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The Role of B Cell Activation State and Sex in Aryl Hydrocarbon Receptor Mediated Induction of Chemokine Receptor 9 and Alpha4Beta7 Expression in Vitro

Logan Bauerle
University of Northern Colorado, rantore@gmail.com

UNIVERSITY OF NORTHERN COLORADO

Greeley, Colorado

The Graduate School

THE ROLE OF B CELL ACTIVATION STATE AND SEX IN ARYL HYDROCARBON RECEPTOR MEDIATED INDUCTION OF CHEMOKINE RECEPTOR 9 AND ALPHA4BETA7 EXPRESSION IN VITRO

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Accepted by the Thesis Committee:

_____

Gregory DeKrey, Ph.D, Chair

_____

Patrick Burns, Ph.D, Committee Member

_____

Nicholas Pullen, Ph.D, Committee Member

_____

Mark Thomas, Ph.D, Committee Member

Accepted by the Graduate School

_____

Jeri-Anne Lyons, Ph.D.
Dean of the Graduate School
Associate Vice President for Research

ABSTRACT

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Defense of mucosal tissues from microbial infection and allergy is reliant on continual production of antibodies. The aryl hydrocarbon receptor (AhR) is known to regulate B cell development and is associated with suppression of systemic humoral immunity. Recent attention has been paid to the role of the AhR in altering expression of cell adhesion molecules (CAMs). B cells express CAMs and chemokine receptors to migrate around the body for localized secretion of antibodies. AhR agonists promote B cell migration to the small intestine through upregulation of chemokine receptor 9 (CCR9) and integrin $\alpha_4\beta_7$. Both the AhR and CAMs are considered as potential therapeutic targets for diseases ranging from inflammatory disorders to autoimmune diseases.

To date, knowledge of AhR control of B cell development and expression of CAMs remains incomplete. Important factors such as state of B cell differentiation and sex have yet to be incorporated into studies on AhR regulation of CAMs. The aim of the current study was to examine how these factors might influence AhR mediated alterations in CCR9 and integrin $\alpha_4\beta_7$ expression by B cells *in vitro*. Here, we report that B cells are sensitive to gut imprinting via the AhR only if AhR and immune activation occur within the first hour of a 24-hour period. Additionally, we observed that B cells derived from females displayed enhanced sensitivity to AhR mediated expression of gut associated migration markers compared to males. These results

together suggest that the AhR exerts control over humoral responses in part through differential regulation of trafficking programs in distinct B cell subsets and in a sex-dependent manner.

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LIST OF TERMINOLOGY

Abbreviation

Ab	Antibody
Ag	Antigen
AhR	Aryl hydrocarbon receptor
AID	Activation-induced cytidine deaminase
AKT	Protein kinase B
ANOVA	Analysis of Variance
APC	Antigen-presenting cell
APRIL	A proliferation inducing enzyme
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASC	Antibody secreting cell
BAFF	B cell activating factor
BCL	B cell lymphoma
BCR	B cell receptor
BLIMP	B-lymphocyte induced maturation protein
BTK	Bruton tyrosine kinase
C region	Constant region
CAM	Cell adhesion molecule
CCL	Chemokine ligand
CCR	C-C chemokine receptor type
CD	Crohn's disease
CD(#)	Cluster of differentiation
CH223191	1-Methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phen yl-1H-pyrazole-5-carboxamide, 2-Methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide
CLA	Cutaneous lymphocyte associated antigen
CM	Complete cell medium
CSR	Class-switch recombination
CXC	C-X-C chemokine receptor type
CXCL	C-X-C chemokine receptor ligand type
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum

Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FcR	Fragment crystallizable receptor
FSC	Forward scatter
GC	Germinal center
GI	Gastrointestinal
IBD	Inflammatory bowel disease
ICOS	Inducible T cell costimulator
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
J chain	Joining chain
KLF	Krüppel-like factor
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MALT	Mucosa-associated lymphoid tissue
MCN	Median channel number
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MZ	Marginal zone
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMPs	Pathogen-associated molecular patterns
pIgR	Polymeric immunoglobulin receptor
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
RAR	Retinoic acid receptor
RPMI	Roswell Park Memorial Institute
S1P	Sphingosine-1-phosphate
SHM	Somatic hypermutation
SSC	Side scatter
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
T_{FH}	Follicular helper T cell
TGF	Transforming growth factor
T_H	T helper cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UC	Ulcerative colitis

V region	Variable region
VCAM-1	Vascular cell adhesion protein 1
xg	Times gravitational force
XRE	Xenobiotic response element

CHAPTER I

INTRODUCTION

In the United States, an estimated 1.3% of the population was diagnosed with inflammatory bowel disease (IBD) in 2015, translating to nearly 3 million cases (Centers for Disease Control and Prevention, 2023). IBD is caused by chronic inflammation of the gastrointestinal (GI) tract, common symptoms of which include diarrhea, abdominal pain, weight loss, fatigue, and GI associated bleeding. IBD encompasses both Crohn's disease (CD) and ulcerative colitis (UC), the former of which is characterized by inflammation of multiple layers of the GI wall and occurs predominately in the small intestine, and the latter of which primarily involves pathology of the large intestine. In either condition, prolonged inflammation can result in permanent GI damage which may necessitate surgical removal of portions of the GI tract (Centers for Disease Control and Prevention, 2023). IBD is suspected to result from an inappropriate inflammatory response to bacteria or viruses by immune cells of the GI tract (Centers for Disease Control and Prevention, 2023).

Regulation of GI microbial populations is heavily dependent on the secretion of antibodies (Ab) by B cells located in the GI tract (Brandtzaeg et al., 1999). Inadequate production of secretory Ab enhances susceptibility to GI infections and may induce persistent inflammatory responses to members of the gut microbiota (Wood, 2011). Thus, IBD has been proposed to result from dysregulation of B cell functions in the context of the GI system (Bamias et al., 2023). B cells and other immune cells are recruited to the GI tract from circulation in the

blood through interaction of receptor proteins with binding partners produced within target tissues in a process called tissue homing (Lycke & Bemark, 2017). Recently, IBD therapies have employed the use of biologics that interfere with the protein interactions that mediate immune cell gut homing to prevent migration of pro-inflammatory immune cells from circulation. An example of this is the Ab, Vedolizumab, which suppresses immune cell gut homing by blocking receptor binding sites on integrin $\alpha_4\beta_7$. While effective in mitigating inflammation, this type of therapy also inhibits recruitment of anti-inflammatory B cells and may compromise resistance to GI infections (Bamias et al., 2023).

Another approach to treat IBD has involved the use of immunomodulatory drugs intended to suppress inflammatory responses, such as corticosteroids (Centers for Disease Control and Prevention, 2023). Other known immunosuppressants are currently being considered as potential therapies for IBD, including those targeting the aryl hydrocarbon receptor (AhR) (Esser & Rannug, 2015). Due to the role of AhR in regulating the functions of many cell types, including immune cells, AhR ligands are being explored as potential therapeutics or preventatives for diseases ranging from inflammatory or autoimmune conditions to cancer and metabolic disorders (Rothhammer & Quintana, 2019). Interestingly, recent studies have demonstrated that AhR can promote expression of proteins involved in immune cells homing to the tissues of the gut, including B cell expression of integrin $\alpha_4\beta_7$ (Lycke & Bemark, 2017; Ortiz, 2021). While immunomodulation by the AhR may provide therapeutic benefit in certain contexts, characterization of the influences on AhR control of tissue homing protein expression by B cells could better inform treatment recommendations in conditions such as IBD (Sondermann et al., 2023).

CHAPTER II

REVIEW OF THE LITERATURE

Overview of Immunity

Pathogens, which are ubiquitous in the environment, must be controlled to prevent potentially lethal or debilitating infections in animals (Lycke & Bemark, 2017). Organisms have developed specialized biological components to defend against illness and infections. This defensive system is commonly referred to as the immune system, and its primary role is to prevent or resolve pathogenic infections, remove foreign materials, neutralize toxins, and heal injuries. The immune system consists of anatomical or physical barriers to infection, specialized cells mostly derived from the hematopoietic lineage (leukocytes), and membrane bound or soluble proteins. A considerable portion of the immune system's functions are determined by inherited genetic code, are static throughout life, and comprise what is known as the innate immune system. The innate immune system serves as the first line of defense against pathogens, and successfully prevents most potential infections (Wood, 2011). When infection occurs, the innate immune system detects the presence of the pathogen and initiates a response. Tissue resident cells of the innate immune system become activated, which promotes their ability to clear pathogens and recruit additional body defenses. If the initial response is insufficient to remove the insult and heal injuries, the other branch of the immune system, known as adaptive immunity, is stimulated by components of the innate immune system to assist in clearing the infection. The adaptive immune system has the unique ability to recognize a large variety of

pathogen derived molecules and can confer resistance to infections that the organisms was initially susceptible to upon subsequent exposures. Once an infection is cleared or if the immune response is ineffective, immune cell activity shifts to promote tissue preservation and healing (Murphy et al., 2019).

Innate Immunity

Barriers to Infection

For an infection to be established, pathogens must first penetrate physical and chemical barriers to colonize body tissues. Intact skin and epithelial tissues provide an important boundary between the body and the environment. If this barrier is compromised by injury or disease, other immune mechanisms may not be able to compensate (Wood, 2011). This is demonstrated by the high susceptibility to infection associated with major burns (Turvey & Broide, 2010). Additionally, all body surfaces contain a diverse flora of microorganisms that are typically nonpathogenic. These microbes can compete with more pathogenic microorganisms for resources and can produce molecules capable of modulating cellular activity in other species, potentially inhibiting infection in the host organism. Pathogens that are unable to mount infections at or penetrate epithelial surfaces are eventually shed from the body during epithelial cell turnover (Murphy et al., 2019)

Bodily secretions can also be used to defend against infection in a variety of ways. Mucosal tissues produce mucus to maintain epithelial cell health, trap microbes, and prevent colonization. Sebaceous glands in the skin secrete oils and other products that help maintain barrier integrity and establish a slightly acidic pH to inhibit microbial growth. Similarly, gastric hydrolases, acids, and bile salts synergize to degrade ingested foods and pathogens alike. Antimicrobial peptides are common constituents of many bodily secretions. Defensin protein

released into the gastrointestinal (GI) and respiratory tract by epithelial or activated immune cells can interfere with microbial plasma membranes or metabolism. Lysozyme secreted in tears and saliva catalyzes hydrolysis of peptidoglycan, resulting in lysis of gram-positive bacteria (Wood, 2011).

Several bodily functions serve to expel pathogens from the body and clean tissue surfaces. The flushing of the conjunctiva through the secretion of tears, the urethra with urine, and the ciliary-mucus escalator of the upper respiratory tract all serve to remove pathogens and other debris from sensitive tissues. Peristaltic contractions of smooth muscle in the GI tract are important for waste secretion and regulation of the gut microflora in homeostatic conditions and can be exaggerated under disease states to promote pathogen excretion in diarrhea. Similarly, noxious stimuli can induce reflexes such as coughing, sneezing, and vomiting for rapid expulsion of pathogens and toxins from the respiratory and GI tracts (Murphy et al., 2019).

Complement

A set of functionally related proteins found in blood and other extracellular fluids are crucial secondary defenses against infiltrating pathogens. Known as the complement system, these proteins consist of a collection of inactive proenzymes produced by the liver that are activated by a series of hydrolytic events. Some complement proteins are hydrolases that serve to activate other components in the cascade, while others perform effector functions by interacting with cell surfaces. The complement cascade can be activated by receptor mediated recognition of foreign molecules, or spontaneous hydrolysis of unstable complement proteins. Inhibitors to complement activation are expressed on healthy self-cell membranes to regulate complement activity and limit autoimmunity (Wood, 2011).

Aggregation of active complement protein complexes on cellular membranes, called complement fixation, functions to promote elimination of pathogens or damaged cells in a variety of ways. Cells and debris coated with complement proteins, specifically protein C3b, can be recognized by phagocytic cells for absorption and degradation. Some products of the cascade remain soluble and act as signaling molecules that promote immune cell activation and recruitment to sites of injury and infection. The final step in the cascade involves polymerization of complement component C9 to form a tunnel through plasma membranes which facilitates targeted destruction through osmotic lysis (Murphy et al., 2019).

Danger Recognition

For immune cells to respond to bodily threats, they must first recognize the presence of danger. Some pathogens can replicate rapidly or produce lethal toxins, so the immune system must remain poised to quickly respond to such threats (Wood, 2011). Pattern recognition receptors (PRRs) are expressed by immune and other cells to detect threats to the organism. Some PRRs function in recognition of cellular damage by binding to molecules associated with cellular stress or lysis, called damage-associated molecular patterns (DAMPs). These can be produced by injury or infection and are often mediated by release of internal cellular components such as heat shock proteins. Other PRRs recognize molecules derived from pathogens that are not expressed in the host organism, called pathogen-associated molecular patterns (PAMPs) (Turvey & Broide, 2010; Wood, 2011).

PRR engagement with DAMPs/PAMPs leads to cellular signaling that promotes immune cell responses, pathogen elimination, and wound healing. Recognition of injury and infection in this way results in activation of defensive responses in epithelial and immune cells. Toll-like receptor 4 (TLR4), for example, is a PRR that can bind to lipopolysaccharide (LPS) molecules

derived from gram negative bacteria. Cellular signaling following TLR4 recognition of LPS results in activation of various immune cells and the release of inflammatory mediators for recruitment of additional soluble and cellular defenses (Murphy et al., 2019). TLR4 is thus important for providing immunity to bacterial infection, but aberrant TLR4 functioning has been implicated in inducing immune related pathologies such as asthma, COPD, and atherosclerosis (Lucas & Maes, 2013).

Leukocyte Migration

Leukocytic progenitors reside in the bone marrow in adults. Most leukocytes must travel to distant tissues to perform effector functions or continue development. Leukocytes use the bloodstream to travel from the bone marrow to distant tissue sites. Transit between the bloodstream to peripheral tissues or sites of inflammation is mediated by cellular penetration of the capillary wall (Murphy et al., 2019). This process requires the release of chemoattractant molecules called chemokines from the target site. Activation of innate immune responses results in localized inflammation through activation of endothelial cells (prostaglandins, IL-1, TNF) and the release of chemoattractant such as IL-8 and the complement proteins C3a and C5a. Inflammation leads to endothelial cytoskeletal remodeling and expression of cell adhesion molecules (CAMs) to facilitate leukocyte attachment to the capillary wall. For example, the CAM P-selectin binds to P-selectin glycoprotein ligand-1 (PSGL-1) expressed by leukocytes. Low affinity interactions between leukocyte and endothelial CAMs result in the leukocyte rolling along the endothelial wall. As the concentration of chemokines increases, expression of additional CAMs is induced through chemokine receptor signaling to provide for high affinity interactions. Leukocytes that bind to endothelium tightly using these high affinity CAM interactions are removed from circulation and extend their plasma between adjacent endothelial

cells to facilitate tissue entry. The leukocyte then continues to follow the chemokine concentration gradient within the tissue to reach sites of injury or infection (Wood, 2011). Leukocyte trafficking can thus be controlled by contextual regulation of chemokine and CAM binding partners in both leukocytes and specific body tissues (Seong et al., 2017).

Innate Responses

Recognition of infection, injury, or disease occurs through PRRs and other receptors. Immune and other cells rapidly produce cytokines to coordinate responses following threat recognition. These include various interferons (IFN), interleukins (IL), chemokines, and members of the transforming growth factor (TGF) and tumor necrosis factor (TNF) superfamilies (Wood, 2011). Cytokines influence immune cell differentiation, activation, and effector functions to promote generation of an effective response. Epithelial, endothelial, or tissue resident so called sentry cells such as macrophages and mast cells are often the first cells to respond to injury or infection. The functions of many cytokines act antagonistically to specifically tailor the nature of the response. Some cytokines regulate endothelial cell functions to modulate inflammation. These include the pro inflammatory cytokines ILs-1, 12, 18, 23, TNF, IFN, and anti-inflammatory IL-10. Inflammation is regulated in this way to allow diversion of soluble and cell based immune defenses to sites of infection or injury while mitigating the potential for inflammatory pathology (Murphy et al., 2019).

Innate immune cell activation occurs through PRR binding to PAMPs or DAMPs and integration of cytokine signaling. Phagocytic cells such as macrophages, neutrophils, and dendritic cells use membrane bound PRRs to recognize foreign or damaged debris in preparation for endocytosis and degradation of these materials. Granulocytes, which include mast cells, basophils, eosinophils, and again neutrophils release inflammatory mediators, microbial growth

inhibitors, and toxins following their activation by PRRs. Natural killer cells mediate killing of self-cells through receptor interactions that promote apoptosis in cancerous or virally infected cells (Wood, 2011). A subset of B cells, known as B1 cells, perform innate as opposed to adaptive immune functions. These B1 cells constantly produce antibodies to highly repetitive molecular sequences such as microbial polysaccharides to aid in pathogen resistance (Haas et al., 2005).

Antigen Presentation

Antigens (Ag) are molecules capable of stimulating an adaptive immune response. Special proteins called major histocompatibility complex (MHC) proteins are used to present bound antigens to immune cells to communicate the presence of infection or disease. MHC class I is used by nucleated cells to present peptide fragments of cytosolic origin to cytotoxic T cells and natural killer cells. The nature of this interaction can indicate the presence of intracellular pathogens, cancerous transformation, and cellular damage. When sufficiently stimulated, cytotoxic cells induce apoptosis in target cells based on the nature of class I MHC-Ag complex and other receptor interactions (Wood, 2011). MHC class II proteins similarly function in presentation of antigens derived from the extracellular environment. This protein is expressed exclusively by what are known as professional antigen-presenting cells (APC), which includes macrophages, dendritic cells, and B cells. Endocytosis and lysosomal degradation of extracellular materials by these cells results in generation of antigens that are presented on class II MHC. These MHC-antigen complexes are crucial for the generation of adaptive immune responses by helper type T cells, which help to direct responses by other immune cells (Murphy et al., 2019).

Adaptive Immunity

Lymphocytes arise from hematopoietic stem cell progenitors and play roles in both innate and adaptive immunity. Natural killer cells, B1 cells, and intraepithelial lymphocytes (IEL) provide immunity but don't exhibit memory responses and are thus considered to have innate functions. These cells provide a similar response upon reinfection with the same pathogen (Wood, 2011). Whereas conventional T and B lineage lymphocytes can produce antigen receptor proteins with variable amino acid sequences and thus unique binding specificities. Initial exposure to a pathogen leads to preservation of lymphocytes capable of recognizing antigens associated with that infection. Re-exposure to the same pathogen can thus stimulate proliferation of these remnant lymphocyte populations, called memory cells, generated upon the initial infection for a more robust and rapid response to secondary infections (Murphy et al., 2019). This is what is meant by the term immunological memory and is used to distinguish the adaptive and innate divisions of the immune system (Martin & Kearney, 2000). Proliferation of populations of lymphocytes that recognize specific antigens is called clonal expansion and is important for timely and effective immune responses to novel and previously encountered infections alike (Wood, 2011).

T Lymphocytes

T lymphocytes begin development in the bone marrow but must travel to the thymus to complete their maturation. Rearrangement of amino acid sequences coding for portions of T cell receptors (TCR) provides each T cell a unique antigen binding specificity. The TCR is used to bind antigens presented on MHC molecules and is necessary for T cell activation and differentiation into effector cells. Importantly, T cells must interact with both the antigen and proper MHC class for activation to occur. Thus, CD8 and CD4 co-receptor proteins, which

recognize MHC class II and class I proteins respectively, are initially co expressed by T cells in the thymus. T cells that recognize MHC I-Ag complexes retain CD8 expression and develop into cytotoxic T cells, while those that recognize MHC II-Ag complexes retain CD4 expression and develop into T helper cells (T_H). Antigens presented on thymic MHC are of self-origin, so developing T cells which bind to MHC-Ag complex too strongly are eliminated by apoptosis in a process called negative selection. T cells which are unable to bind to MHC-Ag or that produce nonfunctional TCRs during genetic rearrangement arrest development and undergo apoptosis. Minimal T cell affinity for MHC-Ag complex in the thymus results in positive selection or survival of the T cell which then travels to secondary lymphoid tissues such as the spleen, lymph nodes, and mucosal lymphoid tissues for subsequent antigen encounter and activation (Murphy et al., 2019).

T Cell Differentiation

Mature T cells circulate between blood and secondary lymphoid tissues in search of antigen encounters with APCs. Antigens can be brought directly into some lymphoid tissues along with extracellular fluids where APCs can present them on MHC following endocytosis. Professional APCs sample antigen in the periphery, particularly in the skin and mucosa where risk of infection is high, and traffic to lymphoid tissues for antigen presentation to T cells if activated. T cell recognition of MHC-Ag complex on APCs through TCR and CD4/CD8 coreceptors initiates formation of an immunological synapse. T cell receptors and integrins bind with corresponding receptors on the APC to stabilize the interaction and mediate activation signaling. Activated T cells undergo proliferation and differentiation into effector cells. If TCR MHC interactions are weak, or regulatory molecules called costimulatory receptors aren’t

expressed by the APC, the T cell will not receive adequate stimulation for survival and activation (Wood, 2011).

T cells that recognize antigens in the context of MHC class I develop into cytotoxic T cells for participation in adaptive, cell-mediated cytotoxicity responses. These T cells are effective against intracellular pathogens and cancers. Those T cells that instead recognize antigen presented by professional APCs on class II MHC develop into helper T cells. Various subsets of helper T cells are generated according to the cytokine environment during T cell activation (Murphy et al., 2019). Each type of helper T cell itself produces a unique repertoire of cytokines and displays specialized functions. T_H1 type helper T cells promote antiviral responses through production of IFN-γ, T_H2 cells assist with clearing helminth infections and produce IL-4 and IL-13, while T_H17 cells are effective against extracellular microbes and can induce inflammatory responses by releasing IL-17 and TNF-α. Follicular helper T cells (T_{FH}) are helper T cells that specialize in assisting B cell development into long lived antibody secreting or memory cells in secondary lymphoid tissues. T_{FH} cell recognition of Ag presented by B cells leads to expression of costimulatory receptors and cytokines which regulate B cell proliferation and maturation through the formation of structures called germinal centers (Wood, 2011). Regulatory T cells are also generated which inhibit T cell activities and control autoimmunity or pathologic inflammation through release of immunosuppressive cytokines such as IL-10 and TGF-β (Bonilla & Oettgen, 2010).

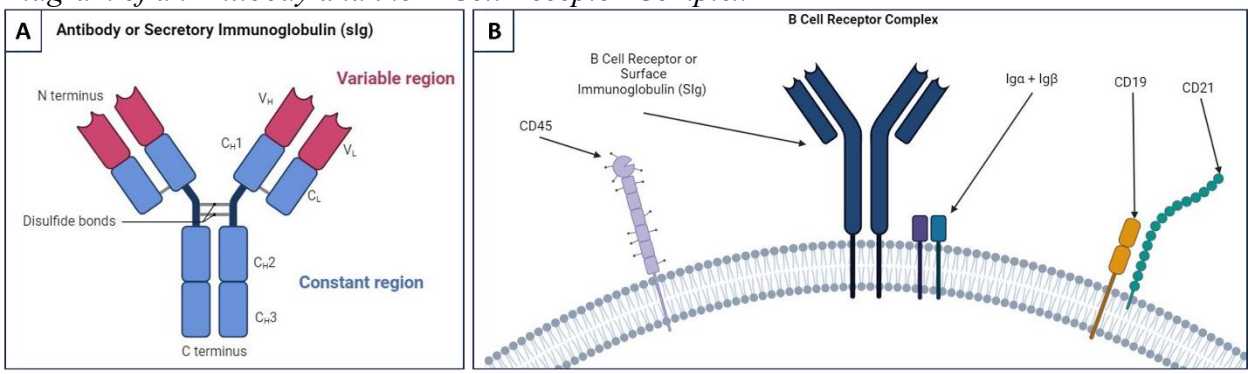
B Lymphocytes

B cells are important immune cells due to their ability to bind antigens using the B cell receptor (BCR). The BCR is used to endocytose encountered antigens for presentation to T cells, making B cells professional APCs capable of activating helper T cells. Additionally, alternative

mRNA processing can result in removal of hydrophobic BCR domains used to anchor the BCR in the plasma membrane, so it can instead be secreted as an antibody (Ab) protein (Wood, 2011). Antibodies secreted in this way form a critical component of humoral immunity, as demonstrated by the prevalence toward immunodeficiencies and autoimmunity associated with various B cell defects (Pieper et al., 2013).

Figure 1

Diagram of an Antibody and the B Cell Receptor Complex



Note. A. Antibodies consist of heavy (H) and light (L) protein chains which contain areas of high (variable; V) or low (constant; C) amino acid sequence diversity. Binding to antigen is determined by the V region and is important for pathogen clearance and initiating immune responses. The C region is used to bind Fc receptors following Ag recognition and can be further modified through addition of proteins or carbohydrates (Wood, 2011). B. The constant region of antibodies can include hydrophobic domains which anchor exported immunoglobulins to B cell plasma membranes. Antibodies anchored in this way are called the B cell receptor (BCR) and interact with other membrane proteins to form the B cell receptor complex. Intracellular signaling through the BCR complex is essential for contextual regulation of B cell development and survival (Bradbury et al., 1992; Wang & Clark, 2003).

B cells can be divided into the B1 and B2 subset. B1 cells produce antibody proteins capable of binding to multiple antigens with low affinity, including some of self-origin. B1 cells are the first mature B cells produced after birth, arise from fetal liver, are replenished in the periphery, and constantly produce antibodies (Rothstein et al., 2013). B1 cells display an inability to enhance BCR specificity in a process called somatic hypermutation (SHM) but have been found to offer protection against bacterial infections such as those caused by *Streptococcus*

pneumoniae (Haas et al., 2005). Additionally, recognition of damaged cellular components by B1 cell antibodies may promote their rapid removal by phagocytes and inhibit generation of autoimmunity (Rothstein et al., 2013).

Immunoglobulins

Antibody production is the primary function of B cells. Ab functions are mediated by their ability to bind to specific molecular sequences and promote pathogen elimination. Ab binding to target molecular sequences can neutralize toxins, inhibit microbial replication, activate complement, and enhance phagocytic activity (opsonization) (Wood, 2011). Major deficiencies in antibody production or B cell development result in primary immunodeficiencies and tendency toward autoimmunity. An appreciation of BCR/antibody structure is thus key to understanding B cell related influences on health and disease (Pieper et al., 2013).

Antibodies or BCR proteins are iconic members of the immunoglobulin protein superfamily and are often simply called immunoglobulins (Ig). Igs consist of heterotetrameric proteins composed of two identical heavy chain proteins bound to two identical light chain proteins. Both heavy and light chain proteins wrap around each other and form a y-shaped overall globular structure. Noncovalent bonds in combination with both intra and inter chain disulfide bonds hold this structure together. The apical arms of the y shaped Ig structure contain portions of the V region that facilitate antigen binding and thus are called fragment antigen-binding regions (Fab). The basal end of Ig structure is made solely of the constant regions of the two heavy chains and is called the fragment crystallizable (Fc) region. This Fc region contains domains that anchor BCR to the plasma membrane and mediates antibody binding with receptors on granulocytes and phagocytes (Murphy et al., 2019).

Each heavy and light chain contains regions of high amino acid sequence variability (V or variable regions) and those of relatively conserved amino acid sequence (C or constant regions) which provide Ig structure and signaling properties. The V regions display tremendous amino acid diversity due to unique gene rearrangement mechanisms that occur during B cell development. Heavy chain Ig proteins are encoded by multiple genes and can be used to construct different classes or isotypes of Igs. The various classes of antibodies have distinct effector functions and tissue distributions (Wood, 2011).

B Cell Development

Lymphocytes commit to the B cell lineage in the fetal liver or bone marrow. Stages of early B cell development are defined by key steps required for expressing functional BCRs. Rearrangement of gene segments coding for the V regions of Ab heavy and light chain proteins provide B cells the ability to recognize a staggering diversity of antigens but must be controlled to prevent production of auto-reactive Ab and resulting autoimmunity (Wood, 2011).

The first stage in B cell development is called the Pro B cell and involves construction of the heavy Ig chain. Specific DNA sequences coding for the V region, called V, D, and J gene segments, are first randomly selected from multiple versions. These are joined together and with the region coding for the C region of the heavy chain protein, with intervening DNA sequences being removed. These gene rearrangement mechanisms are imperfect and can introduce additional sequence variation at adjoining sites, possibly leading to production of dysfunctional heavy chain proteins. Functional heavy chain proteins formed following DNA rearrangement bind with surrogate light chain proteins to form the so-called pre-BCR (Wood, 2011). Pre-BCR mediated signaling terminates heavy chain rearrangements and induces rearrangement of the light chain gene, leading to entry into the next phase of B cell development (Melchers, 2015).

At the Pre-B cell stage, rearrangement of light chain genes occurs in a manner like that for the heavy chain. However, two versions of the light chain gene exist in the genome, kappa (κ) and lambda (λ). Only one of these light chain genes will be incorporated in Igs, with recombination machinery displaying a preference for κ chain gene rearrangement (Liu et al., 1997). Due to the absence of D gene segments in light chain genes, only V and J gene segments are randomly selected to construct the region of DNA encoding the V region. Selected VJ gene segments are first adjoined together and then combined with the region encoding the C region of the light chain with removal of the intervening DNA. Expression of functional heavy and light chain genes leads to production of BCRs and entry into the immature B cell stage of development. Immature B cells then enter the transitional stage, characterized by migration to the spleen (Murphy et al., 2019). There, transitional B cells finalize development and differentiate into marginal zone (MZ), naïve, or follicular B cells (Pieper et al., 2013).

B Cell Differentiation

Fully developed B cells circulate between the blood and secondary lymphoid organs to facilitate encounter with antigens recognized by the BCR. These sites include the spleen, lymph nodes, Peyer's patches in the GI tract, and mucosa-associated lymphoid tissues (MALT). BCR binding with high affinity to soluble antigens or those presented by APCs in secondary lymphoid organs induce BCR and BCR coreceptor signaling events that result in B cell activation (Wood, 2011). BCR coreceptor proteins include Igα, Igβ, and CD19, which modulate BCR activity and are thus important regulators of B cell proliferation and differentiation (Smith & Cunningham-Rundles, 2019; Wang et al., 2012). Activated B cells proliferate and differentiate into effector cells, travel to lymph germinal centers to continue maturation, or are removed by apoptosis (clonal deletion) depending on environmental stimuli (Murphy et al., 2019).

B cells that are sufficiently activated by BCR and other signals undergo several rounds of proliferation called clonal expansion. Progeny resulting from this process are clones of the original B cell and initially express identical Igs and thus recognize the same Ag. This enables some B cells to rapidly differentiate into short lived antibody secreting cells while others develop into follicular B cells which travel to lymph follicles to undergo further maturation. There, follicular B cells present Ag scavenged by endocytosis of BCR-Ag complexes to T_{FH} cells. T_{FH} cells that recognize this antigen through TCR and MHC II interactions can stimulate the B cell to continue clonal expansion and results in formation of a structure called the germinal center (GC). This requires additional signaling through costimulatory receptors such as CD40 or B7 expressed by B cells and CD40L and inducible T cell costimulator (ICOS) proteins on the T_{FH}. In the absence of costimulation, the B cell response is anergic (cell cycle arrest) and no germinal center is formed (Murphy et al., 2019).

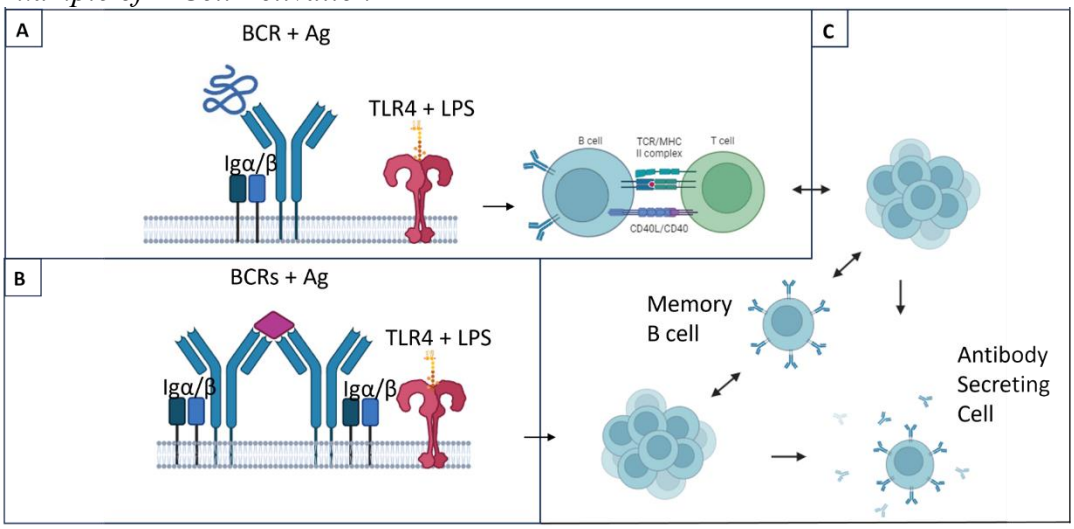
Germinal centers are major sites for several important processes in generation of effective humoral immunity. These include class-switch recombination (CSR), somatic hypermutation (SHM), and generation of long-lived effector cells. CSR is necessary for expression of alternate Ig heavy chain proteins and to facilitate production of diverse antibody isotypes (Wood, 2011). This is illustrated by deficiencies in production of some types of antibodies seen in humans that lack GCs due to impairment of helper T cells (Siegel et al., 1981). SHM introduces point mutations in the variable regions of Ig genes, potentially altering Ab amino acid sequence and thus antigen binding specificity/affinity. An enzyme called activation-induced cytidine deaminase (AID) has been shown to be essential for both SHM and CSR by introducing mutations in Ig genes (Muramatsu et al., 2000).

Activated follicular B cells can form GC in coordination with T cell help. In GC, Ag stimulation induces proliferation of B cells and AID expression. CSR and SHM occur, resulting in changes in Ab isotype and Ag binding specificity/affinity. Competition for Ag and T_{FH} help regulates B cell survival and ensures predominance of clones expressing BCR with high antigen specificity (Zhang et al., 2016). The combination of signals from B cell, T_{FH} interactions and the cytokine environment direct CSR and differentiation of expanding B cells into long-lived ASC (plasma) cells or memory B cells. Long lived plasma cells secrete large quantities of Ab, reside in the bone marrow, and rely on stimulation through CD28 or IL-6 to survive. Memory B cells can localize within lymphoid tissues or enter recirculation, where they await re-activation with Ag to proliferate and mount rapid secondary immune responses. Studies on survivors of the 1918 Spanish flu pandemic suggest that some memory B cells can persist for decades in the absence of Ag restimulation (Pieper et al., 2013).

MZ B cells and B1 cells can mount humoral responses to antigens in the absence of help from T_{FH} cells, or in a T cell independent (TI) fashion (Martin & Kearney, 2000). Antigens with repeating epitopes such as microbial polysaccharides can cross link BCRs and promote TI activation of B cells. Additionally, PRR engagement through surface TLRs such as TLR 4 or TLR 10 is likely required for optimal activation of these populations, a form of costimulatory regulation (Lewis et al., 2019; Wood, 2011). These B cells are important for mounting rapid responses to pathogens by circumventing the lengthy maturation processes that take place in germinal centers (Murphy et al., 2019).

Figure 2

Example of B Cell Activation



Note. Activation of B cells is reliant on recognition of Ag by the BCR and can occur in the presence (A) or absence of T cells (B). Presentation of cognate Ag to follicular helper T cells facilitates activation of B cells through costimulatory receptor interactions such as CD40 binding with CD40L. Crosslinking of BCR-antigen complexes provides a strong activation stimulus and enables activation of B cells without costimulation from T cells. Signaling through PRRs such as TLR4 bound with bacterial lipopolysaccharide further promotes B cell activation. Fully activated B cells (C) proliferate and mature into either memory or antibody secreting cells (Wood, 2011).

Antibody Isotypes

Antibody heavy chain genes contain multiple segments coding for the C region. Expression of specific heavy chain gene loci determines the structure of the Fc portion of antibodies, resulting in production of various classes of Ab that mediate unique effector functions. These are determined by interactions between the Fc portion of the Ab, which is dependent on the Ab isotype, and Fc receptors (FcR) which are expressed by immune cells (Wood, 2011). Five major classes of antibody exist, designated as IgM, IgD, IgG, IgE, and IgA. During early B cell development, IgM is initially expressed for BCR rearrangements. Alternative splicing of heavy chain gene transcripts results in expression of both IgM and IgD in mature, naïve B cells. Peripheral activation of B cells leads to generation of some ASC in the absence of class switching, which secrete either IgM or IgD antibodies (Schatorjé et al., 2014). CSR is

required for production of the other Ab isotypes and is heavily influenced by cytokine signaling. In this process, one C region is selected and adjoined to the V region of the heavy chain gene, so only one Ab isotype is expressed (Murphy et al., 2019).

IgM represents the first class of ab produced in response to infection, as no CSR is required for its production. IgM can be secreted in either monomeric or pentameric form when held together by joining (J) chain proteins. Pentameric IgM has a total of 10 potential Ag binding sites per molecule, providing it high binding avidity. This property also makes IgM the best Ab isotype at activating complement cascades (Wood, 2011).

IgD is unique in that it is co expressed along with IgM by mature, naïve B cells. For this reason, IgD is often used as a marker of B cell development. While IgD is secreted in serum, circulating levels of IgD are very low as it is highly susceptible to degradation by plasmin proteins. Originally, the proposed function of IgD was as a mediator of B cell activation. Studies into its functions using Ig KO mice show that either IgM or IgD alone are sufficient for production of the complete repertoire of ASC populations. Other proposed functions involve roles in innate immunity and defense of the respiratory tract (Edholm et al., 2011).

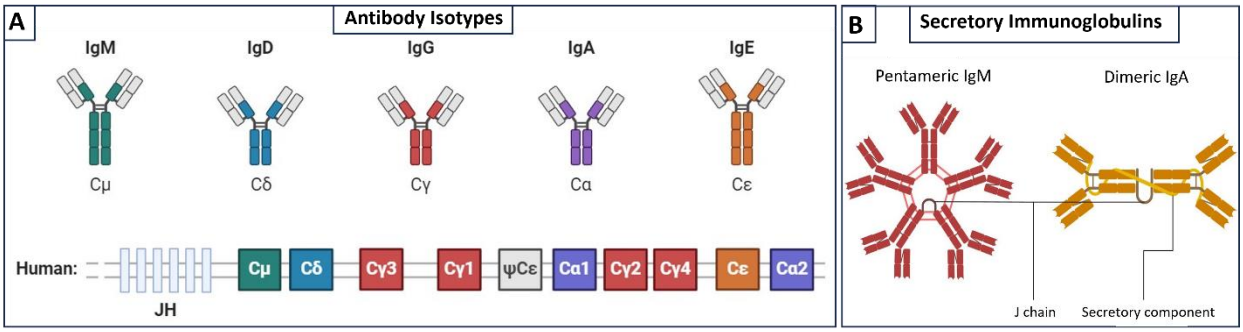
IgG is the most abundant antibody found in serum and in humans can occur in four different versions. CSR to IgG1 is promoted by IL-4 and IL-13, whereas TGF-β promotes CSR to IgG2b, and IFNγ controls CSR to IgG2a and IgG3 (Wood, 2011). IgG is the only class of Ab other than IgM capable of activating complement cascades. IgG binding to Ab complex results in conformational changes in the Ab promoting Fc binding to FcRs on neutrophils, which stimulates release of antimicrobial factors and inflammatory mediators (degranulation) (Murphy et al., 2019).

IgA antibodies can occur in two different versions in humans, IgA1 and IgA2, although these appear to have identical functions. CSR to IgA is regulated by stimulation with IL-5, IL-21, TGF-β, costimulatory receptors, and retinoic acid (Lebman & Edmiston, 1999; Wood, 2011). IgA regulates neutrophils similarly to IgG, but IgA can also be secreted in monomeric or dimeric form. Dimeric IgA is produced with the addition of the J chain as in IgM (Wood, 2011). Another protein called the secretory component is added to dimeric IgA to inhibit enzymatic digestion and increase its half-life. The J chain and secretory component proteins also mediate transport of IgA (and IgM) across adjacent epithelium for secretion into mucus, sweat, saliva, and breast milk. This requires interactions with polymeric Ig receptor (pIgR) to mediate transport across the epithelial cell layer. This function results in IgA, and to a lesser extent, IgM isotype secretion into the mucosal lumen (Murphy et al., 2019). Protection by secretory Igs in this way is important for induction of both immune responses and tolerance to innocuous Ags at mucosal tissues. Inadequate Ig secretion is associated with dysregulation of microbial populations, recurrent infection, pathological inflammation, and loss of barrier integrity, which demonstrates the importance of Ig production and transport (Bamias et al., 2023; Kinoshita et al., 2006).

IgE is present in the serum in the smallest amounts of any Ab but plays a significant role in regulating granulocytes. CSR to IgE is mediated by signaling from IL-4 and IL-13 receptors. Through low affinity Fc-FcR interactions, IgE Ab bound with Ag can trigger release of inflammatory factors by cells called monocytes, as well as secretion of anti-helminth proteins such as major basic protein by eosinophils. IgE is the only class of Ab that binds to high affinity FcR expressed by mast cells and basophils to trigger release of inflammatory histamine and leukotrienes. These functions are responsible for the important role of IgE in certain allergic responses and resistance to parasitic infection (Wood, 2011).

Figure 3

Diagram of Antibody Isotypes



Note. A. The various classes or isotypes of antibody are shown along with a portion of the human immunoglobulin heavy chain gene. The variable region of the heavy chain is constructed through rearrangement of random gene segments from the JH, VH, and DH gene loci. Class switch recombination determines immunoglobulin isotype through expression of a single version of the loci coding for the constant region with excision of the intervening DNA sequence. The potential for further modification and recognition of Fc receptors varies according to the amino acid sequence of the constant region and therefore is unique to each antibody isotype. B. IgM and IgA can alternatively be produced in multimeric form through the addition of a joining (J) protein chain. Multimeric immunoglobulins display enhanced Ag crosslinking and agglutination properties. The J chain additionally promotes transcytosis of multimeric immunoglobulins into bodily secretions through recognition of polymeric immunoglobulin receptors (pIgR) on epithelial cells. Cleavage of a portion of pIgR results in attachment of the secretory component to IgA which inhibits its degradation (Spiegelberg, 1989; Wood, 2011).

Immunoregulation

The immune system must be regulated to promote efficient responses, mitigate pathology, and to facilitate repair of tissue damage. Complex interactions between multiple factors determine the nature of immune responses (Wood, 2011). These include genetics, diet, contents of the microbiota, exercise, sex, and previous exposure to pathogens, as well as various external and internal environmental stimuli. Understanding how these elements shape immunity can provide insights into contributing factors for diseases and can reveal potential targets for therapeutics (Esser et al., 2018).

Intrinsic Immunomodulators

Tolerance

The capacity to generate diverse Ag receptors capable of recognizing the comprehensive repertoire of theoretical Ags (at least 10^{11}) is a hallmark of adaptive immunity (Wood, 2011). Such plasticity inevitably results in construction of Ag receptors capable of binding to Ags of non-pathogenic origin, including self Ags, and provides risk for immunopathology. Mechanisms of tolerance have evolved to regulate lymphocyte responses and survival to mitigate such pathologies. Tolerance thus refers to the ability of adaptive immune cells to tolerate certain Ags in the absence of tissue damage. Central tolerance refers to elimination of self Ag responsive lymphocytes during development in the bone marrow or thymus. Most Ag present at these sites is of self-origin, so lymphocyte clones expressing Ag receptors that bind Ag in these locations with high affinity are generally removed by apoptosis. Peripheral tolerance refers to suppression of lymphocyte activity in secondary lymphoid organs and other tissues (Wood, 2011). Absence of costimulatory signals during APC-lymphocyte interactions and production of immunosuppressive cytokines (i.e. IL-10 and TGF-β) from regulatory immune cells such as T_{Reg} cells are major contributors to peripheral tolerance. Peripheral tolerance functions as a fail-safe for controlling autoreactive lymphocytes that escape central tolerance mechanisms (Murphy et al., 2019). Tolerance is essential to reduce the risk for developing allergic and chronic inflammatory diseases such as asthma and IBD, respectively (Bamias et al., 2023). Through maintenance of microbial populations and Ag scavenging in the absence of P/DAMPs, secretory Igs are important for inducing tolerance to commensal microbes and foods (Brandtzaeg et al., 1999).

B Cell Responses

B cell generation from precursors is constant to maximize the ability to mount rapid responses against any potential antigen. When infection occurs, pathogenic Ag stimulates expansion of Ag specific B cell populations by several orders of magnitude. Clearance of infection should be accompanied by a corresponding decrease in Ag specific ASC populations to avoid redundancy and prevent overcrowding. Memory B cell populations should be maintained, however, for immunity to reinfection. CSR and SHM should occur in some expanding B cells for production of more effective Abs, but not in others so Ab is produced quickly. Proliferation, differentiation, and survival of B cell populations must therefore be highly regulated so that humoral responses are timely and effective, while B cell associated pathologies such as production of auto reactive Ab is mitigated (Murphy et al., 2019).

The most important regulator of B cell survival occurs via BCR or pre-BCR signaling events. Functional BCR signaling is required for proper maintenance of developing B cell populations and initiating B cell activation and differentiation into effectors. While BCR recognition of Ag supports survival in the periphery, strong recognition of what is mostly self-Ag in the bone marrow typically results in B cell anergy or apoptosis as a means of inhibiting generation of auto reactive B cell populations (negative selection) (Wood, 2011). Dysfunctional BCR signaling arrests B cell differentiation at various stages of development, depending on which pathways are impaired. This can be caused by an inability to form BCR or inherited mutations in essential BCR signaling proteins. Somatic recombination of Ig genes can lead to introduction of early stop codons, frameshift mutations, and production of dysfunctional Ig heavy and light chains, which results in clonal deletion due to an inability to form BCRs (Murphy et al., 2019). Similarly, defective surrogate light chain genes prevent pre-BCR

formation and inhibit progression to the Pre-B cell stage. Abnormalities in Bruton tyrosine kinase (BTK) enzyme, which is involved in Ca^{2+} signaling following BCR stimulation, are responsible for the majority of Ab immunodeficiencies in humans (Pieper et al., 2013). BCR signaling is also modulated by the activity of coreceptor proteins, such as Igα, Ig, and CD19. Mutations in any of these genes can also cause impairment in the generation of ASCs. Such conditions are major causes of primary B cell related immunodeficiencies, wherein lack of humoral immunity results in persistent, recurring, and life-threatening infections (Smith & Cunningham-Rundles, 2019).

Genetic rearrangement of the Ab heavy and light chains is regulated by expression of B cell specific recombination enzymes in addition to more universal DNA repair machinery. Rag1 or Rag 2 enzyme expression is induced to promote Ab genetic rearrangement at the Pro and Pre-B cell stages and are downregulated upon completion of these processes to facilitate allelic exclusion (Pieper et al., 2013). The enzyme terminal deoxynucleotidyl transferase (TdT) assists in generating diverse BCR sequences through addition of nucleotides during recombination events in the bone marrow. AID is upregulated upon B cell activation and mediates both SHM and CSR (Melchers, 2015).

Many factors combine to produce microenvironments that regulate B cell transcriptional programs. These include cytokines, growth factors, integrins, chemokines, BCR signaling, and costimulation. Uncharacterized signals from stromal cells in bone marrow initiate stem cell commitment to the B cell lineage in humans, indicated by expression of the transcription factor Pax5 (Melchers, 2015). B cell activating factor (BAFF) and a proliferation inducing enzyme (APRIL) function as important regulators of B cell survival and proliferation through impacts on NF-κB and AKT signaling (Pieper et al., 2013). Clonal expansion appears to be mediated by ILs-2,4, and 5 *in vitro*. B cell activation induces expression of the transcription factor B lymphocyte-

induced maturation protein 1 (BLIMP-1), which is required for differentiation into ASCs and downregulates Pax5 expression. BLIMP-1 prepares B cells for antibody secretions through modifications in the ER and downregulation of the anti-apoptotic factor BCL-6. Maintenance of long-lived plasma cells likely relies on a combination of signals from bone marrow cells and interleukins such as IL-6 (Nutt et al., 2015).

B Cell Trafficking

For B cell development, differentiation, activation, and effector functions to occur appropriately, B cell circulation and localization must be tightly regulated. As with other hematopoietic cells, B cell trafficking is controlled by selective expression of chemokine receptors and CAM binding partners by the B cells themselves and other cells (Habtezion et al., 2016). CAM interactions additionally provide differentiation signals and stabilize interactions with other cells such as formation of immunological synapses with T cells during Ag presentation (Wood, 2011). Importantly, studies characterizing plasma blasts in humans experiencing various states of disease and immune challenge (vaccination) showed that trafficking programming in B cells (i.e. expression of chemokines/CAMs) occurs according to environmental cues from the location of antigenic stimulation. While vaccination at sites less prone to infection produces fewer B cells imprinted to home to more preferrable locations, memory B cells are nevertheless generated that can traffic throughout the body to successfully guard against reinfection (Seong et al., 2017).

Bone marrow cells produce CXCR4 and CXCL12 to promote retention of immature B cells and long-lived ASCs from the blood. Transitional B cell egress from the bone marrow to the spleen occurs in response to chemoattraction to circulating sphingosine-1-phosphate (S1P). Localization of lymphocytes and APCs to the follicular regions of lymphoid tissues is mediated

by signaling through CXCR5 and CCR7 chemokine receptors (Pieper et al., 2013). Studies in mice indicate expression of chemokine receptor 9 (CCR9) and its ligand CCL25, along with integrin $\alpha_4\beta_7$ and its ligand MAdCAM-1 are crucial for B cell migration into the GI tract. Inhibition of β_7 proteins alone was shown to be sufficient to induce susceptibility to GI infections. Alternatively, CCR10 and its ligand CCL28 are associated with trafficking to the respiratory tract, mammary glands, and colon. The L-selectin CD62L and $\alpha_4\beta_1$ integrin along with its ligand VCAM1 are instead implicated in protective and pathological immune responses in the respiratory tract, whereas E selectin and CLA mediate homing to the oral mucosa and skin. Finally, memory B cells express CCR6 which binds CCL20 ligands and is likely involved in localization to lymphoid tissues and sites of infection or inflammation (Seong et al., 2017).

Sex

Sex-based differences in immunity and the contents of the microbiota have been observed (Cho et al., 2019). Females tend to have increased numbers of leukocytes, contents of the microbiota, and mount more robust immune responses than occurs in males (Nunn et al., 2008). Lymphocyte proliferation, activation, and release of inflammatory mediators tends to be elevated in females, with males showing higher levels of anti-inflammatory immune cells (Dodd & Menon, 2022). As a result, females tend to be more resistant to cancer and infections such as Covid-19 but are more susceptible to autoimmune diseases (Cai et al., 2021; Nunn et al., 2008).

Some postulate that this results from the influences of sex hormones, which enhance immune cell survival and function (Bhatia et al., 2014). Immune cells are known to express sex hormone receptors, and endogenous estrogen was found to determine sex-based differences to airway hyper-responsiveness in a model of metacholine induced allergic response (Matsubara et al., 2008). This, however, can be attributed to the activity of vascular smooth muscle as opposed

to that of traditional immune cells. Controversy on the role of sex hormones in sex-based differences in immunity persists, as others point out that evidence supporting the assertion that testosterone is immunotoxic is entirely correlational (Nunn et al., 2008).

Extrinsic Immunomodulation by the Aryl Hydrocarbon Receptor

The AhR is a cytosolic protein expressed by multiple cell types in mammals (Stockinger et al., 2014). Many ligands have been discovered for the AhR, of both endogenous and exogenous origin, with variable affinities and distinct effects on AhR activity (Safe et al., 2016). Some of these ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), activate the AhR once bound, promoting its transit into the nucleus where it functions as a transcription factor (Lamas et al., 2018). This enables the AhR to influence several biological pathways and illicit changes in cellular functions (Holsapple et al., 1986). In this way, AhR plays a role in various processes such as organismal development, cellular proliferation and differentiation, metabolism, and reproduction (Hattori et al., 2018).

Ligands for the AhR include xenobiotics such as polyaromatic hydrocarbons and the drug omeprazole (Jin et al., 2014). Molecules of biological origin, such as certain derivatives of tryptophan, indoles produced by plants, and metabolites of the microbiota have all been shown to activate the AhR. The AhR is thus believed to function as a sort of environmental sensor, detecting the presence of various organic substances to illicit appropriate cellular responses. Additionally, it has recently been appreciated that the AhR plays a key role in the regulation of physiological processes in both homeostatic and disease states (Quintana & Sherr, 2013).

The AhR mediates changes in gene expression in a species, cell type, sex, and context dependent manner (Phadnis-Moghe et al., 2016). Activation of the AhR results in dissociation of cytosolic chaperones such as heat shock protein 90 and translocation of the AhR to the nucleus.

In the canonical signaling pathway, AhR dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT) and binds to sequences on the DNA called xenobiotic response elements (XRE) to alter gene transcription. AhR can regulate expression of non XRE genes by binding with other transcription factors such as estrogen receptor, krüppel-like factor 6, retinoblastoma protein, and retinoic acid receptors. AhR further displays ubiquitin ligase activity and is capable of mediating chromatin remodeling through modifications on histones. Common effects of AhR are inhibition of NF-kB and expression of cytochrome P450 enzymes capable of degrading AhR ligands. Other forms of negative feedback for AhR signaling are mediated by AhR binding the inhibitory proteins aryl-hydrocarbon receptor repressor or hypoxia-inducible factor, and protein kinase C phosphorylation of importin-beta (Rothhammer & Quintana, 2019).

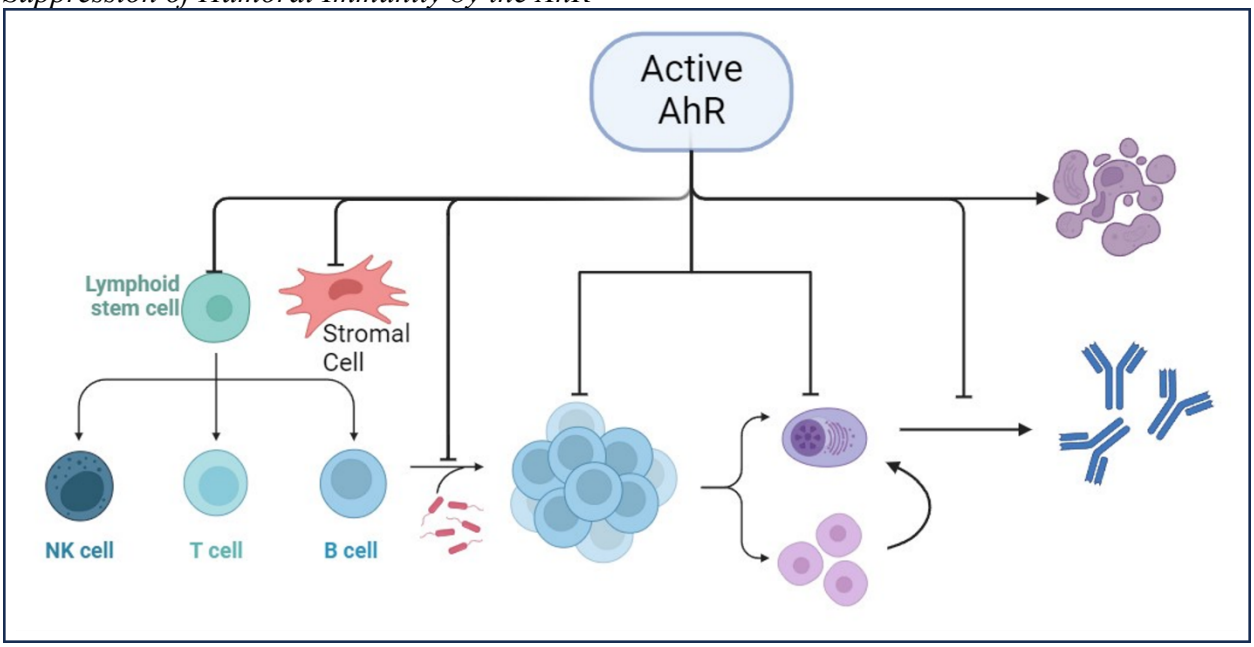
Activation of the AhR with high affinity ligands is associated with general suppression of the immune system (Ehrlich et al., 2017). *In vivo* studies show diminished production of adaptive immune cells and atrophy of the spleen and thymus (McMillan et al., 2007). Such effects can alter susceptibility to diseases (Ehrlich et al., 2017). AhR agonists have been shown to reduce pathology from CD4+ T cells in ocular herpes but also increase mortality rates in models of influenza A infection (Veiga-Parga et al., 2011; Vorderstrasse et al., 2003).

It has been known for some time that exposure to AhR agonists impairs humoral responses (Holsapple et al., 1986). This occurs through inhibition of B cell proliferation, immunoglobulin production, CSR, and SHM. This interferes with B cell differentiation into high affinity antibody secreting plasma cells, resulting in diminished Ab production. This is largely mediated by downregulation of crucial genes for B cell effector functions, such as AID and BLIMP-1 (Vaidyanathan et al., 2016). Others have demonstrated that expression of AhR varies throughout B cell development and according to differentiation status, with relatively low

expression levels in immature B cells and much higher levels of expression following BCR engagement and terminal differentiation into plasma cells (Sherr & Monti, 2013).

Figure 4

Suppression of Humoral Immunity by the AhR



Note. Sustained activation of the AhR suppresses B cell functions at every stage of development. B cell production from hematopoietic stem cells is inhibited by the AhR directly and indirectly via effects on stromal cell populations in the bone marrow. Activation of B cells in response to Ag recognition is additionally impaired in the presence of AhR agonists. Differentiation of activated B cells into antibody secreting cells is suppressed through downregulation of essential factors such as BLIMP-1. B cell survival, proliferation, and apoptosis is influenced by AhR signaling via its control of regulatory proteins such as NF-kB and BCL-6 (North et al., 2009; Rothhammer & Quintana, 2019; Sulentic & Kaminski, 2010; Vaidyanathan et al., 2016).

Rationale of Study

Due to the ability of the AhR to modulate immune functions and suppress inflammation, the AhR has been proposed as a potential therapeutic target for treating inflammatory diseases such as IBD (Rothhammer & Quintana, 2019). Others have proposed inhibiting gut homing proteins to reduce infiltration of pro-inflammatory lymphocytes instead (Bamias et al., 2023). However, deficiency in β_7 has been associated with decreased immune protection in the gut, which could result in persistent infection and inflammation (Seong et al., 2017). Additionally,

suppression of humoral responses by AhR agonists or gut homing inhibitors could result in inadequate Ab secretion into the gut lumen. Such an outcome could exacerbate as opposed to alleviating IBD pathogenesis (Bamias et al., 2023).

Recent studies in our laboratory have shown that among the effects of AhR activation on naïve B cells is upregulation of the gut homing proteins CCR9 and $\alpha_4\beta_7$ (Ortiz, 2021). In an *in vivo* model of cholera toxin immunization, recovery of Ab suppression in response to AhR activation was hastened in the gut compared to what was observed systemically (King et al., 2017). Together, these results suggest that AhR activation of naïve B cells induces preferential migration from the periphery into the gut. This finding provides justification for using the AhR, but not gut homing protein inhibitors, in developing treatments for IBD.

However, as the susceptibility of different B cell populations to induction of gut homing proteins via AhR activation is currently unknown, more research should be done to identify the potential risks and benefits of targeting the AhR to treat inflammatory diseases (Ortiz, 2021; Villa et al., 2016). As B cells differentiate into effector cells following BCR activation, changes in regulation of gene expression could alter their susceptibility to induction of gut homing proteins CCR9 and $\alpha_4\beta_7$ through the AhR (Vaidyanathan et al., 2016).

Additionally, as sexual dimorphism in immunity, drug metabolism, AhR expression, and gene regulation by the AhR has been observed, B cells in individuals of the opposing sex may display unique susceptibility to therapies targeting the AhR (Lee et al., 2015; Lu et al., 2013; Nunn et al., 2008). Such sex-based differences could extend to AhR mediated induction of CCR9 and $\alpha_4\beta_7$ in naïve B cells, and result in different potential risks and benefits in AhR based therapies for IBD between the sexes. Therefore, in the current study, we examined the role of B

cell activation state and sex in induction of CCR9 and $\alpha_4\beta_7$ expression via AhR activation *in vitro*.

Specific Aims and Research Hypotheses

A1 Characterize the influence of B cell activation, differentiation state on AhR mediated induction of CCR9 and $\alpha_4\beta_7$ expression *in vitro*.

H1 Activation of the AhR by TCDD will promote expression of gut associated migration markers, CCR9 and $\alpha_4\beta_7$.

H2 The frequency (%) and intensity (MFI) of CCR9 and $\alpha_4\beta_7$ expression by B cells will be reduced when AhR activation occurs at later stages of differentiation.

A2 Determine if AhR mediated induction of CCR9 and $\alpha_4\beta_7$ expression occurs differentially in B cells based on the sex of the source animal.

H3 Activation of the AhR by TCDD will promote expression of gut associated migration markers, CCR9 and $\alpha_4\beta_7$, regardless of the sex of the source animal.

H4 Induction of CCR9 and $\alpha_4\beta_7$ expression by AhR activation with TCDD will be increased in B cells derived from female C57BL/6J mice relative to those derived from males.

CHAPTER III

METHODOLOGY

B Cell Preparation

Mice

C57BL/6J mice were sourced from the University of Northern Colorado's breeding colonies, which secured original breeding mice from The Jackson Laboratory in Bar Harbor, Maine. Mice were housed using Optimice® system cages and 7099 Tek-Fresh laboratory animal bedding from Envigo. Deionized water and food comprised of Rodent diet 2016 formula from Envigo was available ad libitum. Animals were kept in facilities maintaining 22.2 degrees Celsius in 50% humidity on a 12-hour dark/light cycle. Mice ranging from 6-18 weeks of age were utilized. The sex of each mouse was determined by visual inspection of the distance between the genitourinary tract and anus. Mice were euthanized by carbon dioxide asphyxiation prior to tissue collection. All procedures were conducted in accordance with protocol 2112C approved by the Institutional Animal Care and Use Committee.

Splenocyte Collection

Under aseptic conditions, forceps and scissors were used to collect spleens from euthanized mice. Sterile frosted glass slides were employed to liberate splenocytes into 5 mL of RPMI 1640 (Gibco™, 11-875-119) based medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, SH30088.03), 1% GlutaMAX (Gibco™, 35050061), 1% Gentamycin (Amresco®, E737), 1% Penicillin-Streptomycin (BioWhittaker®, 17-602E), and 50 μM β-

mercaptoethanol (Sigma-Aldrich®, 60-24-2) to generate complete cell medium (CM). Splenocyte solutions were kept on ice during processing and pipetted up and down several times to ensure formation of single cell suspensions. Debris was removed by allowing it to settle in the bottom of the tube and avoiding the last milliliter during collection. Splenocyte cell suspensions were then washed 3 times by placing in a centrifuge at of 370 xg (370 times gravitational force) for 5 minutes followed by removal of the supernatant and CM replacement. The concentration of living cells in suspensions were measured using hemocytometer and Trypan Blue exclusion dye (Gibco™, 15250061) was used to determine non-viable cells.

B Cell Isolation

Splenocyte solutions were adjusted to $1x10^8$ cells/mL using a centrifuge, as described under "splenocyte collection" section of chapter 3 and placed in 1X Mojosort™ buffer (Biolegend®, 480017). Next, isolation of B cells from $2x10^7$ splenocytes was accomplished via negative selection using the Mojosort™ Mouse Pan B cell Isolation Kit (Biolegend®, 480052) as recommended by the manufacturer. Isolated B cells were then placed in a centrifuge for 5 minutes at 278 xg and the supernatant was discarded as described under "splenocyte collection" section of chapter 3. The isolated B cells were then washed two times in 1 mL of fresh, cold CM. Samples of the resulting isolate are reserved to ensure B cell purity via flow cytometric confirmation of a proportion of cells expressing the B cell marker CD19 of at least 94% (Wang et al., 2012).

Experiment 1: Time-Course Study

Culturing B Cells Under Immune Activating Conditions

Setup

B cells were added to 48-well tissue culture plates (5 x 10^5 cells/well) with a final volume of 1 mL of CM per well. The cells were cultured under immune activating conditions based on the protocol of Vaidyanathan et al. (2016) as follows: 300 ng/mL of anti-mouse IgD-Dextran conjugated antibodies (Finabiosolutions LLC, MOS551B), 25 μg/mL of bacterial lipopolysaccharide (LPS; Sigma-Aldrich®, L6529), and 2 ng/mL of recombinant human transforming growth factor-beta (Peprotech®, 100-21).

Anti-mouse IgD antibodies and LPS were employed to facilitate B cell activation thus providing an *in vitro* model of the humoral immune response, while TGF-β was utilized to promote CSR to IgA, the most important Ab isotype for GI defense (Cerutti, 2008; Wood, 2011). The TGF-β was prepared according to manufacturer instructions, which, briefly, involved: dilution of the lyophilized protein to a concentration of 0.2 mg/mL using ultrapure water, generation of aliquots by further dilution to 0.1 μg/mL with phosphate buffered saline supplemented with 0.1% bovine serum albumin, and storage at -20 degrees Celsius. TGF-β aliquots were then thawed and the appropriate volume was dispensed directly into cell cultures.

Treatment and Incubation

The AhR agonist TCDD (Cambridge Isotype Labs., cat. #ED-901-C) and the AhR antagonist CH22319 (Sigma-Aldrich®, C8124), both dissolved in DMSO, were employed to modulate AhR signaling. AhR ligands were added as follows: 100 μM of CH223191 was achieved to generate control cultures, and in those cultures designated for treatment with TCDD during incubation (hours 1,4, or 24). The addition of the AhR antagonist CH223191 to cultures

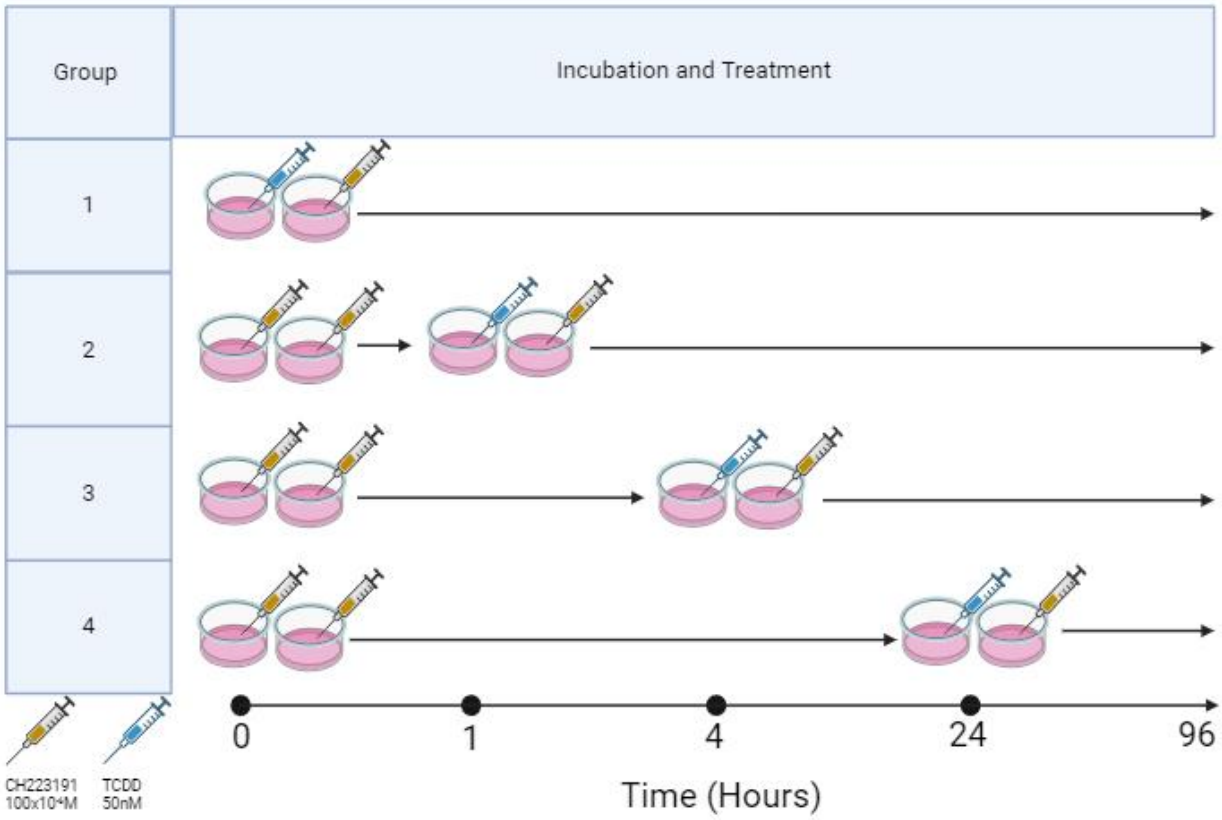
was considered a control because the presence of AhR agonists has been reported in RPMI based culture media (Veldhoen et al., 2008). Samples designated for AhR activation at culture start (hour zero) or at later times (hours 1, 4 or 24) instead received TCDD resulting in a final concentration of 50 nM. Following treatment, B cell cultures were placed into a humidified incubator at 37 degrees Celsius, 5% CO_2 for a total duration of 96-hours.

Treatment During Incubation

Treatment with AhR ligands typically occurred at culture initiation (i.e. hour zero), but certain samples for the time course study and effects of washing experiment received AhR ligands mid culture to facilitate AhR activation at various timepoints after immune activation. For these samples, final concentrations of 100 μM CH223191 or 50 nM TCDD were again employed to modulate AhR signaling. As stated previously, CH223191 was added during culture initiation to generate control samples and for those cultures designated for AhR activation mid culture to inhibit early agonism of the AhR by ligands endogenous to the CM (Veldhoen et al., 2008; Zhao et al., 2010). Cultures designated for treatment with AhR ligands at hours 1, 4, or 24 during incubation were washed two times by transferring to a 5 mL conical tube with immediate centrifugation at 164 xg for 5 minutes followed by CM replacement. The cells were then returned to the 48 well plate and received either CH223191 or TCDD in a final total volume of 1 mL CM. Control samples again received CH223191 in this way to mitigate AhR activation by agonists derived from CM, while experimental samples received TCDD (Veldhoen et al., 2008). Following treatment, all cultures were returned to the humidified incubator at 37 degrees Celsius, 5% CO_2 for the remainder of the 96-hour incubation period.

Figure 5

Experimental Design for Activation of AhR at Various Timepoints



Note. 5x10^5 B Cells/mL were added to 48 well tissue culture plates under immune activating conditions. AhR antagonist (CH223191, 100 μM) was added at start of culture (hour 0). Half of Group 1 received AhR agonist (TCDD, 50 nM) instead of AhR antagonist at hour 0. CM was removed from Groups 2, 3, and 4 by pipette and replaced with CM supplemented with AhR agonist or antagonist at hours 1,4, or 24 since culture start, respectively. All cultures were incubated for 96 hours total prior to culture termination and marker analysis.

Culture Termination and Assessing Cell Recovery and Viability

After 96 hours of incubation, cells were washed three times and counted using a hemocytometer, microscope, and Trypan Blue exclusion dye to measure the concentration of live and dead cells prior to marker analysis. Cell recovery was calculated as the percentage of live cells remaining relative to the initial plating density of 5x10^{5}cells/mL, while cell viability was calculated as the percentage of living cells relative to the total number of cells.

Data Collection

Sample Preparation for Flow Cytometry

Aliquots consisting of $1x10^5$ B cells were transferred to a plastic 96 well plate. These samples were placed into the centrifuge at 164 xg for two minutes and the supernatant was discarded by flicking. Cell Staining Buffer (Biolegend®, 42020) was used as the sample diluent for the remainder of data collection. Fc receptors were blocked through incubation with 2 µg per 100,000 cells of Purified anti-mouse CD16/32 Antibody (Biolegend®, 101302). All incubations were performed in the dark and on ice for a minimum of 15 minutes. Cells were stained using antibody fluorophore/biotin conjugates that specifically bind markers of interest or serve as isotype controls (reagents listed in Table 1). Isotype controls match antibody type/conjugate but lack specific binding to the target protein, which enables exclusion of off-target binding and auto-fluorescence from the data set (Cossarizza et al., 2021). Following 15 minutes of incubation on ice and in darkness, all samples were washed 3 times in Cell Staining Buffer to remove any unbound antibody-fluorophore molecules. Secondary reagents necessary for measuring marker expression (i.e. fluorophore-streptavidin conjugates) were added as appropriate followed by another incubation and 3 washes in Cell Staining Buffer. Sytox Blue Dead Cell Stain from Invitrogen (S34857) was added to samples at a concentration of 0.1µM per 100,000 cells prior to analysis by flow cytometry.

Table 1

List of Reagents Used for Flow Cytometry

Reagent	Specificity	Source	Product #
Anti mouse Antibody-AF647	CD19	Biolegend®	115522
Anti mouse Antibody-AF647	Nonspecific	Biolegend®	400526
Anti mouse CCR9-AF647	CCR9	Biolegend®	128708
Anti mouse CCR9-AF647	Nonspecific	Biolegend®	400234
Anti mouse α4β7-PE	α4β7	Biolegend®	120605
Anti mouse α4β7-PE	Nonspecific	BD Pharmingen™	553930
Anti mouse IgM - BV711	IgM	Biolegend®	406539
Anti mouse IgM - BV711	Nonspecific	Biolegend®	400551
Anti mouse IgA - Biotin	IgA	Biolegend®	407004
Anti mouse IgA - Biotin	Nonspecific	Biolegend®	400404
Streptavidin - AF488	Biotin	Biolegend®	405235

Flow Cytometry

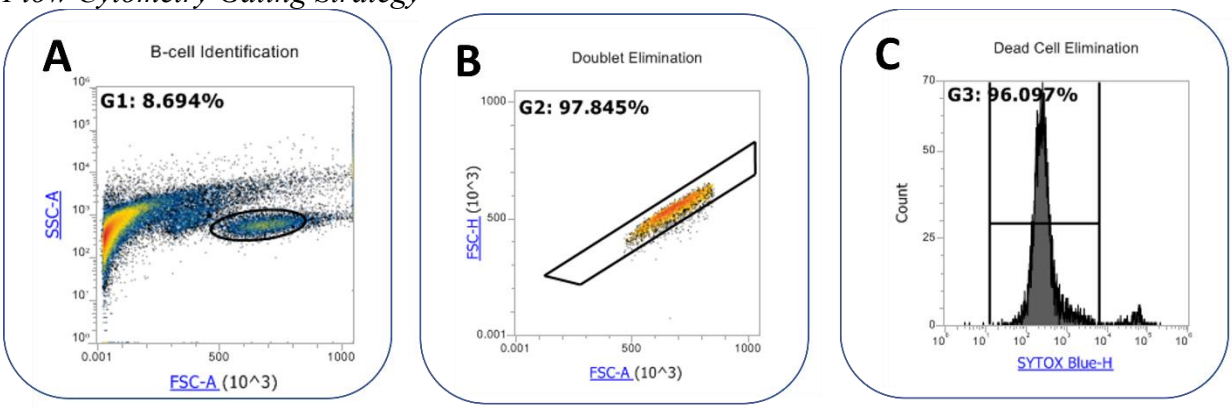
Prepared B cell culture samples were analyzed using the Attune NXT flow cytometer. Compensation parameters were established with UltraComp eBeads™ Plus Compensation Beads procured from Invitrogen (01-3333-42). Unstained samples of B cells were additionally employed to establish proper voltages for lasers prior to data collection. Protein expression was measured by staining cells with antibody-fluorophore conjugates that bind specifically to proteins of interest (Cossarizza et al., 2021).

Event size (FSC) and internal complexity (SSC) characteristics in conjunction with CD19 staining were utilized to determine location of B Cell populations in FSC/SSC plots. This

enabled establishment of Gate 1 (G1) around identified population of B cells and the exclusion of all other events from analysis. Next, doublet elimination was achieved by placing Gate 2 (G2), which is derived from G1 events, only around events that display a proportional relationship in FSC-A vs. FSC-H plots. Dead cells were eliminated from analysis by placing Gate 3 (G3), derived from G2, on events negative for staining with Sytox Blue only. Only live, single cells were analyzed for surface marker expression.

Figure 6

Flow Cytometry Gating Strategy



Note. Methodology for identifying cell populations of interest for analysis. B cells were fluorescently labeled using Sytox Blue viability dye and CD19 specific antibodies prior to analysis by flow cytometry. Events staining positive for CD19 and light scatter properties on SSC-A vs. FSC-A plots were used to identify B cells for inclusion in gate 1 (G1). Events from G1 were used for doublet elimination using FSC-H vs. FSC-A plots to form Gate 2 (G2). Living cells were identified as events from G2 considered negative for Sytox Blue staining and formed Gate 3 (G3). Events from R3 were used for data collection. The percentage of events included in the gate are indicated by the values next to G1, G2, or G3 in the plots.

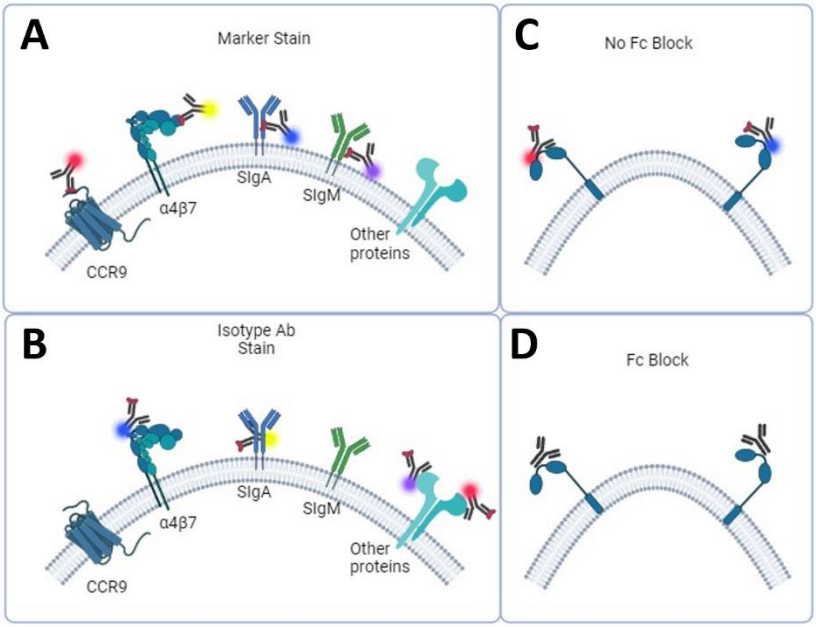
Marker Analysis

Cellular membrane expression of CCR9, $\alpha_4\beta_7$, CD19, IgM, and/or IgA proteins was analyzed by comparison of marker specific stained samples to isotype controls. The boundaries for determining positive versus negative marker expression were established with fluorescence data from isotype controls by setting the upper boundary of the gate to exclude events at the highest channels numbers (i.e. > $10^{5.8}$) and by setting the lower boundary for the gate so that no

more than 1% of events were included within the gate. This gate was used to define the positive population in marker-stained samples. Flow samples identified to contain fewer than 150 total, viable B cells were considered of insufficient sample size to include in the analysis of marker expression (Owens et al., 2000).

Figure 7

Flow Staining and Controls



Note. A. Markers of interest were stained using protein specific Ab-fluorophore conjugates. B. Replicate samples were alternatively stained using comparable Ab-fluorophore conjugates that do not recognize the proteins of interest to exclude fluorescence resulting from autofluorescence and non-specific Ab protein binding in the data analysis. C. The presence of Fc receptors on cells could've contributed to non-specific Ab-protein binding resulting in a false positive signal if insufficiently blocked. D. Antibodies lacking fluorophores were thus employed prior to Ab-fluorophore staining to block the availability of Fc receptors (Cossarizza et al., 2021).

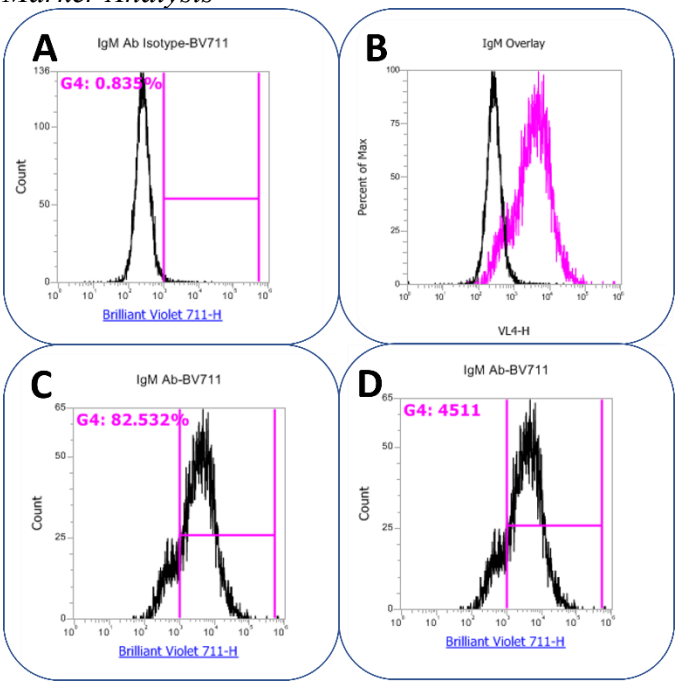
Metrics of Analysis

For each marker of interest, expression data were collected in the form of percent expression, or the proportion of the population of interest considered positive for marker expression, and the median fluorescence intensity (MFI), or the median channel number (MCN) of the emission data for the population considered positive for marker expression. MFI/MCN

represents the brightness of fluorescence for the stained population, and thus correlates with the relative abundance of antibody-fluorophore labeling per cell (Cossarizza et al., 2021).

Figure 8

Marker Analysis



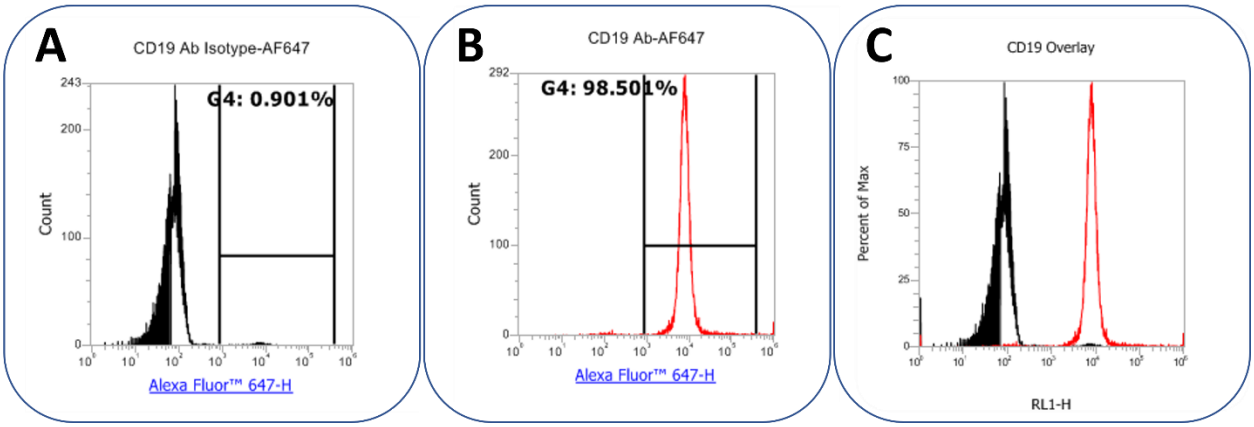
Note. Representative strategy for collecting expression data for markers of interest. A. Antibody isotype controls were used to establish gates for positive expression of marker of interest. B. Antibody isotype control and marker specific stained samples are shown overlaid one another for comparison. The gate from A (i.e. G4) was used to identify the percent expression (C) and mean fluorescence intensity (MFI; D) in marker specific, antibody-fluorophore conjugate stained samples.

Confirmation of B Cell Purity

Expression of the BCR coreceptor protein, CD19, was measured to assess B cell purity prior to culturing and data analysis (Wang et al., 2012). Prepared samples were labeled with anti-mouse CD19 antibodies conjugated to AlexaFluor647 (Biolegend®, 115522). Isotype controls (Biolegend®, 400526) were utilized to set boundaries for negative and positive populations. Samples with proportions of CD19 positive cells less than 94% were considered impure and were excluded from the data set.

Figure 9

Analysis of B Cell Purity



Note. Representation of strategy for assessing sufficient frequency of B cells for analysis. A. Isotype control antibodies were used to establish gate (G4) for markers considered positive for CD19 expression. B. Antibody-fluorophore conjugates specific for binding CD19 were used to measure the proportion of cells expressing membrane CD19 proteins. Samples with ≤ 94% CD19 expression frequency were considered of insufficient B cell purity for further analysis. C. Overlay of CD19 isotype control samples (left peak) with CD19 specific antibody-fluorophore conjugate stained samples (right peak).

Utilized Software

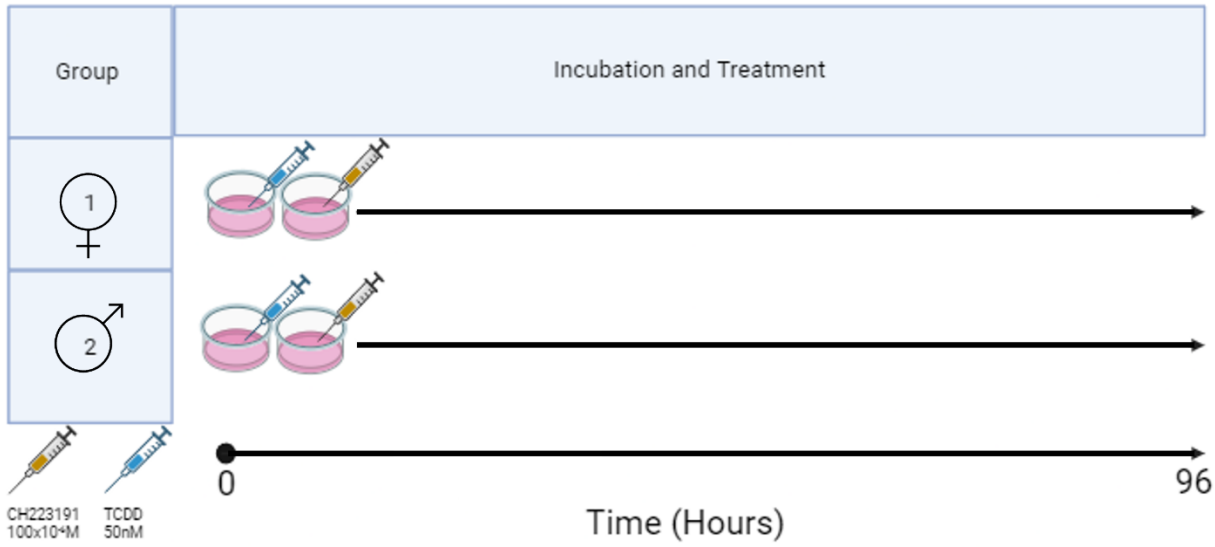
This document, experimental data analysis, and the included figures were generated using Microsoft Office (2010), SigmaPlot (14.0), and the following websites: refworks.proquest.com, citationmachine.net, and BioRender.com.

Experiment 2: Sex-Based Study

Experiment 2 was performed as described under the methods section for experiment 1, with the following adjustments to the experimental design: B cells derived from female and male C57BL/6J mice were utilized for this experiment. All cultures were treated with 50 nM TCDD or 100 µM CH223191 simultaneously to immune activation at the start of culture incubation (hour 0). Cultures were not manipulated in any way during incubation, meaning cultures were not treated with AhR ligands after hour 0 nor were cultures subject to CM replacement or washing mid culture.

Figure 10

Experimental Design for Sex-Based Study



Note. 5x10^5 B cells/mL from female (group1) or male (group 2) C57BL/6J mice were added to 48 well tissue culture plates under immune activating conditions and received either AhR agonist (TCDD, 50nM) or AhR antagonist (CH223191, 100µM) at the start of incubation (hour 0). All cultures were incubated for 96 hours prior to culture termination and marker analysis.

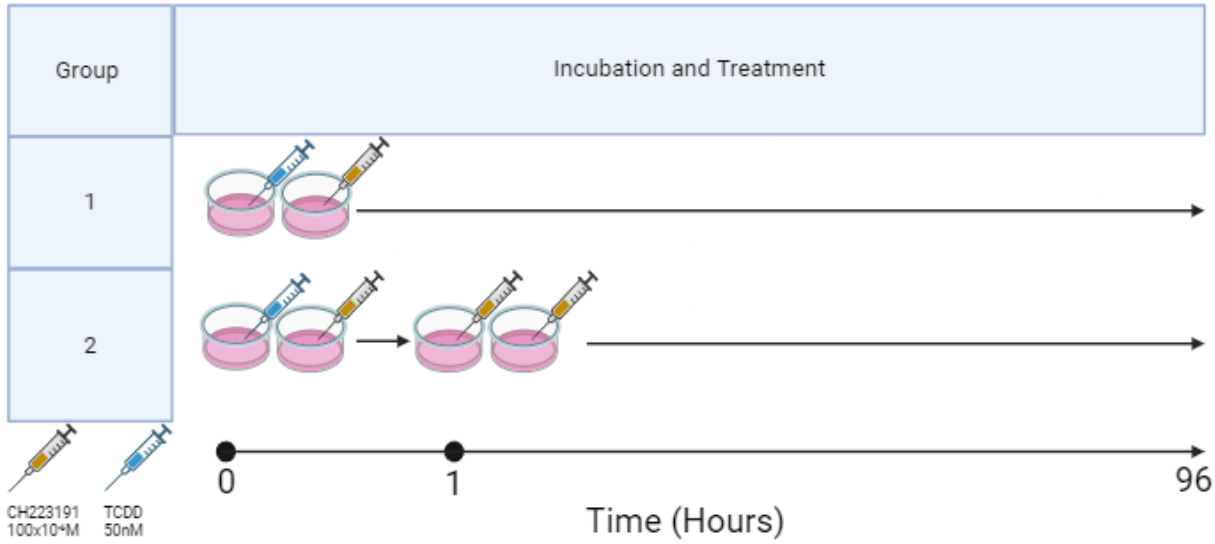
Experiment 3: Effects of Cell Media Replacement

A third study (experiment 3) was conducted to assess the effects of sample handling and media replacement on marker expression. Experiment 3 was performed as described under the methods section for experiment 1 except with the following modifications to the experimental design: male C57Bl/6J mice were used as a source of B cells instead of females. Cells were exposed to TCDD (50 nM) or CH223191 (100 µM) at the start of culture incubation (hour 0) only. After one hour of incubation, some samples were washed twice with CM, and then CH223191 (100 µM) was added to attenuate AhR activation by endogenous CM components (Veldhoen et al., 2008). Additionally, reagents for stimulating immune activation were again added to washed cultures as at hour zero to improve low cell recovery. Other samples (controls) did not undergo washing and CM replacement. All cultures were incubated as described for

experiment 1, including 96 hours of total incubation time prior to analysis of select marker expression via flow cytometry.

Figure 11

Experimental Design for Assessing the Effects of Replacing Cell Media



Note. 5x10^5 B cells/mL from C57BL/6J mice were added to 48 well tissue culture plates under immune activating condition and received either AhR agonist (TCDD, 50nM) or AhR antagonist (CH223191, 100 µM) at the start of incubation (hour 0). After one hour of incubation, AhR agonists and antagonists are removed in interventional cultures (group 2) by CM replacement supplemented with CH223191. Cultures were incubated for 96 hours total and then analyzed by flow cytometry.

Statistical Analysis

To evaluate my hypotheses, the means for the collected data between experimental groups was compared using 2-way analysis of variance (ANOVA) in SigmaPlot software (14.0). The first variable of analysis for all experiments was between samples treated with the AhR antagonist, CH223191 (considered controls), and those exposed to the AhR agonist, TCDD (Lamas et al., 2018; Zhao et al., 2010). The second parameter of analysis varied depending upon the experimental design: experiment 1 – time of treatment with TCDD (hours 0, 1, 4, or 24 of culture incubation), experiment 2 – sex of the mouse utilized (male vs. female), and experiment 3

– culture handling (washed at hour 1 of incubation vs. unwashed). Differences between the means of the groups tested were considered statistically significant at $P \leq 0.05$.

CHAPTER IV

RESULTS

Experiment 1: Time-Course Study

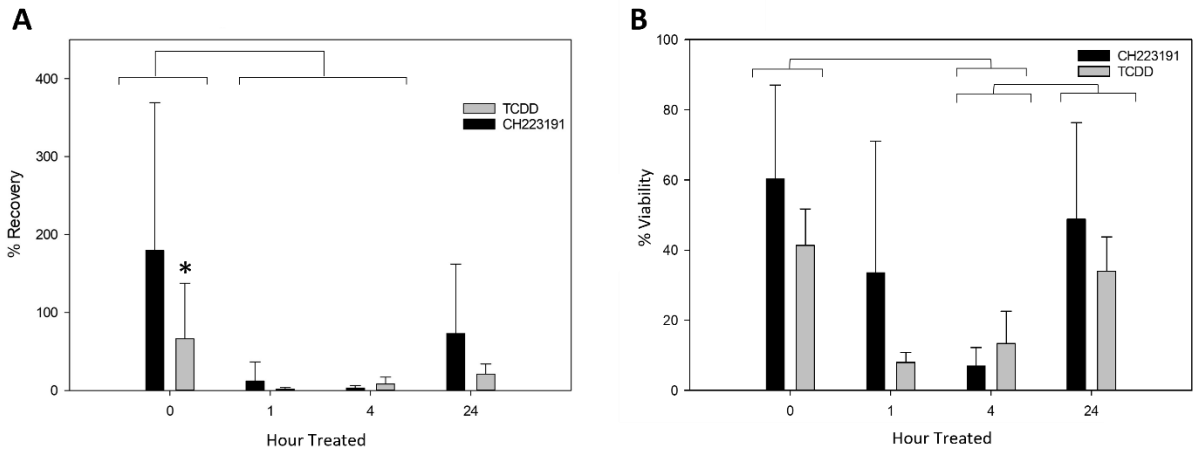
Recovery and Viability of B Cell Cultures

As shown in Figure 12A, when AhR ligands were added at hour 0 of culture, TCDD exposure significantly reduced the recovery of cells after 96 hours by over 2-fold relative to CH223191 exposure. When ligands were added at later time-points, no significant treatment effects were observed. However, manipulation of the cells at hours 1 and 4 significantly reduced cell recovery, independent of other treatments (Figure 12A).

As shown in Figure 12B, cell viability may have been reduced by TCDD exposure at some time-points of addition, but at no time-point was this effect statistically significant. Surprisingly, manipulation of the cells after hour 0 by itself could reduce cell viability at 96 hours (Figure 12B). When compared to hour 0, significantly reduced viability was observed when cells were manipulated at hour 4. Interestingly, manipulation of cells at hour 24 had no significant impact on cell viability.

Figure 12

Recovery and Viability of B Cell Cultures in Time-Course Study



Note. Depiction of pooled cell recovery (A) and viability (B) data from separate experiments (N=4-6). B cells from female C57BL/6J mice were cultured under immune activating conditions for 96 hours. Treatment with AhR ligands occurred at hours 0, 1, 4, or 24 of culture incubation. At culture termination, the concentration of living and dead cells was measured using a hemocytometer, microscope, and trypan blue exclusion dye. All data are depicted as mean value ± SD. Significant differences between treatments with AhR ligands are indicated by "*", while brackets indicate differences between groups based on the time of treatment with AhR ligands.

Gut Migration Marker Expression

Chemokine Receptor 9

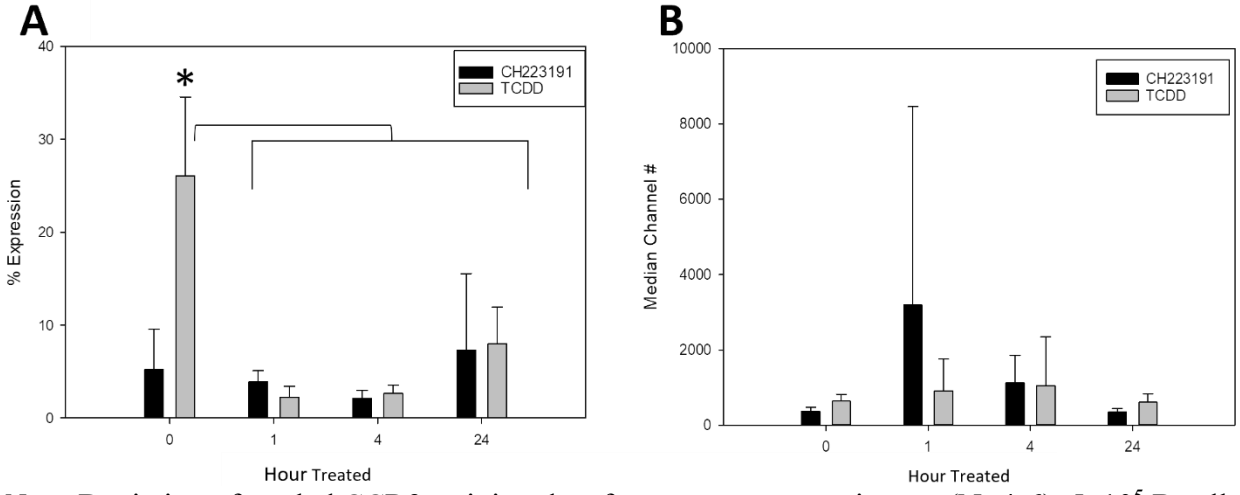
Figure 13A illustrates that treatment with TCDD increased the frequency of CCR9 expression by B cells relative to CH223191 treated cultures at all treatment times tested except for hour 1. The difference in CCR9 expression frequency according to AhR ligand treatment was significant only if treatment occurred simultaneously to immune stimulation (hour 0, $P < 0.001$), wherein a nearly 5-fold increase in the frequency of B cell CCR9 expression was observed following AhR activation. This considerable increase in CCR9 expression frequency by treatment with TCDD occurred only at hour 0, resulting in a significant difference with cultures treated at all other times (hour 0 vs 1 $P<0.001$, hour 0 vs. 2 $P<0.001$, hour 0 vs. 24 $P=0.003$). Notably, statistical analysis of the frequency of CCR9 expression indicates a significant

interaction between treatment with AhR ligands and the timing of such treatment during culture incubation ($P<0.001$).

Figure 13B, in contrast, shows that the relative level of CCR9 expression (MFI) by B cells did not vary considerably based on treatment with either AhR ligand or the timing of such treatment. Notably, a large degree of variation in the MFI data for cultures treated with CH223191 at hour 1 was observed. Such variation was not replicated in any other treatment group, so that no statistically significant differences were identified between cultures based on the time of treatment with AhR ligands nor which AhR ligand was employed.

Figure 13

CCR9 Expression by B cells in Time-Course Study



Note. Depiction of pooled CCR9 staining data from separate experiments (N=4-6). $5x10^5$ B cells from female C57BL/6J mice were cultured under immune activating conditions for 96 hours. TCDD or CH223191 were added at hours 0, 1, 4, or 24 of culture incubation. Membrane CCR9 expression was measured using fluorescently labeled antibodies via flow cytometry at culture termination (hour 96). A: frequency of CCR9 expression by time of treatment. B: Median fluorescence intensity (MFI) of CCR9+ population by time of treatment. Data depicted as mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between groups by time of treatment illustrated by brackets.

Alpha4Beta7

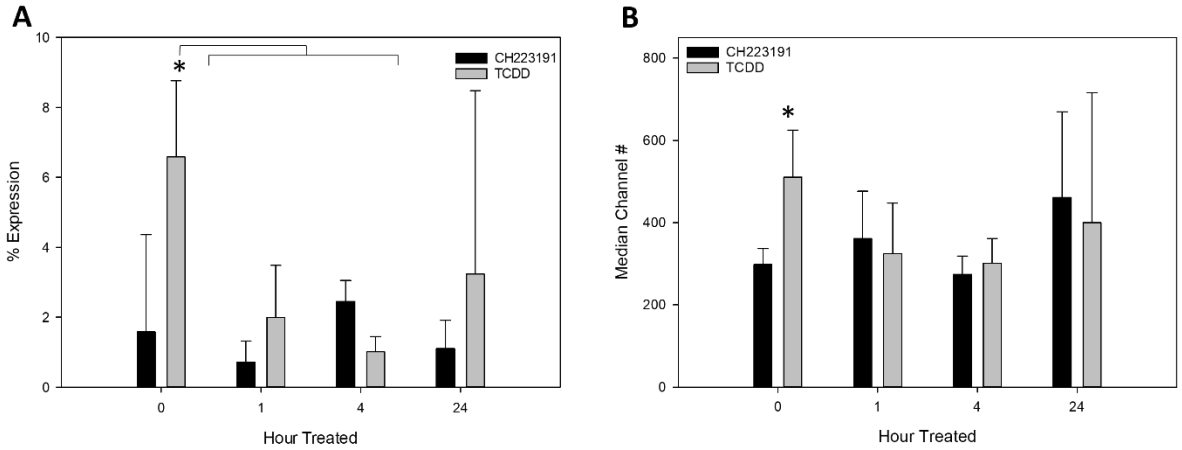
Figure 14A demonstrates that treatment with TCDD resulted in an increase in the proportion of B cells (frequency) expressing $\alpha_4\beta_7$ integrin at all treatment times, except for

during hour 4 of incubation. Differences in expression between cultures based on treatment with AhR ligand were only determined to be significant when treatment occurred simultaneously with AhR activation (treated hour 0, P=0.001). Interestingly, treatment with TCDD at hour 0 resulted in $\alpha_4\beta_7$ expression frequency that was also significantly increased relative to expression frequency in cultures treated with TCDD at hours 1 (P=0.018) and hour 4 (P=0.007), but not those treated at hour 24. Statistical analysis of the frequency of $\alpha_4\beta_7$ expression indicates a significant interaction between treatment with AhR ligands and the timing of such treatment during culture incubation (P=0.046).

Figure 14B indicates that treatment with TCDD resulted in an increase in the relative abundance (level) of $\alpha_4\beta_7$ integrin expression at hours 0 and 4 only. Differences between $\alpha_4\beta_7$ expression levels between cultures based on treatment with AhR ligand were only determined to be significant when treatment occurred simultaneously with AhR activation (treated hour 0, P=0.019). The level of $\alpha_4\beta_7$ integrin expression was comparable among all times of treatment with AhR ligands, with no significant differences observed based on this variable.

Figure 14

$\alpha_4\beta_7$ Expression by B Cells in Time-Course Study



Note. Depiction of pooled $\alpha_4\beta_7$ staining data from separate experiments (N=4-6). $5x10^5$ B cells from female C57BL/6J mice were cultured under immune activating conditions for 96 hours. TCDD or CH223191 were added at hours 0, 1, 4, or 24 of culture incubation. Membrane $\alpha_4\beta_7$ expression was measured using fluorescently labeled antibodies via flow cytometry at culture termination (hour 96). A. frequency of $\alpha_4\beta_7$ expression by time of treatment. B. Median fluorescence intensity (MFI) of $\alpha_4\beta_7$ + population by time of treatment. Data depicted as mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between groups by time of treatment illustrated by brackets.

Immunoglobulin Expression

Immunoglobulin M

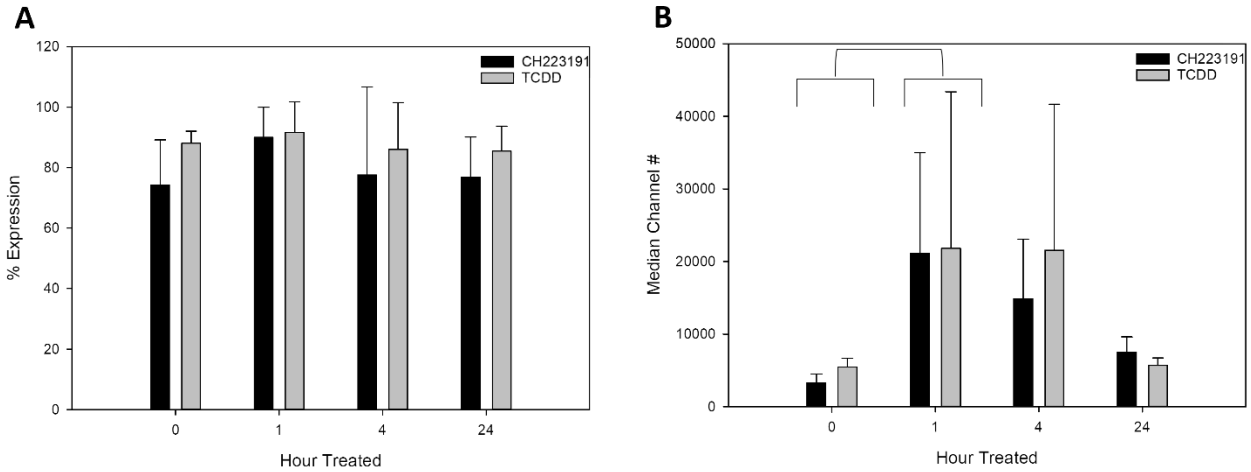
Figure 15A illustrates that exposure to TCDD had no effect on the frequency of membrane bound IgM expression at all treatment times tested. Similarly, the time of treatment with AhR ligands didn't significantly impact the proportion of cells expressing IgM.

Figure 15B shows the relative abundance of cellular membrane bound IgM expression when treated with AhR ligand at various times during culture. Treatment with TCDD increased the abundance of membrane bound IgM molecules to varying degrees at all treatment times except for hour 24. While differences between expression level of IgM between Ahr ligand treatments were of varying magnitude, none of these differences were found to be significant. The level of membrane bound IgM expression increased to varying degrees at later times of

treatment, regardless of AhR ligand. Treatment at hour 1 of incubation resulted in a significant increase in the level of IgM expression relative to groups treated at hour 0 (P=0.034). While the level of IgM expression varied considerably between different treatment times, no other differences between the groups examined were found to be significant.

Figure 15

IgM Expression by B Cells in Time-Course Study



Note. Depiction of pooled immunoglobulin staining data from separate experiments (N=4-6). $5x10^5$ B cells from female C57BL/6J mice were cultured under immune activating conditions for 96 hours. TCDD or CH223191 were added at hours 0, 1, 4, or 24 of culture incubation. Membrane immunoglobulin expression was measured using fluorescently labeled antibodies via flow cytometry at culture termination (hour 96). A. Frequency of IgM expression by time of treatment. B. Median fluorescence intensity (MFI) of IgM+ population by time of treatment. Data depicted as mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between groups by time of treatment illustrated by brackets.

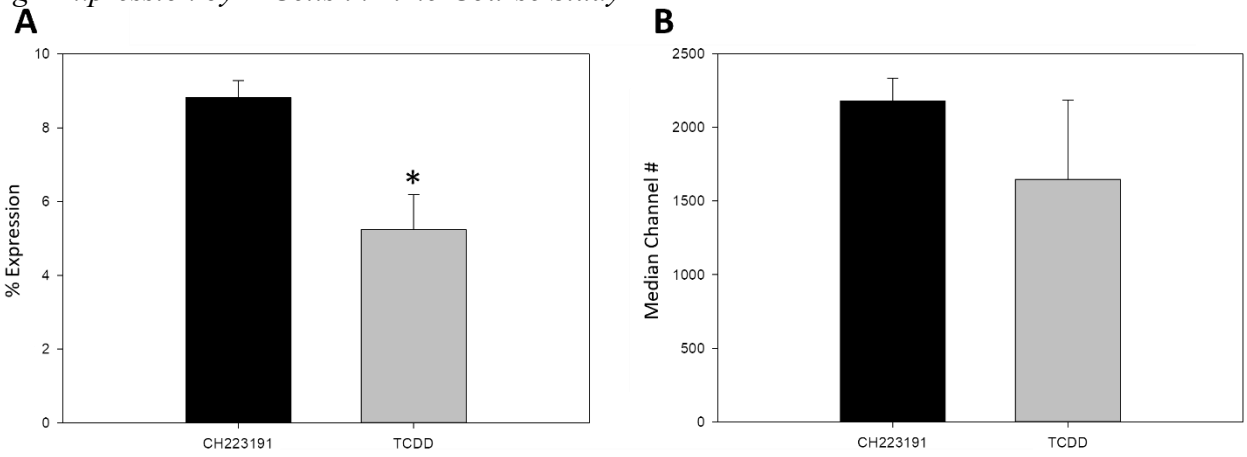
Immunoglobulin A

Figure 16A shows that, among female derived B cell cultures treated with AhR ligands simultaneously with immune stimulation, the frequency of IgA expressing B cells was significantly reduced in TCDD treated cultures (P=0.004).

Figure 16B indicates that cultures treated with TCDD display reduced levels of relative surface IgA expression, although this effect was not found to be significant.

Figure 16

IgA Expression by B Cells in Time-Course Study



Note. Depiction of pooled immunoglobulin staining data from two separate experiments (N=3). B cells from female C57BL/6J mice were given TCDD or CH223191 and incubated under immune stimulating conditions for 96 hours. Surface immunoglobulin expression was measured using fluorescently labeled antibodies via flow cytometry at culture termination (hour 96). A. Frequency of IgA expression by time of treatment. B. Median fluorescence intensity (MFI) of IgA+ population by time of treatment. Data depicted as mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*".

Gut Migration Marker Expression by Antibody Isotype

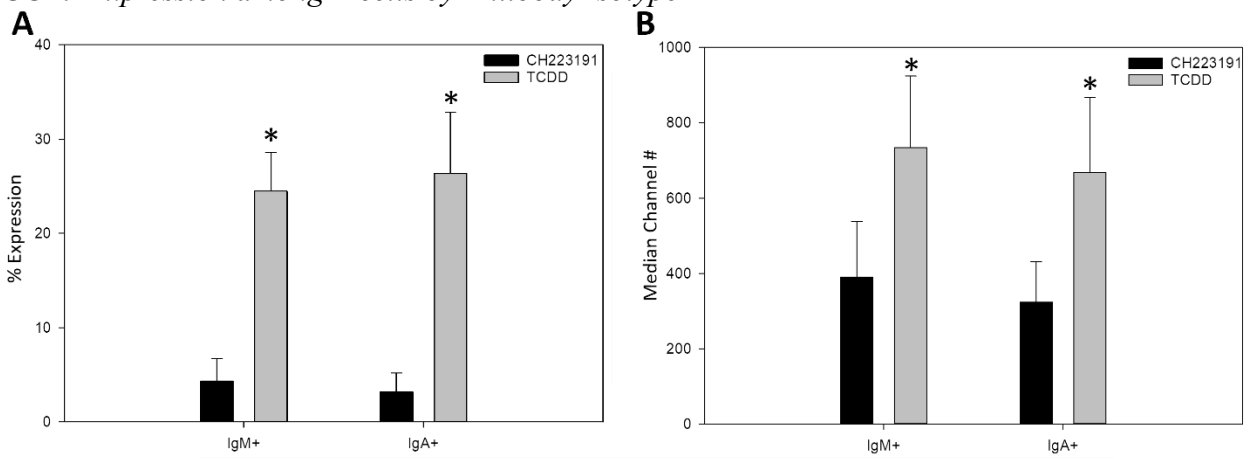
Chemokine Receptor 9

Figure 17A clearly shows that activation of the AhR by treatment with TCDD results in a significant increase in the frequency (%) of CCR9 expression in both IgM+ ($P<0.001$) and IgA+ ($P<0.001$) B cells. While this effect was slightly elevated among IgA+ cells, no significant differences were observed between CCR9 expression frequency based on expression of antibody isotype.

Figure 17B similarly shows that activation of the AhR by treatment with TCDD results in a significant increase in the level of CCR9 expression (MFI) in both IgM+ ($P=0.035$) and IgA+ ($P=0.034$) B cells. Again, this effect is slightly elevated in IgA+ cells, but no significant differences were found in the level of CCR9 expression by identified antibody isotype.

Figure 17

CCR9 Expression among B cells by Antibody Isotype



Note. Depiction of pooled CCR9 staining data from separate experiments (N=3). $5x10^5$ B cells from female C57BL/6J mice were cultured under immune activating conditions for 96 hours. TCDD or CH223191 were added at the start of culture incubation. Membrane CCR9, IgM, and IgA expression was measured using fluorescently labeled antibodies via flow cytometry at culture termination. A. Frequency of CCR9 expression (%) by antibody isotype. B. Median fluorescence intensity (MFI) of CCR9+ population by antibody isotype. Data depicted as mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between antibody isotype illustrated by brackets.

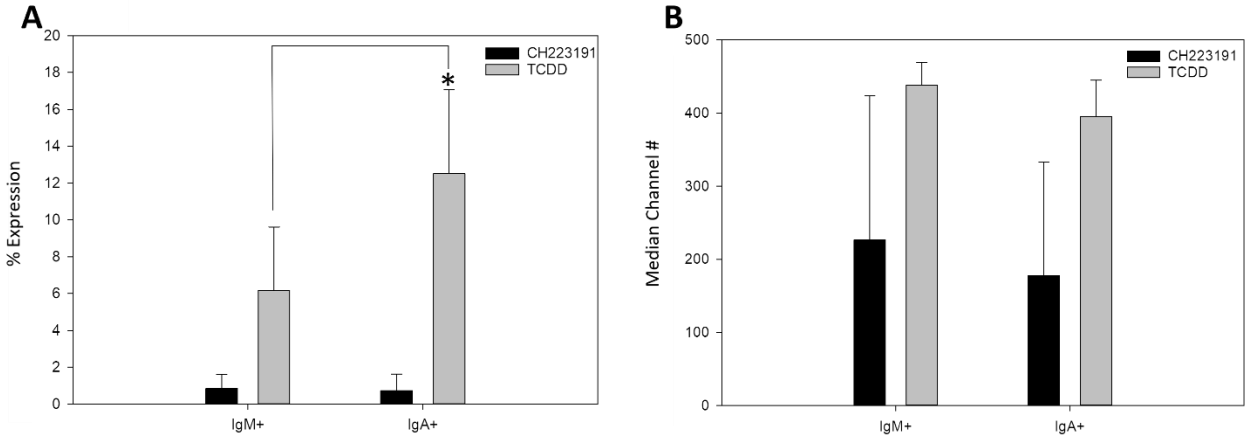
Alpha4Beta7

Figure 18A illustrates that activation of the AhR by treatment with TCDD results in an increase in the frequency (%) of $\alpha_4\beta_7$ expression in both IgM+ and IgA+ B cells. Induction of $\alpha_4\beta_7$ expression frequency by AhR activation was significant in IgA+ cells only (P=0.001), which was also significantly elevated compared to the frequency of $\alpha_4\beta_7$ expression observed among TCDD treated IgM+ B cells (P=0.029).

Whereas Figure 18B shows AhR activation resulted in an increase in the levels of $\alpha_4\beta_7$ expression per cell in both IgA+ and IgM+ B cells, this increase was not determined to be statistically significant regardless of identified antibody isotype.

Figure 18

$\alpha_4\beta_7$ Expression among B Cells by Antibody Isotype



Note. Depiction of pooled $\alpha_4\beta_7$ staining data from separate experiments (N=3). $5x10^5$ B cells from female C57BL/6J mice were cultured under immune activating conditions for 96 hours. TCDD or CH223191 were added at the start of culture incubation. Membrane $\alpha_4\beta_7$, IgM, and IgA expression was measured using fluorescently labeled antibodies via flow cytometry at culture termination. A. Frequency of $\alpha_4\beta_7$ expression (%) by antibody isotype. B. Median fluorescence intensity (MFI) of $\alpha_4\beta_7$+ population by antibody isotype. Data depicted as mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between antibody isotype illustrated by brackets.

Experiment 2: Sex-Based Study

Recovery and Viability of B Cell Cultures

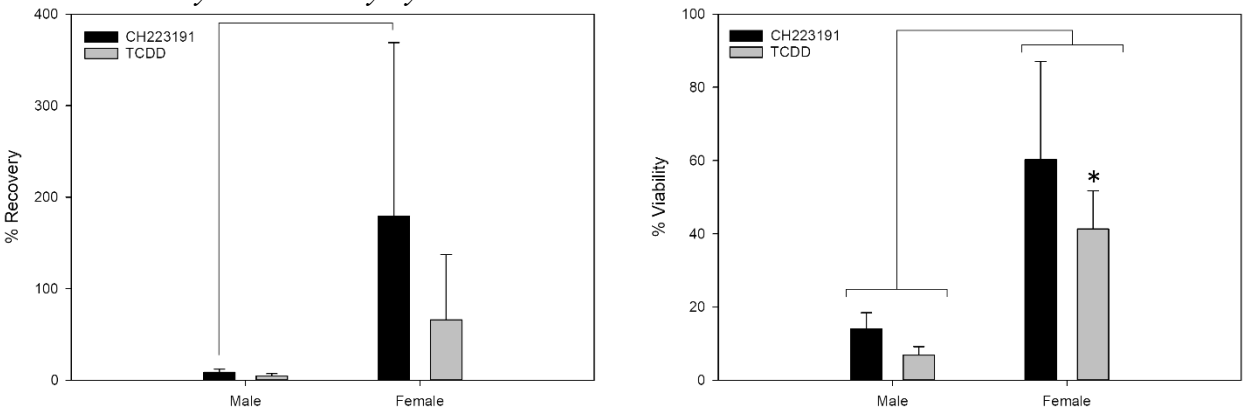
As shown in Figure 19A, the recovery of male derived B cell cultures was much lower than female derived cultures (approximately 20-fold difference). A statistically significant difference in recovery between the sexes occurred among CH223191 treated cultures (P=0.008) but was not observed in TCDD treated cultures. Regardless of sex, treatment with TCDD reduced cell recovery in comparison with CH223191 treated cultures. However, after taking differences in recovery due to sex into account, treatment with TCDD was not found to significantly reduce cell recovery in males or females.

Figure 19B shows that viability of male B cell cultures was significantly lower when compared to female cultures (approximately 5-fold difference), regardless of treatment with AhR

ligands (P<0.001). Treatment with TCDD reduced cell viability in comparison with CH223191 treated cultures in both male and female derived cultures, although this effect was statistically significant only within females (P=0.034).

Figure 19

B Cell Recovery and Viability by Sex



Note. Depiction of pooled cell recovery (A) and viability (B) data from separate experiments (N=6). B cells from female and male C57BL/6J mice were immunologically stimulated and treated with CH223191 or TCDD at the start of culture. After 96 hours of incubation, the concentration of living and dead cells was measured using a hemocytometer, microscope, and trypan blue exclusion dye. All data are depicted as mean value ± SD. Significant differences between groups by treatment with AhR ligands are indicated by "*", while brackets indicate those between groups based on sex.

Gut Migration Marker Expression

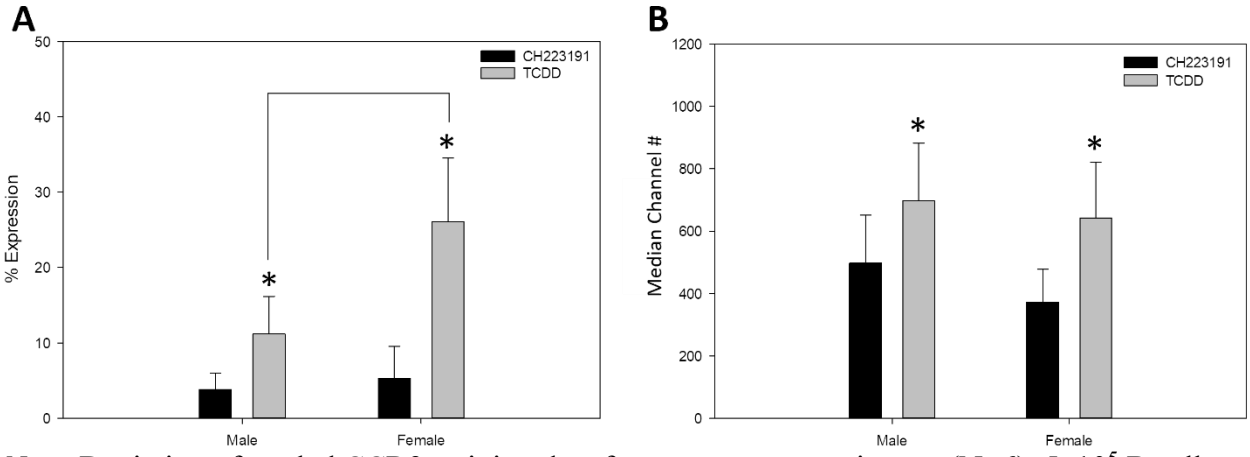
Chemokine Receptor 9

Figure 20A clearly demonstrates that treatment with TCDD at onset of culture incubation significantly increased the frequency of CCR9 expression by B cells in both males (P=0.031) and females (P=0.001). This resulted in an approximately 3-fold increase in the frequency of CCR9 expression in males, and an approximately 4-fold increase in the frequency of CCR9 expression in females. This effect of TCDD on CCR9 expression frequency was significantly different in cultures derived from male and female mice (P=0.020). Cultures treated with CH223191 at culture initiation were not found to be significantly different, regardless of the sex of the source animal.

Figure 20B similarly shows that treatment with TCDD significantly increases the relative level of CCR9 expression in cultures derived from either male (P=0.042) or female (P=0.008) animals. Interestingly, no significant differences were found between the level of CCR9 expression in cultures based on the sex of the source animal.

Figure 20

CCR9 Expression by B Cells in Sex-Based Study



Note. Depiction of pooled CCR9 staining data from separate experiments (N=6). $5x10^5$ B cells from C57BL/6J mice were given TCDD or CH223191 and cultured under immune activating conditions for 96 hours. Membrane CCR9 expression was measured using fluorescently labeled antibodies via flow cytometry at culture termination (hour 96). A. Frequency of CCR9 expression by sex of source animal. B. Median fluorescence intensity (MFI) of CCR9+ population by sex of source animal. Data depicted as mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between groups by sex of source animal illustrated by brackets.

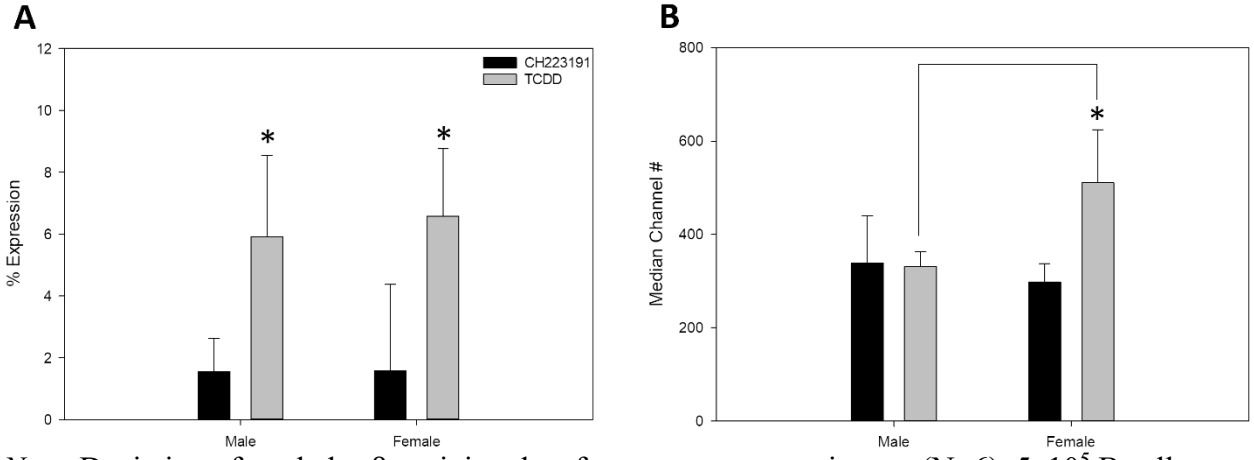
Alpha4Beta7

Figure 21A clearly shows that activation of the AhR resulted in a significant increase in the frequency of B cells expressing $\alpha_4\beta_7$ integrin. In cultures derived from male splenocytes, the percentage of cells expressing $\alpha_4\beta_7$ integrin increased approximately 3-fold (P=0.003), whereas female cultures showed an approximately 2-fold increase (P=0.001) relative to controls. The effect of AhR activation on the percentage of cells expressing $\alpha_4\beta_7$ integrin was not found to differ significantly according to the sex of the source animal for cell culture.

In contrast, Figure 21B illustrates that activation of the AhR induced significantly higher levels of $\alpha_4\beta_7$ integrin expression per cell (MFI) in B cell cultures derived from female mice only (P=0.003). The level of $\alpha_4\beta_7$ integrin expression in female derived cultures treated with TCDD was significantly increased compared to the levels of expression observed in male cultures treated with AhR agonist (P=0.002).

Figure 21

$\alpha_4\beta_7$ Expression by B Cells in Sex-Based Study



Note. Depiction of pooled $\alpha_4\beta_7$ staining data from separate experiments (N=6). $5x10^5$ B cells from C57BL/6J mice were given TCDD or CH223191 and cultured under immune activating conditions for 96 hours. Membrane $\alpha_4\beta_7$ expression was measured using fluorescently labeled antibodies via flow cytometry at culture termination (hour 96). A. Frequency of $\alpha_4\beta_7$ expression by sex of source animal. B. Median fluorescence intensity (MFI) of $\alpha_4\beta_7$+ population by sex of source animal. Data depicted as mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between groups by sex of source animal illustrated by brackets.

Experiment 3: Effects of Cell Media Replacement

Recovery and Viability of B Cell Cultures

Figure 22 depicts cell viability and recovery data for the cell cultures used for the effects of media replacement study. Cultures were treated with AhR ligands at culture start with one experimental replicate undergoing two washes to facilitate media replacement at hour 1 of

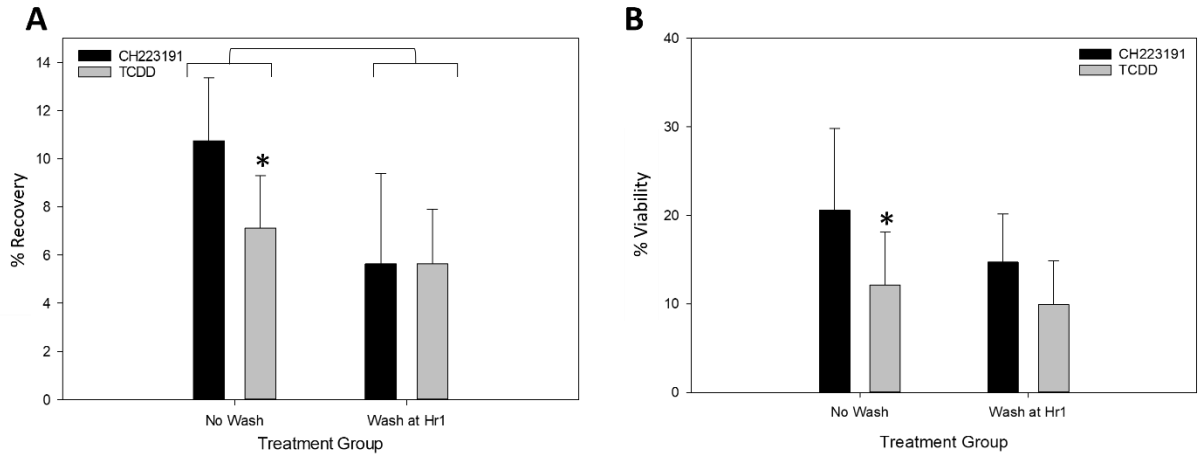
incubation (washed at hour 1). Cell counts and viability were obtained after 96 hours of incubation and prior to analysis of marker expression.

As shown in Figure 22A, replacement of cell media for B cell cultures after one hour of incubation under immune activating conditions significantly reduced cell recovery compared to unwashed samples (P=0.009). Additionally, cultures exposed to TCDD displayed reduced cell recovery relative to controls in unwashed samples only, the magnitude of which was found to be significant (P=0.035).

In Figure 22B, cell viability data for effects of media replacement study are shown. Media replacement at hour 1 of culture incubation resulted in modest reductions in cell viability of CH223191 treated cultures but was less pronounced in TCDD treated cultures. Importantly, the effect of media replacement at hour 1 of incubation was not found to cause a significant reduction in cell viability, regardless of treatment with AhR ligand. Treatment with TCDD, however, significantly reduced cell viability in cultures that did not undergo media replacement (i.e. no wash group; P=0.038) but failed to significantly reduce viability in cultures where media replacement occurred (i.e. wash at hour 1 group).

Figure 22

Recovery and Viability of B Cells in Effects of Media Replacement Study



Note. Representation of pooled cell recovery (A) and viability (B) data from separate experiments (N=6). B cells from male C57BL/6J mice were given TCDD or CH223191 at culture start and incubated under immune stimulating conditions. Experimental group cultures were washed 2x at hour 1 to replace media and remove AhR agonists. CH223191 was again added to cultures after washing. After 96 hours of incubation, the concentration of living and dead cells was measured using a hemocytometer, microscope, and trypan blue exclusion dye. Data show mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between treatment groups illustrated by brackets.

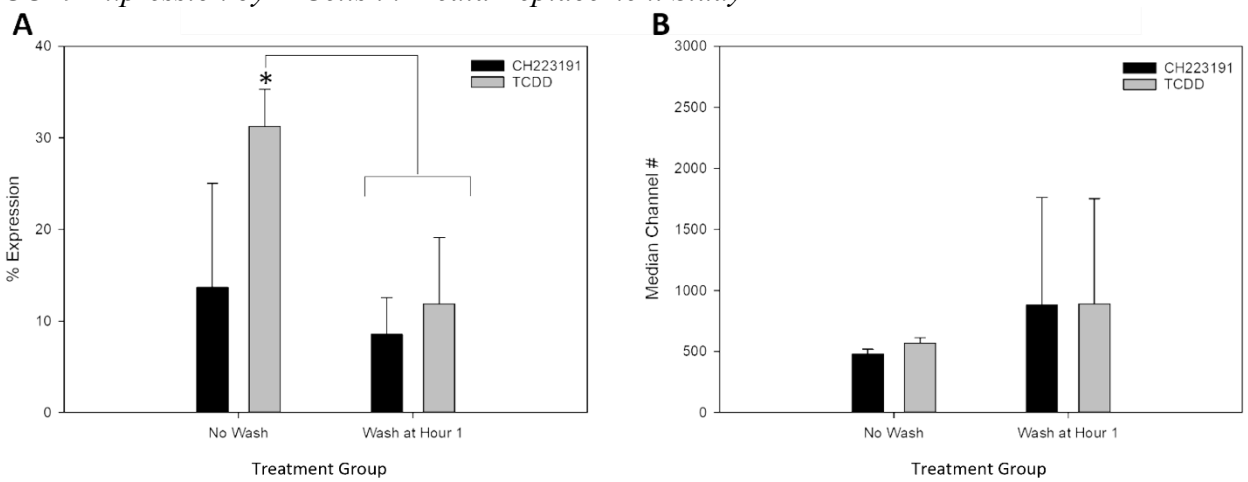
Gut Migration Marker Expression

Chemokine Receptor 9

Figure 23A shows that activation of the AhR via exposure to TCDD results in an increase in the frequency of B cells expressing CCR9 *in vitro*. If TCDD is removed within 1 hour of immune stimulation, this effect was found to be insignificant, while cultures exposed to TCDD throughout incubation display elevated CCR9 expression frequencies compared to controls ($P<0.001$). Notably, a significant interaction between treatment with AhR ligand and media replacement group designation occurred ($P=0.027$). In contrast, Figure 23B shows that neither treatment with AhR ligand nor media replacement at hour 1 of culture incubation had a significant effect on the level of CCR9 expression per cell by B cells *in vitro*.

Figure 23

CCR9 Expression by B Cells in Media Replacement Study



Note. Depiction of pooled data from separate experiments. B cells from male C57BL/6J mice were given TCDD or CH223191 at culture start and incubated under immune stimulating conditions (N=6). Experimental group cultures were washed 2x at hour 1 to replace media and remove AhR agonists. CH223191 was again added to cultures after washing. After 96 hours of incubation, CCR9 expression frequency (A) and median fluorescence intensity (B) were measured by flow cytometry. Data depicts mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between treatment groups illustrated by brackets.

Alpha4Beta7

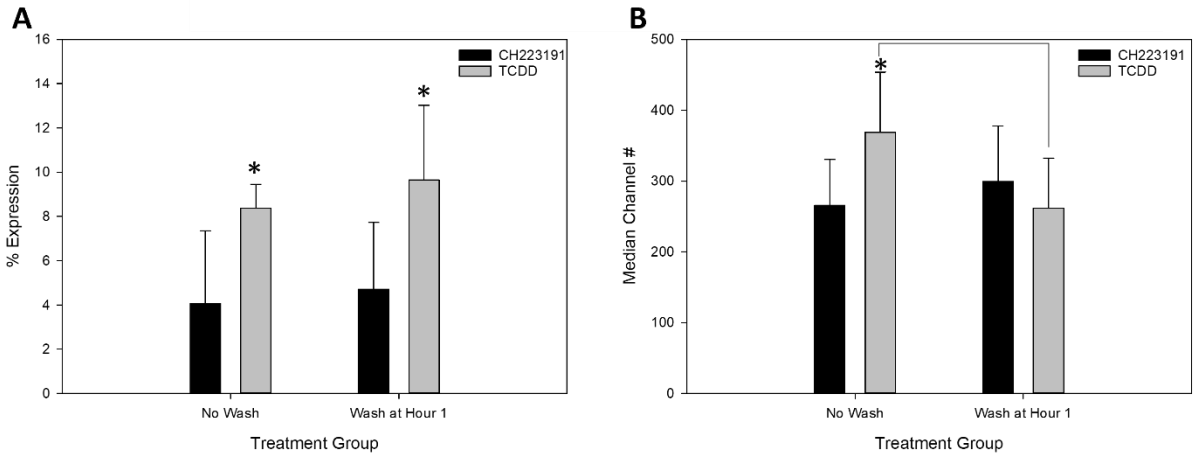
Figure 24A demonstrates that activation of the AhR via exposure to TCDD results in an increase in the frequency (%) of B cells expressing $\alpha_4\beta_7$ integrin *in vitro*. This effect was found to be significant in both cell cultures that underwent media replacement at hour 1 of incubation (P=0.007) and those that didn't (P=0.016). No significant differences in $\alpha_4\beta_7$ expression frequency were observed between cultures that received media replacement at hour 1 of incubation and those that didn't.

Figure 24B, however, shows that activation of the AhR via exposure to TCDD results in an increase in the level of $\alpha_4\beta_7$ integrin expression per cell (MCN) only by B cells that didn't undergo media replacement at hour 1 of culture incubation. This effect was significant in

comparison to comparable CH223191 treated controls (P=0.027) and cultures treated with TCDD that underwent media replacement at hour 1 of culture incubation (P=0.023).

Figure 24

$\alpha_4\beta_7$ Expression by B Cells in Media Replacement Study



Note. Depiction of pooled data from separate experiments. B cells from male C57BL/6J mice were given TCDD or CH223191 at culture start and incubated under immune stimulating conditions (N=6). Experimental group cultures were washed 2x at hour 1 to replace media and remove AhR agonists. CH223191 was again added to cultures after washing. After 96 hours of incubation, $\alpha_4\beta_7$ expression frequency (A) and median fluorescence intensity (B) were measured by flow cytometry. Data depicts mean values ± SD, with statistically significant differences between groups by treatment with AhR ligands indicated by "*", and those between treatment groups illustrated by brackets.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Experiment 1: Time-Course Study

B Cell Activation State and Inhibition of Cellular Proliferation by the Aryl Hydrocarbon Receptor

TCDD has long been known to suppress humoral immunity (Sulentic & Kaminski, 2010). Here, we present similar findings, as AhR activation consistently inhibited B cell responses following stimulation with anti IgD-Dextran and LPS. Exposure to TCDD attenuated B cell recovery and viability in nearly all cell cultures, although not always with statistical significance. However, when AhR activation occurred simultaneously to BCR stimulation, the reduction in B cell proliferation was substantial (approximately 50%). This and similar observations suggest that newly activated B cells display heightened sensitivity to AhR mediated suppression of cell cycling and survival pathways (Holsapple et al., 1986). Thus, the AhR likely exerts its control over B cell proliferation during early BCR signaling events. This could be explained by insufficient activation of pro-survival factors, such as Akt, Erk1/2, or NF-kB (Irish et al., 2006). Similarly, the anti-apoptotic gene BCL-6 is dysregulated by the AhR, possibly contributing to AhR associated loss of cell vitality (North et al., 2009).

B Cell Activation State Affects Gut Imprinting by the Aryl Hydrocarbon Receptor

When AhR activation with TCDD was concurrent to B cell stimulation, expression of the lymphocyte gut homing proteins CCR9 and $\alpha_4\beta_7$ was reliably induced. At later stages of B cell

differentiation, AhR activation failed to illicit a gut homing phenotype. McMillan et al. (2007) reported a similar result in T cells. They observed that mice given TCDD (100 μg/kg) displayed alterations in the trafficking programs of developing T cells. T cell progenitors upregulated β_7 integrin expression and egressed prematurely from the thymus. This aberrant trafficking preceded immune cell loss, was attenuated within 12 hours of TCDD administration, and relied on AhR expression. Interestingly, they associated dysregulation of T cell homing with upregulation of the transcription factor, krüppel-like factor 2 (KLF-2), following AhR activation. Another group of transcription factors, the retinoic acid receptors (RAR), respond to vitamin A and its derivatives and are known to promote gut homing phenotypes in T cells (Manhas et al., 2022). The AhR can interact with retinoic acid receptor signaling, providing an alternative explanation for the induced gut homing phenotype (Rothhammer & Quintana, 2019). Together, these data suggest that lymphocyte sensitivity to AhR related control of gut imprinting depends on the stage of B cell differentiation and is possibly mediated by transcription factors KLF-2 and or RARs.

Immunoglobulin Isotype and Differential Expression of Alpha4Beta7

Immunoglobulin M

Most B cells in this study were found to express surface IgM, ranging from approximately 75-100%. This was expected, as B cell class switching only occurs rarely in the absence of germinal center formation and T cell help (Schatorjé et al., 2014; Wood, 2011). Here, the fraction of cells expressing surface IgM appeared to be unaffected by the nature or timing of AhR ligand addition, which mirrored the results of others (Kashgari, 2015). Interestingly, AhR deficiency has been previously associated with a heightened proportion of IgM+ cell production in response to LPS (Phadnis-Moghe et al., 2016). While antagonism of the AhR didn't replicate

this result in the current study, others have suggested functional differences in regulation of Ig expression in AhR knockdown compared to receptor antagonism (Kashgari, 2015).

Immunoglobulin A

In contrast, AhR activation reduced the fraction of cells expressing surface IgA. Utilizing a similar method to stimulate naïve splenocytes, Vaidyanathan et al. (2016) found comparable levels of IgA expression when AhR activity was at basal levels (~10%), which increased dramatically in AhR knockouts. Negative regulation of essential recombination machinery and resulting inhibition of class switching is known to occur during AhR mediated changes in gene expression (Costa, 2018; Vaidyanathan et al., 2016). Retinoic acid receptor signaling is another important factor for CSR to IgA that cross-signals with AhR pathways, and thus could be impacted by TCDD (Rothhammer & Quintana, 2019; Wood, 2011).

Unfortunately, the interaction between treatment time and AhR activation on IgA expression could not be determined due to the complete absence of class switching to IgA in cultures treated at later stages of B cell differentiation. This could potentially be explained by insufficient levels of IL-21 or retinoic acid, both of which promote CSR to IgA, the former of which through polarization away from TGF-β mediated CSR to IgG and toward IgA (Wood, 2011). CSR to IgA may have additionally been impacted by an outbreak of pinworm that occurred in the mouse colony during portions of this study. Ivermectin treatments and parasitic infection thus may have altered immune cell functions during this incident (Esser, 2016).

Gut Imprinting by Antibody Isotype

Interestingly, IgA+ cells displayed nearly identical proclivities for gut imprinting as IgM+ cells (data not shown) and the general B cell population, except in the case of the proportion of cells expressing $\alpha_4\beta_7$. The fraction of IgA+ cells expressing $\alpha_4\beta_7$ was significantly increased

(approximately two-fold) in comparison with IgM+ cells. As CSR is associated with maturation into effector cells, this could reflect a difference in regulation of trafficking programs between blasting cells and those in a more differentiated state (Seong et al., 2017; Uehara et al., 2002). It also presents an intriguing possibility that a connection may exist between expression of mucosal homing proteins and CSR to the primary secretory Ab IgA, although the lack of clarity on marker expression by Ab isotypes other than IgM or IgA provides little corroboration for this at present (Wood, 2011).

Experiment 2: Sex-Based Study

Sex-Based Differences in B Cell Proliferation

Sexual dimorphism among immune cells is well characterized and was observed in the proliferation of B cells in this study (Bhatia et al., 2014). Male derived cultures displayed profoundly diminished proliferation compared to female cultures, regardless of AhR ligand exposure. AhR activation, despite consistently reducing B cell proliferation in either sex, was a less significant factor than sex in determining B cell recovery and viability, particularly in males.

While females are known to display heightened proliferation and activation of lymphocytes compared to males, the magnitude of the difference observed in this study (approximately 20 fold-difference) implies the possibility of contribution from additional factors (Dodd & Menon, 2022). As these experiments were carried out separately and over a period of several months, variations in the efficacy of immune stimulating reagents, experimental technique, or the condition of the mice were possible. The contribution of such unidentified variables may have exacerbated sex-based differences in B cell proliferation in this study, thereby influencing the degree to which AhR activation was assessed to be responsible for alterations in male B cell proliferation.

Sex-Based Differences in Expression of Gut Migration Markers via the Aryl Hydrocarbon Receptor

While AhR activation promoted CCR9 and $\alpha_4\beta_7$ expression in B cells from both males and females, the effect was much more pronounced in females. This result is consistent with studies showing that AhR mediated changes in gene expression differ among the sexes. Hepatocytes of male and female mice given equivalent doses of TCDD display similar regulation of some AhR mediated genes but differ significantly in others (Lee et al., 2015). While this makes sense, as the liver shows a high degree of sexual dimorphism and sensitivity to TCDD toxicity is partially sex dependent, here our results suggest that this trend applies to AhR regulation of B lymphocyte gut imprinting as well (Nault et al., 2017). Such enhanced gut trafficking may preserve barrier integrity and promote survival by allowing females to maintain much needed resources to maximize reproductive output (Nunn et al., 2008).

Limitations

Effects of Culture Media Replacement During Incubation

Manipulation of B cells during culture incubation, such as during media replacement to facilitate treatment, was found to potentially present a confounding variable in this study. When such manipulation was the only variable in experiment 3, where cultures were treated with TCDD at culture start and cultures were washed at hour 1, media replacement/washing significantly reduced recovery in both control and TCDD treated cultures (Figure 22). Re-addition of immune stimulating reagents after cell washing and media replacement failed to rescue diminished recovery in control or treated cultures (data not shown). Others have noted the fragility of splenic B cells derived from mammals, although viability was not found to be severely impacted by media replacement in this study (Old et al., 2004). However, media

replacement did not significantly affect expression of gut homing proteins in control samples, suggesting that cell loss may not have been accompanied by alterations in B cell trafficking proclivities. Removal of TCDD after one hour of incubation generally attenuated AhR associated induction of gut homing protein expression. AhR activation for one hour proved sufficient to increase the proportion of cells expressing $\alpha_4\beta_7$ (Figures 23, 24). Perhaps this reflects differences in the kinetics of negative regulation of CCR9 and $\alpha_4\beta_7$, or that sustained AhR activation is required to induce high levels of CCR9 expression in proliferating cells but not $\alpha_4\beta_7$.

Cultured Cell Phenotyping

Splenocytes represent a mixed population of leukocytes and other cell types (Ortiz, 2021). While the effectiveness of B cell isolation was confirmed by ensuring a CD19+ population of at least 94%, many B cell subsets are present within the spleen and there is an opportunity for cellular contamination (Lewis et al., 2019; Wang et al., 2012). Cell cultures were shown to be predominately IgM+, but comprehensive B cell phenotyping was beyond the scope of this analysis. As a result, it's difficult to characterize the relative proportions of B cell subtypes present upon analysis, which could include marginal zone B cells, memory cells, and innate like B1 cells in addition to the desired plasmablasts generated from naïve splenocytes (Lewis et al., 2019). Activation of these naïve B cells with anti-IgD antibodies should enrich primarily for this cell type, but some antibody secreting cells express surface IgD and others may have received BCR stimulation *in vivo*, prior to culture initiation (Wood, 2011). Different B cell subtypes would be expected to display unique responses to AhR activation, which could have affected gut migration marker expression, especially in cultures with relatively low recoveries (Sherr & Monti, 2013).

Conclusions

Time-Course Study

Naïve B cells were more susceptible to AhR mediated induction of the gut homing proteins CCR9 and $\alpha_4\beta_7$ during BCR activation than at later times under the conditions tested (acceptance of H1). AhR activation as soon as one hour post BCR stimulation failed to illicit a gut homing phenotype, a phenomenon which persisted for at least 24 hours post B cell activation. This suggests that regulation of trafficking programs by the AhR is at least partially dependent on the stage of B cell activation. This assertion is supported by observations that expression levels of AhR vary among specific B cell populations. Pro- and Pre-B cells in the bone marrow have been shown to express minimal levels of AhR expression, while proliferating cells express slightly increased AhR levels, and transitional and plasma cells display the highest levels of AhR expression (Sherr & Monti, 2013). BCR activation further upregulates the AhR, with the results of this study suggesting that the influence exerted by the AhR over gut homing protein expression is rapidly attenuated during the processes of proliferation and differentiation (Vaidyanathan et al., 2016; Villa et al., 2016). Such variation in B cell sensitivity to control of trafficking programs by the AhR may promote egress of newly formed plasmablasts for immediate participation in immune responses while ensuring a subset of activated B cells remain at the site of Ag exposure to facilitate maturation into longer lived effector cells.

Sex-Based Study

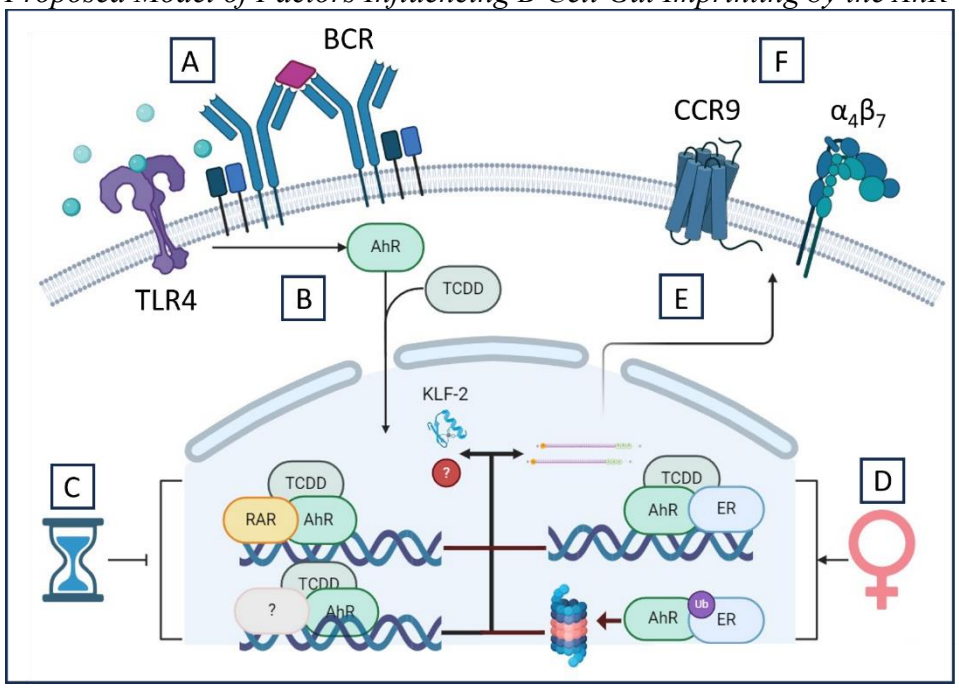
B cells derived from female mice exhibited increased sensitivity to AhR mediated induction of CCR9 and $\alpha_4\beta_7$ than those originating from males in this study (acceptance of H2). Sexual dimorphism in mammals is known to extend to various processes such as responses to chemicals, metabolism, and immunity (Lee et al., 2015; Nunn et al., 2008). Female mice have

been shown to express higher levels of the AhR compared to males, and sex specific responses to AhR activation have been previously observed (Lee et al., 2015; Lu et al., 2013). Others have suggested that sex-based differences in immunity result from the activity of sex hormone receptors, and that the estrogen receptor may play the dominant role (Bhatia et al., 2014; Nunn et al., 2008). Activated AhR is capable of binding to estrogen receptors and regulating expression of estrogen associated genes through direct recruitment to the DNA and by promoting ubiquitination of estrogen receptors (Ohtake et al., 2008). Sustained exposure to AhR agonists diminished sexual dimorphism in murine hepatocyte gene expression over time, possibly due to negative regulation of estrogen receptors by the AhR (Nault et al., 2017). Taken together, these observations seem to indicate that sexual dimorphism in the regulation of immune cells is at least partially reliant on interactions between AhR and estrogen receptor cell signaling pathways.

Hypothesized Mechanism of Action

Figure 25

Proposed Model of Factors Influencing B Cell Gut Imprinting by the AhR



Note. A. B cell activation with LPS and anti IgD antibodies leads to transient upregulation of AhR expression (Vaidyanathan et al., 2016). B. Activation of abundant AhR proteins with agonists results in changes in gene expression which modulate cell proliferation, survival, and upregulate expression of CCR9 and $\alpha_4\beta_7$ (Ortiz, 2021). C. AhR associated changes in gene expression may be rapidly attenuated during maturation into antibody secreting cells or through negative feedback of AhR signaling mechanisms (Henderson & Calame, 1998; Holsapple et al., 1986; Rothhammer & Quintana, 2019). Regulation of gut homing protein expression via the AhR is possibly mediated by interactions with other transcription factors such as the retinoic acid receptor or by an unknown mechanism (Manhas et al., 2022). D. Females likely display enhanced sensitivity to gut imprinting by the AhR because of AhR and estrogen receptor binding interactions or through negative control of ER signaling via ubiquitination (Bhatia et al., 2014; Ohtake et al., 2008). E. AhR mediated changes in gene expression either result in the expression of gut homing proteins directly or do so indirectly through alterations in the activity of KLF-2 or other regulatory factors (McMillan et al., 2007). F. Surface expression of gut homing proteins such as CCR9 and $\alpha_4\beta_7$ prepares B cells for translocation to the gut, particularly the small intestine (Seong et al., 2017).

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

UNIVERSITY OF
NORTHERN COLORADO

Institutional Animal Care and Use Committee

Date: *December 4, 2023*

Principal Investigator: *Greg Dekrey*

Committee Action: **IACUC Protocol- Annual Continuation Approval #2- 2023**

Action Date: December 4, 2023

Protocol Number: 2112C-GD-M-24
Protocol Title: TCDD Exposure Alters Mucosal Immunity by Enhancing IgA Secretion into the Gut Lumen

Expiration Date: December 13, 2024

The University of Northern Colorado Institutional Animal Care and Use Committee (IACUC) has completed an annual review and APPROVED the continuation of animal use protocol *TCDD exposure alters mucosal immunity by enhancing IgA secretion into the gut lumen* – #**2112C-GD-M-24** on December 4, 2023 for another year. Since no changes were incorporated into this protocol at this time, animal use may continue for another year as previously approved.

The committee's review was based on the requirements of the Government Principles, Public Health Policy, USDA Animal Welfare Act and Regulations, the Guide for the Care and Use of Laboratory Animals, as well as university policies and procedures related to the care and use of animals at the UNC. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI is approved to perform the experiments or procedures as described in the protocol as approved by the committee for another year. It is the responsibility of the PI to be familiar with and comply with the protocol and all pertinent institutional, state, and federal rules and policies. Until this protocol expires, annual IACUC review of the protocol is required.

If you have any questions, please contact the UNC Animal Care and Use Program (ACUP) Director, Laura Martin, at 970-351-4313 or via e-mail at laura.martin@unco.edu. Additional information concerning the requirements for the welfare and use of animal subjects can be found at the websites for the University of Northern Colorado ACUP https://www.unco.edu/research/research-

integrity-and-compliance/iacuc/, the NIH's Office of Laboratory Animal Welfare https://olaw.nih.gov/, and the USDA's Animal Plant and Health Inspection Services https://www.aphis.usda.gov/aphis/home/.

Sincerely,

Laura W. Martin
Director of Compliance and Operations
Animal Care and Use Program

OLAW Assurance:
D16-00579

USDA Registration:
84-R-0008

Carter Hall 208 | Campus Box 143 | Greeley, CO 80639 | Office 970-351-4313 | Fax 970-351-1934