2018

Determining the role played by Aryl Hydrocarbon Receptor (AHR) in the colon carcinoma tumor model

Poonam Yakkundi

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DETERMINING THE ROLE PLAYED BY ARYL HYDROCARBON RECEPTOR (AHR) IN THE COLON CARCINOMA TUMOR MODEL

by

Poonam Yakkundi

A dissertation submitted to the

Graduate School

In partial fulfilment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Pharmaceutics and Medicinal Chemistry,
Thomas J Long School of Pharmacy and Health Sciences,

University of the Pacific,
Stockton, California

2018
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by

Poonam Yakkundi
ACKNOWLEDGEMENTS

I thank my advisor Dr. William Chan, Professor at the University of the Pacific, and my industry mentor Dr. Mark Selby, Senior Director at Bristol-Myers Squibb, for their consistent advice, scientific support, and contribution in helping me to develop as an independent scientist. I am indebted to my company, Bristol-Myers Squibb, for giving me the opportunity to pursue my Ph.D. degree. I am grateful to my manager Dr. Maria Galou-Lameyer, and senior leaders of my group Dr. Dan Rohrer and Dr. Keith Joho, for their immense support and encouragement. I also thank my committee members Dr. John Livesey and Dr. Miki Park from the University of the Pacific for consenting to be on my dissertation committee.

I offer gratitude to my ex-colleagues David Klitzing for teaching me the mouse tumor implant technique, Eleanor Gonsalves for husbandry of moAHR mice, and Stephanie Betparoo for helping me with the procurement process of C57BL/6 mice. I thank Miranda Broz, Scientist at Bristol-Myers Squibb, for helping me in assessing the myeloid population in tumors and providing me with the necessary FACS reagents and antibodies. I am grateful to my friends Emanuela Sega, Luhua Zhang, Ralston Barnes and Dong Han, all scientists at Bristol-Myers Squibb, for their constant support and encouragement. I extend my special thanks to my senior colleagues Dr. John Engelhardt, Dr. Francis Rena Bahjat, and Dr. Natalie Bezman for their scientific support.
I thank Kathy Kassab, Program Services Assistant at the University of the Pacific, for her help with documentation and university formalities throughout the course of my graduate studies. Lastly, I owe my deepest gratitude to my family for their never-ending love, support, faith, encouragement, and positive outlook toward life.
ABSTRACT

by Poonam Yakkundi

University of the Pacific
2018

Aryl hydrocarbon receptor (AHR), commonly known as an environmental sensor involved in the metabolism and elimination of xenobiotic substances, is also an important modulator in the development and functioning of the immune system. AHR expression is varied in the T cell subsets with the highest expression in T-helper 17 and T regulatory cells. Work from many researchers has suggested that AHR can act as a tumor promoter or a tumor suppressor depending on the tumor type. Our goal is to understand the role played by AHR in MC38 syngeneic colon carcinoma tumor model. In the absence of AHR, MC38 tumor progresses by an increase in tumor associated macrophages (TAMs), M2 macrophages and a decrease in CD8a positive cytotoxic lymphocytes. Analysis of the intratumoral cytokines reveals a pro-inflammatory phenotype. This has been assessed by pharmacologic blocking of the receptor using CH223191 and in AHR deficient (AHR−/−) mice. Therefore AHR acts as a tumor suppressor gene in colon carcinoma tumor model and silencing it may lead to colon cancer progression.
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<tr>
<td>ADAMs</td>
<td>A disintegrin and metallic proteinases</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AHRR</td>
<td>AHR repressor</td>
</tr>
<tr>
<td>AIP</td>
<td>ARNT interacting protein</td>
</tr>
<tr>
<td>Aldh3a1</td>
<td>Aldehyde dehydrogenase 3 family, member a1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ARNT</td>
<td>AHR nuclear translocator</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix loop helix</td>
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<tr>
<td>CCL5/RANTES</td>
<td>Chemokine (C-C motif) ligand 5</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CH223191</td>
<td>2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl) - amide</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>CXCL5</td>
<td>C-X-C motif chemokine 5</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>Cytochrome P450 1A1</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DIM</td>
<td>3, 3’- diindolylmethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimum eagle’s medium</td>
</tr>
<tr>
<td>DRE</td>
<td>Dioxin response element</td>
</tr>
<tr>
<td>dLN</td>
<td>draining lymph node</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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</table>
EMT  Epithelial mesenchymal transition
EDTA  Ethylene diamine tetraacetic acid
ELISPOT  Enzyme-linked immunospot assay
FACS  Fluorescence activated cell sorting
FBS  Fetal bovine serum
FICZ  6-Formylindolo [3, 2-b] carbazole
Foxp3  Forkhead box p3
GCN2  General control nonderepressible 2
GNF 351  N-(2-(3H-Indol-3-yl) ethyl)-9-isopropyl-2-(5-methyl-3-pyridyl)-7H-purin-6- amine; N-(2-(1H-Indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9H-purin-6-amine
GST  Glutathione S transferase
HAH  Halogenated aromatic hydrocarbons
HCC  Hepatocellular carcinoma
HSP90  Heat shock protein 90
HBSS  Hank’s balanced salt solution
HIF-1α  Hypoxia-inducible factor 1α
IAA  Indole acetic acid
I3C  Indole -3-carbinol
IC2  Indolo [3, 2-b] carbazole
IDO  Indole 2, 3-dioxygenase
IEL  Intraepithelial lymphocytes
IFN-γ  Interferon-gamma
ILC  Innate lymphoid cells
IL-1β  Interleukin-1 beta
IL-6  Interleukin-6
IL-1α  Interleukin-1 alpha
IL-2  Interleukin-2
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<td>Interleukin-17</td>
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<td>Induced Tregs</td>
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<td>ITE</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MNF</td>
<td>3’-methoxy-4’-nitroflavone</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metaloproteinases</td>
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<tr>
<td>MOG (35-55)</td>
<td>Myelin oligodendrocyte glycoprotein</td>
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<tr>
<td>MIG/CXCL9</td>
<td>Monokine induced by gamma interferon</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid derived suppressor cells</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export sequence</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
</tr>
<tr>
<td>Nqo1</td>
<td>NAD (P) H quinone oxidoreductase 1</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PAS</td>
<td>Per-ARNT-Sim</td>
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<tr>
<td>PCB</td>
<td>Polychlorinated bi-phenyls</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PDX</td>
<td>Patient derived xenograft</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>Rosewell Park memorial institute 1640</td>
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<td>SAR</td>
<td>Structure activity relationship</td>
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<td>TCDD</td>
<td>2, 3, 7, 8- Tetrachlorodibenzo-p-dioxin</td>
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<td>TDO</td>
<td>Trp 2, 3-dioxygenase</td>
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<td>T cell receptor</td>
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<td>Type 1 regulatory T cell</td>
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<td>T helper 1</td>
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<td>Tumor microenvironment</td>
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<td>Tumor associated macrophages</td>
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<td>TALEN</td>
<td>Transcription activator-like effector nucleases</td>
</tr>
<tr>
<td>Ugt1a6</td>
<td>UDP glucoronosyl transferase 1 family, polypeptide A6</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
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CHAPTER 1: INTRODUCTION

This dissertation looks into the effects of the aryl hydrocarbon receptor (AHR) on the infiltrating lymphocytes in the tumor environment using a MC38 (mouse colon carcinoma) tumor model. The introduction will provide:

1) An overview of the AHR biology, 2) the immune system, 3) AHR and immune modulation, 4) an in depth review of the T cell subsets activated by AHR and 5) the tumor microenvironment

1.1. AHR biology

Aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor which resides in the cytoplasm in its latent state. It was cloned in 1976 by Poland et al. [1]. It is a member of the basic-helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family of proteins. Toxicology and Pharmacology studies done on AHR revealed it to be an environmental sensor involved in the metabolism of xenobiotics like TCDD (2,3,7,8-Tetrachlorodibenzo-p-dioxin) and PAHs (Polycyclic Aromatic Hydrocarbons) by inducing CYPIA1, belonging to the cytochrome P450 family. As studies on AHR progressed, researchers became aware of the role it played in mediating/regulating the development and function of cells of the innate and adaptive immune system. The latter function of AHR was proposed to be mediated by its response to endogenous ligands generated by
diet, microbiota or physiological processes of the host cell. AHR is now being understood as an
important environmental sensor acting as a physiological mediator of immune cells (function) by providing cues from the outside.

1.2. AHR gene structure

The gene structure is composed of three functional regions –

1. A highly conserved bHLH domain in the N terminal. This has the NLS (nuclear localization sequence) and NES (nuclear export sequence) which mediate the nucleocytoplasmic shuttling of the receptor.

2. A degenerate Per-Arnt-Sim (PAS) domain (PAS A and PAS B). This is the dimer forming and ligand binding domain (amino acids 230 to 420). Structural activity relationship (SAR) analyses of AHR has revealed that the ligand binding pocket can accommodate many planar, hydrophobic compounds explaining its promiscuous nature [2]

3. A poorly conserved, glutamine rich transactivation domain in the C-terminal [2].

1.3 AHR ligands

1.3.1 Exogenous ligands

Halogenated hydrocarbons (HAHs) and polyaromatic hydrocarbons (PAHs) were the first groups of compounds to be identified as AHR agonists [3]. HAHs include – dibenzo-p-dioxins, -dibenzofurans, -axo (xy) benzenes and –napthalenes, are structurally related AHR agonists and enter the environment as pollutants either by an industrial accident or by-product of waste incineration. TCDD (dioxin), one of the most potent
agonists known belongs to this class of compounds. PAHs, another class of AHR agonist, consists of 4 or more benzene rings and are by-products of combustion process found in chimney soot, charbroiled foods and smoke exhaust [3]. Examples of PAHs are benzo[a]pyrene, benzantracene and 3-methylchloanthrene. The reactive intermediates of benzo[a]pyrene are known to covalently bind to macromolecules to form protein adducts and genotoxic DNA. The PAHs are 3 to 4 times less potent than TCDD in inducing AHR signaling [3]. Another class of AHR agonists are polychlorinated bi phenyls (PCBs). These are present in many consumables like insulators, flame retardants and adhesives. Due to their chemical stability and environmental pervasiveness they accumulate in the food chain and cause many health issues in humans and animals. These are about 100 times less potent than TCDD in inducing AHR signaling [3].

1.3.2. Endogenous AHR ligands

- Indigo, indirubin product of indigo plant traditionally used as a textile coloring dye; it has a 100 fold lower potency than TCDD [3].

- ITE 2-(1’4-indole-3’-carbonyl)-thiazole-4-carboxylic acid methyl ester, is isolated from porcine lung tissue and has approximately 100 fold lower potency than TCDD [3].

- Equilenin [3-hydroxy-1, 3, 5(10), 6, 8-estrapentaen-17-one] - This AHR agonist is equine estrogen. It is commonly prescribed by the name Premarin, a hormone replacement drug. This binds to murine AHR with approximately 1/30,000 the affinity of benzo[a]pyrene [3].
- **Arachidonic acid metabolites** – Examples are lipoxin4A, prostaglandins B₂, D₂, F₃α, G₂, H₁ and H₂.

- **Heme metabolites** – There are 3 heme metabolites that mediate CYP1A1 induction, bilirubin > biliverdin > hemin in the order of their potency for AHR activity.

- **Tryptophan metabolites** – metabolites of this amino acid like tryptamine, indole acetic acid (IAA), and kynurenine are endogenous AHR agonists. Photo oxidation of tryptophan metabolism by UV light leads to production of FICZ (6-formylindolo [3, 2-b] carbazole). Kynurenine is produced by the breakdown of tryptophan by enzymes IDO (Indole 2, 3-dioxygenase) / TDO (Trp 2, 3 – dioxygenase). Some of the metabolites of the kynurenine pathway of tryptophan degradation are – N-formylkynurenine, quinolinic acid, kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and NAD [4]. IDO is produced by some tumor cells and immune cells like DCs and macrophages. In the tumor milieu, increased tryptophan metabolites leads to increased AHR activation and increased Treg cell production [5]. Decreased tryptophan concentration leads to an increase in uncharged tRNA in surrounding T cells leading to the activation of GCN2 pathway (an amino acid sensitive stress kinase pathway). GCN2 signaling leads to cell cycle arrest and induces anergy in neighboring T cells [6]. Therefore, accumulation of kynurenine and other tryptophan metabolites leads to cell cycle arrest in effector T cells, creating an environment that promotes suppression and tolerance thereby making the tumors invisible to the immune system. Kynurenine
inhibited T effector cell proliferation and increased the apoptosis of T effectors that express increased levels of AHR [7].

1.3.3. Dietary compounds

- Metabolites from breakdown of cruciferous vegetables like broccoli, brussel sprouts are AHR agonists. Examples are indole-3-carbinol (I3C) and its derivatives, indolo [3, 2-b] carbazole (ICZ), 3-3’-diindolylmethane (LTr-1). Some plant flavonoids (plant polyphenols) like chrysin, galangin, baicalein etc., are also AHR agonists.

Not much is understood about how ligands of AHR activate it. Studies have shown that AHR has a very promiscuous ligand binding pocket because its ligands – be they natural (from diet), endogenous or, synthetic agonists, the prototypical HAH (dioxin) or PAHs have structures with physiochemical properties dramatically different from each other [8].

1.4. AHR signaling pathway

AHR is a member of the basic-helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) protein family. In the latent state it resides in the cytoplasm, is bound to actin filaments and forms an inactive complex with chaperone proteins p23 (PTGES3), HSP90 (heat shock protein, 90KDa) and AIP (AHR interacting protein) /XAP2 / ARA9 [1, 2]. AIP helps maintain steady state AHR levels in the cell and also prevents AHR from being degraded by ubiquitination [4]. AHR signaling could be either genomic or non-genomic.
When an exogenous or endogenous ligand binds to the receptor, there is a conformational change, which exposes its nuclear translocation signal and AHR translocates to the nucleus, forms protein-protein interactions with its partner ARNT (AHR nuclear translocator), in addition to co-activators and transcription factors. This complex regulates the transcription of specific genes [9]. AHR also acts as an E3 ubiquitin ligase that degrades proteins that interact with AHR such as estrogen receptor. An alternative, non-genomic pathway is utilized when unbound AHR helps release cSRC, which then phosphorylates cellular targets. The role for non-genomic AHR signaling on the immune response is unknown [9].

The AHR + ARNT complex binds to specific promoter regions (DRE-Dioxin response elements) with the sequence 5’-T/G/TCGTGA/CG/TA/T-3’ on genomic DNA and promotes the transcription of AHR target genes. These target genes are collectively called the “AHR gene battery” and include the following – *cyp1a1* (cytochrome P450 I family member a1), *cyp1a2, cyp1b1, nqo1* (NAD (P) H quinone oxidoreductase 1), *aldh3a1* (aldehyde dehydrogenase 3 family, member a1), *ugt1a6* (UDP glucoronosyl transferase 1 family, polypeptide A6) and GST-Y group (glutathione S transferase). These genes encode phase I and II metabolizing enzymes that are responsible for the detoxification of xenobiotics in the liver [10]. Bradfield et al. [11] have classified AHR mediated responses to be either adaptive or toxic.
1.4.1. Adaptive response pathway

This is based on the fact that the gene is transcriptionally activated when ligand bound AHR binds to the DRE-containing promoter leading to the expression of gene products that metabolize xenobiotics (containing polycyclic aromatic structures – PAHs). This detoxification mechanism sometimes becomes complicated due to complex nature of the polycyclic compounds rendering them as more harmful electrophiles.

1.4.2. Toxic response pathway

This is the toxic response resulting from receptor mediated xenobiotic metabolism (PAHs and HAHs). Many AHR agonists are common environmental pollutants, resulting from incomplete pyrolysis of carbon sources from diesel exhaust, cigarette smoke, charbroiled foods, etc. Both classes of AHR agonists – PAHs and HAHs are toxic, but the latter are more potent due to their long half-lives leading to prolonged environmental persistence.

1.5. AHR regulation

AHR signaling is regulated at different levels. 1) In the cytoplasm where it is stored in an inactive form bound by hsp90, AIP and p23. 2) AHR is transported back to the cytoplasm after it has initiated the transcription of genes like cyp1a1 via the nuclear export sequence (NES). Once in the cytoplasm AHR is degraded by proteasomes. 3) AHRR (AHR repressor), a member of the bHLH-PAS family, is present in the nucleus.
and as a result of AHR signaling migrates to the cytoplasm and negatively regulates AHR by competing with ARNT for heterodimer formation, which disrupts AHR-ARNT complex and hence AHR signaling [12].

1.6. AHR antagonists

Antagonist is defined as a substance or chemical that interferes with or blocks the physiological action of a receptor (like AHR). The antagonism can be competitive or non-competitive. AHR antagonists are used to reverse the conditions elicited by AHR agonists in research mainly to understand the role played by AHR in disease like cancer or auto immunity (in rheumatoid arthritis (RA), MS, Crohn’s disease etc). AHR antagonists like GNF351, resveratrol (3, 5, 49-trihydroxystilbene) and α-napthoflavone have been used in RA where they reverse AHR ligand mediated effects such as – inhibit TCDD mediated effects such as 1) promoting IL-1β and IL-6 secretion 2) Th17 cell generation and 3) cypla1 activation. GNF351 is a high affinity AHR antagonist binding to the ligand binding pocket of AHR and is known to block the binding of many exogenous and endogenous AHR ligands. Although it is more potent than resveratrol (3, 5, 4′-trihydroxystilbene) and α-napthoflavone [13], GNF351 shows low oral absorption and rapid metabolism in vivo [13]. 3′-methoxy-4-nitroflavone (MNF) and 6, 2’, 4’-trimethoxyflavone (TMF) are two of the most potent flavonoid antagonists. MNF exhibits antagonistic as well as partial agonistic properties and is rapidly metabolized. TMF can antagonize both HAH and non-HAH agonists [14]. Some of the antagonists like flavones and resveratrol have exhibited high affinity for estrogen receptor [15]. CH223191 (2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide) is a potent
and pure AHR antagonist (does not exhibit any agonist properties or affinity towards estrogen receptor), competitively binds to AHR and inhibits AHR-dependent transformation [8]. CH223191 preferentially inhibits ligand binding capacity that would have normally led to AHR activation and signal transduction of TCDD and related HAHs; but has no effect on other agonists like PAHs, flavonoids or indirubin [8]. CH223191 was identified by Kim et al [15], as a result of random screening of a chemical library purchased from Chembridge Corporation. They also showed that this compound successfully suppressed TCDD mediated effects (like cyp1a1 activation) both in vivo and in vitro.

1.7. The immune system

The immune system is the body’s defense system designed to protect the body from invading pathogens and at the same time having the ability to discriminate self from non self. The immune system is classified as innate immune system or cell mediated immunity and adaptive immune system or humoral immunity. The innate immunity is the body’s first line of defense and the adaptive one gives rise to prolonged support and memory a few days later upon B and T cell expansion.

1.7.1. Innate immune system

There are many chemical and biological barriers like tears, saliva, mucous, skin, etc. that protect organisms from pathogens. Innate immune system is triggered when these barriers are compromised by microbes and then recognized by pattern recognition
receptors (PAMPs). The responses of the innate immune system are not specific, i.e., exhibit generic response to all pathogens, the response is short lived and does not result in immune cell memory. When the physical and chemical barriers are compromised, the microbe/pathogen enters into the host tissue. Tissue resident phagocytes recognize the foreign body and immediately destroy it.

Innate immune cells (white blood cells or leukocytes) originate in the bone marrow, some even mature here and get into circulation (either lymphatic or blood) or reside in the tissues. The cells of the innate immune system are innate lymphoid cells, mast cells, natural killer (NK) cells, phagocytes (macrophages/monocytes, granulocytes and dendritic cells).

1.7.1.1. Granulocytes

Granulocytes, which circulate in the blood, are further divided into neutrophils, basophils, eosinophils (collectively termed as polymorphonuclear leukocytes or granulocytes, due to the shape of their nucleus and secretion of granules in the cytoplasm, respectively). Mast cells are tissue resident granulocytes, defend against pathogens, and are important in causing allergic reactions leading to inflammation and autoimmunity. Basophils help combat infections, are less than 2% of all leukocytes. When injured, these cells secrete histamine, resulting in inflammation that helps fight infections. Eosinophils defend against parasites (helminths in particular) by secreting cytotoxic granules. They are mildly phagocytic and make up around 3% of total leukocytes. Natural killer (NK) cells mediate their killing action through cytotoxic granule secretion. They help body’s
defense by rapidly killing (in roughly 3 days) virus infected cells and also tumor cells. They also have the unique ability to detect stressed cells that lack surface MHC I expression.

Neutrophils, the most important and numerous WBCs (comprise 60 to 65% of the total leukocytes) are the first WBCs to arrive at the site of infection or tissue damage. They have a long life – survive for 5 or more days in circulation and for weeks in tissues. They are involved in 1) phagocytosis (removal of dead and infected cells) 2) killing pathogens by releasing peptides and peptidases secreted by their cytoplasmic granules 3) wound healing by helping release cytokines and chemokines by innate and adaptive immune cells. The neutrophils are known to do a reverse migration into the bloodstream from the injury site and 4) act as antigen presenting cells (APCs) to the T cells.

1.7.1.2. Neutrophils in cancer

In the tumor microenvironment, tumor associated neutrophils (TANs) have a conflicting role; either promote tumor growth by invasion and angiogenesis (protumor N2 neutrophils) or inhibit tumor (antitumor N1 neutrophils) based on the environmental cues they receive [16]. TANs produce MMP9 that contributes towards angiogenesis [17]. In the tumor microenvironment (TME), 1) Increased levels of TANs in and around tumors results in poor patient prognosis (increased CXCL5 levels in human HCC patients) 2) neutrophils in tumors recruit many chemokines and cytokines as they would in acute wound responses. This could lead to increased inflammation and amount of chemokines (like CXCL5/CXCR2) involved in epithelial mesenchymal transition (EMT)
leading to tumor invasion 3) TANs in the TME are thought to act as APCs and present antigens to cytotoxic lymphocytes (CTLs) thereby increasing immune surveillance [16].

1.7.1.3. Dendritic cells (DCs)

These cells were discovered by Steinman and Cohn in 1973 and they termed them as dendritic cells because of their dendrite (of neurons) like protrusions. They originate in the bone marrow, come into circulation and then undergo maturation. DCs are characterized by 1) expressing high levels of MHC II and CD11c 2) migrate from non-lymphoid to lymphoid tissues in order to stimulate and prime T cells. Important subsets of DCs are 1) myeloid or conventional DCs (cDCs) with the expression marker CD11b. The cross-presenting DCs express CD103, CD8, CLEC9A, XCR1 and Langerin. The precursors of cDCs exit the bone marrow, migrate to secondary lymphoid and non-lymphoid organs via blood and differentiate to cDCs. 2) plasmacytoid DCs (pDCs) – terminally differentiate in the bone marrow, do not express MHC II and express B220, Siglec-1. 3) Another set of specialized and self-renewing population of DCs are Langerhans cells (of stratified squamous epithelium) and microglia (parenchyma of brain) [18]. Immature DCs express less cell surface MHC, chemokine receptors – CCR1, CCR2 and CCR6 and are highly endocytic in nature. The mature DCs express more MHC II, co-stimulatory markers, CCR7 chemokine receptor and are less endocytic. Endocytic activity is a process by which DCs get rid of debris and pathogens by phagocytosing them, a process specifically termed as ‘macropinocytosis’ and deliver them to MHC II vesicles for presentation to T cells [19]. The DCs are the best antigen presenters of the immune system and often denoted as professional antigen presenting...
cells (APCs). When they encounter an infection, they internalize the pathogen, break proteins into small peptides and present them through the major histocompatibility complex (MHC) molecule, the chemokine receptor expression changes, they migrate to lymphoid tissue rich in T cells and present the antigen and co-stimulatory molecules to stimulate the T cells.

1.7.1.4. Mononuclear phagocytic system

Monocytes, dendritic cells and macrophages comprise the mononuclear phagocyte system (MPS) [20]. Monocytes mature into macrophages, leave the circulation and reside in tissues. Macrophages, neutrophils and DCs have specialized receptors on their surface called pathogen recognition receptors (PRRs) that recognize PAMPs (pathogen associated molecular patterns), antigens from viruses and bacteria, mainly their DNA, RNA and cell membrane. An example of PRRs are toll like receptors (TLRs); there are 11 TLRs in humans (TLR1 through TLR 11). When the macrophages encounter an infection, the macrophages secrete many proteins called cytokines and chemokines. This initiates inflammation and leads to the recruitment of neutrophils, monocytes and antigen presenting cells. The latter leads to activation of adaptive immune system where the B and T cells get activated and recruited to the site of infection. Inflammation is an important response of the innate immune system to combat infection.

1.7.2. Adaptive immune system

This system is comprised of B and T lymphocytes. The B lymphocytes originate and mature in the bone marrow. The T lymphocytes originate in the bone marrow but
migrate to the thymus and mature there. These naïve lymphocytes migrate to the blood, circulate until they encounter an antigen to initiate maturation. When antigen binds to the B cells receptor (BCR), the B cells are activated, proliferate and then differentiate into a plasma cell. The plasma cells secrete antibodies (IgM, IgG, IgD, IgA and IgE). The antibody secreted by the plasma cell is specific to the antigen they have encountered. When the antigen specific antibodies attach to the antigen on the infected cell, the cell gets flagged to be destroyed via processes such as opsonization, neutralization, activation of complement and phagocytosis. When a dendritic cell presents an antigen to the T cell via its MHC molecule, the antigen binds to the T cell receptor (TCR); after receiving a few other activation signals, the T cell becomes activated, proliferates and differentiates into different T cell subsets.

Three types of signals that are provided by APCs are critical for T cells. 1) The first signal is the activation signal provided by the engagement of TCR by the MHC I/II molecule presenting a processed antigen. 2) The second one is the survival signal, co-stimulatory signal – binding of CD28 on the T cell to B7 on the same APC. This induces expression of IL-2 (T cell growth factor) and IL-2 receptor. 3) The final signal is one provided by cytokines secreted from APCs. This leads to the differentiation of the T cells, in particular helps the CD4 + T cells to differentiate into different T cell subsets – T effectors or T regulatory cells. Getting all three signals is very crucial for the survival and proliferation of the T cell, in its absence the T cell will go into a state of anergy. Depending on the cytokines they encounter, the newly activated T cell differentiates into cytotoxic T cell (CTLs), T helper cells or T regulatory cells. The B and T cells mount a
response after the innate immunity has begun to fade and this response lasts for a long time. Some of the T and B cells give rise to memory cells, which become activated when encountered with the same antigen. Apart being in circulation in the blood, the activated/mature B and T cells reside in lymphoid organs. There are two types of lymphoid organs – central or primary and peripheral or secondary lymphoid organs. The former consists of bone marrow and thymus; the latter of spleen, lymph node and mucosal tissue (of the gut, respiratory track etc.).

1.8. AHR mediated effects on immune cells

AHR has a physiological and developmental role in the growth of animals. It is also involved in the terminal differentiation of many immune cells. Its expression is seen in many tissues including liver, lungs, brain, skin, cells of the immune system and so on. In the cells of the innate and adaptive immune system, AHR expression is detected in DCs, macrophages, NK cells and intraepithelial lymphocytes (IELs). Among T cell subsets, the AHR gene is transcriptionally silent in naïve, Th1 and Th2 cells, but the expression is high in Th17, Tr1 and Treg cells. Quintana et al., have shown that AHR affects Treg and Th17 cell differentiation in a ligand-dependent manner -treating mice with TCDD led to increased Tregs population and ameliorated the autoimmune disease EAE. Treatment with FICZ led to increased Th17 cells and exacerbated EAE [21]. Treatment with ITE, an endogenous AHR ligand resulted in increased Foxp3+ Tregs, indirectly converted DCs to tolerogenic DCs, thereby reducing EAE symptoms [22].
1.8.1. Dendritic cells

AHR mediated activation of DCs rendered them tolerogenic. Mice that were treated with MOG 35-55 to induce EAE, then treated with ITE (endogenous AHR ligand) exhibited decreased CD86, MHC II and increased CD103 expression in splenic DCs. There was also low expression of pro-inflammatory cytokines like IL-6, IL1β, IL-2, IL-23 and increased expression of anti-inflammatory cytokines like IL-10 and TGF-β. EAE symptoms were also ameliorated [22]. TCDD mediated AHR activation of DCs altered DC activation, differentiation and immunoregulatory functions but not their antigen presenting capacity to T cells [23]. AHR activated by TCDD also increases DC maturation, induces increased production of IDO and increased accumulation of NF-κB subunit RelB which is essential for the development and function of DCs. Indeed, RelB null mice are deficient in myeloid DCs. Nuclear expression of RelB is one of the hallmarks of DC differentiation and AHR/RelB dimerization directly correlates to their degree of maturation and antigen presenting capacity [24].

1.8.2. Natural Killer (NK) cells

AHR is essential for the cytolytic activity of NK cells. For example, there is increased tumor burden in an MHC-1 deficient RMA-S mouse tumor model in AHR -/- mice compared to WT. AHR is expressed in NK cells from spleen upon stimulation with cytokines IL-2, IL-15 or IL-12. When RMA-S tumor bearing wild type mice were treated with FICZ (endogenous ligand, a tryptophan derivative) there was reduced tumor burden and that coincided with increased NK cell cytolytic activity and IFN-γ production [25].
1.8.3. T regulatory cells

T regulatory cells (Tregs) can be divided into natural Tregs that are Foxp3+ (Forkhead box p3) and induced Tregs (iTregs) that are either Foxp3+ or Foxp3-. Also known as suppressor cells, they are a subset of CD4+ T cells that keep the immune system in check and are responsible for 1) maintaining peripheral tolerance 2) preventing autoimmunity and 3) reducing chronic inflammation. In the process of suppressing the immune system and the effector T cell proliferation, Tregs inhibit 1) antitumor immunity and 2) sterilizing immunity [26]. Tregs express cell surface markers CD4 and CD25 and the intracellular marker Foxp3. In vitro, naïve CD4+ cells in the presence of cytokines TGF-β and anti-CD3 + anti-CD28 stimulation give rise to Treg populations [27]. Foxp3 is a transcription factor present in Tregs and is critical for their development, survival and function. Foxp3 -/- mice develop severe autoimmune-like lymphoproliferative disease, - a fatal autoimmune disease with hyper-responsive CD4 + T cells. These mice, termed scurfy mice, carry a spontaneous loss of function mutation. Humans with no functional Foxp3 develop an autoimmune disease termed IPEX (Immunodysfunction polyendocrinopathy and enteropathy, an x-linked syndrome) [26]. It has been suggested by Vignali et al, that Treg cells use the following mechanisms to bring about immune tolerance. 1) Inhibitory cytokines such as IL-35, IL-10 and TGF-β are secreted by Tregs and suppress T effectors either by preventing their infiltration or by reducing inflammation. 2) Tregs are thought to secrete Granzyme A/B to kill T effectors via caspases or perforin enzymes stored in the granules. 3) Metabolic suppression – whereby increased CD25 expression in Tregs (CD25\textsuperscript{high}) allows them to consume more IL-2, thereby reducing IL-2 needed for the survival of T effectors.
4) By targeting DCs, Tregs inhibit the maturation and function of DCs via binding of CD80/CD86 to CTLA4 molecules which deprives effector T cells of CD28 positive co-stimulation. Tregs induce DCs to secrete IDO which also leads to the suppression of T effectors.

1.8.4. Tr1 cells

These cells mature in the peripheral lymphoid organs and are very important in maintaining immune tolerance by keeping the immune system in check. Tr1 cells secrete IL-10, an anti-inflammatory cytokine that dampens the antigen presenting capacity of DCs and antigen-specificity of T effectors and are thus involved in suppressing tissue inflammation and autoimmunity. In vitro, naïve CD4+ cells can be cultured in the presence of cytokines TGF-β + IL-27 and anti-CD3 + anti-CD28 antibodies to give rise to immune suppressive IL-10 secreting Tr1 cells [27]. IL-27 is thought to induce AHR, upon which AHR combines with c-Maf, and the AHR + c-Maf complex transactivates Il-10 and Il-21 promoters in Tr1 cells inducing the later to secrete IL-10 cytokine [28].

1.8.5. Cytotoxic lymphocytes (CTLs or CD8a positive cells)

There are two types of T cell co-receptors that T cells express – CD8 positive and CD4 positive T cells. The CD8 + cells express the cell surface marker CD8 which is a 2 chain glycoprotein. It exists as a heterodimer (CD8αβ) and a homodimer (CD8αα), of which the former is present on most T cells. MHC I binds with greater affinity to CD8 αβ than CD8αα. These cells are important in modulating T cell responses during cancer, auto-immune reactions and transplant. CD8 positive T cells constitute what are called
cytotoxic T lymphocytes (or CTLs). They act by releasing cytotoxins – perforin, granzymes and granulysin present in the granules of their cytoplasm. These enzymes trigger the caspase cascade, a series of cysteine proteases that induce apoptosis in the infected cell. Paige et al have demonstrated that AHR activation by pollutants early in life leads to reduced CD8 + T cell responses to a viral infection (influenza A virus) later in life. This was suggested to be due to DNA methylation resulting from AHR activation; increased methylation in the DNA encoding the CD8 gene reduced its gene expression following an infection [29].

1.8.6. T helper 17 (Th17) cells

These cells express the T cell co-receptor CD4 on their cells surface (CD4 + T cells). These CD4 + T helper cells are pro-inflammatory in nature, they defend the host against extracellular pathogens by recruiting neutrophils and macrophages to the affected area. They are controlled by the transcription factor ROR-\(\gamma\)T. They secrete cytokines – IL-17A and IL17 F. They play an important role in exacerbating autoimmune responses and worsen autoimmune diseases like EAE, MS, Crohn’s disease. In vitro, naïve CD4+ cells in the presence of cytokines (TGF-\(\beta\) + IL-6) and anti-CD3 + anti-CD28 stimulation give rise to the Th17 population [27]. In cancer they play a conflicting role – they adopt a pro or anti-tumorigenic role based on the stimulation (cytokines, co stimulatory molecules and cell-cell interactions) they encounter. When a naïve T cell is stimulated with TGF-\(\beta\) + IL-6, it differentiates into a Th17 cell (regulatory Th17 cell) with surface expression of CD39 and CD73 (convert ATP to immunosuppressive adenosine) and secrete IL17 and IL-10. This results in uncontrolled tumor growth. When a naïve T cell is stimulated with
IL-1β, IL-6 and IL-23, results in an effector Th17 cell that secretes IL-17 and IFN-γ and less of IL-10. Such a Th17 cell promotes tumor regression [30].

1.9. The Tumor Microenvironment (TME)

The tumor environment is more than just an accumulation of cancer cells – it is very complex and can be broadly classified into cancerous or transformed cells and stroma. Stroma provides supportive framework to the tumor and consists of 1) extracellular matrix (ECM) – made up of fibrous proteins, hyaluronic acid and proteoglycans. It also consists of cancerous cells, proteolytic enzymes like matrix metalloproteinases (MMPs), a disintegrin and metallic proteinases (ADAMS). These proteolytic enzymes are involved in ECM remodeling, influence their growth, metastatic potential, differentiation as well as immune cell infiltration. 2) Fibroblasts, endothelial cells, pericytes, mesenchymal and immune cells 3) peptides like chemokines, cytokines, and enzymes. Enzymes such as IDO/TDO are secreted normally by DCs and promote the breakdown of the amino acid tryptophan. The metabolites of tryptophan, including kynurenine recruit Tregs into the tumor environment and have deleterious effects on Th1 and CD8+ T cells and 4) metabolites secreted by cancerous cells, stromal cells. Fibroblasts form majority of the stromal cells and secrete chemokines and cytokines [31].

Robert Schreiber proposed “The three E’s of cancer immunoediting” where in tumors undergo 1) immune elimination by cancer cells 2) immune equilibrium between cancer and immune cells and 3) immune escape by cancer cells [32].
Inflammation plays a major role in tumor progression, affecting tumor infiltration, progression all the way to metastasis. In the tumor environment, both pro-tumor and anti-tumor signals exist. If the pro-tumor signals supersede the latter, tumor growth progresses. Increased expression of oncogenes like \textit{ras} and \textit{myc} family of genes contribute to TME remodeling leading to the recruitment of leucocytes, or secretion of chemokines and cytokines that are protumorigenic. Mutations (single mutation is insufficient; at least 4 are required for tumor initiation) and genomic instability also aid in tumor progression.

The tumor environment also constitutes immune cells. In mouse tumor models, some tumors (MC38, mouse colon carcinoma) are heavily infiltrated by immune cells while others (B16F10, mouse melanoma) are not. The immune infiltrates comprise of 1) innate immune cells - cells of mononuclear phagocytic lineage (MPS) – TAMs (tumor associated macrophages), tumor DCs, monocytes - these do not present antigens, but are thought to be precursors to TAMs and DCs, Neutrophils, mast cells, NK cells 2) adaptive immune system - B and T lymphocytes. All the cells in the TME communicate with each other in an autocrine and or paracrine fashion and modulate tumor growth. Tumor progression or regression is definitely modulated and directed by the abundance and activation status of these various immune cells in the TME [34].
Tumor associated macrophages (TAMs) are most frequently found in the TME. These are differentiated myeloid populations with immune suppressive properties. TAMs block anti-tumor immunity, promote angiogenesis, reactivate EMT and aid the secretion of immunosuppressive cytokines IL-10 and TGF-b. They can be identified by the cell surface markers CD11b and F4/80 [33]. Other cells of the innate immune system present in the TME are neutrophils, myeloid derived suppressor cells (MDSCs), DCs and NK cells. All of them except NK cells have shown to be pro-tumor or anti-tumor. NK cells have always proved to be anti-tumor, killing tumor cells by their cytolytic activity. The macrophages can be of two phenotypes M1 and M2. The former is an immune promoter, with an anti-tumor phenotype and the latter is an immune suppressor with pro-tumor phenotype. The cells of the adaptive immune system constitute the CD4+ positive and CD8+ T cells. CD4+Foxp3+Tregs are pro-tumor and suppress anti-tumor immune responses. Other CD4+ T cells like Th17, Th1, Th2, and CD8+ T cells, which are traditionally promoters of immunity and protect the host in case of viral or pathogen invasion, can switch roles and become either tumor promoters or suppressors [34].

Immune and stromal cells, like fibroblasts and endothelial cells of the TME secrete many cytokines and chemokines that either promote or regress the tumor progression. These proteins help regulate cell activation, differentiation, survival and trafficking of immune cells into and out of the tumor. Depending on the cytokines secreted by the tumor, could lead to either anti-tumor or pro-tumor environment in the
There is enough evidence to support the fact that excessive amounts of anti-inflammatory cytokines in the TME leads to increased malignancy. This is dependent on their receptor expression, relative concentrations and the activation state of the cells. [35]. Macrophages secrete high levels of cytokines and chemokines in the TME. M1 macrophages produce IFN-γ and pro-inflammatory cytokines such as IL-6, IL-12, IL-23 and TNF-α while M2 macrophages secrete inhibitory IL-10 and TGF-β. Oncoproteins like Ras, Myc etc., when upregulated, aid the stimulation of pro-inflammatory cytokines and chemokine pathways leading to increased secretion of IL-6, IL-8, IL-1β, CCL2 and CCL20. A chronic pro-inflammatory environment can result in the production of free radicals that could potentially lead to DNA damage and eventually mutations leading to tumor initiation/progression. IL-12 is a cytokine that is involved in the differentiation of Th1 cells. The p40 subunit of IL-12 is involved in the IFN-γ production and activation of anti-tumor immunity. MDSCs produce arginase 1 and IDO which suppress anti-tumor immunity by indirectly interfering with T cell activation. Various pro-angiogenic genes get activated, secrete VEGF, IL-8, HIF-1α, which promote angiogenesis that could lead to tumor progression [34].

AHR acts as either a tumor promoter or suppressor depending on the tumor type. Its role in epithelial-mesenchymal transition and progression is also controversial. When looked into tumor vs. normal tissues for multiple tumors, differential AHR expression was seen; it can be considered either a negative or positive prognostic factor.
The reason for the dual role of AHR in cancer is not known. Some of AHR agonists like TCDD are known carcinogens. Kynurenine, an endogenous ligand, produced from IDO-mediated tryptophan metabolism, indirectly leads to cancer progression by increasing Tregs and suppressing T effectors. Ligands like β-napthoflavone, I3C (from cruciferous vegetables) and its dimer DIM exhibit promising anti-tumor activities. The role of others like FICZ are not known. There is evidence for unknown endogenous ligands that prevent cancer. For example, AHR prevents liver carcinogenesis [37], colon cancer [38] and ovarian cancer [39]. In lung cancer, increased AHR expression decreased autophagy and inhibited migration of lung cancer cell lines [40]. In certain cancers AHR signaling promotes them, as seen in the case of gliomas [41] and breast cancer [42].

Mice models are used in research as there is roughly 99% similarity with humans in terms of pathology and physiology; there is homology in the nervous, skeletal, cardiovascular, immune systems etc. Although mouse models do not fully recapitulate the characteristics of human cancers in terms of interactions within the tumor microenvironment, between tumor-host and responses towards drugs and drug resistance, they are still a very good and affordable tool to study mechanistic pathways concerning basic research and drug development [50].

Many mouse models are used in research and they come with their own sets of benefits and limitations [50].
1) **GEMM** (genetically engineered mouse model): These are derived by inducing genetic mutations through CRISPR/Cas or TALENs approaches. They are expensive, time consuming, have off target effects and could harbor lethal mutations.

2) **PDX/CDX** (patient derived xenografts or cell derived xenografts): These are derived by implantation of human cells/tissues from either patients or cell lines into immunocompromised mice. These models are quick and easy to generate. Although, some of the drawbacks are the lack of a durable graft immune system and graft-mediated effects on the host.

3) **Humanized mouse models**: These mice are manipulated such that mouse genes are knocked out and replaced by human genes so as to make human proteins. For example, replacement of the mouse immune system genes by human genes. These models are very complex, expensive and time consuming to generate.

4) **Syngeneic mouse models** - These models are mostly used in cancer research; the transplant is histocompatible with the host and injected in immunocompetent mice, with an intact immune system. The transplants are often cell lines that were generated from mice with tumors induced by cancer promoting chemicals. These are either subcutaneously implanted on the flank to obtain a palpable and visible tumor or injected systemically (intravenously) to study metastases. These models are very useful in understanding the role of immune system in cancer modulation and immune-based therapies [51]. Some of the commonly used syngeneic tumor models in cancer research are CT26 (colon carcinoma; BALB/c mice), MC38 (colon carcinoma; C57BL/6 mice),
B16F10 (mouse melanoma; C57BL/6 mice), 4T1 (breast cancer model using BALB/c).

Immunotherapy based on blocking immune checkpoint inhibitors like CTLA4 (cytotoxic T lymphocyte associated protein 4) and PD-1 (programed death 1) for the treatment of cancers like metastatic melanoma, renal cell carcinoma etc. (by Bristol-Myers Squibb/ Medarex), were supported from data from mouse tumor models, namely – MC38, B16F10 and CT26. Immunotherapy utilizes the potential of the immune system to recognize and abrogate tumors by limiting inhibitory interactions that reduce antitumor responses.

Based on the dual role of AHR in cancer, I sought to understand the role it plays in a MC38 mouse colon carcinoma model and B16F10 (mouse melanoma model). Changes in tumor burden after pharmacologically activating the receptor using TCDD, pharmacologically blocking the receptor using antagonist CH223191 and in AHR – deficient (AHR-/-) mice were determined. The tumor microenvironment was investigated in TILs along with cytokine and chemokine profiling to determine if they contribute to changes in tumor burden.

I propose that, AHR activation by TCDD, results in increased Tregs and decreased CD8+ T effector cells which may promote tumor progression.
Hypothesis 1

T regulatory cells suppress anti-tumor immunity by inhibiting cytotoxic T lymphocytes (CD8+ T cells). Activation of the AHR by agonists increases the differentiation of naïve T cells into T regulatory cells. Therefore, activation of AHR in tumor bearing mice should increase the tumor burden.

Hypothesis 2

In the absence of the receptor (AHR-/-) mice, the tumor burden should be less in AHR+/+ counterparts.
CHAPTER 2: MATERIALS AND METHODS

Tumor study design

2.1. MC38 tumor study

The MC38 cells that were ready to be implanted were kept on ice until the implant was completed. The cells were implanted within 10 minutes of their harvest. On day 0, 25 gauge sterile needle (from Becton Dickenson) was fitted to a sterile 1ml syringe (BD Luer-Lok, from Becton Dickenson), then filled with $2 \times 10^6$ cells in 200 µl volume of 1X PBS and implanted subcutaneously on the right flank of the mouse. This procedure was done in a bio-safety cabinet. While implanting the cells subcutaneously, a clearly visible bleb should be seen, if not the tumor growth may not be visible, may result in a delayed growth or no growth at all. A palpable tumor was seen on day 7 after implant. On day 7 after the implants, the weight of the mouse and tumor burden were recorded using a database called Studylog (from Studylog Systems Inc.). The height, width and length of the tumors were measured using a caliper and the tumor burden was calculated using the formula $L \times W \times H / 2$. The mice were randomized based on the tumor burden and divided into the control (vehicle/DMSO in corn oil) and the treatment group (agonist – TCDD or antagonist – CH223191) such that both groups had the same average tumor burden. TCDD was mixed with corn oil and administered in mice at a concentration of 1µg/mouse and depending on the experiment, was administered either once or three times per study. AHR antagonist CH223191 was dissolved in DMSO and administered every
day at 10mg/kg body weight (200 µL volume) per mouse. The drug was administered orally starting on day 7 till the end of the study. The tumor study involving AHR WT and AHR KO mice did not receive any treatment.

Body weight and tumor burden were monitored on days 7, 10, 14, 17, 20 and 23. The study ended on day 26 or sooner if the study endpoint was reached. Mice were sacrificed if they exhibited significant weight loss (>20%), have a tumor volume which is at or near the maximum allowed (2000mm$^3$ or 10% of the body weight), the tumors were ulcerated or the mice are in poor health (lethargic, moribund, failure to thrive, inability to drink or access food, posture, vocalization and tented skin). The health of the mice and husbandry was monitored on a daily basis as per the guidelines of Bristol-Myers Squibb animal facility. After the tumor study was completed, the mice were euthanized using CO$_2$.

For the immuno-phenotyping experiment, on day 17 after tumor implants, the tumors were excised from the mouse and stored in 50 mL conical tubes containing 5 mL of MC38 cell culture media. The tumor draining lymph nodes comprising of the axillary, brachial and inguinal lymph nodes on the tumor bearing side were harvested in a 24 well cell culture plate containing 1.5 mL of MC38 cell culture media. These procedures were performed in a bio-safety cabinet and the harvested tissues were kept on ice.

### 2.2. B16F10 tumor study

The B16F10 cells that were ready to be implanted were kept on ice until the implant was completed. The cells were implanted within 10 minutes of their harvest. On day 0, 25 gauge sterile needle (from Becton Dickenson) was fitted to a sterile 1ml syringe
(BD Luer-Lok, from Becton Dickenson), then filled with 1X10^6 cells in 200ul volume and implanted subcutaneously on the right flank of the mouse. This procedure was done in a bio-safety cabinet. On day 7 after the implants, the weight of the mouse and tumor burden were recorded using a database called Studylog (from Studylog Systems Inc.). The height, width and length of the tumors were measured using a caliper and the tumor burden was calculated using the formula L x W x H /2. The mice were randomized based on the tumor burden and divided into the control (vehicle/DMSO) and the treatment group such that both groups had the same average tumor burden. AHR antagonist CH223191 was dissolved in DMSO and administered every day at 10mg/kg body weight per mouse. The drug was administered orally starting on day 7 till the end of the study.

The tumor study involving AHR WT and AHR KO mice did not receive any treatment. Body weight and tumor burden were monitored on days 7, 10, 14, 17, 20 and 23. The study ended on day 26 or sooner if the study endpoint was reached. Mice were sacrificed if they exhibited significant weight loss (>20%), have a tumor volume which is at or near the maximum allowed (2000mm3 or 10% of the body weight), the tumors were ulcerated or the mice are in poor health (lethargic, moribund, failure to thrive, inability to drink or access food, posture, vocalization and tented skin). The health of the mice and husbandry was monitored on a daily basis as per the guidelines of Bristol-Myers Squibb Animal facility. After the tumor study was completed, the mice were euthanized using CO2.
2.3. Animals

For all tumor burden studies, the C57BL/6 mice, with wild type AHR gene, were purchased from The Jackson Laboratories. These mice underwent a 3 week quarantine and acclimatization period in the Bristol-Myers Squibb Company Animal facility after which they were transferred to the protocol room. The mice used on the study were female and 8 weeks of age at the time of tumor implants.

For the studies involving AHR WT and AHR KO (−/−) mice, the AHR WT and KO were obtained from matings of male and female AHR heterozygous (+/−) mice (were littermates) that were purchased from The Jackson Laboratories. Some of WT and KO mice were also obtained from AHR (−/−) × AHR (−/−) and AHR (+/+ × AHR (+/+ matings respectively. The KO mice from both sets of mating did not exhibit any differences in their tumor burden. These mice were housed and bred at the Bristol-Myers Squibb Animal facility. Female mice between 8 to 12 weeks of age at the time of tumor implants were used in the study. All mice WT and KO that were put on a tumor study had an acclimatization period of 5 days in the protocol room.

2.4. MC38 cell culture (for tumor study)

The cells are grown in Corning cellgro RPMI 1640 medium, 1X with L-glutamine (Thermo Fisher) supplemented with heat inactivated 10% fetal bovine serum – FBS (from Gibco, Thermo Fisher) in a 5% CO₂ incubator at 37°C. The cells were passaged three times a week. Heat inactivated FBS was obtained by heating for 30 minutes at 56°C. The FBS consists of a lot of nutrients including complement. Heat inactivation denatures
the complement proteins as these proteins could affect immunological studies by interfering with the host’s immune response. For tumor implants the cells were split into half one day before. The cells were counted using Vi-CELL XR (from Beckman Coulter), washed once with 1X PBS (from Corning cellgro cell culture Phosphate Buffered Saline, 1X, without calcium and magnesium) and re-suspended in 1X PBS at a concentration of 2X10^6 cells in 200ul of 1X PBS. The cells were in culture for not more than a month after which a new thaw of cells were used.

2.5. B16F10 cell culture (for tumor study)

The cells were obtained from a frozen cell bank located at Bristol Myers Squibb in Redwood City. They were grown in Corning 1X DMEM with L-glutamine, 4.5g/L glucose and sodium pyruvate (Fisher Scientific) with 10% heat inactivated FBS (from Gibco, Thermo Fisher) in a 5% CO₂ incubator at 37°C. The cells were passaged three times a week. For tumor implants the cells were split into half one day before. The cells were counted using Vi-CELL XR (from Beckman Coulter), washed once with 1X PBS (from Corning cellgro cell culture Phosphate Buffered Saline, 1X, without calcium and magnesium) and re-suspended in 1X PBS at a concentration of 1X10^6 cells in 200ul of 1X PBS. The cells were in culture for not more than a month after which a new thaw of cells were cultured.
2.6. Preparing TCDD for intra-peritoneal administration in mice

TCDD dissolved in DMSO (at 50µg/mL concentration) was purchased from Cambridge Isotope Laboratories. This was mixed with corn oil - NF grade (CO136-25ML), purchased from Spectrum Chemicals. The mixture was loaded into sterile 1mL syringes (BD Luer-Lok, from Becton Dickenson) and 1µg/mouse was injected intra-peritoneally in mice using a 20 gauge sterile needle (from Becton Dickenson). The administration was done on day -1, a day prior to MC38 tumor implant.

2.7. Preparing AHR antagonist to be administered orally in mice

CH223191 (C8124-5MG) and DMSO (Dimethyl Sulfoxide-HybriMax, sterile filtered, D2650) were both purchased from Sigma-Aldrich. Corn oil, NF grade (CO136-25ML) was purchased from Spectrum Chemicals. CH223191 was stored in 4°C as per manufacturer’s instructions. The oil was stored at room temperature, but once open was stored at 4°C. The preparation that was administered to the mice was prepared in a Bio-Safety cabinet and kept sterile at all times. The mixture was fed to the mice using sterile feeding needles – disposable AFN 20G plastic animal feeding needles (from Cadence Science). CH223191 was dissolved in DMSO, under sterile conditions to obtain a concentration of 25 mg/mL. This working stock was dissolved in corn oil to obtain a mixture of 10 mg/mL of CH223191 in corn oil.
2.8. AHR KO mice breeding

The mice were bred at Bristol-Myers Squibb Company Animal facility in Redwood City, CA. The tails (2 mm in size) were clipped at the time of weaning on day 21 after birth for genotyping.

2.9. Genotyping protocol to distinguish between AHR WT and KO mice

The genotyping protocol was obtained from The Jackson Laboratories. The DNA was extracted from mouse tail snips using Extract N Amp tissue DNA extraction kit from Sigma.

2.9.1. DNA extraction protocol

4 parts of extraction buffer was mixed with 1 part of tissue extraction solution. 100ul of this solution was added to the tails, so that the tail clips were immersed in the buffer. The tails were incubated at room temperature for 10 minutes. Then incubated at 95°C heat block for 10 minutes. 100ul of neutralization buffer was added and the crude DNA was transferred to a fresh box – DNA box. The DNA was then stored at 4°C until used for PCR set up.

2.9.2. PCR set up

The PCR was set up using PCR buffer from Sigma. 1X concentration of this buffer was mixed with forward and reverse PCR primers (to obtain a final concentration of 10uM). The PCR primers were purchased from Life Technologies (Thermo Fisher). Below are the PCR primers and PCR cycling conditions.
Table 1: PCR set up protocol using PCR buffer from Sigma

<table>
<thead>
<tr>
<th>Composition</th>
<th>Volume (μl)</th>
<th>Starting concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer (1X)</td>
<td>10</td>
<td>2X</td>
</tr>
<tr>
<td>Forward primer (10μM)</td>
<td>0.2</td>
<td>100μM</td>
</tr>
<tr>
<td>Reverse primer (10μM)</td>
<td>0.2</td>
<td>100μM</td>
</tr>
<tr>
<td>Water</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Genotyping primer sequences for identifying homozygous AHR KO (-/-), AHR WT (+/+) and heterozygous AHR mice (+/-).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' --&gt; 3'</th>
<th>Primer Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>oIMR0443</td>
<td>GGATTGACTTAATCCTTCAGCGG</td>
<td>Wild type reverse</td>
</tr>
<tr>
<td>oIMR0444</td>
<td>TCTTGGGCTCGATCTTGTGTCAGGAACAGG</td>
<td>Common</td>
</tr>
<tr>
<td>oIMR08162</td>
<td>TGGATGTTGAATGTTGTCGAGG</td>
<td>Mutant Reverse</td>
</tr>
</tbody>
</table>

Table 3: PCR cycling conditions for genotyping AHR WT and KO mice

<table>
<thead>
<tr>
<th>Cycling Step #</th>
<th>Temp °C</th>
<th>Time</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>3 min</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>30 sec</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>30 sec</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1 min</td>
<td>repeat steps 2-4 for 35 cycles</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>2 min</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>-</td>
<td>hold</td>
</tr>
</tbody>
</table>
Below is a picture of the PCR amplicons run on a 3% agarose gel (with ethidium bromide from Bio-Rad) for 14 minutes at 165 Volts. The gel was imaged using AlphalImager (from Protein Simple). The mutant band size is 450bp and the wild type band size is 669bp. Samples that showed one mutant band of 450bp were identified as homozygous KO, one wild type band of 669bp as homozygous for wild type and two bands – mutant (450bp) and WT (669 bp) were identified as heterozygous mice. Sample that was previously tested as positive and negative were used as positive and negative controls, respectively.

Figure 1: 3% agarose gel depicting amplicons after PCR run to identify homozygous AHR KO (−/−), AHR WT (+/+) and heterozygous AHR mice (+/−).
2.10. RT-PCR

2.10.1. MC38 cell culture

The cells were obtained from a frozen cell bank located at Bristol Myers Squibb in Redwood City. The cells were grown in Corning cellgro RPMI 1640 medium, 1X with L-glutamine (Thermo Fisher) with 10% fetal bovine serum (FBS) (from Gibco, Thermo Fisher) in a 5% CO₂ incubator at 37°C. FBS was heat inactivated by heating for 30 minutes at 56°C. The FBS consists of a lot of nutrients including complement. Heat inactivation kills the complement proteins as these proteins could affect immunological studies by interfering with the host’s immune response. The cells were grown to reach 90% confluence and were split three times a week. The cells were in culture for not more than a month after which a new thaw of cells were cultured.

2.10.2. B16F10 cell culture

The cells were obtained from a frozen cell bank located at Bristol Myers Squibb in Redwood City. They were grown in Corning 1X DMEM with L-glutamine, 4.5g/L glucose and sodium pyruvate (Fisher Scientific) with 10% heat inactivated FBS in a 5% CO₂ incubator at 37°C and split three times a week. The cells were in culture for not more than a month after which a new thaw of cells were cultured.

2.10.3. Hepa1c1c7 cell culture

A frozen aliquot of these cells were obtained from Dr. William Chan’s lab (University of the Pacific). They were grown in alpha minimum essential medium (MEM) with 10% FBS in a 5% CO₂ incubator at 37°C and split three times a week.
2.10.4. RT-PCR

For the gene expression experiment, the cells were counted using Vi-CELL XR (Beckman Coulter) and plated in a 6 well plate at 1X10⁶ cells and incubated overnight in a 5% CO₂ incubator at 37°C. TCDD dissolved in DMSO at a concentration of 1nM per well or equivalent volume of DMSO was added per well, with a n=3 per condition. The cells were incubated in a 5% CO₂ incubator at 37°C. After 3, 6, 18 and 24 hours of incubation they were washed with 1XPBS, harvested in Trizol (Zymo Research), were flash frozen on dry ice and stored in -80 deg C freezer until RNA extraction.

RNA was extracted using Direct-zol Total RNA extraction kit (Zymo Research) by following the instructions provided by the manufacturer. RNA quantitation was done using a QIAxpert (Qiagen). 1µg of RNA per reaction was used to set up the cDNA reaction. cDNA was prepared immediately from RNA using the SuperScript III first strand synthesis super mix for qRT-PCR (Thermo Fisher Scientific) following manufacturer’s instructions. The prepared cDNA was immediately used to set up the RT-PCR reaction. 2 µL of cDNA was used per sample to set the RT-PCR reaction with three technical replicates. Real time PCR was done using Sso advanced SYBR green master mix from Bio-Rad. The samples were run on the LC480 cycler from Roche. GAPDH was used as the reference gene. All the primers for the assay were ordered from IDT (Integrated DNA Technologies). The assay was set up in a 384 well plate. Below is an example of the plate layout, PCR assay set up and PCR cycling conditions.
### Table 4: RT-PCR assay – 384 well plate set up, PCR set up protocol and PCR cycling Conditions

#### 2.10.5. PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahr</td>
<td>GCCCTTCCCCGCAAGATGTTAT</td>
<td>CAGGGGTGGACTTTAATGCAA</td>
</tr>
<tr>
<td>cyp1al</td>
<td>CAATGAGTTTTGGGAGGTTACTG</td>
<td>CCCCTCTCAAATGTCTCTGTAGTG</td>
</tr>
<tr>
<td>ugt1a</td>
<td>GCTTCCTCGTACCTCTGTGTG</td>
<td>GCTGCTGAATAACTCCAAGCAT</td>
</tr>
<tr>
<td>nqo</td>
<td>AGGATGGGAGGTACTCGAATC</td>
<td>TGCTAGAGATGACTCGGAAGG</td>
</tr>
<tr>
<td>ahrr</td>
<td>ACATACGCCGGTAGGAAGAGA</td>
<td>GGTCCAGCTCTGTATTGAGGC</td>
</tr>
<tr>
<td>cyp1a2</td>
<td>AGTACATCTCCTTAGCCCACAG</td>
<td>GGTCGGGTGAGTTCTTCAG</td>
</tr>
<tr>
<td>cyp1bl</td>
<td>CACCAGCTTATGGCAGACAG</td>
<td>GAGGACCACCGTTTCCGTGG</td>
</tr>
<tr>
<td>gapdh</td>
<td>TCTCCCTCAAAATTCATCCCAAG</td>
<td>GGTTGCAAGCGAATTTATTGATGG</td>
</tr>
</tbody>
</table>

Table 5: Forward and reverse primer sequences used for AHR gene battery expression
2.11. RT-PCR from liver samples

Upon termination of the study, mice were euthanized using CO2. The livers were harvested in RNA later (from Ambion, Thermo Fisher Scientific), stored at 4°C until total RNA was extracted from them. Prior to RNA extraction, the tissue was ground in Trizol (from Zymo Research) in M-tubes (from Miltenyi Biotech) using GentleMacs™ (a tissue grinder from Miltenyi Biotech). Total RNA was extracted using the kit Direct-zol Total RNA extraction kit (Zymo Research) by following the instructions provided by the manufacturer. RNA quantitation was done using QIAxpert (from Qiagen). 2.5 µg of RNA per reaction was used to set up the cDNA reaction. cDNA was prepared immediately from RNA preparation using the SuperScript III first strand synthesis super mix for qRT-PCR (Thermo Fisher Scientific) following manufacturer’s instructions. Real time PCR was set up using SYBR chemistry (Sso Advanced Universal SYBR Green Supermix from Bio-Rad) and PCR oligos from Thermo Fisher Scientific. For each time point there were three mice and three technical replicates per mouse. The samples were run on the LC480 cycler from Roche. GAPDH diluted at a ratio of 1:500 was used as the reference gene. The primer sequences, PCR cycling parameters and RT-PCR assay set up are outlined in table 4. The RT-PCR data was obtained by calculating ΔCt (Ct of target gene-Ct of reference gene), ΔΔCt (ΔCt of target gene-ΔCt of control sample) and then finding the $2^{-\Delta\Delta Ct}$.
2.12. LPS administration and ELISPOT assay

ELISPOT assay

The principle of ELISPOT assay is very similar to that of a sandwich ELISA (Enzyme-linked immunosorbant assay) technique. The assay is both qualitative and quantitative, and is used to determine the number of antibody or cytokine cells. The monoclonal or polyclonal antibodies of interest are pre-coated to the microplate, on to which the stimulated cells are added. The binding occurs in a humidified chamber at 37°C for a specified amount of time (should be determined). The cells are washed and the antibody/cytokine producing cells are determined using a biotinylated ab/AP-strepatvidin conjugate/substrate solution. The blue-black spots that are formed correspond to the antibody or cytokine localization and can be counted with an automated ELISPOT reader.

Lipopolysaccharide (LPS) was purchased from Sigma Aldrich and was dissolved in 1XPBS (from Corning cellgro cell culture Phosphate Buffered Saline, 1X, without calcium and magnesium) to obtain a stock of 5 mg/mL. TCDD dissolved in DMSO (50 µg/mL) was from Cambridge Isotope Laboratories. Mice used for the experiment were C57BL/6 purchased from The Jackson Laboratories. Murine IgM ELISPOT (Immunospot) kit for detecting IgM producing plasma cells was from CTL (Cellular Technology Limited). DMSO Dimethyl Sulfoxide-HybriMax, sterile filtered (D2650) was from Sigma-Aldrich. Corn oil, NF grade (CO136-25ML) was purchased from Spectrum Chemicals.
Some preliminary experiments were done using the IgM ELISPOT kit to obtain optimum cell seeding density and incubation time (of cells with capture Ig). Spleens were harvested from an un-stimulated mouse. Splenocytes were obtained using gentleMACS™ tissue dissociator (from Miltenyi Biotech) in C-tubes (for tissue grinding, from Miltenyi Biotech). The cells were counted using Vi-CELL XR (from Beckman Coulter). This was compared to the stimulated splenocytes (as they secrete more IgM than unstimulated ones. Stimulation was done by treating splenocytes with B-Poly S (provided with the ELISPOT assay kit) for 3 days in a 5% CO2 incubator at 37º C. Before plating, the cells were washed 3 times with CTL media, to remove any IgM present on the cell surface. Both stimulated and un-stimulated splenocytes were plated at the density listed below in table 6 on the ELISPOT plate. The latter had been coated with capture Ig the previous night. The plates were incubated for either 12 or 20 hours in a 5% CO2 incubator at 37º C. Next day the plate was washed and ELISPOT protocol was conducted as per manufacturer’s instructions.
Table 6: Cell counts of splenocytes with stimulated and unstimulated B cells.

![Image of ELISPOT assay results]

<table>
<thead>
<tr>
<th>No stimulation cell count</th>
<th>B-cell stimulation cell count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A 1x10^6</td>
<td>1x10^5</td>
</tr>
<tr>
<td>B 500,000</td>
<td>50,000</td>
</tr>
<tr>
<td>C 250,000</td>
<td>25,000</td>
</tr>
<tr>
<td>D 125,000</td>
<td>12,500</td>
</tr>
<tr>
<td>E 6,250</td>
<td>625</td>
</tr>
<tr>
<td>F 3,125</td>
<td>312.5</td>
</tr>
<tr>
<td>G 1,562.5</td>
<td>1,562.5</td>
</tr>
<tr>
<td>H Blank</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2: Stimulated and unstimulated B cells from ELISPOT assay after 12 hour incubation.

2.13. ELISPOT assay on mice treated with LPS and TCDD

C57BL/6 mice were injected with 1µg per mouse of TCDD dissolved in corn oil.

DMSO dissolved in corn oil was used as the vehicle. LPS was administered at a concentration of 25 µg per mouse [15]. Before LPS was administered to mice on the
study, a pilot run was conducted to see if LPS administration caused any adverse effects in mice. The mice (n=3 per group) were treated with 25 µg or 35 µg LPS or 1X PBS and monitored for 30 minutes. The spleens were harvested and IgM secreting plasma cells were looked into.

TCDD (1µg /mouse) or DMSO in corn oil was administered on day -4. 25 µg of LPS was administered on day 0. Administration of LPS was considered as day 0. Three days later spleens were harvested, splenocytes obtained using gentleMACSTM and plated on Murine IgM ELISPOT assay plate that was coated the previous night with capture Ig. The cell dilutions used for plating were 62,500, 31,250, 15,625 and 7,812.5 cells per well. The cell dilution of 15,625 per well was used for calculating the spots which are IgM secreting plasma cells. There were 4 treatment groups – LPS alone, LPS + TCDD, TCDD alone, DMSO alone and untreated group. The assay had an n=5 in all treatment groups and an n=3 in the untreated group. The assay plate was incubated for 12 hours in a 5% CO2 incubator at 37degC. Next day the plate was washed and ELISPOT protocol was conducted as per manufacturer’s instructions. The plate was read in Immunospot analyzer.


The spleens from mice with the following treatment groups were harvested – LPS alone, LPS + TCDD, DMSO + LPS and untreated. The splenocytes were obtained by grinding the tissues in C-tubes using gentleMACS. Red blood cells from the ground spleens were lysed using 1X RBC lysis buffer (from Sigma Aldrich). The cells were counted using Vi-CELL XR (from Beckman Coulter). The cells were plated in a U-
bottom 96 well plate (from Corning), washed once with FACS buffer (1X PBS + 2% FBS) and blocked using Fc block (2.4 G2, anti-mouse CD16/CD32 from Bio X Cell) at a concentration of 1:1000 for at least 5 minutes. The cells were then stained with 100ul of fluorescent antibodies in FACS buffer as listed in the table below.

<table>
<thead>
<tr>
<th>Company</th>
<th>Cat#</th>
<th>Lot#</th>
<th>Clone</th>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invitrogen</td>
<td>L34555</td>
<td>1189794</td>
<td>live/dead</td>
<td>live/dead</td>
<td>PacBlue</td>
<td>700</td>
</tr>
<tr>
<td>BioLegend</td>
<td>103116</td>
<td>-</td>
<td>30-F11</td>
<td>a-mouse CD45</td>
<td>APC/Cy7</td>
<td>200</td>
</tr>
<tr>
<td>BioLegend</td>
<td>100540</td>
<td>-</td>
<td>RM4-5</td>
<td>a-mouse CD4</td>
<td>PerCP/Cy5.5</td>
<td>200</td>
</tr>
<tr>
<td>eBioscience</td>
<td>12-0193-85</td>
<td>E014445</td>
<td>eBic1D3</td>
<td>a-mouse CD19</td>
<td>PE</td>
<td>0.5ul per million cells</td>
</tr>
<tr>
<td>BD biosciences</td>
<td>559562</td>
<td>77866</td>
<td>281-2</td>
<td>a-mouse CD138</td>
<td>APC</td>
<td>0.06ul per million cells</td>
</tr>
<tr>
<td>eBioscience</td>
<td>11-5321-82</td>
<td>E016385</td>
<td>M5/114:15.2</td>
<td>a-mouse MHC II</td>
<td>FITC</td>
<td>0.1ul per million cells</td>
</tr>
</tbody>
</table>

Table 7: List of fluorescent antibodies for identifying CD138+ plasma cells. The cells were stained and fixed as per the FACS protocol. The samples were run on FACS Canto (from BD Biosciences).

The cells were stained for 30 minutes at 4°C in the dark. The cells were washed twice with FACS buffer, resuspended in 300ul of FACS buffer and analyzed by FACS on BD Canto from BD Biosciences. Proper compensation controls were prepared using splenocytes to set the PMT voltages on the FACS machine. The data was analyzed using FlowJo software.

2.15. Flow cytometry for TILs and tumor draining lymph nodes

The tumors and tumor draining lymph nodes were harvested on day 17 after tumor implants, for immuno-phenotyping. Mice were euthanized using CO₂ and the tumors were excised and stored in 50 mL conical tubes containing 5mL of MC38 cell
culture media. The tumor draining lymph nodes comprising of the axillary, brachial and inguinal lymph nodes on the tumor bearing side were harvested in a 24 well cell culture plate containing 1.5 ml of MC38 cell culture media. These procedures were performed in a bio-safety cabinet and the tissues were kept on ice as they were being harvested. The tumors were weighed on a balance, weight recorded and roughly 0.3 to 0.4 g of tumor tissue was used for immunophenotyping studies. The remaining tissue was flash frozen on dry ice and stored at -80°C for future use.

2.16. Tumor and lymph node digestion

Tumors and lymph nodes were digested with enzymes – Dnase I (Sigma Aldrich) and Collagenase IV (from Worthington) using the digest buffer to help release the dendritic cells and other myeloid cells from fibroblasts.

The weighed tumors were transferred to a gentleMACSTM C-tube (from Miltenyi Biotech) with 1.5ml of digest buffer (1.5ml for 0.5g of tissue). Below is the recipe for preparing digest buffer. All the contents of the digest buffer except for the enzymes were kept at room temperature.

<table>
<thead>
<tr>
<th>Colagenase IV (250U/ml)</th>
<th>DNAse1 (100ug/ml)</th>
<th>CaCl2 (5mM)</th>
<th>FBS (5%)</th>
<th>HBSS (up to)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.89 ml</td>
<td>0.46 ml</td>
<td>0.92 ml</td>
<td>2.31 ml</td>
<td>39.62 ml</td>
</tr>
</tbody>
</table>

Collagenase IV was purchased from Worthington Biochemical (CLS-4), Dnase I from Sigma Aldrich, HBSS – Hank’s balanced salt solution from Thermo Fisher and FBS
from Gibco, Thermo Fisher. Tumor tissue was chopped 20 to 30 times with a pair of scissors to little chunks. The tubes were placed in gentleMACSTM tissue grinder (protocol used: m_tumor_imp_03) to grind the tumors. The samples in C-tubes were incubated at 37°C incubator for 30 minutes with constant shaking. Tubes were then vortexed on high for 5 seconds. For lymph nodes digestion, the lymph node capsule was torn using a pair of scissors, then crushed with the flat end of a sterile syringe and pipetted with 500 µl of digestion buffer to tease out all the lymphocytes. These were incubated at 37°C for only 15 minutes and then quenched with the quench buffer. The tumors and lymph nodes were treated in a similar fashion. The digestion enzymes were quenched with serum and EDTA containing media by adding 7mL of cold quench buffer. The quench buffer was made of Corning cellgro RPMI 1640 medium, 1X with L-glutamine (Thermo Fisher) with 10% fetal bovine serum (FBS) (from Gibco, Thermo Fisher) + 2mM EDTA from Corning cellgro. From here onwards all steps were performed on ice to maintain the integrity of the leucocytes. The cells were passed through a 100µM filter (Falcon cell strainer, 100µM pore size, from Fisher Scientific) to remove debris and hair. A small aliquot of cells (around 500ul) was taken for counting on a cell counter (ViCell from Beckman Coulter). In the meantime, cells were spun at 400g for 5 minutes at 4°C and washed once with 1X PBS. 1 million cells were taken per well per sample for live dead and other antibody staining. The cells were plated in a 96 well U-bottom plate for the convenience of staining, fixing and running samples in HTS mode on the BD Fortessa. Zombie aqua (from Biolegend) at a concentration of 1:1000 was used as the live dead stain. Zombie aqua is a fixable, viability dye that is amine-reactive. Therefore the cells had to be washed thoroughly with 1X PBS to remove all proteins. The samples were incubated
with 100ul of the stain for 30 minutes in the dark at 4°C. The staining was stopped by the addition of Fc block buffer, which also block Fc receptors that are highly expressed in tumors. The recipe for Fc block buffer is FACS buffer (1X PBS + 2% FBS + 2mM EDTA) along with 4% rat serum (from Jackson Immunoresearch) and 1:250 mouse FcX (Trustain fcX, anti-mouse CD16/CD32 from Biolegend, clone 2.4G2). The Trustain fcX block CD16 (low affinity IgG Fc receptor III) and CD32 (Fc receptor II) receptors. The antigen-antibody immune complexes bind to Fc receptors and mediate adaptive immune responses. These receptors are present on many cells like DCs, mast cells, B cells, NK cells, macrophages etc. Using this blocking antibody prevents non-specific binding of the cell surface antibodies to the Fc receptors. The samples were incubated for another 15 minutes with 100ul per well of Fc block in the dark at 4°C. All centrifugation steps were done at 400g for 5 minutes at 4°C. The supernatant was decanted, the cells were re-suspended in 50ul of the staining cocktail which constituted of different cell surface antibodies specific for tumor infiltrating lymphocytes. There were 2 staining panels – one for staining of the myeloid cell populations (table 8) and the other for NK + T cell populations (table 9).
### 2.17. Fluorescent antibody panel for myeloid cell analysis

<table>
<thead>
<tr>
<th>Cell surface marker</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4/80</td>
<td>Perccy5.5</td>
<td>BM8</td>
<td>BioLegend</td>
<td>1 to 250</td>
</tr>
<tr>
<td>CD80</td>
<td>Alexa 488</td>
<td>16-10A1</td>
<td>BioLegend</td>
<td>1 to 250</td>
</tr>
<tr>
<td>CD11c</td>
<td>PEcy7</td>
<td>N418</td>
<td>BioLegend</td>
<td>1 to 500</td>
</tr>
<tr>
<td>CD86</td>
<td>PE</td>
<td>GL-1</td>
<td>BioLegend</td>
<td>1 to 300</td>
</tr>
<tr>
<td>Ly6G</td>
<td>PE texasRed</td>
<td>1A8</td>
<td>BioLegend</td>
<td>1 to 1000</td>
</tr>
<tr>
<td>CD90.2</td>
<td>Alexa 700</td>
<td>30-H12</td>
<td>BioLegend</td>
<td>1 to 250</td>
</tr>
<tr>
<td>CD19</td>
<td>Alexa 700</td>
<td>6D5</td>
<td>BioLegend</td>
<td>1 to 250</td>
</tr>
<tr>
<td>MHCII</td>
<td>BV421</td>
<td>M5/114.15.2</td>
<td>BioLegend</td>
<td>1 to 500</td>
</tr>
<tr>
<td>CD11b</td>
<td>BV605</td>
<td>M1/70</td>
<td>BioLegend</td>
<td>1 to 2000</td>
</tr>
<tr>
<td>Ly6C</td>
<td>BV711</td>
<td>HK1.4</td>
<td>BioLegend</td>
<td>1 to 1000</td>
</tr>
<tr>
<td>CD45</td>
<td>BUV395</td>
<td>30-F11</td>
<td>BD</td>
<td>1 to 500</td>
</tr>
</tbody>
</table>

Table 8: Fluorescent antibody panel for myeloid staining

### 2.18. Fluorescent antibody panel for NK and T cell analysis

<table>
<thead>
<tr>
<th>cell surface marker</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>Perccy5.5/PE-cy5</td>
<td>RM4-5</td>
<td>ebioscience</td>
<td>1 to 250</td>
</tr>
<tr>
<td>CD8a</td>
<td>PE-cy7</td>
<td>5.3-6.7</td>
<td>ebioscience</td>
<td>1 to 400</td>
</tr>
<tr>
<td>Foxp3</td>
<td>FITC/Alexa 488</td>
<td>FJK-16s</td>
<td>ebioscience</td>
<td>1 to 300</td>
</tr>
<tr>
<td>LIVE DEAD</td>
<td>BV510</td>
<td></td>
<td>Biolegend</td>
<td>1 to 1000</td>
</tr>
<tr>
<td>CD45</td>
<td>APC/Cy7</td>
<td>30-F11</td>
<td>ebioscience</td>
<td>1 to 200</td>
</tr>
<tr>
<td>Ki67</td>
<td>PE</td>
<td>SolA15</td>
<td>ebioscience</td>
<td>1 to 250</td>
</tr>
<tr>
<td>NK1.1</td>
<td>BV421</td>
<td>PK136</td>
<td>ebioscience</td>
<td>1 to 200</td>
</tr>
<tr>
<td>CD90.2</td>
<td>Alexa 700</td>
<td>30-H12</td>
<td>Biolegend</td>
<td>1 to 250</td>
</tr>
<tr>
<td>CD19</td>
<td>Alexa 700</td>
<td>6D5</td>
<td>Biolegend</td>
<td>1 to 300</td>
</tr>
<tr>
<td>CD27</td>
<td>APC</td>
<td>LG.7F9</td>
<td>ebioscience</td>
<td>1 to 250</td>
</tr>
<tr>
<td>CD11b</td>
<td>BV605</td>
<td>M1/70</td>
<td>Biolegend</td>
<td>1 to 2000</td>
</tr>
<tr>
<td>FoxP3 TF Staining Kit</td>
<td></td>
<td></td>
<td>00-5523-00</td>
<td></td>
</tr>
</tbody>
</table>

Table 9: Fluorescent antibody panel for NK and T cell staining
The cells were stained with cell surface antibodies for 30 minutes in the dark at 4°C. After staining, they were washed twice with Facs buffer. The cells were fixed overnight in the dark at 4°C in Foxp3 fix-perm buffer, essential for staining intracellular antibodies like Foxp3 and CD206, which was done for 30 minutes at 4°C in the dark. After fixing the cells, washes were done in 1X perm buffer, in order to keep the cells in a permeable state for the intracellular antibodies to enter the cell. For analysis on the flow cytometer the cells were re-suspended in 300 to 500 µL FACS buffer and run in HTS mode on BD Fortessa. For setting up PMT voltages, compensation controls were prepared. Either cells from tumor/lymph nodes or compensation beads were used with CD4 or CD19 antibodies for the fluorophores being used. The FACS data was analyzed using FlowJo 10.08 software.

2.19. Intratumoral cytokine/chemokine analysis

Cytokines and chemokine analysis was done using the “MILLIPLEX MAP mouse cytokine/chemokine magnetic bead panel – Immunology Multiplex Assay” from Millipore. Multiple cytokine and chemokine markers from tumor supernatant were analyzed by bead-based Multiplex assay from Luminex Technology. The samples were processed as per the manufacturer’s instructions. The assayed beads were then read on a Magpix plate reader.

2.20. Statistical analysis

This was done using GraphPad PRISM. Error bars represent SEM calculated using PRISM. For looking into the differences in tumor burden between groups, one way
and two-way ANOVA (with Bonferroni posttest) was used. For comparing the
differences between AHR WT vs AHR KO mice and Vehicle vs CH223191 treated mice
for different lymphocyte subsets, data analysis was done by the Student’s t test (unpaired)
using GraphPad PRISM to compare the means of the two study groups. A p value of
<0.05 was considered as a significant difference. * p < 0.05, ** p <0.005, *** p < 0.0005,
and ns (not significant) represents p > 0.05
CHAPTER 3: RESULTS

3.1. AHR in MC38 cells drives a modest and short lived \textit{cyp1a1} gene expression when exposed to TCDD

The aim of this experiment was to elucidate whether MC38, B16F10 and Hepa1c1c7 cells expressed AHR and if the receptor was functional, i.e., can drive the expression of \textit{cyp1a1} and other genes of the AHR gene battery. The nuclear AHR + ARNT complex binds to specific promoter regions (DRE-Dioxin response elements) with the sequence 5′-T/G/TCGTGA/CG/TA/T-3′ on genomic DNA to enhance the transcription of AHR target genes. These are collectively called as the “AHR gene battery” – \textit{cyp1a1}, \textit{cyp1b1} (cytochrome P450 I family member a1 and b1), \textit{nqo1} (NAD (P) H quinone oxidoreductase 1), \textit{aldh3a1} (aldehyde dehydrogenase 3 family, member a1), \textit{ugt1a6} (UDP glucoronosyl transferase 1 family, polypeptide A6) and GST-Ya (glutathione S transferase). These genes encode phase I and II metabolizing enzymes that are responsible for the detoxification of xenobiotics in the liver. Apart from this, AHR plays a role in mediating xenobiotic-independent and physiological functions. To list a few, it is involved in reproduction, cell proliferation, apoptosis, tumor suppression or progression, and differentiation of immune cells [43].

\textit{Cyp1a1} is the quintessential biomarker that is expressed upon exposure to halogented hydrocarbons such as TCDD. MC38 (colon carcinoma) and B16F10 (melanoma) were two transplantable mouse cell lines used for tumor model generation.
After treatment with 1nM TCDD in vitro, mRNA levels of *ahr* and *cyp1a1* were evaluated in these two cell lines. Hepa1c1c7 (mouse hepatoma) cells which have been shown to express *ahr* and are highly inducible for *cyp1a1* expression with TCDD treatment, was used for comparison. The mRNA levels of the AHR gene battery were measured only in MC38 cells.

The *cyp1a1*, *cyp1b1* and *ahr* message levels of Hepa1c1c7 (Fig 3, 4 & 5) in comparison to that of MC38 were measured at 6 and 24 hours in the presence or absence of 1nM TCDD. The induced expression level of *cyp1a1* in Hepa1c1c7 is much higher than that of MC38 cells (Fig. 3). There is roughly a two fold increase in the *cyp1b1* and *ahr* transcripts in Hepa1c1c7 cells at 24 hours (Fig. 4 and 5). The data was normalized to the control (DMSO) group.

![Graph showing *cyp1a1* mRNA levels measured in MC38 and Hepa1c1c7 cell lines, at 6 and 24 hours after 1nM TCDD treatment. Data were normalized to the control group, n=3 per experiment.](image)

**Figure 3:** *cyp1a1* mRNA levels measured in MC38 and Hepa1c1c7 cell lines, at 6 and 24 hours after 1nM TCDD treatment. Data were normalized to the control group, n=3 per experiment.
Figure 4: *cyp1b1* mRNA levels measured in MC38 and Hepa1c1c7 cell lines, at 6 and 24 hours after 1nM TCDD treatment. Data were normalized to the control group, n=3 per experiment.

Figure 5: *ahr* mRNA levels measured in MC38 and Hepa1c1c7 cell lines, at 6 and 24 hours after 1nM TCDD treatment. Data were normalized to the control group, n=3 per experiment.
To determine the consequences of TCDD incubation of MC38 and to compare to B16F10, MC38 and B16F10 cells were exposed to 1nM TCDD. The expression of *ahr* and *cyp1a1* were measured at 3, 6, 18 and 24 hours. The message levels of *ahr* were very similar in the two cell lines, but higher in MC38 cells at the 3 hour time point (Fig.6).

![Figure 6: ahr mRNA levels measured in MC38 and B16F10 cell lines, at 3, 6, 18 and 24 hours after 1nM TCDD treatment. Data were normalized to the control group, n=3 per experiment.](image)

There is little induction of the *cyp1a1* message in MC38 cells; it is highest at the 3 hour period and tapers down thereafter to 24 hours. This suggests that AHR, in the presence of TCDD drives a modest *cyp1a1* expression (Fig.7).
By contrast, the cyp1a1 levels of B16F10 were quite steady over the 24 hour period and significantly higher than that of MC38 (Fig.8).
The message levels of \textit{ugt1a}, \textit{ahrr}, \textit{cyp1a2}, \textit{nqo}, \textit{cyp1b1} were measured in MC38 cells 24 hours after 1nM TCDD treatment and no detectable expression in the members of the AHR gene battery were seen (Fig.9). The mRNA levels at earlier time points were not measured. Like \textit{cyp1a1} expression in MC38 cells, perhaps the expression for these genes occurs for a very short duration (either at 3 hours or within 0 to 24 hour period) and if this is the case, the AHR gene battery is active for a very short period and the changes might have been missed due to the transient nature of induction. If a sustained AHR induction is required, then AHR may not elicit normal functions in MC38 cells and perhaps AHR expression in MC38 cells is irrelevant and thus, any effects on tumor burden are mediated by the host AHR.

![Message levels in MC38 cells after exposure to 1nM TCDD for 24 hours](image)

Figure 9: AHR gene battery mRNA levels measured in MC38 cell line, at 24 hours after 1nM TCDD treatment. Data were normalized to the control group, n=3 per experiment.
3.2. In vivo TCDD efficacy studies

According to the stated hypothesis, activation of AHR by its ligands increases Treg differentiation; increased Treg differentiation leads to increased immune suppression and therefore increased tumor burden. TCDD is a very potent ligand of AHR and even small quantities of the toxin can activate AHR, both in vivo and in vitro. TCDD is also a potent immune suppressor and is known to increase Treg differentiation in many in vivo models [21]. Therefore, TCDD was used as an AHR ligand for in vivo studies. The dosage of 1µg/mouse was based on the Quintana et. al paper [21].

Before administering TCDD in the tumor model, the effects and duration of TCDD in vivo were explored. As *cyp1a1* is the classic biomarker that is expressed upon exposure to TCDD, mice were injected with 1µg/mouse TCDD (administered intraperitoneally). Livers were harvested at 72 hours, 7 days and 14 days after TCDD administration. RNA was extracted and message levels of *cyp1a1* and *ahr* were measured.
The cyp1a1 mRNA levels were measured till day 14 after TCDD treatment from livers of treated mice (Fig. 10), demonstrating TCDD activated liver AHR expression until at least 14 days. There is more than 2-fold induction on day 14 in comparison to day 3 (or 72 hours).

In two separate experiments the cyp1a1 levels were measured at 48 hours (Fig. 12) and again on day 24 (Fig. 11) after TCDD administration from MC38 tumor-bearing mice. The RT-PCR data from all these experiments demonstrated that TCDD activated the receptor as early as 2 days and up to at least 24 days, thus, throughout the duration of the tumor burden experiment.
**cyp1a1 RNA measured in liver 24 days after TCDD treatment**

![Graph showing cyp1a1 RNA levels in liver 24 days after TCDD treatment.](image)

Figure 11: Expression levels of cyp1a1 gene in liver of MC38 tumor bearing C57BL/6 mice treated with 1µg/mouse TCDD, harvested 24 days later. RNA was normalized to the control group.

**cyp1a1 RNA measured in liver 48 hours after TCDD treatment**

![Graph showing cyp1a1 RNA levels in liver 48 hours after TCDD treatment.](image)

Figure 12: Expression levels of cyp1a1 gene in liver of C57BL/6 mice treated with 1µg/mouse TCDD, harvested 48 hours later. RNA was normalized to the control group.
3.3. Pharmacologic activation by TCDD (1 dose) does not alter MC38 tumor growth

The tumor model used to prove the aforementioned hypothesis is the MC38 syngeneic tumor model. MC38 is a colon adenocarcinoma tumor model on the C57BL/6 background. This cell line was generated by Corbett et. al in 1975 by chemically inducing tumors in C57BL/6 mice. Tumor studies were performed as indicated in the schematic below (Fig. 13). TCDD was administered one day prior to MC38 cell implantation so as to have AHR activated pre-implantation. The experiment was repeated at least 5 times with n = 8 to 12 animals per experiment.

Figure 13: Schematic of MC38 tumor study on C57BL/6 mice; AHR was pharmacologically activated by 1ug/mouse TCDD. Arrows indicate either the day of treatment or tumor measurement

Tumors were measured twice weekly using Calipers and the data captured in STUDYLOG software. Data from the TCDD treated group was compared to the vehicle group, 2% DMSO + corn oil (pharmaceutical grade). The treatment was administered once, one day prior to the MC38 implantations. The average tumor burden was the same on day 7 for both groups (67mm$^3$). Based on the tumor burden results below there was no
significant change in the tumor burden with the treatment of one dose of 1µg/mouse with TCDD.

Figure 14: MC38 tumor burden in C57BL/6 mice measured on day 7, 10, 14, 17, 21, 24 and 26 in vehicle and treatment groups. This data is a representation of one tumor burden experiment.

3.4. T cell analysis by FACS

Although there was no change in tumor burden, the T cell compartment in spleen and the tumor implant (infiltrating lymphocytes) were characterized on day 14. The T cell subsets evaluated were effector CD4+, CD8a and CD4+ Foxp3+Treg populations. The
CD8a+ to Foxp3+Treg ratios were also calculated. Analysis of the T cell subsets in spleen showed that there was a significant reduction in the effector CD4+ T cells in the TCDD treated group in comparison to the vehicle group. The effector CD4+ T cells were gated on the total CD45+ lymphocyte population after gating out lymphocytes stained for the CD8a+ and CD4+ Foxp3+ Treg lymphocytes. There was a significant increase in the splenic Foxp3+ Tregs in the TCDD treated group indicating a peripheral immune suppressive phenotype (1.2 fold increase). There was a significant decrease in the CD8a to Foxp3+Treg ratio in the TCDD treated spleens suggesting that there was an increased number of Foxp3+ Tregs than the CD8a+ cells indicating a peripheral immune suppressive phenotype (1.4 fold decrease). There was no change in the percentage of the CD8a+ cells in the two groups of mice.

Analysis of tumor infiltrating lymphocytes revealed that there was no change in the effector CD4+ T cells, Foxp3+ Tregs and CD8a to Treg ratio cells in the tumors from TCDD and vehicle treated mice. However, there was a significant reduction in the infiltrating CD8a+ lymphocyte populations in the tumors from TCDD treated mice.
Figure 15: Analysis of T cell subsets in spleen of TCDD and vehicle treated mice with MC38 tumor implants. Data are representative of one of the three experiments, with n = 8 animals per group. * p < 0.05, *** p < 0.0005, and ns (not significant) represents p > 0.05.
Figure 16: Analysis of the tumor infiltrating lymphocytes from MC38 tumors treated with TCDD and vehicle treated mice. Data are representative of one of the three experiments, with n = 8 animals per group. * p < 0.05, and ns (not significant) represents p > 0.05.

The tumor burden and FACS analyses data are consistent with TCDD administration causing a peripheral immune suppression in the mouse as indicated by the
increase in Foxp3+ Tregs and decrease in the CD8a to Treg ratio, but is neither reflected in the T cell subsets of the tumor microenvironment nor the tumor volume. This leads to the question if the administration of TCDD – 1ug per mouse given intraperitoneally, was sufficient to cause immune suppression. This question was addressed by using the LPS mouse model, where humoral responses were measured in response to LPS administration in the presence and absence of TCDD.

**3.5. Effect of TCDD on LPS elicited humoral challenge**

LPS (Lipopolysaccharide) is a component of the bacterial cell wall and an endotoxin which elicits an innate immune response via TLR4 pathway and also a humoral immune response mediated by CD4+ T cells. This experiment was based on the paper from Kaminski et. al, [44]. The idea behind using this model was to determine if the TCDD dosage of 1ug per mouse could suppress activation of humoral responses promoted by 25ug of LPS.
Figure 17: Schematic of the TCDD-mediated suppression of the humoral response due to administration of 25µg of LPS in C57BL/6 mice.

Figure 18: Humoral response measured by the frequency of IgM producing plasma cells after LPS and TCDD challenge. N = 6 mice / group, *** p < 0.0005
Figure 19: Reduced plasma cell numbers in spleen after TCDD and LPS treatment. A. FACS plot showing CD19+ CD138+ plasma cells in splenocytes from mice treated with TCDD, LPS and controls. B. CD138 + plasma cell numbers after TCDD and LPS challenge N = 6 mice / group, ** p <0.005

The experiment was done as described in Fig. 17. Mouse splenocytes were plated into a 96 well ELISPOT assay plate and the frequency of the IgM-producing plasma cells was measured. There was an increase in the number of IgM-producing plasma cells upon LPS challenge. This response was reduced by almost three-fold in mice that were exposed to TCDD prior to LPS challenge. In the DMSO + LPS group that received no TCDD, the humoral response was similar to the LPS alone group. The untreated group did not elicit any humoral response.

Next, CD138+ plasma cells were enumerated by FACS. Plasma cells are terminally differentiated B cells that produce specific antibodies for the antigen they have encountered. CD138 (Syndecan-1) is a specific plasma cell surface marker which B cells
acquire after antigen-driven differentiation. CD19 was also used as a B-lymphocyte marker.

In Fig. 19A, LPS challenge in the LPS and DMSO + LPS groups, resulted in increased numbers of CD138\textsuperscript{hi} plasma cells. In Fig. 19B, plasma cell populations identified as CD19\textsuperscript{hi}CD138\textsuperscript{hi} cells were measured and a 3-fold reduction was observed in the TCDD-treated group. Therefore, TCDD at 1\mu g/mouse suppressed the humoral response elicited by LPS challenge as measured by CD19\textsuperscript{hi}CD138\textsuperscript{hi} cells.

With the FACS analysis of B cells from the LPS challenge and the T cell subsets (from spleen and tumor), 1\mu g/mouse TCDD dose is sufficient to suppress an LPS elicited humoral response, the effector CD4\textsuperscript{+} T cells in the spleen and the CD8\textsuperscript{+} T cells in the tumor. However this dosage has no effect on tumor volume. Therefore, it was decided to increase the TCDD dosing frequency, but below the lethal dose of TCDD (LD\textsubscript{50}), namely 160\mu g/kg for C57BL/6 [29].

3.6. Pharmacologic activation by TCDD (administered 3 times) suppresses MC38 tumor growth

The tumor model was generated in a similar fashion to the schema described in Fig. 13, except for the TCDD dosing regimen. TCDD was administered on days 7, 10 and 15 at 1\mu g/mouse. The body weights of these mice were measured along with the tumors twice weekly; there were no gross changes in body weight or the apparent health of the mice. The tumor burden in the group that received TCDD started to show reduced
Figure 20: MC38 tumor burden with TCDD dosed on days 7, 10 and 15. This experiment was done twice with n= 16 mice per group. *** p < 0.0005, * p < 0.05

Figure 21: MC38 tumor burden comparing mice dosed with TCDD days 7, 10 and 15 with three different days. Each group had n = 16 mice.
tumor growth starting day 17 and there was a significant reduction in tumor burden in comparison to the vehicle group starting day 21.

The body weights of mice were measured each time the tumor was measured. This was done to ensure that the TCDD administration and tumor implantation did not affect the health of the mouse. The graph below is one representative of multiple studies. In Fig. 22, all mice seemed to be healthy except for one mouse (received 3 doses of TCDD) that was sacrificed on day 14 for exhibiting moribund symptoms and 2 mice that were sacrificed around day 20 due to losing more than 20% of the body weight; one mouse received 1 dose of TCDD and reached a tumor endpoint (2084 mm$^3$ volume) and the other one received 3 doses of TCDD.

Figure 22: Body weight (in grams) of all the mice on study were measured using a balance and recorded in STUDYLOG.
The tumor infiltrating lymphocytes on day 14, i.e., 7 days after the first TCDD treatment were evaluated. Among the T cell subsets analyzed, there was a change only in CD8a+ lymphocytes (Fig. 24) - there was a reduction in CD8a+ T lymphocytes in the groups of mice that received two doses (where a significant reduction in tumor burden was seen) and one dose of TCDD (where no change in tumor burden was seen) in comparison to the vehicle group. Therefore, there is no correlation between the reduced tumor infiltrating CD8+ T cells to changes in tumor burden. The CD8+ T cells are very sensitive to TCDD and these reductions could be a direct result of TCDD-mediated immune suppression [45].

Figure 23: Graph compiling all the tumor infiltrating T cell subsets analyzed on day 14 (or 7 days after TCDD treatment) in mice that received 2 doses of TCDD, 1 dose of TCDD and the vehicle group.
3.7. **MC38 tumor burden experiments with different vehicles**

Differences in MC38 tumor burdens in AHR WT mice between TCDD and different vehicles were evaluated to rule out any effects caused by DMSO and corn oil on tumor burden. This experiment was done once with an \( n = 12 \) animals per group. The TCDD and the vehicles were administered intraperitoneally on day 7 after the mice were randomized by tumor volume and divided into 4 groups with the same average tumor burden per group. There were no statistically significant differences between any of the groups and thus no consequence of the vehicle to tumor growth. Notably, the 1X PBS group trended towards a higher tumor burden than the other groups for an unknown reason.

Figure 24: Graph compiling the tumor infiltrating CD8a + T cells analyzed on day 14 (or 7 days after TCDD treatment) in mice that received 2 doses of TCDD, 1 dose of TCDD and the vehicle group. * \( p < 0.05 \)
Figure 25: MC38 tumor burden experiment done with TCDD and different vehicles to rule out any vehicle mediated effect on the tumor growth. No statistically significant differences were seen between the groups.

3.8. AHR absence either genetically or by pharmacologic blockade showed enhanced MC38 tumor growth

Experiments with the AHR agonist TCDD have shown that increasing the dosing frequency reduces the MC38 tumor burden. This prompted experiments to determine the consequence to MC38 tumor burden in 1) the context of pharmacological blockade of the receptor using an antagonist and 2) in the absence of the receptor, by using AHR KO mice. The next set of experiments address these issues.
3.9. CH223191 blocks TCDD mediated cyp1a1 induction in liver

Before the AHR antagonist molecule CH223191 was used, preliminary studies were undertaken to define the dosing regimen and route of administration. CH223191 at 10mg/kg body weight was administered 48 hours prior to TCDD administration (which amounts to 0.5µg of CH223191 per mouse). Three mice per group were used. Livers from mice were harvested 3 and 6 hours post TCDD administration.

Total RNA was extracted and RT-PCR performed to quantitate cyp1a1 and ahr message levels. CH223191 blocked TCDD mediated cyp1a1 induction as early as 3 hours post-administration but reached significance only at 6 hours.
As such, CH223191 was used at 10mg/kg body weight for all the subsequent tumor experiments. In the tumor studies, because no TCDD was administered, CH223191 is blocking endogenous AHR ligands.

Figure 27: Structure of AHR antagonist – CH223191 (- 2-Methyl-2H-pyrazole -3-carboxylic Acid (2-methyl-4-o-tolylazo-phenyl)-amide)

Below is the schematic of the MC38 tumor burden study used for antagonizing AHR.

3.10. MC38 tumor burden studies by antagonizing the receptor

Figure 28: Schematic of tumor study. Note the daily administration of CH223191, indicated by black arrows.
CH223191 was orally administered daily using feeding needles, starting from day 7 until the end of study. CH223191 was dissolved in 2% DMSO + corn oil which was also the vehicle control. CH223191 was kept at 4°C as a 25 mg/mL stock and a fresh solution was prepared for every administration. These tumor burden studies were repeated at least three times with an n = 8 to 12 mice per group. 2 X 10^6 MC38 cells were implanted on the right flank of the mice and tumors and body weights measured twice weekly.

The mice on the study were in good health and the administration of the compound did not cause any adverse effects in mice. The mice that exhibited ulcerated tumors were removed from the study and were excluded from the tumor volume measurements. In the tumor burden data represented in Fig. 26, tumors on 3 mice (out of 10) were found ulcerated on day 17 and were removed from the experiment. We started seeing significant changes in the tumor burden starting on day 17. The study was terminated around day 21 as most of the mice in the study had reached the maximum tumor volume of 2000mm^3 or exhibited tumor ulcerations. Therefore, antagonizing the AHR through an antagonist increased the MC38 tumor burden in comparison to the control group.
Figure 29: Pharmacologic blocking of AHR by CH223191 increased MC38 tumor burden in C57BL/6 mice. N = 10 mice per group on day 0.

3.11. MC38 tumor burden experiments with AHR deficient mice

The tumor burden experiments using AHR KO and WT mice were done as described in the schematic Fig.30
The mice used for the study were bred at BMS animal facility and were originally created in Chris Bradfield’s lab [46]. On day 7, the first day of the measurement, mice were not randomized but the average tumor burdens between the WT and the KO mice were similar after excluding low and high tumor burden outliers, which resulted in 8 to 10 mice per group. Differences in tumor burden were observed on day 17 and were significant on day 21. Indeed, the tumor burden in the AHR KO mice was almost twice the volume as compared to their WT counterparts. The experiment was repeated at least three times and each time the same trend was observed.
Figure 31: MC38 tumor burden is increased in AHR deficient mice in comparison to AHR proficient mice. 2 X 10⁶ MC38 cells were implanted on day 0. Tumor volumes were determined twice weekly.

3.12. B16F10 tumor model

To confirm and extend to a different tumor model, studies were performed using the B16F10 tumor model in both AHR antagonist treated and AHR deficient (AHR KO) mice. B16F10 is an aggressive C57BL/6 melanoma cell line that is considered poorly immunogenic in vivo. A pilot study was conducted to determine what cell number would be sufficient, so as to have palpable tumors by day 6 or 7. It was empirically determined that one million cells would result in palpable tumors by day 6. The experiment was done
once each with the antagonist and once in AHR deficient mice. A starting number of 12 mice per group were used in each experiment.

3.13. B16F10 tumor-bearing mice treated with AHR pharmacologic blockade did not exhibit any significant differences in the tumor burden

Figure 32: B16F10 tumor burden in C57BL/6 mice in the presence of AHR antagonist CH223191 did not result in any significant changes in tumor volume. N = 12 was used per experiment.

The study shown in figure 31 was done using one million cells for implantation. On day 7, tumors were measured, mice were randomized based on tumor burden, then divided into the treatment and control groups. The antagonist (CH223191) or vehicle was administered every day orally starting from day 7 and continued until the end of the study. There were no significant changes in the tumor burden until day 16 post-implantation. The study had to be prematurely terminated because many mice developed
ulcerated tumors and their health status began to decline. The latter was monitored by the animal weight and general health (lethargy, moribund, failure to thrive, posture, tented skin and/ or weight loss were monitored).


One million B16F10 cells were implanted in AHR WT and AHR KO (AHR-/-) mice bred at the Bristol-Myers Squibb Company animal facility. Genotypes of the mice used in the study were confirmed by PCR using DNA extracted from tails and specific primers. The tumor measurements started on day 7 and the co-housed mice did not receive any treatments. The study was done once with 10 to 11 mice per group.

![Figure 33: B16F10 tumor burden in AHR deficient mice resulted in significant increase in tumor volume with the absence of the receptor. N = 11 was used per experiment.](image-url)
There were significant changes in the tumor burden on day 16 after the subcutaneous implants ($P = 0.002$). The tumors grew faster in the absence of AHR in comparison to their WT counterparts. The study had to be terminated by day 16, because many mice developed ulcerated tumors and their health status began to decline. The latter was monitored by the animal weight and general health (exhibited signs of lethargy, moribund, failure to thrive, posture, tented skin and or weight loss).

### 3.15. Immune profiling of the MC38 tumor microenvironment

Based on the tumor burden data obtained from the agonist, antagonist and the KO studies, the data suggest that absence of AHR signaling increases the MC38 tumor burden. The tumor environment was analyzed to determine if the increased tumor burden could be attributed to the changes in the infiltrating lymphocytes and cytokines in the tumor milieu.

Immune phenotyping of the T, NK and myeloid populations were done. Immune cells are distinguished based on a signature through the expression of specific surface proteins. Immune phenotyping is a technique of identifying cells by their cell surface and cytoplasmic protein expression using a specific antibody tagged to a fluorescent moiety. The stained samples are then run through a flow cytometer, a laser based instrument that counts cells based on the gate set for the fluorescent tagged antibodies. The samples were run on either a BD Fortessa or BD Canto and data analyzed using FlowJo software version 10.3.
As stated earlier, differences in MC38 tumor burden in the studies with AHR KO mice and the antagonist CH223191 became noticeable starting day 17. The tumors and draining lymph nodes were harvested on day 17. Two FACS antibody staining panels – one for the myeloid cells/dendritic cells and the other for T/NK cells – were used. Tissues were harvested in RPMI + 10% FBS and homogenized using the gentleMACS™ tissue grinder (Miltenyi Biotech). The disrupted tissues were then digested with a mixture of collagenase IV and DNase I enzymes, to release myeloid cell populations embedded in the tissue. The samples used for T/NK cells were also treated similarly and notably the enzyme digest did not adversely affect the detection of cell surface staining markers.

### 3.16. Myeloid population gating strategy

In the myeloid population, cells were first gated on singlets (doublets were excluded from the analysis), then the live population followed by CD45 staining lymphocytes. T and B cells were excluded using the dump channel for CD90.2 and CD19 antibodies, respectively. From the T and B cell excluded population denoted as ‘minus T cells’, neutrophil and monocytes (Ly6C positive) were identified. From the remainder of the cells, MHC II positive cells were gated. Thereafter, F4/80 positive tumor-associated macrophages (TAMs) and CD206 positive M2 macrophages were gated. Dendritic cells, identified by the markers CD11b, CD24 and CD103 were also enumerated in addition of the evaluation of dendritic cell activation markers CD80 and CD86. The myeloid cell gating strategy is shown below (Fig. 34) using a single cell suspension of tumor cells from a tumor-bearing mouse.
Figure 34: Myeloid cell gating strategy for AHR WT, AHR KO and WT +/- CH223191 treated mice
3.17. T cell gating strategy

Figure 35: T cell gating strategy for AHR WT, AHR KO and WT +/- CH223191 treated mice

From the tumor single cell suspension, cells were first gated on singlets (doublets were excluded from the analysis), then the live population and then CD45 positive lymphocytes. CD4 positive and CD8a positive T cells were gated from the CD45 positive lymphocyte population. The Foxp3 positive T regulatory cells were gated from the CD4+ T cell population. To identify proliferating Foxp3+ T regulatory and CD8a + cells, the
cell surface marker Ki67 was used. The proliferating Foxp3^{hi} T regs were noted to be Ki67^{hi}. The proliferating CD8a^{hi} cells were also Ki67^{hi}.

3.18. NK (Natural killer) cell gating strategy

![Figure 36: NK cell gating strategy for AHR WT, AHR KO and WT +/- CH223191 treated mice](image)

From the singlet cell gate (doublets were excluded from the analysis), live cells and then lymphocytes were gated. From the lymphocyte population the T and B cells and NK1.1^{+} cells were gated. The T and B cells were excluded using the dump channel for CD90.2 and CD19 antibodies, respectively. The NK1.1^{+} population were further gated on CD11b and CD27 positive NK cells, which are NK cell maturation markers.
3.19. Immune phenotyping results in AHR WT and AHR KO mice

FACS analysis of tumor implants and draining lymph nodes (dLN)s harvested on day 17 from AHR WT and KO mice revealed increased MC38 tumor burdens resulted in a modest, but not significant increase, in Foxp3+ Tregs in the tumors but not in dLN>s.
Figure 37: A) Increase in MC38 tumor burden in AHR KO did not result significantly increased Foxp3+ Tregs. B) Number of Foxp3+ Tregs in AHR WT and AHR KO mice in draining lymph nodes (dLNs). C) Increased MC38 tumor burden in AHR KO mice resulted in a reduction of CD8a + T cell infiltration. D) Number of Foxp3+ Tregs in AHR WT and AHR KO mice in dLNs. E) CD8a to Foxp3+ Treg ratio significantly reduced with increase in tumor burden. These data are a representative of 2 experiments. * p < 0.05, **** p < 0.0001, and ns (not significant) represents p>0.05
In tumors there was a significant decrease in CD8a+ T cells and the CD8a to Foxp3+ Treg ratio associated with increased tumor burden. By contrast a significant increase in the CD8a+ T cells was detected in the dLNs of AHR KO mice. The proliferating marker Ki67 was used to stain Foxp3+ Tregs and CD8a+ T cells and there were no differences in its expression in the tumors from AHR WT versus KO mice. This suggests that there is no increased proliferation of these two cell populations but the increased numbers seen in the tumor may have resulted from increased infiltration.

Analysis of NK cells using the cell surface markers – NK1.1 and CD27 revealed reduced numbers with increased tumor burden in the AHR KO mice.

![Figure 38](image)

Figure 38: A) NK1.1 cells reduce in numbers with increase in tumor burden. B) Reduced CD27+ NK1.1 cells in AHR KO mice with increase in MC38 tumor burden. These data are a representative of 2 experiments.

Next, the myeloid populations in the MC38 tumor microenvironment were characterized. There was no change in the infiltrating monocytes (Ly6C positive) or neutrophils. The dendritic cell subsets represented by the cell surface markers CD103,
CD11b, CD80 and CD86 were evaluated and there were no changes in the two groups of mice. However, there was a significant increase in the F4/80 positive TAMs and the CD206 positive M2 macrophages, although there was also no change in their MHCII. The tumor burden experiments involving the AHR WT and KO mice were repeated twice with a starting n = 10 per group in each experiment.
Figure 39: A, B, C, D) No change in monocyte, neutrophil, CD11b DCs and CD103 DCs with increased MC38 tumor burden in AHR KO mice. E) F4/80+ TAMs increased with increased tumor burden. F) CD206 macrophages increased with increased MC38 tumor burden. These data are a representative of 2 experiments. *** p < 0.0005, and ns (not significant) represents p > 0.05
3.20. Immune phenotyping results in AHR WT mice treated in the presence and absence of the antagonist CH223191

Figure 40: A) Increased MC38 tumor burden in CH223191 treated mice did not result in increased Foxp3+ Tregs. B) Number of Foxp3+ Tregs in the dLN of c57BL/6 mice +/- CH223191. C) Increased MC38 tumor burden in CH223191 treated mice associated with a reduction in CD8a+ T cell infiltration. D) Number of CD8a+ cells in the dLN of the two treatment groups. E) CD8a to Foxp3+ Treg ratio significantly reduced with increased tumor burden. These data are a representative of 5 experiments, each starting n=10 per group per experiment. * p < 0.05, and ns (not significant) represents p > 0.05
FACS analysis of tumors and dLN s on day 17 after tumor implants in MC38 tumor-bearing mice treated with CH223191 and vehicle, revealed that increased tumor burden was associated with a very modest, but insignificant increase in Foxp3+ Tregs in the tumors, although there was a significant decrease in the CD8a+ T cells with increased tumor burden in the antagonist treated mice. However there were no changes in the CD8a+ population in the dLN s. There was a significant decrease in the CD8a to Foxp3+ Treg ratio with increased tumor burden. There was no change in Ki67 staining in Foxp3+ Tregs and CD8a+ T cells from mice treated with the antagonist and the vehicle. This suggests that again, there is no increased proliferation of these two cell populations. The increased numbers seen in the tumor may have resulted from increased infiltration.

Reduced NK cell numbers were seen with increased tumor burden in the antagonist treated mice, which was measured by the NK cell marker, NK1.1.
Figure 41: A) NK1.1 cells reduce in numbers with increase in tumor burden. These data are a representative of 4 experiments, each with a starting n=10 per group per experiment.
Figure 42: A, B, C, D) No change in monocyte, neutrophil, CD11b DCs and CD103 DCs with increase in MC38 tumor burden in CH223191 treated mice. E) F4/80+ TAMs increase with increase in tumor burden. F) CD206 macrophages increase with increase in MC38 tumor burden. These data are a representative of 5 experiments with a starting n = 10 per experiment per group. *p < 0.05, and ns (not significant) represents p > 0.05
Figure 43: Surface expression of activated DC markers CD80 and CD86 showing no changes in the CH223191 treated group with increased MC38 tumor burden.

The myeloid populations in the MC38 tumors from mice treated with either CH223191 or vehicle were similar to that of AHR WT and KO mice: there were no changes in infiltrating Ly6C positive monocytes and DC subsets, represented by the cell surface markers CD103, CD11b, CD80 and CD86 (gated from MHC II high population) in the two groups of mice. However, there was a significant increase in the F4/80 positive tumor associated macrophages and the CD206 positive M2 macrophages. The tumor...
burden experiment involving mice treated with either CH223191 or vehicle was repeated 5 times with a starting n = 10 animals per group in each experiment.

3.21. Intratumoral cytokine and chemokine analysis

The tumors from AHR WT, AHRKO, vehicle and CH223191 treated mice were harvested in RPMI-1640 medium using the gentleMACSTM tissue grinder. The samples were then centrifuged, and supernatants flash frozen on dry ice and stored at - 80°C freezer for cytokine/chemokine analysis. These analyses was done on thawed samples using Millipore’s bead based Luminex assays and run on Magpix. Cytokines secreted by Th17 cells, namely IL17A, IL 17F, IL21 and IL22, were analyzed but due to low concentrations could not be plotted. Among other measurable cytokines, the levels of eotaxin a chemokine secreted by T cell types in response to IL-4 and IL-13 (secreted by Th2) cells, that helps mobilize eosinophils from bone marrow into the circulation was detectable.
Figure 44: Amount of intratumoral cytokines/chemokines compared between AHR WT vs AHR KO mice and Vehicle vs CH223191 treated mice. A) CD40L. B) Eotaxin, C) IFN-γ and D) RANTES (CCL5). Statistical analysis was done using unpaired student’s t test. * p < 0.05, **** p < 0.0001, and ns (not significant) represents p > 0.05
In the data shown in Fig.43, the levels of eotaxin were significantly lower for the AHR KO mice than the WT mice, although there were no difference in tumors from mice treated with vehicle and antagonist. The levels of IFN-γ and RANTES (CCL5) were significantly reduced with increased tumor burden in both groups.
Figure 45: Amount of intratumoral cytokines/chemokines compared between AHR WT vs AHR KO mice and Vehicle vs CH223191 treated mice. A) IL-1α. B) IL-1β. C) IL-6. D) IL-10. Statistical analysis was done using the unpaired student’s t test. * p < 0.05, ** p < 0.005, *** p < 0.0005, **** p < 0.0001, and ns (not significant) represents p > 0.05
Figure 46: Amount of intratumoral cytokines/chemokines compared between AHR WT vs AHR KO mice and Vehicle vs CH223191 treated mice. A) IL-12p70. B) IL-13. C) IP-10. D) MIG. Statistical analysis was done using the unpaired student’s t test.
Table 10: The amount of cytokines/chemokines (in pg/ml) in AHR WT vs AHR KO mice and vehicle (Veh) vs CH223191 (CH) treated mice. Statistical analysis was done using the unpaired student’s t test. * p < 0.05, ** p < 0.005, *** p < 0.0005, **** p < 0.0001, and ns (not significant) represents p > 0.05

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Based on the graphs in figures 43, 44 and 45 and table 10, there were no changes in the levels of IL-1α, IL-2, IL-13, IL-10, MIG (CXCL9) and VEGF. There was a significant increase in the pro-inflammatory cytokine IL-6; a reduction in IL-1β and TNF-α in the AHR KO and antagonist-treated mice as tumor burden increased. Conversely, there was a reduction in the amount of IL-10, an anti-inflammatory cytokine, in the AHR KO and antagonist-treated mice as tumor burden increased. IL-12 is a cytokine involved in the differentiation of Th1 cells, mainly secreted by the DCs, that
helps activate effector T cells and NK cells, is involved in the IFN-γ production and activation of anti-tumor immunity. The amount of IL-12p40 subunit cytokine was reduced in both groups with increased tumor burden but it was significantly reduced in the antagonist-treated group. The IL-12p70 subunit reduced in both treatment groups with increased tumor burden. RANTES/CCL5 is a chemoattractant for T cells, basophils and other leucocytes. CCL5 expression is known to increase in tumors, causes inflammation and apoptotic killing of CD8+ T cells.
CHAPTER 4: DISCUSSION

AHR is known to play a dual role in cancer progression and the tumor progression or suppression is related to the target cell phenotype. There are not many in vivo tumor studies analyzing the role of AHR in colon carcinogenesis. Although AHR (-/-) mice have an increased incidence of intestinal inflammation and colo-rectal tumors, this pathology could be reversed with treatment by AHR-agonists [38]. To elucidate the role of AHR in cancer, a mouse colon carcinoma syngeneic model was used. The following hypotheses were proposed based on AHR-research findings that, AHR activation by TCDD, results in increased Tregs and decreased T effectors such as CD8+ T, cells leading to tumor progression.

Hypothesis 1

T regulatory cells suppress anti-tumor immunity by inhibiting cytotoxic T lymphocytes (CD8 + T cells). Activation of AHR by agonists increased the differentiation of naïve T cells into T regulatory cells. Therefore activation of the AHR with an exogenous agonist in tumor-bearing mice should increase the tumor burden.

Hypothesis 2

If endogenous ligands help to maintain tumor surveillance to suppress tumor growth, the tumor burden should be lower in wild-type mice when compared to AHR KO mice.
Testing the hypothesis

MC38 and B16F10 syngeneic models were employed to explore the relevance of AHR in MC38 and B16F10 tumor progression. AHR activity in MC38 and B16F10 cells was assessed by measuring the expression of two AHR-responsive genes, *cyplal* and *cyp1bl*. *Cyplal* is the classic marker of this receptor that is expressed upon exposure to TCDD, a potent AHR agonist. In vitro tumor cells were exposed to 1nM TCDD and *cyplal* expression was determined at 0, 3, 6, 18 and 24 hour post treatment. In MC38 cells, *cyplal* expression was highest at the 3 hour time point and tapered down over 24 hours. Contrary to this, the expression in B16F10 cells was quite steady over 24 hours. The *cyplal* expression in MC38 cells is short lived and lower in comparison to that of B16F10.

After nuclear localization of ligand-bound AHR, a set of genes is induced, termed as the AHR gene battery. These genes co-induced as a result of AHR activation vary depending on the cell type and species [47]. TCDD activation of AHR led to altered expression of 379 genes in kidney and 471 in liver [48]. The expression of the following AHR battery genes, - *cyplal, la2, lb1, nqo, ahrr* and *ugt1a6* were measured in MC38 cells after exposure to 1nM TCDD for 24 hours and their expression was not detectable. Perhaps their expression like *cyplal* is for a shorter duration, within the 0 to 24 hour period. If so, then AHR gene battery is active for a very short period and AHR may not elicit normal functions in MC38 cells. Therefore, any changes in the tumor burden will likely be mediated by AHR from stromal cells and tumor infiltrating lymphocytes without contribution from MC38 cells.

It has been reported that AHR (-/-) mice are refractory to TCDD exposure and do not activate the AHR target genes [49].
TCDD, which is a high affinity AHR ligand, was employed in the MC38 tumor burden studies to activate the AHR pathway. TCDD was administered intraperitoneally at 1ug per mouse, one day prior to the MC38 implants. Because TCDD is a hydrophobic compound, it was dissolved in DMSO and administered with corn oil as the vehicle. The control group was DMSO in corn oil. No modulation in tumor burden was observed between the tumor bearing treatment and control groups of mice. T cell analysis by flow cytometry from spleens and tumors were done on day 14 after tumor implants. These revealed increased Foxp3+ Tregs, decreased CD8a: Treg ratio and CD4 effectors in spleen, and a decrease in CD8+ T cells in the tumors of dioxin-treated mice. Tregs are important mediators of immune suppression and peripheral tolerance, helping maintain homeostasis in the immune system which is essential to prevent auto-immunity and alleviate inflammation after infection or injury. Increased Tregs have been seen in tumors and immune suppression, tumor progression and poor prognoses of tumors have been attributed to increased Tregs [52]. Based on the increased Treg numbers by FACS, in spleen and not tumors is an indication that there is immune suppression in the mouse but not in the MC38 tumor environment.

This led to the question whether TCDD dose of 1ug/mouse was sufficient enough to cause immune-suppression in the mouse. The lipopolysaccharide (LPS) model was used to address this question [44]. Here, humoral responses are elicited by IgM- producing plasma cells in response to LPS (25ug per mouse, i.p. administration). LPS is a bacterial endotoxin, a component of the cell wall of gram negative bacteria, and is highly immunogenic. It activates innate immunity via the TLR4 pathway leading to increased activation of the transcription factors – NF-kB/AP-1. This leads to activation of humoral
immunity by increased antigen presentation through APCs. There was a 3-fold reduction in IgM producing plasma cells in the TCDD treated mice in comparison to the LPS alone and DMSO + LPS groups (Fig. 17). This was accompanied by a roughly 3-fold reduction in the CD19hi CD138hi cells in the TCDD-treated mice (Fig 18). The 1ug/mouse TCDD dose and IP route of administration causes immune suppression and this same dose did not modulate tumor burden.

Perhaps TCDD is not reaching the MC38 tumor and therefore increasing the TCDD dosing frequency was evaluated. One microgram of TCDD was administered IP on three days-7, 10 and 14 after MC38 implantations. Dosing mice three times resulted in a significant decrease in tumor volume by day 21 in the dioxin-treated group. It is assumed that the decreased tumor volume resulted from receptor agonism as this was not formally demonstrated herein.

As MC38 tumor volumes decreased with increased AHR agonism, tumor burden studies were done to further explore this observation. Tumor burden studies in AHR-deficient mice (AHR KO) and by pharmacologic blockade of the receptor were evaluated. AHR WT and AHR KO mice in the tumor study did not receive any therapeutic treatments. There was a nearly three-fold increase in the tumor burden in the AHR KO mice as compared to the AHR WT mice. To pharmacologically block the receptor, the MC38 tumor bearing mice were treated with 10 mg/kg of the CH223191 antagonist, administered orally daily, starting from day 7 until the end of the study. CH223191 is a small molecule antagonist of AHR, discovered from a random library screening campaign [15]. It is a pure antagonist in that concentrations up to 100 µM did not exhibit agonist-like activity or bind to other nuclear receptors such as the estrogen receptor. It brings about its antagonistic
function by blocking the binding of TCDD to AHR thereby preventing the TCDD-mediated nuclear translocation [15]. There was a significant increase in the tumor burden on day 17 (one-fold) and day 21 (1.5-fold) in mice that received CH223191 treatment. The data presented herein show the tumor burden increases in AHR-deficient mice and also when the receptor is pharmacologically blocked, suggesting a role for AHR in modulating MC38 tumor growth. Increased tumor growth with AHR antagonist is consistent with a role for endogenous ligands to maintain tumor burden under some level of control. Lack thereof allows tumors to grow faster. Increased tumor burden resulting from AHR blockade with CH223191 suggests the presence of endogenous AHR ligands that inhibits tumor growth. These endogenous ligands could be derived from a dietary source or produced by the animal.

Tumor studies were also performed on B16F10, a melanoma model to confirm the results seen in MC38 studies. There was a significant increase in the B16F10 tumor burden in AHR KO mice on day 16 after the implant. This is similar to that seen in MC38 tumors, where tumors grew faster in the absence of the receptor in AHR KO mice in comparison to WT mice. Increased B16F10 tumor growth in AHR KO mice was also reported by Sunwoo and colleagues [25]. The antagonist was administered daily at 10 mg/kg body weight, starting day 7 until the end of the study. No changes in tumor burden were seen in B16F10 model when the receptor was pharmacologically blocked by CH223191. This could be because the B16F10 tumors are resistant to treatment [53] and perhaps the antagonist dose was not sufficient to reduce tumor burden.

A comprehensive panel of TILs comprising of NK cells, T cell subsets and myeloid cell populations from AHR-deficient and AHR antagonist-treated mice along with their
wild type counterparts were analyzed by flow. The T cell, myeloid and NK cell gating strategy is shown in figures 33, 34 and 35. There was a reduction in total NK cell numbers and CD27+ activated NK cells (CD27 data with CH223191 treatment has not been shown here) with increased tumor burden. The data agree with those shown by Sunwoo et.al, [25] in that AHR was shown to be essential for the cytolytic activity of NK cells. This was manifested by an increased tumor burden in RMA-S mouse tumor model in AHR -/- mice. Analysis of the myeloid populations demonstrated that there were no changes in the infiltrating Ly6C + monocytes or neutrophils. Dendritic cells are the primary antigen presenting cells and there was also no change in the infiltrating DCs, the classical DCs (CD11b+), cross presenting DCs (CD103) and activated DCs (CD80+ and CD86+) with increased tumor burden. However, a significant increase in F4/80 positive tumor-associated macrophages (TAMs) and CD206 positive macrophages was seen. TAMs are most frequently found in the TME and express CD206, CD163 and CD68 as cell surface markers. These are differentiated myeloid populations with immune suppressive properties; they secrete anti-inflammatory cytokines like interleukin-10 (IL-10) and tumor growth factor-β (TGF-β). Macrophages can be re-programed; for example, treatment of macrophages in 4T1 and B16 tumor-bearing mice with antibodies to MARCO receptor, suppressed tumor growth [33]. The CD206 positive macrophages or M2 macrophages are thought to be anti-inflammatory, immune suppressive and promote angiogenesis and tumor growth [54]. Goudot et. al, have shown in in-vivo and in-vitro that AHR regulates monocytes to differentiate into monocyte-derived dendritic cells as opposed to monocyte macrophages via MAF B and IRF 4 [55]. In the data shown herein, increased tumor burden
In AHR KO and AHR WT-CH223191 mice led to increased F4/80 + TAMs and increased M2 macrophages.

In-vitro, monocytes can be differentiated into macrophages by stimulating them with M-CSF (macrophage-colony stimulating factor) and then polarizing them to either M1 or M2 macrophages. Use of IFN-γ leads them to differentiate into the M1 phenotype and IL-4 / IL-10 leads them to the M2 phenotype [54].

T cell analysis in tumors revealed a significant decrease in cytotoxic T lymphocytes (CD8a positive) and the CD8/Treg ratio with increase in tumor burden. There was a modest increase in the number of Treg with increase in tumor burden however this was not significant. One of the reasons for reduced Foxp3+ Tregs could be due to reduced kynurenine levels in the MC38 tumor microenvironment (TME). The kynurenine levels in the tumors were not measured but notably MC38 cells do not express IDO, the enzyme involved in the metabolism of tryptophan. Kynurenine is an endogenous AHR ligand produced by tryptophan metabolism and is known to mediate the differentiation of Treg cells [56]. The lack of difference in the Foxp3+ Treg numbers between the different treatment groups could also be due to their reduced infiltration into tumors. The proliferation of Foxp3 + Tregs and CD8a+ T cells was assessed using Ki67 (data not shown) proliferating marker. Ki67 is a cellular marker for proliferation, present in the G (1), S, G (2) and mitosis, active phases of the cell cycle, but absent in G (0). Therefore, only proliferating cells will express this marker. There was no change in the expression of Foxp3hi Ki67hi and CD8a hi Ki67hi in AHR WT versus KO and in AHRWT+/− CH223191 mice. This suggests that the reduced cytotoxic lymphocytes is due to reduced infiltration of these cells into the tumor environment and not due to reduced proliferation. The role
AHR plays in CD8 positive cytotoxic lymphocytes is not very well understood. Traditionally CD8+ TILs recognize the tumor antigens and mediate the killing of tumor cells through the catalytic action of secreted cytotoxins, such as perforin, granzyme B and granulysin. But, this has proved to be controversial as they are known to switch roles and become either tumor promoters or suppressors [34]. These molecules were not evaluated in the studies described herein. From human tumors, CD8+ TILs have been found to be functionally inactive by virtue of reduced proliferation and secretion of less IFN-γ [57]. CD8+ populations in tumors are known to exhibit exhaustion, anergy and senescence phenotypes [58], rendering them incapable of immune surveillance. The CD8+ TILs from AHR KO and AHR WT-CH223191 treated MC38 tumor bearing mice need to be further characterized to better understand their phenotype.

Intratumoral cytokine analysis of the MC38 tumor microenvironment from AHR KO and CH223191 treated wild-type mice, revealed an increase in pro-inflammatory cytokine interleukin -6 (IL-6) and a reduction of tumor necrosis factor – α (TNF-α) and interleukin-1β (IL-1β) with increased tumor burden. There was a reduction in the amount of interleukin-10 (IL-10), an anti-inflammatory cytokine, in the AHR KO and antagonist treated mice as tumor burden increased. The levels of interleukin-10 are usually increased in the TME with increase in tumor burden [33]. But reduced IFN-γ and IL-10 levels have been seen in CH223191 antagonist treated lymphokine activated killer cells or NK cells. Also, NK cells from AHR(-/-) mice exhibited a defect in IL-10 expression with T.gondii infection [59]. Therefore, reduced IL-10 and IFN-γ levels could be a result of reduction or lack of AHR activity. The reduced IFN-γ levels in the absence of AHR agrees with that reported by Sunwoo et al, [25], as well as increased tumor burden with reduced AHR
mediated NK cell activity. IL-12 is a cytokine that is involved in the differentiation of Th1
cells, mainly secreted by the DCs, which helps activate effector T cells and NK cells, and
is involved in the IFN-γ production and activation of anti-tumor immunity. The amount of
IL-12p40 subunit cytokine does not change in the AHR WT vs AHR KO mice, however
the levels are significantly lower in the CH223191 treated group with increased tumor
burden. IL-12p70 subunit was reduced in both treatment groups with increased tumor
burden. The role AHR plays in IL-12 secretion is not well understood, although high levels
of IL-12 have been reported in AHR null mice when splenocytes were stimulated with ova
peptide [60]. RANTES/CCL5 is a chemoattractant for T cells, basophils and other
leucocytes. CCL5 expression is known to increase in tumors, causes inflammation and
apoptotic killing of CD8 + T cells [61]. Reduced CCL5 levels with increased AHR-
mediated MC38 tumor burden were observed. The role of AHR in mediating CCL5
expression in the context of the tumor environment is not well understood, although AHR
ligands (FICZ and BaP) reduced CCL5 mRNA and protein levels in the HaCaT (human
keratinocyte) cell line [62]. Cytokines secreted by Th17 cells, namely IL17A, IL 17F, IL21
and IL22 were also evaluated but were out of range of the standard curve and could not be
plotted. Levels of Eotaxin or CCL11 were found to be significantly reduced in the AHR
KO mice. There were no changes in their levels in the tumors of AHR +/- CH223191
treated mice. CCL11 is a chemokine that helps in the mobilization of eosinophils. There
were no changes in the levels of IL-13, IL-15, VEGF-A, IP10 (CXCL-10) and MIG
(CXCL9) with increased tumor burden.

The data herein show a a pro-inflammatory tumor environment with increased IL-
6 levels and a reduced IL-10 and IFN-γ levels with increased tumor burden. A chronic pro-
inflammatory environment is thought to result in an increase in free radicals, hence DNA damage and mutations, leading to tumor initiation and or progression.

AHR is expressed in the intraepithelial lymphocytes (IELs) and innate lymphoid cells (ILCs) along with AHR ligands derived from diet, are essential for maintaining an intact gut lining. The lumen of the intestine is comprised of many species of commensal bacteria in a symbiotic relationship with its host. The gut’s immune system has a challenging role in maintaining a barrier by providing protection to the host and at the same time maintaining tolerance towards these beneficial bacteria [12]. Loss of ILC3, IELs, IL-22 and dysregulation of intestinal bacteria has been observed in the absence of AHR. AHR KO mice also have reduced peyer’s patches, compromised gut biota and are prone to intestinal infections [63]. As microbiota of the gut plays a crucial role in shaping systemic immune responses; manipulating it could be used to modulate cancer immunotherapy [64]. The microbiota of MC38 tumor bearing AHR KO and CH223191-treated wild-type mice were not evaluated in studies reported herein. Experiments involving modulating of the gut microbiota would be interesting to do in the future.

**Conclusion:** Based on the results described here, AHR plays a crucial role in the MC38 tumor burden, the absence of which leads to tumor progression by reducing the infiltration of cytotoxic T lymphocytes and recruitment of F4/80 + tumor associated macrophages (TAMs6) and CD206 + M2 macrophages.
CHAPTER 5: REFERENCES


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