CH223191. Both antagonists also decreased Cyp1a1 levels (Figure 2.4a, right panel).

Next, we examined whether exposure to a SAhRM, SGA360, decreases Cxcl5 expression. SGA360 exhibited a 3-fold reduction of IL1B-induced levels of Cxcl5 after 24 h of exposure (Figure 2.4b, left panel). To determine the ability of AHR ligands to influence CXCL5 protein levels, an ELISA was performed using media collected from primary keratinocytes treated for 24 h. SGA360 significantly reduced both basal (2-fold reduction) and induced (2.7-fold) CXCL5 protein levels, while TCDD increased levels of CXCL5 protein in the media (Figure 2.4b, right panel). These data demonstrate that pharmacological inhibition of AHR is effective in decreasing gene expression of Cxcl5 in primary mouse keratinocytes under basal and induced conditions, and that an AHR agonist and antagonist can increase and reduce CXCL5 protein levels, respectively.
Figure 2.4: Antagonism of AHR activity represses basal and IL1B-induced Cxcl5 levels in primary mouse keratinocytes.
(a) Keratinocytes from Ahr+/+ mice were pre-treated for 1 h with vehicle, GNF351 or CH223191 followed by treatment with PBS or IL1B for 4 h. Gene expression was determined by qRT-PCR. (b) Keratinocytes from Ahr+/+ mice were treated with vehicle, TCDD or SGA360 for 1 h followed by treatment with PBS or IL1B for 23 h. Cells were re-dosed with vehicle or SGA360 at 12 h. Expression of Cxcl5 was determined by qRT-PCR (left panel). Media was collected from keratinocytes and used for ELISA (right panel. Letters (i.e., “a”, “b”) denote compared columns within each individual graph, with the significance indicated by number of asterisks as noted in Materials and Methods.
**AHR-mediated synergistic induction of Cxcl5 is enhanced under high calcium conditions**

It has been shown previously that exposing keratinocytes to differentiation culture media enhances nuclear translocation of AHR [23]. As AHR-mediated induction of Cxcl5 is regulated through the canonical DRE pathway, we wanted to determine if differentiation culture media (0.12 mM Ca$^{2+}$) would induce a greater synergistic induction of Cxcl5 compared to keratinocytes grown in proliferation media (0.05 mM Ca$^{2+}$). Exposure to differentiation culture media induced a statistically significant increase in TCDD and IL1B exposed cells compared to keratinocytes exposed to proliferation media that were treated in the same manner (Figure 2.5, left panel). Additionally, the TCDD-induced increase in Cyp1a1 was significantly enhanced under higher calcium culture conditions compared to lower calcium culture conditions (Figure 2.5, right panel). Therefore, media conditions which promote enhanced differentiation of primary keratinocytes also contribute to a higher induction of synergistic Cxcl5 expression in an AHR-mediated manner.
**Figure 2.5: Differentiation media enhances AHR-driven synergistic Cxcl5 induction.**

Ahr<sup>+/+</sup> keratinocytes were exposed to proliferation media conditions (0.05 mM Ca<sup>2+</sup>) or differentiation media conditions (0.12 mM Ca<sup>2+</sup>) for 7 h, followed by treatment with vehicle or TCDD for 1 h, then treatment with PBS or IL1B (4 h) for a 12 h total exposure. Expression of Cxcl5 (left) and Cyp1a1 (right) was determined by qRT-PCR. Letters (i.e., “a”, “b”, “c”) denote compared columns within each individual graph, with the significance indicated by number of asterisks as noted in Materials and Methods.

**AHR mediates Cxcl5 expression after UVB exposure in vivo**

To determine the in vivo consequences of AHR activation under conditions of increased inflammation, mice were exposed to UVB, followed by topical application of FICZ. UVB exposure significantly induced Cxcl5 in the absence of ligand treatment in the skin of Ahr<sup>+/+</sup> mice, but when combined with FICZ the level of induction of Cxcl5 was enhanced compared to UVB exposure alone (Figure 2.6a, left panel). UVB exposure alone did not increase Cyp1a1 expression in the skin of the exposed mouse ears, while FICZ with or without UVB exposure significantly induced Cyp1a1. Notably, UVB exposure significantly decreased FICZ-induced Cyp1a1 induction, in contrast to previous studies in which CYP1A1 is increased by UV (Figure 2.6a, right panel). In Ahr<sup>-/-</sup> mice, neither UVB nor FICZ exposure significantly increased Cxcl5 in the exposed ears (Figure
2.6b, left panel). Cyp1a1 expression was not influenced by UVB or FICZ exposure in Ahr−/− mice (Figure 2.6b, right panel). These data demonstrate that AHR is an important mediator of UVB-induced Cxcl5 expression in vivo.

**Figure 2.6: The AHR regulates UVB-mediated Cxcl5 expression in vivo.**

Mice (n=4-5) were anesthetized followed by sham or UVB exposure. UVB-exposed mice received a total dose of 360 mJ/cm² UVB. Immediately following exposure, the left ears received vehicle treatment in acetone, and the right ears received 100 ng of FICZ in acetone. Treatments were reapplied every 8 h, and the mice were sacrificed at 24 h after the initial exposure. Whole ears were collected and used for analysis. Expression of Cxcl5 and Cyp1a1 was analyzed by qRT-PCR in Ahr+/+ (a) and Ahr−/− (b) mice. Letters (i.e., “a”, “b”) denote compared columns within each individual graph, with the significance indicated by number of asterisks below the letter as noted in Materials and Methods.
2.5 DISCUSSION

In this study, we have identified Cxcl5 as a novel AHR target in primary mouse keratinocytes. A physiological role for the AHR in the regulation of this gene in the skin was previously suggested by the results of a DNA microarray conducted on mice expressing a constitutively active form of the AHR in keratinocytes. These mice developed inflammatory skin lesions resembling atopic dermatitis. Among the chemokines analyzed in the microarray, Cxcl5 was the most highly expressed [24], but the authors did not investigate whether the increase in Cxcl5 was due to direct AHR regulation. A DRE-dependent mode of regulation was hypothesized upon mouse Cxcl5 promoter analysis, when the presence of nine adjacent DRE sequences was noted upstream of the transcription start site. We demonstrate that AHR-mediated regulation of Cxcl5 is most likely due to transcriptional activation by AHR via direct DRE binding, as opposed to an alternative pathway such as the interaction of AHR with another transcriptional mediator. It should be noted that the close proximity of the DRE sequences precludes the possibility that each DRE is functional. Site-directed mutagenesis could be used to determine how many and which specific DREs contribute to the ability of AHR to induce Cxcl5 expression.

In addition to the direct regulation of Cxcl5 by AHR, this study provides further evidence for a model by which agonist-activated AHR potentiates inflammatory gene expression in a synergistic manner in the presence of additional stimuli, including the pro-inflammatory mediator IL1B, and under elevated calcium concentrations. Calcium levels in the epidermis regulate the terminal differentiation of keratinocytes [25], so the inflammatory status of the skin as well as the degree of differentiation could mediate the
extent by which AHR agonists can induce Cxcl5 expression. Our previous work established this model of regulation for IL6 in human tumor cells lines in the presence of IL1B [14] and for Ccl20 in primary mouse peritoneal macrophages in the presence of LPS [15], establishing this as a cross-species phenomenon. The effect was first observed when it was noted that human tumor cell lines minimally responsive to IL1B-mediated gene induction of IL6 were able to robustly induce expression in the presence of both TCDD and IL1B [14]. The mechanism for this effect was further determined to be due to the binding of AHR to nonconsensus DREs upstream of the IL6 transcriptional start site, which in turn allowed a conformational change to occur rendering the promoter more accessible for the IL1B-induced binding of NFκB elements to their response elements. In addition, the dismissal of histone deacetylase 1 (HDAC1), and reduction of HDAC3 on the promoter following combinatorial treatment with TCDD and IL1B allowed for increased binding of NFκB elements [26]. Though additional work must confirm that a similar mechanism governs the regulation of Cxcl5 in mouse keratinocytes, it is a probable explanation due to the presence of a RelA binding site in the promoter of Cxcl5 in addition to the sequential DREs discussed previously (Figure 2.1a). This observation promotes the likely scenario that other inflammation-related genes may be regulated in this manner and need to be identified and characterized in humans and mice. We therefore propose the term “xenokine” to describe this class of genes which exhibit synergistically increased regulation in the presence of AHR agonists and inflammatory stimuli (Figure 2.7). As more studies indicate an imperative role for AHR in immune regulation, identification of xenokine genes will aid in elucidating the role of AHR under
specific pathological conditions. As further functions of AHR are defined, pharmacological intervention can be used to manipulate AHR activity.

Figure 2.7: AHR-mediated regulation of “xenokine” gene expression.
Schematic depiction of the synergistic regulation of responsive inflammatory genes in an AHR-dependent manner.

Compounds which inhibit the binding of AHR to DRE sequences are effective in decreasing levels of Cxcl5 induction, and this provides further evidence for a DRE-dependent mechanism of regulation. The two AHR antagonists GNF351 and CH223191 and the SAhRM, SGA360, were able to significantly reduce both basal and IL1B-induced Cxcl5 levels. Though SGA360 was characterized previously for its ability to inhibit DRE-mediated effects of AHR while retaining its cytosolic functions, in the case of Cxcl5 inhibition, it is likely that its antagonistic ability is responsible and it is therefore operating in the context of these experiments as a typical AHR antagonist. The ability of AHR antagonists to mediate reduction in levels of Cxcl5 indicates that the development of therapeutics inhibiting AHR function could be useful in reducing excessive neutrophil chemotaxis in the skin. The reduction of basal levels of Cxcl5 by these antagonists
indicates the likely presence of an endogenous AHR agonist. The ability of AHR to mediate expression of Cxcl5 therefore has broader potential physiological implications. The characterization of tryptophan products such as indole, kynurenine, indoxyl sulfate, and FICZ as endogenous ligands of the AHR, and their importance in a variety of processes, means that AHR control of xenokines could be an important implication in many biological functions. The AHR-mediated control of xenokine gene expression adds to an already complex narrative on the beneficial or detrimental nature of AHR activation in the skin. Increased expression of xenokine gene expression could imply the need to inhibit excessive AHR activation in the skin, but AHR also has beneficial properties in regards to the maintenance of a healthy skin phenotype. A recent study highlighted the importance of AHR ligands in the diet for the maintenance of proper skin barrier integrity. Upon the removal of dietary AHR ligands, transepidermal water loss (TEWL), a measure of skin barrier integrity, was similar to the impaired levels observed in Ahr−/− mice. When AHR is specifically knocked out in keratinocytes, increased levels of TEWL were observed, further demonstrating the protective role of AHR in the keratinocytes in this model. The addition of AHR ligands to the diet rescued barrier integrity in these mice [27]. Agonist-activated AHR is also involved in keratinocyte differentiation, a process that is essential for proper barrier function of the skin [28], [29], [23]. Impaired differentiation and inflammation are hallmarks of inflammatory skin diseases such as psoriasis and atopic dermatitis, and therefore agonist-driven AHR is postulated to have a protective effect under these disease conditions. The seeming contradictory nature of AHR activation in the skin in regards to disease treatment is well recognized, and has been addressed in a recent review [30]. The factors governing whether AHR activation in
the skin is beneficial or detrimental likely depend on whether or not a disease state is present, and if there is disease, whether it is an acute or chronic condition, and which factors are causing and mediating the pathological state.

This study has established a physiological, *in vivo* relevance for AHR-mediated regulation of xenokine gene expression, as mice exposed to UV and treated directly after with FICZ demonstrated a greater induction of *Cxcl5* gene expression in the skin. This is the first of our studies involving xenokines to demonstrate an *in vivo* effect. Additionally, previous studies have begun to characterize a role for AHR in neutrophil biology, particularly in the lungs. In an influenza model of lung inflammation, neutrophil recruitment to the lungs was enhanced upon TCDD exposure, which was due to AHR activation at the site of infection rather than AHR in the neutrophils [31]. However, earlier studies from the same group determined that the AHR-dependent increase in neutrophil infiltration to the lungs in this model was not due to enhanced levels of neutrophil chemoattractants in the bronchoalveolar lavage fluid, including CXCL5 (aka LIX) [32]. In contrast, it was observed in a different study that *Ahr*−/− mice had a more inflammation-inducible phenotype, in that they exhibited more lung inflammation upon cigarette smoke exposure when compared to controls, as well as enhanced neutrophilia upon endotoxin challenge [33]. A recent review explains this seeming contradiction by postulating that sustained exposure to TCDD in the influenza study may mimic an AHR-null phenotype [34]. However, the complexity which governs AHR activation precludes making an overarching conclusion about the role of the receptor in neutrophil recruitment; it is likely that cell-type, and context-specific effects are present.
Future studies are needed to determine the relevance of AHR activation in the skin and other barrier tissues for direct recruitment of neutrophils. Whether the increased expression in the skin of Cxcl5 leads to direct physiological consequences (i.e., enhanced neutrophil chemotaxis to the skin) is the subject of current study in our laboratory. As specific effects of AHR are highly context-dependent, our observation of AHR-mediated control of Cxcl5 expression could be specific to keratinocytes. Further work will elucidate whether the synergistic induction of Cxcl5 we observed is also present in other epithelial barrier cells, such as alveolar epithelial type II cells or enterocytes.
2.6 BIBLIOGRAPHY


CHAPTER 3: CHARACTERIZATION OF A MOUSE MODEL EXPRESSING THE HIGH AFFINITY ALLELE OF THE ARYL HYDROCARBON RECEPTOR ON A HAIRLESS SKH1 MOUSE BACKGROUND

3.1 ABSTRACT

The laboratory mouse strain SKH1 is broadly used in skin research due to its hairless phenotype and intact immune system. Due to the complex nature of AHR function in the skin, the development of additional in vivo models is necessary to study its function in dermal homeostasis and pathology. The outbred SKH1 mice express both the $Ah^{b-2}$ and $Ah^d$ alleles. SKH1 mice were crossed with C57BL/6J mice, which harbor the $Ah^{b-1}$ allele, which expresses a high-affinity binding form of the receptor. SKH1 mice were bred to express either the $Ah^{b-1}$ or $Ah^d$ allele, to create useful models for studying endogenous AHR function in the skin. Allelic status was confirmed by Western blot and DNA sequence analysis. Differential basal AHR activation between the strains was determined by assessing $Cyp1a1$ expression levels in the small intestine, liver, and skin of the SKH1-$Ah^{b-1}$ mice compared to SKH1-$Ah^d$ mice. To determine if SKH1-$Ah^{b-1}$ mice are more prone to a pro-inflammatory phenotype in response to UVB, gene expression of inflammatory mediators was analyzed. SKH1-$Ah^{b-1}$ mice expressed enhanced gene levels of the chemotactic factors $Cxcl5$, $Cxcl1$, and $Ccl20$, as well as the inflammatory signaling factors $S100a9$ and $Ptgs2$, compared to SKH1-$Ah^d$ mice. These data support the hypothesis that SKH1-$Ah^{b-1}$ mice have enhanced inflammatory signaling upon UVB exposure. Future studies are needed to determine the physiological relevance of the increased level of inflammatory signaling mediated by the presence of the high-affinity ligand binding form of the AHR.
3.2 INTRODUCTION

The mouse strain SKH1 (Crl: SKH1-hr) is the most common hairless line used in skin-related research. Its phenotype is due to an autosomal recessive mutation in the \textit{hairless} (\textit{Hr}) gene, which encodes a 130 kDa transcriptional co-repressor. The SKH1 mouse is immunocompetent and unpigmented, and is used in a wide array of dermatological studies, including UV exposure, wound healing, and carcinogenesis. However, its outbred status complicates its use in research due to its undefined genetic background [1]. Due to the ease of both the topical application of chemicals and observation of the phenotypic consequences of exposure, SKH1 mice, as well as the inbred hairless mouse line HRS/J, have previously served as models to study the effects of AHR activation in the skin. In particular, hairless mice were used as models to study the development of chloracne, a skin condition that arises due to sustained activation of the AHR by aromatic hydrocarbons [2], [3], [4], [5].

A preliminary study in our laboratory indicated that the wild-type SKH1 strain expressed an AHR protein of a larger size than that found in C57BL/6J mice, a strain known to express the high affinity \textit{Ah}^{b-1} allele [6]. This led to the possibility of the original (wild-type) SKH1 line expressing either the \textit{Ah}^{b-2}, \textit{Ah}^{b-3}, or \textit{Ah}^{d} allele, due to the similar size between these three forms of the AHR. The vast majority of studies examining the AHR in mice utilize C57BL/6J mice. Therefore we wished to develop hairless mouse models to study the role of AHR in UVB-mediated inflammation that expresses either the \textit{Ah}^{b-1} or \textit{Ah}^{d} allele to determine endogenous ligand-mediated effects. The AHR has been implicated in the mediation of various skin pathologies, including atopic dermatitis, psoriasis, UV-mediated carcinogenesis, and chemical carcinogenesis,
and the positive or negative consequences of AHR activation on disease phenotype are often highly context-dependent [7, 8]. The development and characterization of this model will help to elucidate the role of the AHR in these various skin conditions.
3.3 MATERIALS AND METHODS

Animals and husbandry
All mouse lines were bred in-house after acquisition. C57BL/6J mice and $Ah^d$ congenic mice on a C57BL/6J background were originally purchased from the Jackson Laboratory (Bar Harbor, ME). Wild-type SKH1 mice were a kind gift from Dr. Jeffrey M. Peters (The Pennsylvania State University). Animals were housed in specific pathogen-free conditions under a 12 h light/dark cycle with ad libitum access to standard chow and water in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University.

Genotyping of hairless mouse strains
SKH1-$Ah^{b-l}$ mice were generated by mating wild-type SKH1 mice with C57BL/6J mice in house. Resulting heterozygous pups were mated with wild-type SKH1 mice for five generations to generate a hairless phenotype. Heterozygote F5 littermates were mated, and resulting SKH1-$Ah^{b-l}$ and SKH1-$Ah^d$ homozygotes were selected and mated, followed by colony expansion. Mice were selected for breeding pairs based on Western blot analysis for AHR and PCR based genotyping using the following primers: OL4064: 5´-CAGTGGGAATAAGGCAAGAGTGA-3´ and OL4066: 5´-AGGGAGATGAAGTATGTGTATGTA-3´, which dependent on the allelic form of the AHR yield a different product size.

Sequencing of hairless mouse strains
RNA was isolated from livers and converted to cDNA to be used for $Ah$ allele determination. Liver samples from the BALB/c mice were a kind gift from Dr. Robert Paulson (The Pennsylvania State University). cDNA was further amplified through
additional PCR cycles using the following primer set: F - 5' -
AAGAAAGGAAGGACGGAGC - 3' and R - 5' - GGCTTGAAGGAGGACACAGA -
3'. Sequencing of DNA from all strains of mice was performed at the Penn State
Genomics Core Facility (University Park, PA), using the primers listed above.
Sequencing results were translated using the online ExPASy Translate Tool
(http://web.expasy.org/translate/) (Swiss Institute of Bioinformatics) and compared to
amino acid sequences for Ahb-1, Ahb-2, Ahb-3, and Ahd mice previously published by
Poland, Palen, and Glover [9].

UV irradiation time course
Age-matched male mice were i.p. treated with avertin prior to irradiation with a dose of
3.6 kJ/m² UVB from UV bulbs (American Ultraviolet Light Co.) covered with cellulose
triacetate (Kodak) to allow only UV wavelengths between 280 and 320 nm. Output of the
UV bulbs was measured using a UVX radiometer (UVP, Upland, CA). Mice were
sacrificed by CO₂ asphyxiation at 6, 12, 24, or 48 h after initial UV exposure. Skin
samples were collected from the exposed back of the mice, snap-frozen in liquid
nitrogen, and stored at -80°C until further analysis.

RNA isolation and quantitative reverse transcription PCR
Total RNA was isolated from tissue samples using TRI Reagent (Sigma-Aldrich, St.
Louis, MO), and then converted to cDNA using a High Capacity cDNA Reverse
Transcription Kit (Applied Biosystems). Quantitative real-time PCR was conducted using
PerfeCTa SYBR Green Supermix for iQ (Quanta Biosciences, Beverly, MA) on a CFX
Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Primers used for
real-time PCR are listed in Table 1. Ribosomal protein, large, P0 (Rplp0) was used to normalize gene expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5' → 3')</th>
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<td>Cyp1a1</td>
<td>CTCTTCCTGGATGCCTTCAA</td>
<td>GGATGTGGCCCTTCTCAAATG</td>
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<td>Cxe15</td>
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<tr>
<td>Cxe12</td>
<td>AGACAGAAGTGATAGGCACTGAA</td>
<td>CTCCTTTCAGGGTTCTAGGC</td>
</tr>
<tr>
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<td>ATGGCCCTGCGGTGGCAAGCGTCTG</td>
<td>TAGGCTGAGGAGTTTCACAGCCCT</td>
</tr>
<tr>
<td>Il6</td>
<td>ATCCAGTTGCCCCTTGTTGGGACTGA</td>
<td>AAAGCTCCGACTTGTGAAGTGCTG</td>
</tr>
<tr>
<td>Il1b</td>
<td>AGCTTTCTTGTGCAAGTGCTT</td>
<td>GACAGCCCAGGGTTCTAGG</td>
</tr>
<tr>
<td>Tnf</td>
<td>GTGCCTATGTCTCGCTAGCCTCTT</td>
<td>GCCATGAACTGATGAGGAGCCG</td>
</tr>
<tr>
<td>S100a9</td>
<td>TCATCGACACCTCCCATCAA</td>
<td>TTACTCCACAGCCCTTTC</td>
</tr>
<tr>
<td>Ptgs2</td>
<td>CGTACCCCAAGGGCTCAAAT</td>
<td>ACCTTCAACAAATGACCTGA</td>
</tr>
<tr>
<td>Rplp0</td>
<td>CGTCTCGTGGAGGTGACAT</td>
<td>TAGTGGACTTCCAGGTACG</td>
</tr>
</tbody>
</table>

**Table 3.1: Mouse primer sequences used for qRT-PCR.**

**Western blot analysis**

Skin samples were excised for protein analysis and homogenized in MENG buffer (20 mM MOPS, 2 mM EDTA, 0.02% sodium azide, 10% Glycerol, pH 7.4) plus 20 mM molybdate, 500 mM NaCl, and cOmplete Mini protease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany.) Samples were resolved by 8% tricine-SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Rpt1 antibody was used to probe for AHR [10]. Biotin-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) and 125I-streptavidin were used for detection and radioactivity was visualized using BioMax film. 125I-streptavidin was prepared as described previously [11].
Statistical analyses

GraphPad Prism 5 software was used to conduct all statistical analyses. Data were analyzed using unpaired, two-tailed Student’s t-test. For the UV time course experiment, significance was determined by comparing gene expression levels between SKH1-Ah\textsuperscript{b-1} and SKH1-Ah\textsuperscript{d} mice at each time point using unpaired, two-tailed Student’s t-test. Data represent mean ± S.E.M., with $p$-value ≤ 0.05 (*), $p$-value ≤ 0.01 (**), $p$-value ≤ 0.001 (***).
3.4 RESULTS

Selective breeding results in a mouse expressing the high affinity \( \text{Ah}^{b-1} \) allele on an SKH1 background

SKH1 mice have a mixed genetic background and express the \( \text{Ah}^d \) and/or \( \text{Ah}^{b-2} \) alleles. Inbred C57BL/6J mice, harboring the \( \text{Ah}^{b-1} \) allele, and out-crossed SKH1 mice were crossbred to generate a mouse strain expressing the high affinity \( \text{Ah}^{b-1} \) allele on a hairless, immunocompetent background. Heterozygote pups expressing the \( \text{Ah}^{b-1} \) allele from the F1 hybrid generation were selected and mated with SKH1 mice. For generations F2 through F4, heterozygote pups expressing the \( \text{Ah}^{b-1} \) allele were again selected and mated with SKH1 wild-type mice in order to generate a mouse with a full hairless phenotype. Heterozygous littermates from the F5 generation were mated and homozygote \( \text{Ah}^{b-1} \) allele offspring were selected and bred to colony status. Mice homozygous for the \( \text{Ah}^d \) allele found in the SKH1 mice were also selected and bred to colony status to use as a strain that would exhibit reduced AHR activation potential (Figure 3.1a). It is worth noting that these mice cannot be considered congenic due to the outbred nature of the SKH1 mouse strain. \( \text{Ah} \) allelic status did not impact overt dermal phenotype, as no gross pathological conditions were observed, and all strains exhibited similar levels of health and breeding capabilities.

Western blot analysis of AHR expression in the skin of the generated hairless mouse strains demonstrated that the hairless mice selectively bred to express the \( \text{Ah}^{b-1} \) allele (hereafter referred to as SKH1-\( \text{Ah}^{b-1} \) mice) expressed a protein smaller than that expressed by the selectively bred controls (hereafter referred to as SKH1-\( \text{Ah}^d \) mice). The SKH1-\( \text{Ah}^d \) mice expressed a protein of a similar size to the original SKH1 founder mouse line used to establish these strains (hereafter referred to as SKH1 wild-type (WT) mice)
(Figure 3.1b). This is consistent with a previous study which found that the $Ah^{b-1}$ allele encodes a 95 kDa protein, whereas the other $Ah$ alleles encode larger proteins ($Ah^d$ and $Ah^{b-2}$ alleles each encode a 104 kDa protein, while the $Ah^{b-3}$ allele encodes a 105 kDa protein) [6].

Figure 3.1: Selective crossbreeding of C57BL/6J and SKH1 mice results in hairless mice expressing either the $Ah^{b-1}$ or $Ah^d$ allele.

(a) Schematic diagram depicting the breeding protocol used to produce mouse strains expressing either the $Ah^{b-1}$ or $Ah^d$ allele on an SKH1 background. (b) Western blot analysis of AHR in the skin of SKH1-$Ah^{b-1}$, SKH1-$Ah^d$, and SKH1-WT mice. (n=2 mice/genotype).
To definitively establish the Ah allelic status for the generated mice, livers were harvested from two mice of each strain as well as established mouse strains known to harbor three of the Ah alleles, C57BL/6J mice (Ah\textsuperscript{b-1}), C57BL/6J mice congenic for the Ah\textsuperscript{d} allele, and BALB/c mice (Ah\textsuperscript{b-2}) and used for sequencing. The resulting amino acid sequences were compared to the previously published sequences as determined by Poland and colleagues [9]. The mice used in our analysis were homozygous for the point mutations indicated as there were no additional significant peaks in the DNA sequencing results. Notable amino acid polymorphisms in the Ah allele sequences according to Poland include: isoleucine (I) at residue 324 in the Ah\textsuperscript{b-1} allele, with methionine (M) at that position in the other three alleles; phenylalanine (F) at residue 348 in the Ah\textsuperscript{b-2} allele with leucine (L) at that position in the other three alleles; and valine (V) at residue 375 in the Ah\textsuperscript{d} allele with alanine (A) in all of the Ah\textsuperscript{b} alleles (Figure 3.2a).

The sequencing results for the established mouse lines agree with the results of Poland at the residues of interest noted above. C57BL/6J mice harbor I324, L348 and A375, consistent with the Ah\textsuperscript{b-1} allele, C57BL/6J mice congenic for the Ah\textsuperscript{d} allele harbor M324, L348 and V375, consistent with the Ah\textsuperscript{d} allele, and BALB/c mice harbor M324, F348 and A375, consistent with the Ah\textsuperscript{b-2} allele (Figure 3.2b). The SKH1-Ah\textsuperscript{b-1} mice were confirmed to harbor the Ah\textsuperscript{b-1} allele (I324, L348, and A375), and the SKH1-Ah\textsuperscript{d} mice were confirmed to harbor the Ah\textsuperscript{d} allele (M324, L348 and V375). Two of the mice from the original SKH1 WT line used to establish the SKH1-Ah\textsuperscript{b-1} and SKH1-Ah\textsuperscript{d} mice were found to harbor the Ah\textsuperscript{b-2} allele (M324, F348 and A375) (Figure 3.2c). These results confirm the successful generation of a hairless SKH1 mouse strain expressing either the Ah\textsuperscript{b-1} allele or Ah\textsuperscript{d} allele.
Figure 3.2: Sequencing reveals variation in the amino acid sequence for the Ah allele across different mouse strains.

(a) Amino acid sequences for the four mouse Ah alleles as previously published by Poland and colleagues [9]. Amino acid sequences for C57BL/6J, C57BL/6J-Ah<sup>d</sup>, and Balb/c mice (b) and hairless SKH1-Ah<sup>b-1</sup>, SKH1-Ah<sup>d</sup>, and SKH1-WT mouse strains (c). (n=2 mice/genotype). Point mutations are highlighted with bold type. Because the SKH1 outcrossed strain carries two alleles of the Ahr, the WT SKH1 mice sequenced here were homozygous for the Ah<sup>b-2</sup> allele.

Endogenous Cyp1a1 expression differs between SKH1-Ah<sup>b-1</sup> and SKH1-Ah<sup>d</sup> mice in small intestine, liver, and skin.

The ability of the AHR<sup>b-1</sup> to bind ligands with higher affinity, relative to the AHR<sup>d</sup>, may lead to increased basal AHR target gene induction in tissues. To investigate this possibility, differences in basal expression of the AHR target gene Cyp1a1 in various tissues of the SKH1-Ah<sup>b-1</sup> and SKH1-Ah<sup>d</sup> mouse strains was assessed. Cyp1a1 induction was increased in the duodenum and ileum and decreased in the liver and skin in the SKH1-Ah<sup>b-1</sup> mice compared to SKH1-Ah<sup>d</sup> mice (Figure 3.3), demonstrating that differences in basal AHR activity exist between the two strains.
Figure 3.3: Basal levels of Cyp1a1 differ in tissues of SKH1-Ah\(^{b-l}\) and SKH1-Ah\(^{d}\) hairless mice.

Duodenum, ileum, liver and skin tissues were collected from SKH1-Ah\(^{b-l}\) (black) and SKH1-Ah\(^{d}\) (white) mice and Cyp1a1 expression levels were assessed by qRT-PCR and normalized to Rplp0. A statistically significant difference between the two genotypes is indicated by number of asterisks as noted in Materials and Methods. “ns” denotes no significant difference.

**SKH1-Ah\(^{b-l}\) mice exhibit enhanced inflammatory gene expression levels after acute UVB exposure**

The AHR ligand FICZ is a high-affinity endogenous agonist which is formed upon tryptophan exposure to UV [12]. Due to the role of the AHR in modulating inflammation, we wanted to determine if the mice with different alleles of the Ahr respond differently to UVB exposure in the skin, possibly due to the presence of endogenous AHR ligands such as FICZ [7]. Mice were therefore exposed to UVB to
determine if the $Ah$ allele status is influential in the inflammatory response at 6, 12, 24, and 48 h after initial exposure. Members of the CXC subfamily of chemokines are important in the initial response of the skin to UVB exposure due to their role in neutrophil chemotaxis to sites of injury or infection. Since AHR was previously shown to regulate $Cxcl5$ under pro-inflammatory conditions (see Chapter 2), we wanted to determine if $Ah$ allelic status influenced the expression of this family of chemokines.

Acute UVB exposure increased expression of the CXC subfamily chemokines $Cxcl1$, $Cxcl2$, and $Cxcl5$ in both strains compared to sham irradiated mice, with $Cxcl5$ peaking at 48 h and $Cxcl1$ and $Cxcl2$ both peaking at 24 h (Figure 3.4a-c, right panels). SKH1-$Ah^{b-l}$ mice exhibited an elevated response to UVB as exhibited by enhanced chemokine expression when compared to SKH1-$Ah^d$ mice. $Cxcl5$ was increased approximately 12.5-fold at 6 h, 7-fold at 12 h, and 3-fold at both the 24 h and 48 h time points in SKH1-$Ah^{b-l}$ mice compared to SKH1-$Ah^d$ mice (Figure 3.4a, right panel). In the absence of the inflammatory stimulus provided by UVB, SKH1-$Ah^{b-l}$ mice exhibited elevated basal levels of $Cxcl5$, with a 3.5-fold induction compared to SKH1-$Ah^d$ mice (Figure 3.4a, left panel). $Cxcl1$ was increased approximately 3-fold at 6 h, and 3.5-fold at 12 h and 24 h in SKH1-$Ah^{b-l}$ mice compared to SKH1-$Ah^d$ mice (Figure 3.4b, right panel), but there was no significant difference in $Cxcl2$ between the two genotypes (Figure 3.4c, right panel). $Ccl20$, a member of the CC subfamily of chemokines, was not enhanced to the degree of the CXC chemokines after UVB exposure, but there was a 2-fold increase at both the 6 h and 24 h time points in the SKH1-$Ah^{b-l}$ mice compared to SKH1-$Ah^d$ mice (Figure 3.4d, right panel). No significant basal difference in $Cxcl1$, $Cxcl2$, or $Ccl20$ was observed between the two strains (Figure 3.4b, c, and d, left panels).
Figure 3.4: SKH1-Ah<sup>b-l</sup> mice exhibit greater induction of chemokine gene expression after UVB exposure compared to SKH1-Ah<sup>d</sup> mice.

SKH1-Ah<sup>b-l</sup> (black) and SKH1-Ah<sup>d</sup> (white) mice were sham or UVB irradiated and sacrificed at 6, 12, 24, or 48 h post-UVB exposure (n=4-5 mice/genotype/time point). After sacrifice, back skin was excised, used for RNA isolation, and converted to cDNA. Relative gene expression levels of the chemokines Cxcl5 (a), Cxcl1 (b), Cxcl2 (c), and Ccl20 (d) were determined by qRT-PCR and normalized to the internal control gene Rplp0. Bar graphs depict gene expression levels of sham irradiated mice. “ns” denotes no significant difference.

Due to the differences observed in chemokine expression levels after UVB exposure, we wanted to determine if other cytokines were differentially regulated between the two mouse strains. Therefore, expression levels of interleukin 6 (Il6), interleukin 1 beta (Il1b), and tumor necrosis factor (Tnf) were assessed in the skin of the mice. Il6 (Figure 3.5a) and Il1b (Figure 3.5b) were elevated in the skin after UVB exposure in both mouse strains (right panels) with a peak induction at 24 h in the SKH1-Ah<sup>b-l</sup> mice and at 6 h in SKH1-Ah<sup>d</sup> mice for Il6 and a peak induction at 24 h for Il1b in
both strains. Only \textit{Il6} was significantly different between the two strains at 6 h, with a 2-fold increase in the SKH1-\textit{Ah}^d mice compared to the SKH1-\textit{Ah}^{b/l} mice (Figure 3.5a, right panel). \textit{Tnf} expression peaked in both strains at 6 h, and was significantly different between the two strains only at 12 h, with a 1.2-fold difference in SKH1-\textit{Ah}^{b/l} mice compared to SKH1-\textit{Ah}^d mice (Figure 3.5c, right panel). No significant difference existed in basal induction of these genes between the different strains.

The levels of the inflammatory mediators S100 calcium binding protein A9 (calgranulin B) (\textit{S100a9}) and prostaglandin-endoperoxide synthase 2 (\textit{Ptgs2}) (also known as cyclooxygenase-2 or COX-2) were also assessed in the skin of the strains for differences in expression levels. UVB increased \textit{S100a9} in both strains with the maximum induction achieved at 48 h (Figure 3.5d, right panel). SKH1-\textit{Ah}^{b/l} mice exhibited greater induction of \textit{S100a9} at all time points after UVB compared to SKH1-\textit{Ah}^d mice, but with significantly different levels of induction at 6 h (96-fold), 24 h (12-fold), and 48 h (9-fold). \textit{Ptgs2} was elevated in both strains after UVB exposure, with a peak at 48 h in both, and levels were significantly different between the two strains at every time point after UVB, with a 32-fold difference at both 6 h and 12 h, a 15-fold difference at 24 h, and a 5-fold difference at 48 h (Figure 3.5e, right panel). \textit{S100a9} and \textit{Ptgs2} levels were basally elevated in SKH1-\textit{Ah}^{b/l} mice compared to SKH1-\textit{Ah}^d mice, but only \textit{Ptgs2} was statistically significant, with a 9.5-fold difference (Figure 3.5e, left panel). These data demonstrate that the SKH1-\textit{Ah}^{b/l} mice exhibit a more pro-inflammatory phenotype after UVB exposure as measured by gene expression levels of inflammatory mediators.
Figure 3.5: SKH1-Ah\textsuperscript{b-I} mice exhibit greater induction of inflammatory gene expression after UVB exposure compared to SKH1-Ah\textsuperscript{d} mice.

SKH1-Ah\textsuperscript{b-I} (black) and SKH1-Ah\textsuperscript{d} (white) mice were sham or UVB irradiated and sacrificed at 6, 12, 24, or 48 h post-UVB exposure (n=4-5 mice/genotype/time point). After sacrifice, back skin was excised, used for RNA isolation, and converted to cDNA. Relative gene expression levels of the inflammatory mediators \textit{Il6} (a), \textit{Il1b} (b), \textit{Tnf} (c), \textit{S100a9} (d), and \textit{Ptgs2} (e) were determined by qRT-PCR and normalized to the internal control gene \textit{Rplp0}. Bar graphs depict gene expression levels of sham irradiated mice. “ns” denotes no significant difference. Note: At the 6 h and 12 h time points for \textit{S100a9} and \textit{Ptgs2}, n = 2-3 values for SKH1-Ah\textsuperscript{d} mouse results due to lack of amplification of the additional samples.
3.5 DISCUSSION

The aim of this study was to generate a hairless, immunocompetent mouse model expressing the high affinity-binding form of the allele encoding the AHR (SKH1-Ah$^{b-1}$). The hypothesis driving the development of this model postulates that the skin of an SKH1-Ah$^{b-1}$ mouse would be more highly susceptible to the pro-inflammatory effects of UVB exposure compared to the wild-type SKH1 strain due to the ability of the Ah$^{b-1}$ allele to bind AHR ligands with greater affinity. The high affinity agonist FICZ and other potential AHR agonists generated after UVB exposure would more highly activate the AHR in these mice, leading to the enhanced expression of inflammation-related genes, since AHR is a mediator of inflammatory gene expression. The genes in this study were chosen either because AHR was previously demonstrated to be involved in their regulation, or because they were previously shown to be enhanced in the skin after acute UVB exposure; some of the genes analyzed fit both criteria. Cxcl5 is regulated by AHR under basal and pro-inflammatory conditions (see Chapter 2), but is also a mediator of UVB-induced pain in the skin [13]. Ptgs2 (also known as Cox2) contributes to inflammation through the production of PGE2 and the development of UVB-induced skin cancer in vivo [14]. In addition, the AHR contributes to Ptgs2 expression, as Ahr$^{-/-}$ mice exposed to UVB did not induce COX2 protein expression [15]. AHR is also involved in the regulation of Ccl20 [16], Cxcl1 [17], Il6 [18], Il1b [19], and Tnf [20]. Il6, Ccl20, S100a9, Ptgs2, Cxcl5, Cxcl1, and Cxcl2 were among the top fifty genes increased 48 h after UVB exposure in human skin [21]. Keratinocytes secrete IL1B in response to UVB [22], and TNF is produced in epidermal cells in response to UV [23]. The gene expression results support the hypothesis because the SKH1-Ah$^{b-1}$ mice exhibit elevated levels of inflammatory genes in response to UVB, particularly Cxcl5, Cxcl1, Ccl20,
S100a9, and Ptgs2. Future analysis of protein levels by Western blot or ELISA for these increased factors in the skin of SKH1-Ah<sup>b-l</sup> would establish whether the increase in mRNA of these genes leads to functional consequences. Additionally, flow cytometry analysis or activity assays for these mediators would increase evidence to support the hypothesis.

UV light induces expression of the prototypical AHR response genes, *CYPIA1* and *CYP1B1* in human epidermis [24] and aryl hydrocarbon hydroxylase (AHH) activity (i.e., CYP1A1 activity) was induced in response to UVB in neonatal rat skin [25]. One possible explanation for this observed induction is that photoproducts generated from exposure to UV act as AHR agonists. Potential AHR ligands formed after UVB exposure include tryptophan-derived photoproducts [12] and photo-oxidized skin surface lipids [26]. While the tryptophan UV-derived photoproduct FICZ is a strong inducer of AHR, its effects are transient compared to other ligands such as TCDD, with a maximum induction in *CYPIA1* levels at 3 h in a human keratinocyte cell line [27]. If the UVB-induced levels of Cxcl5, Cxcl1, Ccl20, S100a9, and Ptgs2 observed in the skin of SKH1-Ah<sup>b-l</sup> mice are due to the generation of an endogenously derived ligand, FICZ is most likely not the only mediator over the entire 48 h time course due to its rapid metabolism. Additionally, though there is evidence that FICZ could be generated *in vivo* [28], whether it is produced under physiologically relevant conditions in the skin and whether sufficient cellular tryptophan levels exist to enable its biosynthesis are questions requiring further study [29]. An additional complicating factor is that IL1B represses Cyp1a1 and Cyp1b1 [30], so these genes may not always represent ideal indicators of endogenous AHR activation under conditions of injury, infection, or stress.
Due to the complicated nature of determining if and how endogenous AHR ligands activate the receptor in vivo under basal and pathological conditions, the hairless mouse model characterized here may prove valuable due to the expression of the high affinity $Ah^{b-1}$ allele. Although an SKH1-Ahr$^{-/-}$ mouse has been developed [31], a benefit of our model compared to a complete knockout is that the effects of in vivo activation of AHR by endogenous ligands can be studied without the negative health consequences and developmental problems found in the Ahr$^{-/-}$ mouse. Due to the hairless phenotype of the SKH1-Ah$^{b-1}$ mouse, inflammation caused by hair removal will not impact experimental results. This mouse is an ideal model for the study of UV-mediated effects, as this study demonstrates, including photocarcinogenesis, as well as chemical carcinogenesis.

The SKH1-Ah$^{b-1}$ mouse was confirmed to harbor the Ah$^{b-1}$ allele through Western blot and DNA sequencing analysis. The translation of the ORF of the Ah alleles for established and the generated hairless mouse strains from the sequencing results confirmed the presence of the Ah$^{b-1}$ allele in the SKH1-Ah$^{b-1}$ mice, but also yielded a surprising result. Before the generation of this mouse, it was known that the wild-type SKH1 mouse did not harbor the Ah$^{b-1}$ allele due to a protein size difference determined by analysis via Western blot between the wild-type SKH1 mouse and a C57BL/6J mouse (see Figure 3.1). However, due to the similar size of the AHR protein between the Ah$^{b-2}$, Ah$^{b-3}$, and Ah$^{d}$ alleles (104 kDa, 105 kDa, and 104 kDa, respectively) [6], it was unknown which version of the Ah allele was carried by the wild-type SKH1 mice, though it was presumed to be the Ah$^{d}$ allele. According to the generated sequences in this study, the control SKH1 littermates of the generated SKH1-Ah$^{b-1}$ mice harbor the Ah$^{d}$ allele,
because they carry the M324, L348 and V375 residues, matching the residues found in the $Ah^d$ allele as previously published by Poland [9], and confirmed in our own study by comparison to C57BL/6J-$Ah^d$ congenic mice (Figure 3.2). However, the sequencing results for the original parent SKH1 WT line, which was bred and maintained separately from the SKH1-$Ah^{b-1}$ and SKH1-$Ah^d$ mice after the initial breeding pairs were established, was determined to harbor the M324, F348 and A375 residues, consistent with the $Ah^{b-2}$ allele. This unexpected result is most likely explained by the possibility that in the original SKH1 parent line, heterozygous alleles for AHR exist for the $Ah^{b-2}$ and $Ah^d$ alleles, and our breeding regime selected for the $Ah^d$ allele-expressing mice. The SKH1 mouse line is outbred, which means that it is a stock that does not have a fully characterized genetic profile [32], which could potentially explain our result. It is worth noting that Poland had previously characterized the SKH1 mice as harboring an $Ah^b$ allele, based on the degree of hepatic AHH activity by BNF, and by the affinity of cytosolic AHR for TCDD [5]. However, this study did not clarify which of the three $Ah^b$ alleles was carried by SKH1 mice.

Differences in $Cyp1a1$ levels in the skin, liver, and small intestine in the SKH1-$Ah^{b-1}$ and SKH1-$Ah^d$ mice without exogenous ligand exposure could indicate that the ligand-binding ability of the $Ah^{b-1}$ and $Ah^d$ alleles impacts the ability of AHR to be activated by endogenous ligands, though these differences still need to be confirmed at the protein level by measuring CYP1A1 in these tissues. AHR ligands are present in standard laboratory rodent chow, which could explain the elevated $Cyp1a1$ levels in the duodenum and ileum of SKH1-$Ah^{b-1}$ mice. The potential decreased affinity for dietary ligands in the SKH1-$Ah^d$ mice could lead to the increase in $Cyp1a1$ levels observed in the
liver due to the decreased metabolism of the ligands by CYP1A1 in the small intestine of the SKH1-\(Ah^d\) mice. However, the \(Ah^d\) allele has primarily been characterized in terms of its diminished response to AHR ligands such as TCDD and 3MC. The human AHR also exhibits a reduced affinity for TCDD, at approximately 10-fold less than the \(Ah^{b-l}\) allele in mice, which has sometimes led to the conclusion that the human AHR is most similar to the mouse \(Ah^d\) allele. Both the human and \(Ah^d\) allele forms of the AHR contain a valine residue in the ligand binding domain (V381 in human AHR, V375 in the mouse \(Ah^d\) allele, compared to A375 in the mouse \(Ah^{b-l}\) allele). However, direct comparison should not be drawn between the human AHR and the AHR encoded by the mouse \(Ah^d\) allele, as mutation of V381 in the human AHR to alanine did not improve binding of the human receptor. Additionally, the C-terminal domain of the AHR, which contains the transactivation domain, is only 58% similar between the human and mouse AHRs, indicating that other differences between these two forms may influence AHR activity [33]. Furthermore, human AHR exhibits higher potential for ligand binding of endogenous ligands such as those derived from indole [34]. The relative affinity with which mouse AHRs encoded by the \(Ah^{b-l}\) and/or \(Ah^d\) allele binds to endogenous ligands such as FICZ has yet to be established. Additional comparative studies are needed between the high- and low- affinity alleles to determine the binding properties for specific AHR agonists, including dietary ligands.

AHR allele status may impact levels and availability of endogenous ligands in different tissues. A recent study supports this idea, when it was shown that a diet deficient in AHR ligands decreased skin barrier integrity, a phenotype also associated with \(Ahr^{-/-}\) mice, but the barrier could be restored by the addition of an AHR ligand to the
diet [35]. Additionally, mice in which Arnt was knocked out in the intestinal epithelium exhibited increased Cyp1a1 expression in many non-gut tissues including the liver and skin, an effect which was lost when AHR ligands were removed from the diet [36]. Conversely, the increase in inflammatory gene expression in the skin of SKH1-Ah$^{b-1}$ mice after UVB exposure is most likely due to local generation of photoproducts which act as agonists for the AHR. This same effect could potentially be observed due to ligands produced by skin resident microbes.

Due to the contradictory nature of AHR activation in the skin, the development of mouse models such the one described here are important for determining the role of AHR in skin homeostasis and disease. The SKH1-Ah$^{b-1}$ mouse model will be useful for studies involving pharmacological manipulation of the AHR by topically applied therapeutic agents, as well as studies involving the further elucidation of the role of AHR in a variety of skin processes and diseases, including keratinocyte differentiation, the response to UVB- and microbe-derived ligands, psoriasis, and carcinogenesis.
3.6 BIBLIOGRAPHY


CHAPTER 4: THE ROLE OF INTESTINAL EPITHELIAL ARYL HYDROCARBON RECEPTOR EXPRESSION ON INTESTINAL HOMEOSTASIS

4.1 ABSTRACT
Recent *in vivo* experimental evidence indicates that agonist-activated AHR is protective in the gastrointestinal tract upon insult. However, many of these studies have focused on AHR expression and function in intestinal immune cells. We wanted to test whether AHR expression and activation in intestinal epithelial cells may also be responsible for the promotion of a healthy intestinal environment. To examine this hypothesis, a mouse line was generated with a specific deletion of the AHR in the intestinal epithelial cells of the small and large intestines (AhrΔIE). Successful deletion of the AHR in the intestinal epithelium was confirmed with qRT-PCR analysis of intestinal scrape samples. Though loss of AHR in the intestinal epithelial cells did not render the AhrΔIE mice more susceptible to *Citrobacter rodentium* infection or DSS-induced colitis, disturbance of enterohepatic signaling was evident through changes in gene expression levels of Cyp7a1 and several other mediators of enterohepatic signaling. AHR in the intestine may have a role in maintaining whole-body homeostatic conditions, and its role in the maintenance of intestinal health through expression in the intestinal epithelial cells warrants further investigation.
4.2 INTRODUCTION

AHR expression has been shown to be protective during the course of *in vivo* models of intestinal disease, such as DSS-induced colitis [1], TNBS-induced colitis [2], and *C. rodentium* infection [3], and in particular, through activation by agonists. Though some of the beneficial effects of AHR ligands have been determined to act through AHR expression in gut immune cell subsets, particularly the intestinal intraepithelial lymphocytes [4] and the intestinal innate lymphoid cells [5], the contribution of AHR expression in the intestinal epithelium remains relatively unexplored. Indeed, intact and properly functioning intestinal epithelial cells (IECs) are essential components of the healthy gut environment due to their role in barrier function, absorption of nutrients and water, and response to and maintenance of gut microbes. AHR expression is high in barrier tissues [6], supporting the hypothesis that AHR expression is important for the maintenance of proper intestinal homeostasis. This hypothesis led us to test whether the lack of AHR in the intestinal epithelium may lead to detrimental effects under homeostatic and disease conditions. To meaningfully investigate this hypothesis, a mouse model was generated in which the AHR gene is specifically deleted from the intestinal epithelium. While the results of the current study do not indicate an essential role for AHR expression in the IECs upon insult, further studies are needed to further clarify the relative contributions of different intestinal cell subtypes to the beneficial effects of AHR activation.
4.3 MATERIALS AND METHODS

Animals, husbandry, and genotyping

All mouse lines were bred in-house after acquisition. B6.Cg-Tg(Vil1-cre)997Gum/J (Villin-Cre) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ahr-floxed (Ahr<sup>F/F</sup>) mice were kindly provided by Dr. Christopher Bradfield (University of Wisconsin, Madison, WI). Villin-Cre expression status was verified using the following primers: Forward primer: 5´ – GTGTGGGACAGAAGAAACC – 3´, Reverse primer: 5´ – ACATCTTCAGGTCTGCGGG – 3´, and Ahr-floxed status was confirmed using the following primers: Forward 1: 5´ – GTCACCTCAGATTACACTTTTCA – 3´, Forward 2: 5´ – CAGTGG GAATAAGGGCAAGTGGA – 3´, Reverse: 5´ – GGTACAAGTGCAGATGCCTGC – 3´. Animals were housed in specific pathogen-free conditions under a 12 h light/dark cycle with ad libitum access to standard chow and water in accordance with protocols approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University.

*Citrobacter rodentium*-infection model

Age-matched male Ahr<sup>F/F</sup> and Ahr<sup>ΔIE</sup> mice (n=15/genotype) were infected with 5 x 10<sup>9</sup> CFU in 200 µL of *Citrobacter rodentium* strain ICC169 by oral gavage, and bacteria were kindly provided by Dr. Margherita Cantorna (The Pennsylvania State University). *C. rodentium* cultures were grown one day prior to inoculation in Luria Bertani broth supplemented with 50 µg/mL nalidixic acid. Body weight was measured and feces were collected and homogenized on the days indicated, and serial dilutions were grown on plates containing nalidixic acid. Colonies were counted the following day [7, 8].
**Dextran sodium sulfate (DSS)-induced colitis model**
Immediately before the beginning of DSS exposure, mice were i.p. treated with either vehicle (corn oil) or TCDD (0.833 µg in 100 µL of corn oil per mouse). The TCDD was a kind gift from Dr. Stephen Safe (Texas A&M University). Experimental colitis was induced in male $Ahr^{F/F}$ and $Ahr^{ΔIE}$ mice (n=5 mice/genotype/treatment group) through *ad libitum* access to 3.5% dextran sodium sulfate (DSS; Dextran sulfate sodium salt reagent grade (MP Biomedicals, LLC; MW = 36,000-50,000)) in autoclaved, filtered tap water for six days followed by two days of autoclaved, filtered tap water only. Control mice received autoclaved, filtered tap water only for the duration of the experiment. Mice were monitored daily for changes in weight, stool consistency, presence of blood in the stool, rectal bleeding, and general mobility.

**RNA isolation and quantitative reverse transcription PCR**
Total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO), and was then converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was conducted using PerfeCTa SYBR Green Supermix for iQ (Quanta Biosciences, Beverly, MA). Primers used for real-time PCR are listed in Table 1. $Rpl13a$ was used to normalize gene expression.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5' → 3')</th>
</tr>
</thead>
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<tr>
<td>Ahr</td>
<td>GCGTCAGCTACCTGAGGGGACA</td>
<td>GGGCCATGGGCTTCGTCAC</td>
</tr>
<tr>
<td>Cyp1a1</td>
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<tr>
<td>Fxr</td>
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<td>Rpl13a</td>
<td>TTCGCTGGAAGCTACCAGAAGT</td>
<td>GCACTTTGGCTTTTCTCGGT</td>
</tr>
</tbody>
</table>

Table 4.1: Mouse primer sequences used for qRT-PCR.

Western blot analysis

Liver samples were used for protein analysis and homogenized with a 2 mL tissue grinder (Wheaton, Millville, NJ) in cold MENG buffer (20 mM MOPS, 2 mM EDTA, 0.02% sodium azide, 10% Glycerol, pH 7.4) with eComplete Mini protease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany) so that each sample contained approximately 100 mg liver/mL of buffer. Lysate was collected after centrifugation at 20,000 g for 15 min. Samples were resolved by 8% tricine-SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Primary antibodies for CYP7A1 (ab65596, Abcam, Cambridge, MA) and p23 (Prod #MA3-414, Pierce, Thermo Fisher Scientific, Waltham, MA) were used for protein detection. Biotin-conjugated secondary antibodies (Jackson
Immunoresearch, West Grove, PA) and $^{125}$I-streptavidin were used for detection and radioactivity was visualized using BioMax film. $^{125}$I-streptavidin was prepared as described previously [9]. Western blot bands were quantified by filmless autoradiographic analysis using a Cyclone storage phosphor screen instrument (PerkinElmer Life and Analytical Sciences) in conjunction with densitometric analysis using Optiquant software.

**Statistical analyses**
GraphPad Prism 5 software was used to conduct all statistical analyses. Data were analyzed either using one-way ANOVA followed by Tukey’s multiple comparison test, or by using unpaired, two-tailed Student’s $t$-test. Data represent mean ± S.E.M., with $p$-value $\leq 0.05$ (*), $p$-value $\leq 0.01$ (**), $p$-value $\leq 0.001$ (***)
4.4 RESULTS

Ahr expression is absent in the intestinal epithelium of Ahr<sup>ΔIE</sup> mice

An intestinal epithelium-specific knock-out of AHR (Ahr<sup>ΔIE</sup>) was generated by mating mice with a Cre recombinase under the direction of the mouse villin 1 promoter (Villin-cre) to Ahr floxed (Ahr<sup>F/F</sup>) mice. Villin is primarily expressed in all IECs along the horizontal axis (duodenum to colon) and vertical axis (crypt to tip of villi) [10, 11]. Therefore, the resulting progeny should lack Ahr expression in the gut epithelium. Sibling matings were set up using the F1 progeny, and mice that were Cre<sup>+</sup> and homozygous for Ahr<sup>F/F</sup> were bred to colony status, as well as Cre<sup>-</sup>, homozygous Ahr<sup>F/F</sup> littermates for use as controls (Figure 4.1a). To determine if Ahr expression was successfully deleted in the intestinal epithelium of the Ahr<sup>ΔIE</sup> mice, intestinal scrape samples were collected from the duodenum, jejunum, ileum, and colon and qRT-PCR was used to assess gene expression levels. Ahr expression was absent in intestinal tissue samples from Ahr<sup>ΔIE</sup> mice, while the Ahr<sup>F/F</sup> mice expressed Ahr in all intestinal sections. It is worth noting that Ahr is at its highest level of expression in the duodenum, and decreases down the length of the intestinal tract. Villin-Cre expression did not affect Ahr expression in the liver, as there was no difference in levels between Ahr<sup>F/F</sup> and Ahr<sup>ΔIE</sup> mice (Figure 4.1b).
Figure 4.1: Use of a mouse expressing Cre recombinase under the control of the Villin promoter crossed with an Ahr<sup>F/F</sup> mouse generates a mouse with an intestinal epithelium-specific deletion of the Ahr gene.

(a) Schematic diagram depicting the breeding protocol used to produce a mouse strain in which the Ahr is knocked out specifically in the IECs. (b) Liver tissue and intestinal scrape samples from the duodenum (Duod), jejunum (Jejun), ileum, and colon were collected from Ahr<sup>F/F</sup> mice (black) and Ahr<sup>ΔIE</sup> mice (white) (n=3 mice/genotype) and Ahr expression levels were assessed by qRT-PCR and normalized to Rpl13a expression.

**Intestinal epithelium-specific knockout of Ahr does not affect body, liver, or spleen size.**

To determine if loss of AHR in the intestinal epithelium leads to any general abnormalities, body, liver, and spleen weights were determined for Ahr<sup>F/F</sup> and Ahr<sup>ΔIE</sup> mice. Ahr<sup>ΔIE</sup> mice did not differ from Ahr<sup>F/F</sup> control mice for these parameters (Figure 4.2).

Figure 4.2: Body, liver, and spleen weights do not differ between Ahr<sup>F/F</sup> and Ahr<sup>ΔIE</sup> mice.

Body, liver, and spleen weights were compared between male Ahr<sup>F/F</sup> (n=10) and Ahr<sup>ΔIE</sup> (n=14) mice. Liver and spleen weights are expressed as a percentage of body weight.
Loss of AHR in the intestinal epithelium does not lead to impaired response to an intestinal pathogen

Since the intestinal epithelium is essential for maintaining proper barrier function against pathogenic microflora and the AHR has been shown to be protective in the course of intestinal disease, $Ahr^{F/F}$ and $Ahr^{ΔIE}$ mice were infected with *Citrobacter rodentium*, an attaching and effacing bacterial strain used to model human enteropathogenic *Escherichia coli* and enterohaemorrhagic *E. coli* infections [12] to determine if $Ahr^{ΔIE}$ mice are more susceptible to mucosal infections. Differences between $Ahr^{F/F}$ and $Ahr^{ΔIE}$ in body weight loss (Figure 4.3a) and fecal bacteria shedding (Figure 4.3b) were statistically insignificant. While both $Ahr^{F/F}$ and $Ahr^{ΔIE}$ mice initially lost weight in response to *C. rodentium* infection, the pattern of weight loss differed between the two strains. $Ahr^{F/F}$ mice experienced the greatest decrease in weight at day 2 post-infection, while the $Ahr^{ΔIE}$ mice did not experience the greatest decrease in weight until day 14. $Ahr^{F/F}$ mice also had the initial drop in weight on day 2, after which they experience a period of recovery, followed by another decrease from days 9 to 14, followed by a recovery period from days 14 to 18. $Ahr^{ΔIE}$ mice slowly declined until day 14, when they experienced the greatest decrease in weight, and then recovered from day 14 to day 18.

Both strains of mice had a final decrease in weight from day 18 to day 21 (Figure 4.3a). In contrast, the shedding patterns as determined by colony forming units (CFU) in the feces of the mice did not differ between the two strains during the post-infection period (Figure 4.3b). These data demonstrate that under basal conditions the loss of AHR in the IECs of $Ahr^{ΔIE}$ mice does not lead to increased susceptibility to an intestinal infection within the parameters measured.
Figure 4.3: Loss of Ahr in IECs does not increase susceptibility to *C. rodentium* infection.

*Ahr*<sup>F/F</sup> and *Ahr*<sup>ΔIE</sup> mice were infected with *C. rodentium* as described in Materials and Methods. (a) Percentage weight loss was calculated as compared to weight at day 0. (b) Shedding curve calculated by analysis of CFU in the feces.

Exposure to TCDD results in less weight loss in *Ahr*<sup>ΔIE</sup> mice

Previous studies have established that agonist-activated AHR is protective in chemically-induced colitis models [13, 14]. To determine if the beneficial effects of agonist exposure are due to AHR expression in the intestinal epithelium or immune cells, *Ahr*<sup>F/F</sup> and *Ahr*<sup>ΔIE</sup> mice were subjected a DSS-induced colitis model. A primary phenotypic effect of DSS exposure is weight loss, and TCDD and other AHR agonists are able to reduce weight loss due to DSS in this model. DSS caused weight loss in both genotypes from days 5 to 8 after initial DSS exposure compared to mice that received water only. A significant difference in weight loss under basal conditions (i.e., in the absence of TCDD exposure), occurred only at day 5, when *Ahr*<sup>F/F</sup> lost more weight than *Ahr*<sup>ΔIE</sup> mice (Figure 4.4a). At day 5, vehicle-treated *Ahr*<sup>F/F</sup> mice exposed to DSS exhibited a significant loss of weight compared to *Ahr*<sup>F/F</sup> mice treated with TCDD.
However, from days 6 to 8, the weight loss difference between vehicle- and TCDD-treated \( Ahr^{F/F} \) mice was the same (Figure 4.4b). By day 6 and 7 after initial DSS exposure, \( Ahr^{AIE} \) mice treated with TCDD lost significantly less weight that \( Ahr^{AIE} \) mice treated with vehicle, though by day 8 there was no significant difference (Figure 4.4c).

Another parameter associated with severity of DSS-induced colitis is a decrease in colon length. A significant decrease in colon length was observed in DSS-exposed \( Ahr^{AIE} \) mice compared to DSS-exposed \( Ahr^{F/F} \) mice under basal conditions (Figure 4.5a). Exposure to DSS decreased colon length in both \( Ahr^{F/F} \) and \( Ahr^{AIE} \) mice, but exposure to TCDD was not able to rescue this phenotype for either strain (Figure 4.5b).
Figure 4.4: TCDD exposure leads to reduced weight loss in $Ahr^{\Delta IE}$ mice after DSS treatment.

Mice received vehicle or TCDD by i.p. injection followed by control or DSS exposure as detailed in Materials and Methods. Weight was monitored daily and change in body weight was calculated as a percentage by comparison to the weight of each mouse at experimental day 0. All data presented are from the same experiment. Both genotypes given water or DSS in the absence of TCDD are depicted in (a), all treatment groups for $Ahr^{FF}$ mice are depicted in (b), and all treatment groups for $Ahr^{\Delta IE}$ mice are depicted in (c). Statistics represent unpaired, two-tailed Student’s t-test analysis of the compared DSS-exposed groups for each graph.
Figure 4.5: Exposure to TCDD does not improve colon length in Ahr$^{+/+}$ or Ahr$^{ΔIE}$ mice exposed to DSS.

Mice were treated with or without TCDD and received tap water or DSS as described in Materials and Methods. Upon termination of the experiment, colon lengths were measured for all mice. All data presented are from the same experiment. Letters (i.e., “a”, “b”, “c”, “d”) denote compared columns, with the significance indicated by number of asterisks as noted in Materials and Methods using one-way ANOVA followed by Tukey’s multiple comparison test.

Loss of AHR in the intestinal epithelium impairs enterohepatic signaling

A previous study found that loss of ARNT in the intestinal epithelium led to whole-body changes in Cyp1a1 expression [15], including a marked increase in Cyp1a1 in the livers of Arnt$^{ΔIE}$ mice. To determine if a similar mechanism occurs when Ahr is selectively knocked out in the intestinal epithelium, Cyp1a1 levels were measured in Ahr$^{ΔIE}$ mouse livers. Similar to the results observed with Arnt$^{ΔIE}$ mice, Cyp1a1 levels were increased in the Ahr$^{ΔIE}$ livers, with a 2-fold induction compared to Ahr$^{+/+}$ livers (Figure 4.6a).

Enterohepatic circulation of bile acids represents an important mode of gut-liver communication, and CYP7A1 is a rate-limiting enzyme in the bile acid biosynthetic pathway in the liver [16]. Since loss of AHR in the intestinal epithelium leads to altered
gut-liver signaling as exemplified by changes in \textit{Cyp1a1} expression in the liver, \textit{Cyp7a1} levels were compared in the livers of \textit{Ahr}/F/F and \textit{Ahr}/AIE mice to determine if additional pathways are altered by loss of AHR in IECs. \textit{Cyp7a1} levels were increased in the periportal livers of \textit{Ahr}/AIE mice 2.4-fold compared to \textit{Ahr}/F/F mice (Figure 4.6b), and CYP7A1 protein levels were increased 1.2-fold in \textit{Ahr}/AIE mouse livers compared to \textit{Ahr}/F/F mice (Figure 4.6c,d). This demonstrates that loss of AHR in the intestinal epithelium could lead to altered regulation of the enterohepatic circulation of bile acids.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure46.png}
\caption{\textit{Cyp1a1} and \textit{Cyp7a1} expression levels are increased in the periportal liver of \textit{Ahr}/AIE mice.}
\end{figure}

Periportal liver tissue was collected from \textit{Ahr}/F/F (n=10) and \textit{Ahr}/AIE (n=14) mice and \textit{Cyp1a1} (a) and \textit{Cyp7a1} (b) expression levels were assessed by qRT-PCR and normalized to \textit{Rpl13a} expression. Liver tissue from \textit{Ahr}/F/F and \textit{Ahr}/AIE mice (n=4/genotype) was used for Western blot analysis of CYP7A1 and p23 was used as a loading control (d). CYP7A1 bands were quantified and normalized to p23 (c).
Due to the increased expression of both gene and protein levels of CYP7A1 in the livers of Ahr<sup>ΔIE</sup> mice, other genes involved in bile acid synthesis and transport or lipid transport were evaluated for changes in expression levels. As shown in Figure 4.6b, Cyp7a1 was the only gene involved in either the central or alternative bile acid biosynthesis pathway that was significantly altered in the livers of Ahr<sup>ΔIE</sup> mice, though the levels of the genes encoding the other enzymes involved (Cyp8b1, Cyp27a1, and Cyp7b1) did trend higher in Ahr<sup>ΔIE</sup> mice (Figure 4.7a). After biosynthesis, conjugated bile salts are secreted into the canaliculi by transporter proteins located on the apical surface of the hepatocyte. ABCB11, also known as the bile salt export pump (BSEP), transports the bile salts, while ABCB4 transports phospholipids, and ABCG5 and ABCG8 transport cholesterol with a requirement for ABCB4 [16]. Of the genes encoding these transporters, only Abcg5 and Abcg8 were significantly increased in the livers of Ahr<sup>ΔIE</sup> mice (Figure 4.7b).

Repression of the bile acid synthesis pathway can occur through the production of FGF15 in the intestine, which then travels through the blood and binds to the FGFR4/β-Klotho receptor complex, leading to the inhibition of bile acid synthesis [17]. Expression levels of Fgfr4 are significantly induced in Ahr<sup>ΔIE</sup> mouse livers compared to Ahr<sup>F/F</sup> mice, and while the expression of the gene encoding β-Klotho (Klb) was also increased, this did not achieve statistical significance (Figure 4.7c). Additionally, the exporters Mrp2 and Mrp3 were significantly elevated in the livers of Ahr<sup>ΔIE</sup> mice (Figure 4.7d). These data demonstrate that loss of AHR in the intestinal epithelium leads to altered regulation of factors involved in bile acid and lipid transport and cycling in the liver.
Figure 4.7: Expression levels of genes involved in bile acid and lipid pathways are altered in the periportal liver of Ahr\textsuperscript{ΔIE} mice.

Periportal liver tissue was collected from Ahr\textsuperscript{+/+} (n=10) and Ahr\textsuperscript{ΔIE} (n=14) mice and levels of genes involved in the bile acid biosynthetic pathway (a), lipid transport (b), the FGF15 receptor complex (c), and basolateral export transporters (d) were assessed by qRT-PCR and normalized to Rpl13a expression. “ns” denotes no significant difference.

The farnesoid X receptor (FXR, also known as the bile acid receptor), sterol regulatory element-binding protein 1 (SREBP-1), and sterol regulatory element-binding protein 2...
(SREBP-2) are transcription factors which regulate genes involved in the bile acid pathway, lipogenesis, and cholesterol metabolism, respectively. *Srebp1* and *Fxr* were significantly induced in the livers of *Ahr<sup>ΔIE</sup>* and *Srebp2* was increased, but not to a significant degree. The FXR target gene, small heterodimer partner (*Shp*), which represses expression of *Cyp7a1*, was not significantly different between the *Ahr<sup>F/F</sup>* and *Ahr<sup>ΔIE</sup>* mice (Figure 4.8).

![Graph](image)

**Figure 4.8: Srebp1 and Fxr are increased in the periportal liver of Ahr<sup>ΔIE</sup> mice.** Periportal liver tissue was collected from *Ahr<sup>F/F</sup>* (n=10) and *Ahr<sup>ΔIE</sup>* (n=14) mice and relative gene expression levels for *Srebp1*, *Srebp2*, *Fxr*, and *Shp* were determined using qRT-PCR and normalized to *Rpl13a* expression. “ns” denotes no significant difference.

Control of bile acid synthesis also relies on signals from the small intestine. Since *Cyp7a1* was increased in *Ahr<sup>ΔIE</sup>* livers, *Fgf15* was evaluated in the ileal tissue of the mice. *Fgf15* expression was significantly decreased in *Ahr<sup>ΔIE</sup>* mice. However, *Slc10a2*, which encodes a protein also known as the apical sodium–bile acid transporter (ASBT) or the ileal bile acid transporter (IBAT), though decreased in *Ahr<sup>ΔIE</sup>* ileal tissue, was not significantly different than *Ahr<sup>F/F</sup>* mice. Additionally, *Fxr* expression was unaltered between the two strains (Figure 4.9). These results demonstrate that enterohepatic signaling is altered in *Ahr<sup>ΔIE</sup>* mice.
Figure 4.9: *Fgf15* expression is decreased in the ileum of *Ahr*ΔIE mice.

Ileal tissue was collected from *Ahr*°F/F (n=10) and *Ahr*ΔIE (n=14) mice and relative gene expression levels for *Fgf15*, *Slc10a2*, and *Fxr* were determined using qRT-PCR and normalized to *Rpl13a* expression. “ns” denotes no significant difference.
4.5 DISCUSSION

The $Ahr^{ΔIE}$ mouse model described in this study was generated to determine if AHR expression in the intestinal epithelium contributes to the protective function of agonist-activated AHR which has recently been described in the primary literature. Many of these previous studies have focused on the role of AHR in gut immune cell subsets, particularly in the innate lymphoid cells. AHR agonists were repeatedly demonstrated to protect against the effects of chemically-induced colitis. The hypothesis driving this study is that AHR expression in the intestinal epithelium may contribute to its protective effects as observed in intestinal disease models, in addition to the contribution of immune cells.

The successful generation of the $Ahr^{ΔIE}$ mice was confirmed with qRT-PCR of intestinal scrape samples taken from the small and large intestines of the mice and compared with $Ahr^{F/F}$ mice. $Ahr$ expression levels were absent in these samples, confirming the successful knockout of AHR in the intestinal epithelium.

Due to the increased mortality of $Ahr^{-/-}$ mice exposed to DSS colitis [18] and the increased protection from colitis-related symptoms afforded by AHR agonist treatment, it was expected that $Ahr^{ΔIE}$ mice would suffer more detrimental health consequences when exposed to intestinal disease models, even in the absence of overt AHR ligand exposure. However, when the mice were exposed to the intestinal pathogen $C.\ rodentium$, increased mortality was not observed, and the $Ahr^{ΔIE}$ mice did not exhibit any significant differences in weight loss or bacterial shedding in the feces compared to $Ahr^{F/F}$ mice. Additionally, under basal conditions (i.e., no ligand treatment) the response of the $Ahr^{ΔIE}$ mice to DSS colitis did not significantly differ overall from $Ahr^{F/F}$ mice. While it is possible that alterations in disease model parameters could lead to different results (such
as changes in dose and/or duration of *C. rodentium* or DSS colitis exposure), these results suggest that under basal conditions (i.e., in the presence of endogenous ligands) AHR expression in the intestinal epithelium is not the primary mediator of the protective functions of AHR in the gut. One complication for determining the role of endogenous AHR function using the *Ahr*<sup>F/F</sup> mouse as a control is that the *Ahr*<sup>F/F</sup> mice express the low-affinity *Ah<sup>d</sup>* allele [19]. Therefore, effects of endogenous ligands may be obscured in these experiments. However, whether there is indeed differential activation of the *Ah<sup>b</sup>* and *Ah<sup>d</sup>* alleles by endogenous ligands has not been established.

The result of the DSS experiment contrasts with the conclusions of other labs using *Ahr<sup>ΔIE</sup>* mice, in which it was found that *Ahr<sup>ΔIE</sup>* mice were more susceptible to DSS-induced colitis compared to control mice, and this was associated with increased caspase-3 or TUNEL-positive cells, as well as the activation of JNK and p38 MAPK pathways in the colonic IECs [20]. In the current study, when the *Ahr<sup>ΔIE</sup>* mice were exposed to TCDD, their degree of weight loss was significantly improved on days 6 and 7 compared to *Ahr<sup>ΔIE</sup>* mice not exposed to TCDD. This result could indicate that the beneficial effect of AHR activation is indeed most likely due to cells of the immune compartment. However, this result contrasts with a study in which kynurenine is used to activate the AHR. This study determined that the disease-improving aspects of kynurenine exposure during DSS colitis were dependent on AHR expression in the IECs, as kynurenine did not improve colitis in *Ahr<sup>ΔIE</sup>* mice. In fact, kynurenine-treated *Ahr<sup>ΔIE</sup>* mice displayed more severe colitis progression than control mice. IL-10R1 expression was also not increased in isolated colon epithelial cells from *Ahr<sup>ΔIE</sup>* mice exposed to DSS, which is one possible mechanism by which kynurenine induces its beneficial effects in mice in which AHR is
expressed in the intestinal epithelium [21]. Differences in factors such as intestinal microflora and dietary components of the mice described in this study and the mice in previous studies could account for some of the differences observed. Further analysis of colonic samples collected during the study described here may help to clarify the results and determine if TCDD treatment could improve other disease markers in this model, such as the reduced expression of inflammatory mediators.

A study using Arnt\textsuperscript{ΔIE} mice demonstrated that loss of ARNT in the intestinal tract leads to changes in Cyp1a1 induction levels systemically [15]. Gene expression analysis of liver and ileal tissues of Ahr\textsuperscript{ΔIE} mice demonstrated that enterohepatic signaling may be altered due to lack of AHR signaling in the intestinal epithelium of the small intestine. The enhanced CYP7A1 gene and protein levels of Ahr\textsuperscript{ΔIE} mice could be due to the decreased levels of Fgf15 produced in the ileum of the mice. The biological consequences of these changes, and of the alterations of other factors mediating bile acid and lipid cycling in the liver and intestine, as well as other systemic effects, need further investigation before any conclusions may be drawn. Additionally, the time of day may influence experimental results in terms of the bile acid pathway [22].

Evidence from our studies indicates that lack of AHR in the intestinal epithelium does not lead to direct morbidity. Parameters measured indicate no significant loss of barrier function; however, this does not conclusively mean that no impairment of barrier function exists. Since AHR expression influences microbial populations in the intestine [23], determining the contribution of AHR expressed in the intestinal epithelium to microbial communities could help clarify its role in the gut. The subtle changes observed in this model, and emerging data indicating a role for AHR in the regulation of metabolic
function, points to a role for AHR in the intestinal epithelium that has yet to be elucidated.
4.6 BIBLIOGRAPHY


CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

The development and use of appropriate *in vitro* and *in vivo* models is essential for the understanding of the complex physiological role of the AHR under homeostatic and pathological conditions in barrier tissues. The use of *in vitro* models in the form of primary cell culture allows for the investigation of the consequences of AHR activation in specific cell types. The use of *in vivo* models can determine if the observations in cell culture occur in a whole organism. However, when animal models are utilized for research, it is important to take into account the genetic background of the mice being used, and in the case of AHR research, the allelic status of the strains, when interpreting experimental results. The use of *in vivo* mouse models is important for the study of AHR function in physiology, as cell culture experiments do not always replicate what occurs in a whole organism. However, both models are useful for the full understanding of the complexity of AHR biology.

5.1 FUTURE DIRECTIONS

Future directions and experiments are detailed for each chapter:

CHAPTER 2: EXPRESSION OF THE NEUTROPHIL CHEMOATTRACTANT GENE (C-X-C MOTIF) LIGAND 5 IS REGULATED BY THE ARYL HYDROCARBON RECEPTOR IN KERATINOCYTES

Though the experiments conducted thus far support a DRE-based mechanism for the regulation of *Cxcl5* by AHR, the use of chromatin immunoprecipitation (ChIP) would provide more evidence for direct binding of AHR to the DRE elements in the *Cxcl5* promoter within the mouse primary keratinocytes, because the EMSA analysis provides
evidence within the in vitro system but does not reflect endogenous conditions. Using primary keratinocytes for ChIP analysis would demonstrate that the DRE elements upstream of Cxcl5 are functional for AHR binding within those particular cells. Additionally, mutagenesis experiments can be used to determine how many and which DREs are functional in the mouse Cxcl5 promoter. The mechanisms by which AHR regulates Cxcl5 under pro-inflammatory conditions also need to be explored in further detail. In particular, it will need to be determined if regulation of Cxcl5 is governed in a similar manner to human IL6 [1].

Additional future experiments could include determining if the increase in Cxcl5 expression and protein content observed in the keratinocytes could potentially lead to increased infiltration of neutrophils. An in vitro chemotaxis assay using media collected from keratinocytes treated with AHR ligands and IL1B could be used with neutrophils to determine if migration is enhanced. To determine in vivo relevance, flow cytometry, histological analysis of skin sections, or a myeloperoxidase (MPO) assay could all be used to determine if neutrophil migration in the skin upon combination of UV exposure and AHR ligand treatment is enhanced. Different inflammatory models could also be used to determine in vivo relevance, including infection models in both skin and other barrier tissues. Additionally, the use of antagonists or SAhRMs in the UVB exposure model can determine if the in vitro results in primary keratinocytes are applicable to the skin in vivo. Since AHR is expressed in other barrier tissues, it would also be interesting to explore the possibility that AHR-mediated Cxcl5 regulation occurs in other tissues such as the intestine or lung.
Due to the possibility of "xenokine" expression influencing local infiltration of immune cell subsets, DNA microarray analysis and/or ChIP-sequencing should be performed on mouse primary keratinocytes treated with AHR agonists and IL1B to determine if other genes besides the previously characterized xenokine genes are regulated in this manner. Additionally, the mouse and/or human genome could be analyzed for similar sequences of sequential, overlapping DREs similar to the sequence present in the mouse Cxcl5 promoter.

CHAPTER 3: CHARACTERIZATION OF A MOUSE MODEL EXPRESSING THE HIGH AFFINITY ALLELE OF THE ARYL HYDROCARBON RECEPTOR ON A HAIRLESS SKH1 MOUSE BACKGROUND

SKH1-\textit{Ah}\textsuperscript{b-1} mice exhibited increased induction of inflammatory gene expression after UVB exposure, but the physiological consequences of this induction remain to be explored. Western blot or ELISA analysis for the elevated inflammatory mediators determined by qRT-PCR analysis should be used to verify whether or not protein levels are also elevated as a result of increased mRNA. Due to the enhanced levels of \textit{Ptgs2} expression in the skin of SKH1-\textit{Ah}\textsuperscript{b-1} mice, levels of PTGS2 protein and its enzymatic product, prostaglandin E2, could also be assessed in the skin of the mice. Additionally, flow cytometry can be used to determine whether the increase in gene levels of the inflammatory mediators leads to an increase in immune cell populations in the skin of SKH1-\textit{Ah}\textsuperscript{b-1} mice compared to SKH1-\textit{Ah}\textsuperscript{d} mice. Alternatively, histological analysis of skin sections can detect levels of markers such as myeloperoxidase (MPO), a component of neutrophils, and other specific immune cell markers. A DNA microarray could also be
conducted using skin samples from SKH1-Ah<sup>bl</sup> mice compared to SKH1-Ah<sup>d</sup> mice to determine the full extent of differential regulation of inflammatory mediators between the two mouse lines. A long term UV-induced carcinogenesis study would determine the physiological consequences of chronic UVB exposure and whether the increased inflammatory signaling in the skin of the SKH1-Ah<sup>bl</sup> mice could also lead to increased dermal tumorigenesis. The topical application of antagonists which are able to be absorbed by the skin could also determine if pharmacological intervention through the use of AHR inhibitors could lead to decreased inflammatory signaling after UVB. Due to their hairless phenotype, the SKH1-Ah<sup>bl</sup> and SKH1-Ah<sup>d</sup> mice would also be useful in other skin-related studies, such as studies determining the effects of AHR activation in psoriasis and wound healing models. The SKH1-Ah<sup>bl</sup> and SKH1-Ah<sup>d</sup> mouse models could also be used for comparative studies to determine binding affinities of endogenous (particularly skin-relevant) ligands between the Ah<sup>bl</sup> and Ah<sup>d</sup> alleles.

CHAPTER 4: THE DEVELOPMENT OF A MOUSE MODEL TO DETERMINE THE ROLE OF ARYL HYDROCARBON RECEPTOR EXPRESSION IN THE INTESTINAL EPITHELIUM

Gene and protein expression analysis provided evidence that the regulation of enterohepatic signaling in the livers of Ahr<sup>IE</sup> mice is disrupted. The hypothesis is that due to these gene expression and protein changes, levels of bile acids and/or lipids could be altered in the hepatic tissue of Ahr<sup>IE</sup> mice compared to Ahr<sup>FF</sup> mice. To determine if this leads to physiologic changes in the livers and ileal tissues of these mice, mass
spectrometry analysis of the livers should be conducted to investigate potential changes in bile acids and lipids.

Additionally, tissues samples, including colon and liver, will be analyzed from the DSS experiment using $Ahr^{F/F}$ and $Ahr^{ΔIE}$ mice, to determine if TCDD treatment improved experimental parameters, including decreased inflammatory gene and protein expression.
5.2 BIBLIOGRAPHY

CONTRIBUTIONS

I would like to recognize those who made significant experimental contributions to this work. For the experiments detailed in Chapter 2, Figure 2.1: Dr. Iain Murray conducted the mouse Cxcl5 promoter analysis (Figure 2.1a), Elif Muku conducted the Cxcl5 reporter assay (Figure 2.1b), and Jacob Boyer conducted the mouse Cxcl5 EMSA analysis (Figure 2.1c,d). In Chapter 3, Figure 3.3: Angela Alnemri and Shana Santarelli contributed to the Cyp1a1 expression analysis in tissues of SKH1-Ah<sup>b-l</sup> and SKH1-Ah<sup>d</sup> mice.
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