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Genomic vs. Non-genomic Role of the AhR in Human Immunoglobulin Expression

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GENOMIC VS. NON-GENOMIC ROLE OF THE AHR IN HUMAN IMMUNOGLOBULIN EXPRESSION

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Nasser Alhamdan ENTITLED Genomic vs. Non-genomic Role of the AhR in Human Immunoglobulin Expression BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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The immunoglobulin heavy chain gene (*Igh*) in various animal models is regulated by numerous regulatory elements including the 3'*Igh* regulatory region (3'*Igh*RR). Several transcription factors are involved in modulating the 3'*Igh*RR including the aryl hydrocarbon receptor (AhR). The AhR is a ligand-activated transcription factor that mediates the transcription of genes involved in the metabolism of environmental toxicants such as TCDD. TCDD binds AhR and regulates immunoglobulin (Ig) expression in B cells. This modulation appears to be directly mediated by binding of the AhR to dioxin response elements (DRE) within the 3'*Igh*RR. In human B cells, IgG secretion inhibited by TCDD and increased by chemical antagonist of the AhR (AhRA). AhR can interact with different transcription factors like NFκB and AP-1, and modulate signaling pathways such as Src and Akt pathways. the human CL-01 cells express a nonfunctional transactivation domain (AhR TA) in one of its alleles. Therefore, we hypothesized that in human cells the AhR regulates *IGH* expression by altering 3'*IGH*RR activation through both genomic and non-genomic mediated mechanisms. The current study focuses on determining the potential non-genomic effects of the AhR on the activation of cytosolic signaling proteins such as Src and Akt, and transcription factors like NFκB and AP-1. In addition, it evaluates the genomic effects of the nonfunctional AhR TA on the 3'*IGH*RR reporter activity and evaluates the role of AhR in the effect of the TCDD and AhRA on Ig expressing and 3'*IGH*RR activity. Our results showed that the AhR has non-genomic effects by activating cytosolic signaling protein like Akt and increasing the $I\gamma_3$-3'*IGH*RR reporter activity in

response to AhRA that could lead to an increase in IgG secretion. The nonfunctional AhR TA decreased the AhR binding to DRE sites as demonstrated in a DRE reporter but it did not affect 3'*IGH*RR activity in response to TCDD or AhRA. AhR knockdown by gene editing demonstrated inhibition of IgG secretion and AhRA had no effect on $I\gamma_3$-3'*IGH*RR activity, supporting the suggested a physiological non-genomic role of the AhR in the expression of IgG. Determining the role of the 3'*IGH*RR and its potential difference in sensitivity to the AhR could provide new insight into potential environmental triggers of immune disorders and provide new targets for drug development.

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I. INTRODUCTION

The immune system

The immune system is a host defense system that consists of a cooperative network between cells, organs and tissues to protect the body. This network has mechanisms that identify and neutralize different threats to the human body. Threats can be arise from harmful alterations happening inside the body such as dysregulation of a normal cell into a tumor cell or that may enter the body from the outside such as infectious bacteria or toxic agents. The immune system has the ability to differentiate self-organisms and molecules from nonself and contains two lines of defense against threats. The first line is the innate immune system that include a group of well-maintained chemical, mechanical and microbiological barriers that prevent pathogens from entering the body. In the event that these barriers are penetrated, the nonspecific components of the innate immune system, then the second line, which is the specific adaptive immune system, are activated (Parham, 2009).

Each defense system has special recognizing mechanisms that has advantages and disadvantages. The mechanisms of innate immune system are ready for action. It has fast response that just takes a few hours to distinguish pathogens. Some of the innate immunity mechanisms are constantly work even if there is no pathogen, for example antimicrobial proteins continuously secreted into gut. This system is non-specific because it responds the same to a variety of antigens and it attacks all pathogens equally. The core components of innate immunity include macrophages, neutrophils and natural killer cells. Once the pathogen invades the body, the innate effector cells recognize pathogen-associated

molecular patterns (PAMPs) through pattern recognition receptors (PRR) expressed on the surface of innate effector cells. Pathogen recognition induces innate effector cells to secrete inflammatory mediators such as cytokines and chemokines. In contrast, the adaptive immune system has slower reactions and it may takes days or even weeks to recognize antigens and support an immune response, but antigens are selected by highly specificity. The adaptive immunity generates an immune response by antibody-mediated immunity or cell- mediated immunity as well as immunological memory, which gives the host long-term protection from reinfection by the same specific pathogen (Parham, 2009). Innate and adaptive immunity cooperate by a lot of cross-reaction between the two mechanisms. For example, some of the innate system cells are critical for activation of the adaptive system cells and can in turn have their activity elevated and directed by activated cells from the adaptive system (Doan, et.al, 2008).

The adaptive immune system has unique cells and molecules. It is the second level of defense against potential threats to the body, following the barriers and the innate immune system. It can eliminate specific antigens by either cell-mediated immunity or antibody-mediated (humoral) immunity. Cell-mediated immunity is dominated by T cells that bind to antigens presented on the cell surface via major histocompatibility complex (MHC). T cells express $CD4^+$ (T helper) that bind to antigens presented by MHC molecules class II (MHC II). These molecules are exclusively expressed on antigen-presenting cells, which include macrophages, dendritic cells, and B-cells. Binding of $CD4^+$ T-helper cells with (MHC II) molecules leads to proliferation and differentiation of the T-helper cells into memory, effector or regulatory T helper cells. Moreover, T cells expressing CD8 (cytotoxic T cells) bind to an antigen presented by MHC class I, which is expressed by nearly all

nucleated cells of the body. Cytotoxic T-cells are specialized to recognize and fight cancer cells and intracellular infection by destroying cells presenting foreign intracellular antigen in their MHC I. On the other hand, antibody-mediated immunity is mediated by B cells that differentiate into antibody-forming cells (plasma cells), which produce specific antibodies against a particular epitope: the part of an antigen that is recognized by antibodies (Parham, 2005).

B cells

B cells are the main effectors of the antibody-mediated response of adaptive immunity. Like other cells, B cells are created in the bone marrow and then circulate as immature or mature B cells through blood and lymph nodes to the secondary lymphoid tissue like the spleen. B cells have transmembrane proteins called B-cell receptor (BCR) located on the surface of B cells. Each BCR has only one isotype of a membrane-bound immunoglobulin (Ig), and all isotypes on the same B cell have the same antigen-binding site. Mature B cells that express IgM and IgD on their surface and have not been exposed to antigens are called naïve. They circulate in the bloodstream and lymph as inactive B cells, and once they recognize specific antigens, they become activated and differentiate into antibody-secreting cells (plasma cells).

In order to activate B cells, a signal provided by antigen and interaction with helper T cells stimulates B cells to proliferate and differentiate into plasma cells or long-lived memory B cells. Plasma cells secrete a huge amount of antibodies, the soluble form of Ig, in response to antigen and release them into the circulation. The amount of antibodies production decrease as the stimulus of infection is removed by the elimination of the pathogen from the body. The antibodies can be present in the circulation for several months

afterward. The IgM antibody isotype mediates the primary immune response when mature B cells encounter antigen for the first time and differentiate to produce memory cells (long-lived cells) and plasma cells. On the other hand, the secondary immune response mediated when memory B cells are re-expose to the same antigen, is a faster immune response and these cells produce a huge amount of specific and high-affinity antibodies of different isotypes such as IgG.

Immunoglobulins

Ig proteins are the critical ingredients at every stage of the humoral acquired immune response. Antibodies are the Ig that are secreted from B cells and travel through the circulation to bind to the specific antigens that triggered their production (Parslow et.al, 2001). Ig contains four polypeptides chains: two identical light chains (IgL) and two identical heavy chains (IgH) linked by disulfide bonds to form a monomeric subunit (Fig. 1). The antigen-binding site (epitope-binding site) at the tip of the arm is formed by a single heavy chain and a single light chain. A single antibody subunit is bivalent due to the two arms, which have identical antigen-binding sites. Each chain has a C-terminal end with a constant amino acid sequence (constant region) and an N-terminal end with a variable amino acid sequence (variable region), which is responsible for the huge diversity of the antigen binding site. Heavy chains contain one variable (V_H) domain and three or four constant (C_H) domains (Fig. 1). There are five isotypes of Ig (IgM, IgD, IgG, IgE and IgA), each isotype is encoded by a specific constant region within the Ig heavy chain gene: Cμ, Cδ, Cγ, Cε, and Cα are encoded for isotypes IgM, IgD, IgG, IgE, and IgA respectively. In human, there are four IgG subclasses IgG1, IgG2, IgG3 and IgG4 encoded by $C\gamma_1$, $C\gamma_2$, $C\gamma_3$ and $C\gamma_4$ heavy chain genes, respectively and two IgA subclasses IgA1 and IgA2

encoded by $C\alpha_1$ and $C\alpha_2$ heavy chain genes, respectively. Normally, humans produce two types of light chain, kappa (κ) or lambda (λ). Light chains contain one variable (V_L) domain and one constant (C_L) domain. In an individual B cell, each Ig monomer contains two identical kappa (κ) or two identical lambda (λ) but never both (Doan, et.al, 2008).

The IgM isotype is the first Ig formed and is found as a monomer on the B-cell surface. Once B cells differentiate into plasma cell, IgM is secreted as a pentamer resulting in a total of 10 antigen-binding sites. IgM is the primary antibody secreted in a primary response. As the response progresses somatic hypermutation and class switch recombination are engaged, which will result in a change in the antibody isotype and in the immune response according to the type of encountered antigen.

The IgD isotype has a monomeric structure and is mostly displayed on the B-cell surface. It works as a co-receptor along with IgM and it can activate basophils and mast cells to coordinate an immune response that eliminates bacteria (Parham, 2005).

The IgG isotypes (IgG1-4) are secreted as monomers and they are the most abundant antibody in the serum. IgG is produced by B cells in a huge amount during a secondary immune response and it is the only Ig isotype that can cross the placental barrier to give immunity to the fetus. It can activate complement, and neutralize microorganism and viruses (Doan, et.al, 2008).

The IgA isotypes (IgA1-2) are the main antibody found in body secretions. IgA can be secreted as a monomer as found in the serum or as a dimer as found in secretions, including milk, saliva, sweat, tears, and intestinal secretions. IgA provides protection against microbes in body secretions.

The IgE isotype is secreted as a monomer and binds to mast cells, eosinophils, and basophiles in the absence of antigen. When antigen binds to IgE on eosinophil, a strong physical reaction and inflammatory reaction is triggered to kill parasites, while IgE on mast cells and basophils triggers the release of histamine leading to immediate hypersensitivity responses (Doan, et.al, 2008).

Somatic recombination or VDJ recombination happen during early B lymphocyte development in the bone marrow to form the antigen-binding sites. It is assembly of the variable (V), diversity (D) and joining (J) gene segments that gives rise to the variable region exons of the antigen receptor. The V and J gene segments encode the light chain variable region (V_L), while the heavy chain variable region (V_H) also has the D segments along with V and J. VDJ recombination needs recombination activating genes 1 and 2 (RAG-1 and RAG-2) to complete this process. In the heavy chain (V_H), random D and J gene segments recombine together and the intervening DNA sequences between them are deleted. Eventually, the D-J recombination is joined to a V gene segment to get a rearranged VDJ gene segment. In the light chain variable region (V_L), only one recombination V to J takes place. V(D)J recombination helps to produce high diversity of antigen binding sites. When mature B cells interact with antigen, they undergo two more forms of genetic change within the Ig genes: somatic hypermutation (SHM) and isotype class switch recombination (CSR). In somatic hypermutation, point mutations are induced within the DNA sequences of the heavy and light chain variable regions with the aim of increasing Ig affinity for a particular antigen. Class switch recombination refers to isotype switching from IgM to IgG, IgE or IgA isotypes. CSR occurs only in the heavy chain constant region (C_H) by joining the rearranged variable region exon to a different

downstream constant region (i.e. $C\gamma_{1-4}$, $C\varepsilon$, or $C\alpha_{1-2}$), and delete the intervening germline DNA. These events enable a rearranged V-region to be used with other heavy chain C-regions in order to express different antibody isotypes with the same antigen specificity (Parham, 2005).

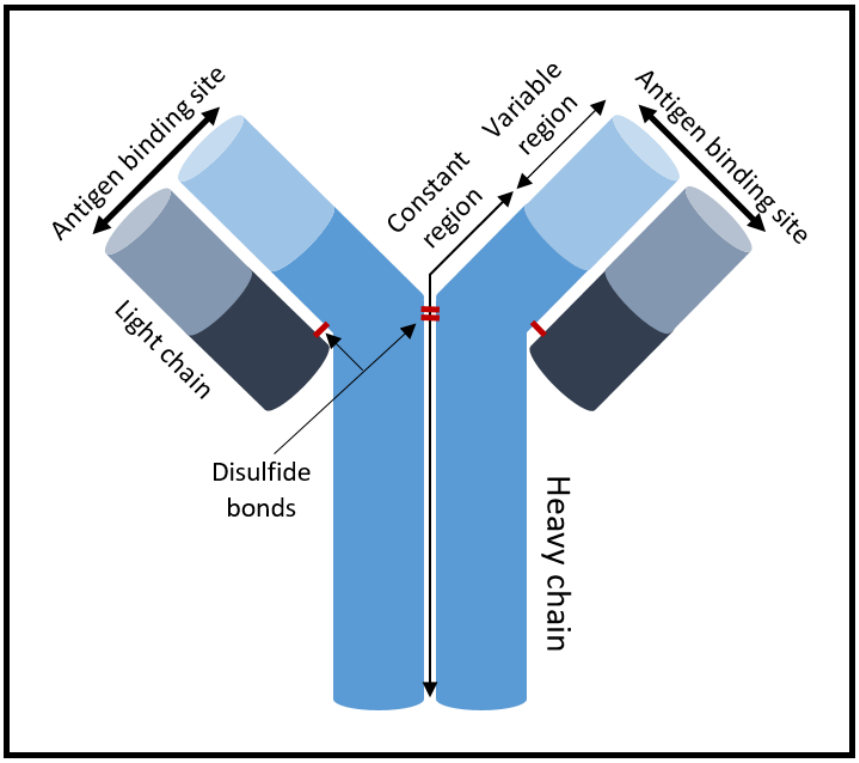


Figure 1. Structure of Immunoglobulin. Four polypeptide chains consisting of two identical light chains and two identical heavy chains linked by disulfide bonds form the Ig monomeric unit. Each chain has a variable region that forms the antigen binding sites, and each heavy chain contains a constant region that determines the function of Ig.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

Dioxins refer to a group of toxic chemicals that consist of halogenated aromatic hydrocarbons (HAH). They have similarities in structure, properties, and mechanisms. HAH includes polychlorinated biphenyls (PCB), polychlorinated dibenzofurans (PCDF), and polychlorinated dibenzodioxins (PCDD). Dioxins have a long half-life and hydrophobic properties make them resistance to metabolism. This tend to bio-accumulate of dioxins in adipose tissues, especially with long-term exposure, and persist as environmental contaminants (Mandal 2005; Van den Berg et al., 1998).

The most studied PCDD is the potent and highly toxic dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). It is a planner molecule ($C_{12}H_4Cl_4O_2$) and insoluble in water (Fig. 2). In addition, it has no odor or color at room temperature and it is not intentionally produced by industry except in a limited quantity for research purposes. TCDD is produced from many industrial processes that have incomplete combustion of organic materials in presence of chlorine such as plastic production, herbicides and pesticides. Also it is produced from improper disposal of medical waste, metal smelting, diesel trucks, burning treated wood and forest fires (Malisch & Kotz, 2014; Strucinski et al., 2011). Biological effects of TCDD are dependent upon many factors including exposure duration and dose as well as species. In fact, exposure to TCDD could be through ingestion of contaminated food or water, inhalation of contaminated dust or cigarette smoke, and direct contact with incorporated soil or surfaces (Mandal, 2005).

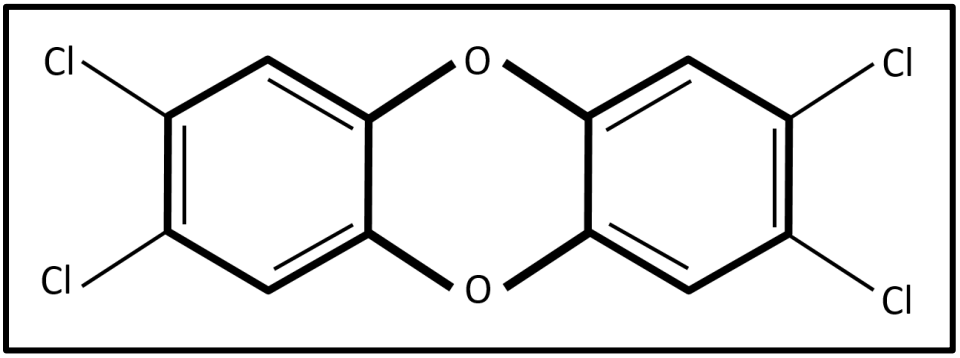


Figure 2. Chemical structure of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD).

During the Vietnam War (1961-1971), TCDD was discovered to be a contaminant in the herbicide Agent Orange when American military forces sprayed it on foliage to expose Vietnamese soldiers (Karch, et al., 2004). Additionally, in Seveso, Italy (1976), thousands of inhabitants were exposed to TCDD after an accidental explosion of a pressure tank containing substances contaminated with TCDD leading to the highest residential exposure to TCDD (Mandal 2005). Another incident happened in Vienna 1997 when two women were poisoned at their workplace with the highest measured dose of TCDD in human (144 ng/g of fat) (Geusau et al., 2001). The second highest exposure happened in Ukraine during the 2004 election campaign when Viktor Yushchenko was poisoned with TCDD (108 ng/g of fat) after a failed assassination attempt (Sorg et al. 2009). Humans seem to be less sensitive to toxic effects by TCDD than many laboratory animals (Poland & Knutson, 1982). In animal studies, exposure to TCDD caused immune dysfunction, endocrine disruption, teratogenesis, hepatic damage and tumorigenesis (Mandal 2005).

TCDD is not acutely life-threatening in humans but has been shown to induce death in sensitive animal species. For example, the lethal dose 50% (LD50) for TCDD varies from 1 μg/kg for guinea pig, the most sensitive animal species, to >5000 μg/kg for hamster, the most resistant (Poland & Knutson, 1982). The main effects of TCDD on human in short-term include blurred vision, nausea and development of severe chloracne. Whereas the long-term effects include vascular ocular changes, signs of neural system damage and immunological effects include effects on B cell lead to low level of immunoglobulin IgG in the circulation (Silbergeld & Gasiewicz, 1989; Dertinger et al., 2001; Marinkovic et al., 2010). Additionally, TCDD has been classified as a human carcinogen because it promotes cancer development (IARC, 1997). The mechanism of TCDD toxicity in human is not clear yet, but the biological effects are thought to be mediated by the aryl hydrocarbon receptor (AhR) signaling pathway.

The Aryl Hydrocarbon Receptor (AhR) Signaling Pathway

The aryl hydrocarbon receptor (AhR) is a cytosolic receptor protein. It is a ligand-activated transcription factor that is encoded by the *Ahr* gene and consists of 848 amino acids. The structure of AhR comprises of a basic-helix-loop-helix domain followed by Per-ARNT-Sim protein domain (PAS). Therefore, it is part of the bHLH/PAS family of transcription factors (Gu et al., 2000). In mouse, the high concentrations of AhR have been found in most tissues especially thymus, liver and kidney, while in humans, it can be found in the placenta, lung, and liver, and low concentrations in brain, muscle and kidney (Carlstedt-Duke, 1979; Carver et al., 1994a; Dolwick et al., 1993; Yamamoto et al., 2004). The AhR is activated through binding to a variety of endogenous or exogenous ligands. A number of endogenous compounds such as indoles, 7-ketocholesterol, bilirubin and tetrapyroles have been identified as AhR ligands (Denison & Nagy, 2003). The majority of high-affinity AhR ligands are exogenous ligands like polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs). The ligand with the highest AhR binding affinity is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Fig. 2). It is part of the HAH family and the prototypical ligand used in AhR studies (Abel and Haarmann-Steeman, 2010).

In the absent of a ligand, AhR is an inactive protein located in the cytosol and bound to protective proteins to stabilize the whole complex. This complex consists of a dimer of heat-shock proteins (Hsp90) that protects from cell temperature elevation; co-chaperone p23, which prevents non-specific binding to ARNT; and XAP2, which binds to AhR and Hsp90 (Whitlock, 1999). Once ligand (e.g. TCDD) binds to the AhR in the cytoplasm, the AhR protein goes through a conformational change leading to its dissociation from the

complex of protective cytosolic proteins (Fig. 3). Then the TCDD-AhR complex translocates from the cytoplasm into the nucleus where it binds to the aryl hydrocarbon receptor nuclear translocator (ARNT) to form a heterodimer. The TCDD-AhR-ARNT heterodimer complex binds to specific DNA sequences (5'-TNGCGTG-3') called dioxin responsive elements (DRE). Since DREs are located within the promoter or enhancer regions of target genes, the heterodimer complex causes upregulation or downregulation of gene expression (Abel & Haarmann-Stemmann, 2010) (Fig. 3). If the AhR ligand, which activates AhR signaling, is no longer present, the activation of the AhR can be controlled by either of two regulatory pathways; negative feedback inhibition mediated by the AhR transcriptional repressor (AHRR) or cytosolic export of the AhR and degradation by ubiquitination and proteasome pathways (Abel & Haarmann-Stemmann, 2010).

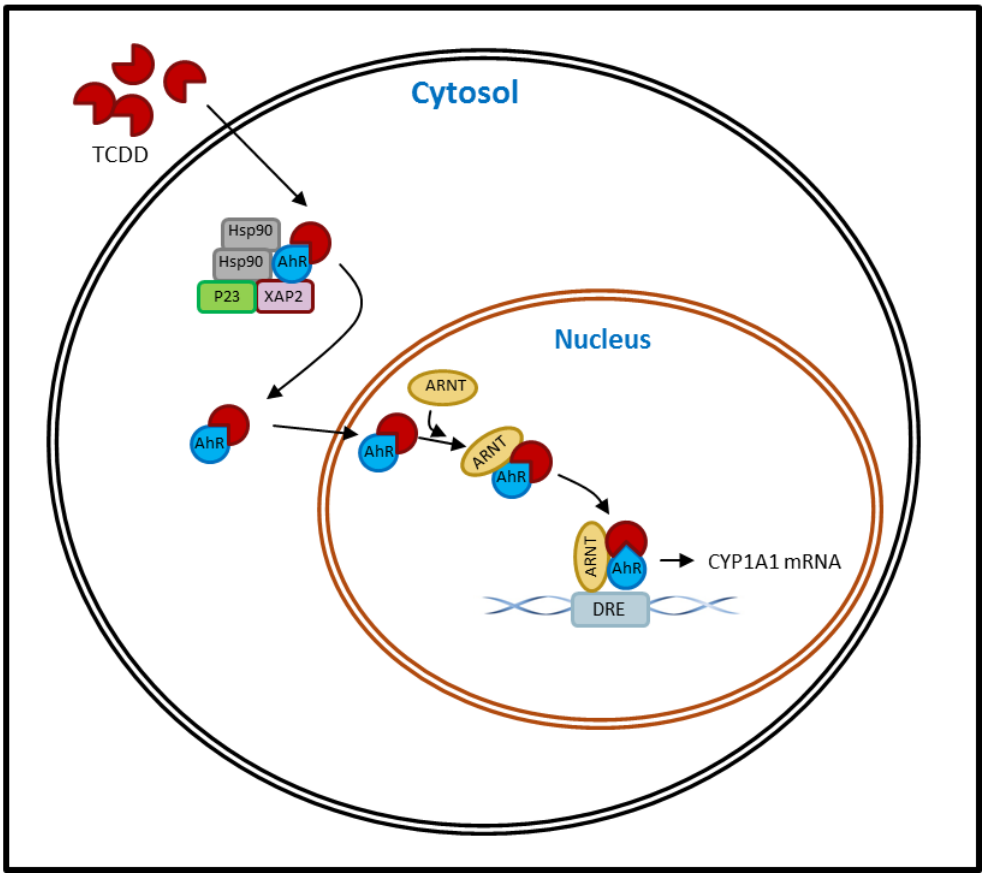


Figure 3. Aryl hydrocarbon receptor signaling pathway. TCDD binds to the AhR and cytoplasmic proteins dissociate. TCDD-AhR translocates to the nucleus and binds ARNT. The complex of TCDD-AhR-ARNT binds to DRE sequences and modulates transcription. CYP1A1 is a sensitive indicator of AhR activation.

The AhR has an important role in the regulation of xenobiotic metabolizing enzymes and drug metabolizing enzymes such as cytochrome P450A1 (CYP1A1) (Fig. 3). CYP1A1 is highly induced by TCDD, and it is one of the most characterized genes regulated by the AhR. It has been used as a biomarker for AhR activation (Hansen et al., 2014; Whitlock, 1999). In addition to drug metabolism, the AhR signaling pathway is involved in crosstalk pathways with steroid receptors of androgen, estrogen and progesterone hormones by co-regulating gene transcription and expression (Ohtake et al., 2008). Moreover, the AhR plays an important role in proliferation, differentiation, apoptosis and survival of cells. Also, it has effects in the immune system, it mediates immunological responses by inhibiting inflammation through induction of interleukin-22 (IL-22) and down-regulation of Th17 and $CD4^+$ T cell response (Monteleone et al., 2011; Wei et al., 2014), and the AhR has main role in inhibition of Ig production through suppression of B-cell proliferation and differentiation (Sulentic & Kaminski, 2011).

Mouse AhR studies show that the AhR is highly responsive to TCDD and the mouse AhR has ten-fold higher affinity compared to the human AhR (Denison et al., 1986). In addition, the human and mouse AhR gene have single nucleotide polymorphisms (SNPs) in different locations and they only share about 58% amino acid sequence identity in the C-terminal of the gene, which includes the transactivation domain (Flaveny et al. 2008). The SNPs in exon 10 of the human AhR have been reported in association with decreased CYP1A1 induction by AhR in response to TCDD (Wong, et al., 2001). The lower affinity of the human AhR and the exon 10 SNPs may explain why animal models are more sensitive to TCDD than human. Interestingly, the exon 10 SNPs did not affect the ligand

binding affinity, also the SNPs located in the ligand-binding domain did not affect the ligand-induced CYP1A1 expression (Harper et al., 2002).

The Aryl Hydrocarbon Receptor Antagonist (AhRA)

In animal models, the AhR plays an important role in mediating toxicity and inducing activity of the TCDD; therefore inactivation of the AhR signaling pathway would be estimated to offer protection from TCDD toxicity. High affinity ligands for the AhR include toxic compounds such as halogenated aromatic hydrocarbons HAHs, polycyclic aromatic hydrocarbons PAHs and PAH-like compounds (Denison et al., 1998). Other natural and endogenous AhR ligands have been identified but they have different chemical structures and characteristics from HAH and PAH AhR ligands, which suggests that the ligand-binding pocket of the AhR is very promiscuous (Denison et al., 1998). Since structurally diverse ligands can bind to different amino acids within the ligand-binding pocket of the AhR, it could be possible to determine an antagonist that could affect one or a group of ligands over another (Zhao et al. 2010). Some of the AhR antagonists lack of specificity due to two reasons; they have binding affinity to the estrogen receptor that enhance the estrogen-related effects or they are partial agonists such as flavones that at high doses act as AhR agonists (Zhang et al., 2003; Amakura et al. 2002). The CH223191 compound has been identified as a pure AhR antagonist, which competes and inhibits binding of TCDD to the AhR, and prevents the AhR from translocating to the nucleus and binding the DNA (Fig. 4) (Kim et al. 2006). It is a selective AhR antagonist for HAH compounds while no antagonism effect on AhR activated by different concentration of PAH or non-PAH AhR agonists (Zhao et al. 2010). In addition, it does not show AhR agonist-like activity at high doses or any binding affinity to the estrogen receptor, therefore

CH223191 (AhRA) is the best candidate compound to elucidate the role of the AhR when it is activated by TCDD (Zhao et al. 2010). This compound has been tested in our lab with the mouse B-cell line (CH12.LX) and it inhibits the AhR signaling pathway and decreases the expression of CYP1A1 in response to TCDD (Wourms & Sulentic, 2015). In another study on the CL-01 human B-cell line, the AhRA and TCDD demonstrated inconstant effects on IgM secretion but they antagonized each other's effect on IgG secretion. Surprisingly, activated CL-01 cells by the AhRA induced IgG secretion by two-fold (Burra, 2015 data not published).

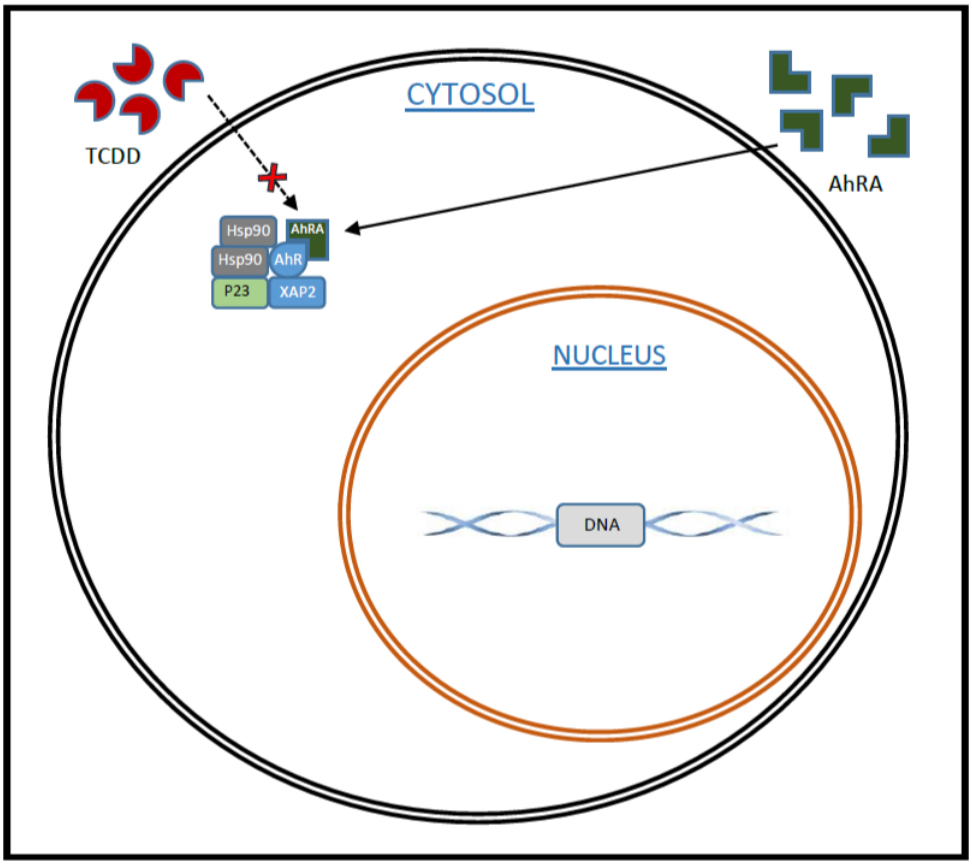


Figure 4. AhR antagonist (AhRA) inhibits translocation of AhR into the nucleus. AhRA competes and blocks TCDD binding to the AhR as well as TCDD-mediated nuclear translocation and DNA binding activity of the AhR.

The Immunological effects of TCDD

The immune system is an early and sensitive target to TCDD-induced toxicity. A wide range of TCDD concentrations can cause significant immunological dysfunction including suppression of both innate and adaptive immunity and inhibition of cell-mediated and humoral immunity (Mandal, 2005; Abel & Haarmann-Stemmann, 2010). In animal studies, acute or chronic exposure to TCDD causes thymic atrophy, endocrine disruption, hepatic damage and immunological effects include immune-cell dysfunction and suppression of immune responses that lead to susceptibility to infectious diseases (Kerkvliet, 2002; Mandal, 2005; Safe, 1998). TCDD improved the induction of T_{reg} cells, which suppressed T-cell proliferation (Quintana et al. 2008), and TCDD inhibited Ig production through suppression of B-cell differentiation (Sulentic & Kaminski, 2011). As mentioned the effects of TCDD on humans are less sensitive than animals (Denison et al., 1986). In human, several epidemiological studies found that an association between people exposed to TCDD and non-Hodgkin's lymphoma or multiple myeloma (Becher et al., 1996; Hooiveld et al., 1998). Among people living in highly TCDD contaminated area, there was 2.3 times greater incidence of non-Hodgkin's lymphoma than people living in low TCDD contamination (Floret et al., 2003). Another study found that prenatal exposure to TCDD affects humoral immune status of their children after birth through demonstrated of reduced vaccine titers and increased incidence of otitis and chicken pox (ten Tusscher et al., 2003). Moreover, exposure to TCDD for a long time mediates B-cell immunotoxicity such as markedly decreased the IgG levels in circulation (Baccarelli et al., 2002). TCDD also increased the production of only IgE isotypes from B cells isolated from atopic patients (Kimata, 2003).

TCDD-Induced B-cell Dysfunction

B cells are an essential component of the humoral immune response and are a sensitive direct target of TCDD. In mouse models, TCDD affects B-cell maturation and gene regulation that lead to suppression of immunoglobulin production (Lu et al. 2010; Sulentic and Kaminski 2011). TCDD inhibits B-cells differentiation into antibody-forming cells mediated by the AhR (Wourms & Sulentic, 2015; Vorderstrasse et al., 2001). TCDD inhibits B-cell differentiation induced with T-helper function (T-dependent) by activating B-cell with sheep red blood cells or without T-helper function (T-independent) using lipopolysaccharide (LPS) for activating B-cell (Holsapple et al., 1986). In a mouse B-cell line (CH12.LX) stimulated with LPS, TCDD in a concentration-dependent manner (0.03-30nM TCDD) decreased IgM secretion, whereas it did not affect IgM secretion in an AhR-deficient B-cell line (BCL-1) stimulated with LPS, suggesting that AhR plays an important role in inhibition of IgM secretion by TCDD (Sulentic et al., 1998). Moreover, in an IgA secreting B-cell line that has the AhR knocked down by shRNA or the AhR inhibited by an AhR antagonist prevented TCDD-induced inhibition of IgA secretion (Wourms & Sulentic, 2015). Another study with $AhR^{-/-}$ mice showed normal immune function and Ig production after treatment with TCDD (Vorderstrasse et al., 2001). These studies support an important role of the AhR in TCDD-induced inhibition of Ig and B-cell differentiation and inactivation of the AhR has no effect in normal immune function (Wourms & Sulentic, 2015; Vorderstrasse et al., 2001).

Human B-cells appear to have a variable response to TCDD. In human primary B-cells, TCDD suppressed IgM in nine donors while having no effect in two donors and increased IgM in one donor (Lu et al., 2010). In addition, it suppressed the differentiation

of human primary B cells by inhibiting the activation markers CD69, CD86 and CD80 (Lu et al. 2011). In a study of people who live in TCDD-contaminated areas including residents in Seveso, Italy and Korean veterans, decreased plasma IgG levels were reported (Baccarelli et al., 2002 ; Kim et al., 2003). In another study on human B cells, TCDD had variable effects on IgM secretion and μ IGH functional transcripts while IgG secretion and $\gamma_{1\text{-}4}$ germline/functional transcripts were inhibited (Burra 2015, unpublished data). Whereas in tonsillar mature B cells, TCDD induced IgE production without affecting production of other Ig isotypes (IgG, IgM or IgA) in patients with allergic rhinitis, atopic eczema/dermatitis syndrome, or bronchial asthma with no effects on Ig isotypes in other patients, suggesting that allergic diseases could be aggravated by TCDD (Kimata, 2003). Additionally, the Ig heavy chain regulatory region (3'*Igh*RR) mediates TCDD-induced suppression of B-cell differentiation in mouse models but its role in humans is unknown (Wourms & Sulentic, 2015; Fernando et al., 2012; Sulentic et al., 1998). Overall, the mechanism of TCDD and AhR in mediating B-cell dysregulation by TCDD in mouse is relatively well defined whereas the mechanism in humans is still unclear.

Immunoglobulin Heavy Chain (*IgH*) Locus

The human Ig heavy chain (*IGH*) gene is located on chromosome 14 and the mouse Ig heavy chain (*Igh*) gene is located on chromosome 12. They encode the heavy chain of antibodies during early B-cell development and differentiation by DNA remodeling events such as VDJ recombination, class switch recombination (CSR), and somatic hypermutation (SHM) (Pinaud et al. 2011). The rearranged Ig heavy chain (*IGH*) gene consists of variable heavy chain promotor (V_H), variable region (VDJ), the heavy chain intronic enhancer (Eμ), the heavy chain constant region genes (C_H), and the 3' immunoglobulin heavy chain

regulatory region (3'*IgH*RR) (Fig. 5) (Mills et al. 1997). The V_H promoter initiates and regulates the transcription of the *IGH* locus and gene expression, while the VDJ gene encodes the antigen binding sites. The intronic enhancer (Eμ) is important for efficient VDJ recombination and Cμ expression (Perlot et al. 2005). In Burkitt's lymphoma, translocation of the 3'*IGH*RR with the cellular oncogene *MYC* deregulates gene expression (Yan et al., 2007). In addition, the chromosomal translocation between *BCL-2* gene and 3'*IGH*RR in follicular lymphomas deregulates gene expression and increases cell death resistance (Heckman et al., 2003). Other studies showed that *Igh* transcription in a mutant myeloma cell line was significantly inhibited after deletion in a sequence downstream of Cα leading to the discovery of the 3'*Igh*RR (Gregor & Morrison, 1986). The mouse and human heavy chain locus are 90% homologous but there are some differences (Mills et al. 1997). The human *IGH* has two groups of constant regions with two 3'*IGH*RRs each has three enhancers (hs3, hs1.2, hs4) while the mouse *Igh* has one group of constant region with one 3'*Igh*RR that has four enhancers (hs3a, hs1.2, hs3b, hs4) (Fig. 5). In human *IGH*, upstream of each 3'*IGH*RR are constant region genes that encoding for specific heavy chain isotypes. The first set of heavy chain constant region (C_H) genes include Cμ, Cδ, $C\gamma_3$, $C\gamma_1$, Cψε and $C\alpha_1$, followed by the first regulatory region 3'*IGH*RR-1. The second set of constant region genes located downstream of 3'*IGH*RR-1 that include $C\gamma_2$, $C\gamma_4$, Cε and $C\alpha_2$, followed by the second regulatory region 3'*IGH*RR-2 (Mills et al. 1997). The mouse *Igh* has one set of C_H genes that include Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε and Cα followed by one regulatory region 3'*Igh*RR (Fig. 5) (Madisen and Groudine 1994).

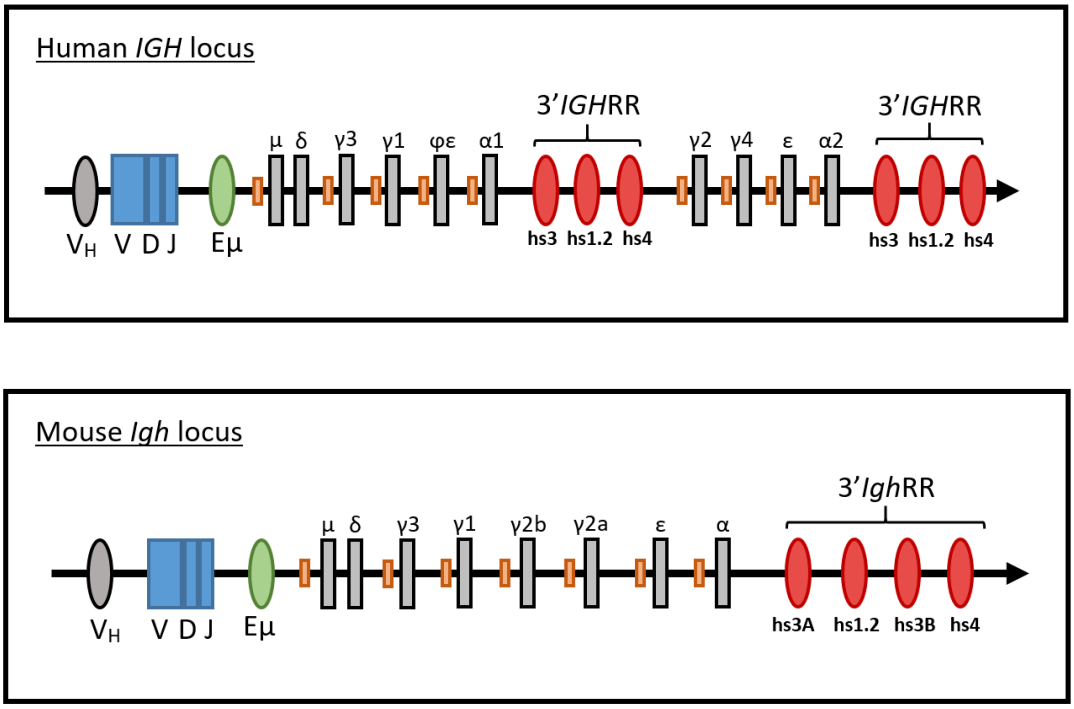


Figure 5. Human and mouse immunoglobulin heavy chain (*Igh*) gene locus. V_H, variable heavy chain promoter; Eμ, intronic enhancer; long rectangles, *IgH* constant region genes; short rectangles, germline promoters; 3'*Igh*RR, regulatory region; hs, hypersensitivity site.

The mouse 3' Immunoglobulin Heavy Chain Regulatory Region (3'*Igh*RR)

The mouse *Igh* locus has one 3'*Igh*RR and this region is about a 40 kb DNA segment. The main role of the 3'*Igh*RR is to control and regulate the transcription of the *Igh* gene and class switch recombination (CSR), but it does not appear to play a role in VDJ recombination (Cogne et al., 1994; Ju, Chatterjee, & Birshtein, 2011). It has four major DNAase I hypersensitive elements (hs3a, hs1.2, hs3b, and hs4), which are regions of chromatin that are sensitive to cleavage by the DNase I enzyme (Fig. 5). The hs1.2 enhancer has transcriptional activity in a mature B cell and plasma cell, the hs3a and hs3b enhancers have minor activity in B cells or plasma cells, while the hs4 has transcriptional activity throughout B-cell development especially pre-B cell and plasma cells. Replacement of mouse hs1.2 enhancer with neomycin resistance gene resulted in decreased CSR into IgG2a, IgG2b, IgG3 and IgE (Cogne 1994). On the other hand, the four enhancers display maximum activity at all stages of B-cell development when all four enhancers are link together (Matthias and Baltimore, 1993; Madisen and Groudine, 1994; Chauveau et al., 1998; Saleque et al., 1997). They have several binding sites for transcription factors including DRE, NF-κB, Octamer (OCT), NF-αP, AP-1/Ets, and Pax5 that regulate 3'*Igh*RR activity (Fig. 6). Therefore the mouse 3'*Igh*RR could be a sensitive target of several exogenous and endogenous toxic chemicals such as the AhR-ligand TCDD.

Past research shows that transcriptional activity of mouse 3'*Igh*RR in CH12.LX cells stimulated with LPS is inhibited by TCDD (Sulentic et al., 2004a). This effect matches the suppression of *Cμ* gene expression and IgM secretion by TCDD (Sulentic et al., 2000). The mouse 3'*Igh*RR has a DRE motif in the most transcriptional active enhancers hs1.2 and hs4, and TCDD was shown to induce binding of AhR/ARNT to DRE sites within these

enhancers (Salisbury & Sulentic, 2015; Sulentic et al., 2000). Suggesting that mouse 3'*Igh*RR is a transcriptional target of the AhR signaling pathway, and because this region is important for CSR and *Igh* transcription, it could mediate the TCDD-inhibitory effect on the Ig expression (Sulentic et al., 2004a). However, 3'*Igh*RR enhancers react differently with TCDD when evaluated in isolation. When the CH12.LX cell line was transfected with a plasmid reporter regulated by either the hs4 or the hs1.2 enhancer, the transcriptional activity of the hs4 enhancer was increased, while the hs1.2 enhancer was suppressed by TCDD (Sulentic et al., 2004b; Fernando et al., 2012). Moreover, the deletion of the hs4 enhancer does not affect class switch recombination (CSR) or Ig production in mouse cells stimulated with LPS or cytokines (Vincent-Fabert et al., 2009). These outcomes are against the effect of TCDD on the full length 3'*Igh*RR suggesting that the hs4 enhancer has an unrelated or different role with TCDD while inhibition of the hs1.2 enhancer by TCDD may be responsible for the inhibitory effect on 3'*Igh*RR transcriptional activity. Interestingly, the mouse hs1.2 enhancer has two binding sites for paired box protein (Pax5) but the human hs1.2 enhancer does not have any Pax5 binding sites (Fig. 6) (Schneider et al., 2009). The Pax5 transcription factor plays a main role in early stages of B-cell development and must be downregulated to allow for differentiation of B cells into plasma cells (Cobaleda et al., 2007). Pax5 levels are decreased in LPS-stimulated mouse B-cell, but TCDD co-treatment inhibits this down-regulation (Yoo et al., 2004; Schneider et al., 2008). Deregulation of Pax5 expression after TCDD treatment implies that Pax5 plays a role in TCDD-mediated inhibition of Ig expression and B-cell differentiation (Schneider et al., 2009).

The Human Immunoglobulin Heavy Chain Regulatory Region (3'*IGH*RR)

The human *IGH* locus has two regulatory regions: 3'*IGH*RR-1 located downstream of $C\alpha_1$ and the 3'*IGH*RR-2 located downstream of $C\alpha_2$. Each regulatory region is composed of three DNAase I hypersensitive elements (hs3, hs1.2 and hs4) (Fig. 5) (Chauveau & Cogne, 1996; Mills et al., 1997). The duplication of the 3'*IGH*RR in the human locus is thought to be functionally significant, but no studies have investigated the specific roles of the two 3'*IGH*RRs in antibody expression. The three human enhancers, hs3, hs1.2 and hs4, share 74%, 90% and 76% similarity, respectively to the mouse enhancers. TCDD treatment was expected to result in reduced activity of the 3'*IGH*RR since TCDD is known to act as an immunosuppressant in both mouse and human B cells. In luciferase reporter studies, when the human 3'*IGH*RR plasmids with different promotors (i.e. V_H, $I\gamma_3$ and $I\varepsilon$) were transfected into B cells and treated with TCDD, they exhibited highly variable and statistically insignificant change in activity (Alfaheeda 2016, unpublished data). In addition, several lymphomas, which are B-cell malignancies, have chromosomal translocation between oncogenes like *C-MYC* or *BCL-2* and the heavy chain regulatory region 3'*IGH*RR that lead to cancer such as Burkitt's lymphoma or multiple myeloma (Heckman et al., 2003).

Among the three human enhancers, the human hs1.2 enhancer is the most important one and it plays a main function in the control of transcription (Guglielmi et al., 2004; Cogne et al., 1994). Unlike the mouse hs1.2, the human hs1.2 enhancer actually decreases activity in response to human B-cell stimulation and increases activity in response to TCDD in both a mouse (CH12.LX) and human (IM-9) B-cell line, which is exactly the opposite of the stimulatory effect of cellular activation on the mouse hs1.2 and inhibition

by TCDD (Fernando et al., 2012). Additionally, the activity of the human hs4 enhancer alone is slightly reduced by TCDD unlike the stimulatory effect of TCDD on mouse hs4 activation (Alfaheeda 2016, unpublished data). These conflicting results between hs1.2 and hs4 enhancers could partially explain why 3'*IGH*RR plasmid did not respond to TCDD at all. The AhR antagonist was used to confirm that TCDD-induced activation of human hs1.2 enhancer requires the AhR (Fernando et al. 2012). Other studies have shown that the AhR can bind to other transcription factors like SP1, NF-κB and AP-1 (Suh et al., 2002; Tian et al., 1999; Kobayashi et al., 1996) therefore, the AhR could be directly or indirectly involved in the hs1.2 activation by TCDD.

The hs1.2 enhancer in mouse and human are almost the same and similar in about 90% of the DNA sequences, but there are many important differences between them that could explain the different effects by TCDD (Mills et al., 1997). The human hs1.2 enhancer has a polymorphic region consisting of a 55 bp repeated sequence called the invariant sequence (IS), which can be repeated up to four times, and appears to alter transcriptional activity (Fig. 6). This polymorphism does not occur in the mouse hs1.2 enhancer (Denizot et al., 2001) so all studies involving the mouse *Igh* locus have missed any functional significance of the human polymorphism. Moreover, the IS has binding sites for several transcription factors including NF-κB, NF-1, AP-1 and the AhR (Fig. 6). Unlike the mouse enhancers, the human enhancers have NF-1 binding sites but they do not have binding sites for Pax5 transcription factor, which is a negative regulator of B-cell differentiation into antibody-secreting cells (Fernando et al. 2012; Chen and Birshtein 1997; Denizot et al. 2001). The transcription factors binding sites are increased with each additional IS, which is correlated with an increase in the basal transcriptional activity of the hs1.2 enhancer

(Cogné et al., 1994). The NF-1 binding site could mediate the TCDD-induced activation of human hs1.2 enhancer since the mutation of the NF-1 binding site has a lower sensitivity to TCDD (Snyder 2016, unpublished data). In contrast, another study found that mutation of the AP-1 and NF-κB site increased the human hs1.2 enhancer activity by TCDD suggesting that these sites have repressor roles (Ochs 2012, unpublished data). The polymorphic region in the human hs1.2 enhancer makes the human heavy chain locus more complex than the mouse locus. This polymorphism has drawn particular interest, because it has been associated with Burkitt's lymphoma and a number of autoimmune disorders such as celiac disease, psoriatic arthritis, IgA nephropathy, rheumatoid arthritis, systemic sclerosis, and plaque psoriasis. The hs1.2 allele 2, which has two IS repeats, has been correlated with enhanced prevalence or severity of the previous disorders (Tolusso et al., 2009; Giambra et al., 2009; Cianci et al., 2008; Aupetit et al., 2000; Frezza et al., 2004).

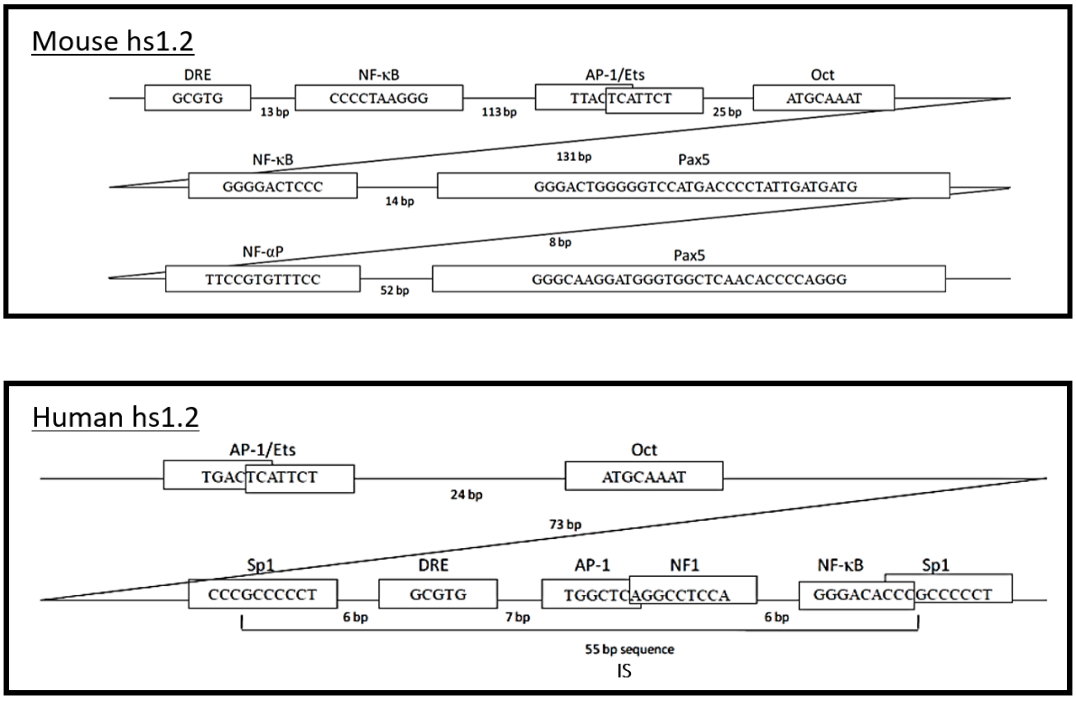


Figure 6. Differences between mouse and human hs1.2 enhancers. The hs1.2 enhancer is densely packed with transcription factor binding sites. A 55 base pair invariant sequence (IS) in human hs1.2 enhancer is polymorphic and can be repeated up to four times. AP-1 activator protein 1; Oct, octamer; Sp1, specificity protein 1; NF-κB, nuclear factor kappa B cells; DRE, dioxin responsive element; NF-1, nuclear factor 1; Pax5, paired box 5**.**

AhR interacts with cytosolic signaling proteins.

In the absent of a ligand, AhR is an inactive protein located in the cytosol and bound to protective proteins complex. Once ligand binds to the AhR in the cytoplasm, the AhR protein goes through a conformational change leading to its dissociation from the complex of protective cytosolic proteins (Abel & Haarmann-Stemmann, 2010). An additional mechanism for the AhR activity is through protein-protein interactions with regulatory proteins such as Src protein kinase and NFκB after dissociation from the proteins complex in the cytosol (Enan and Matsumura, 1996; Tian et al., 1999). Moreover, the AhR signaling pathway has been shown to modulate a number of signaling pathways such as tyrosine kinases associated with the epidermal growth factor receptor (EGFR) and Akt/PKB (Marlowe & Puga, 2005). Therefore, the biological effects of the AhR could occur via at least two general mechanisms: direct influence on gene transcription by binding to DRE motifs and/or modulate signaling pathways by protein–protein interaction (Suh et al., 2002).

Src family kinases are cytoplasmic proteins that are associated with the plasma membrane via lipid anchors at their N termini. The Src tyrosine kinase is overexpressed and activated in B-cell malignancies and dysregulation of Src activity is thought to be the key contributor to oncogenesis (Ye et al., 2016). Src family kinases (Lyn, Fyn, Fgr, Blk) play a role in initial phosphorylation of cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) of the Igα and Igβ that activate Syk, which is the dominant tyrosine kinase activity in B cells, leading to B-cell activation (Xu et al.,2012). Indeed, Src deficient mice displayed a profound block in B-cell development (Stepanek et al., 2013). Moreover, Park et al. (2007) indicated that TCDD-induced activation of Src kinase is

mediated by the cross-talk between the ligand-activated AhR in the cytosol and the cytosolic Src chaperone complex (Src-cdc37-Hsp90 complex). In another study, Src protein kinase is associated specifically with the cytosolic AhR complex along with hsp90 and upon ligand binding to the AhR, Src is activated and released from the complex (Enan & Matsumura, 1996).

Consistent with Src kinase, the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway plays a crucial role in multiple cellular processes, including cell proliferation, angiogenesis, metabolism, differentiation and survival. The members of the PI3K/Akt pathway are dysregulated in a wide spectrum of human B-cell cancers. Akt signaling triggers a cascade of responses, from cell growth and proliferation to survival and motility, which drive tumor progression (Vivanco, & Sawyers, 2002). Phosphorylated Akt activates the mTOR complex, causing increased mRNA translation, protein synthesis and cellular proliferation (Agata et al., 2010). Akt/forkhead box O (FoxO) pathway is a major pathway involved in B-cell differentiation and function. Activation of BCR by antigen leads to the transformation phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3) that phosphorylates Akt. The p-Akt enters the nucleus to inactivate FOXO protein, which blocks cell cycle progression and promotes stress resistance, that help in B-cell prolifration (Limon & Fruman, 2012). Basal expression of p-Akt was observed in the CA46 Burkitt lymphoma cell line under untreated conditions (Huang et al., 2012). Many studies have shown that AhR regulates Akt activation. Lack of the AhR leads to impaired activation of Akt and enhanced sensitivity to apoptosis in a hepatoma cell line (LA1) (Wu et al., 2007). Another study showed that the AhR signaling pathway controls breast cancer cell proliferation and development through inhibition of the

phosphatase and tensin homolog (PTEN) and activation of β-Catenin and Akt pathways (Al-Dhfyan et al., 2017).

The nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) protein is sequestered in the cytoplasm and their activity is regulated by binding of inhibitory κB factors (IκB). Upon activation, NFκB dimers dissociate from IκB and translocate to the nucleus then bind to the NFκB binding sites (κB site) in promoters and enhancers of NFκB responsive genes, consequently activating their transcription (Doi et al., 1997). The NFκB/Rel plays a main role in regulation of B-cell activation and diffrentiation (Gerondakis & Siebenlist, 2009). RelA/p50 is the major NFκB in pre-B cells and plays an important role in certain steps of B-cell development (Liou et al., 1994). RelA-deficient mouse B cells have the ability to secrete all immunoglobulins except IgG1 and IgA were reduced (Doi et al., 1997). The AhR and NFκB pathways interact by physical association of Re1A and AhR, and this interaction is associated with modulation of gene expression (Tian et al., 1999). The κB binding site is close proximity to the DRE binding site within the hs1.2 enhancer while it overlaps the DRE site within the hs4 enhancer (Sulentic et al., 2000). Binding of both the AhR and NFκB cooperatively influenced hs4 reporter activity (Sulentic et al., 2004b). Moreover, another study suggested that interaction between the AhR and NFκB proteins mediates the inhibitory effects of TCDD on 3'*Igh*RR and Ig expression (Salisbury & Sulentic, 2015).

AP-1 is another transcription factor binding site in the hs1.2 enhancer of the 3'*IGH*RR (Pinaud et al., 2011). B-cell signaling initiated at TLRs activates Jun N-terminal kinase (JNK). JNK phosphorylates c-Jun and c-Fos, which are components of AP-1, increasing the AP-1 transcription activity that lead to plasmacytic differentiation and Ig

production (North et al., 2010). AP-1 forms a transcriptional complex with ETS in activated B cells that bind to an ETS/AP-1 motif in the mouse 3'*Igh*RR enhancers that plays a main role in class switch recombination (Grant et al., 1995). It has a main role in the regulation of B-cell activation and differentiation since the surface IgM cross-linking or activation of CD40 induced the expression of AP-1 (Chan et al., 1993). In addition, AP-1 is involved in Ig production by transactivation of the promoter for κ light chain (Schanke et al., 1994). Another study showed that inhibition of AP-1 activity by TCDD is AhR-dependent and partially contributes to TCDD-mediated suppression of IgM expression in mouse B cells (Suh et al., 2002).

Hypothesis and Objectives:

In a mouse B-cell line, TCDD suppresses B-cell differentiation and inhibits IgM secretion (Sulentic & Kaminski, 2011). This suppression is mediated by the AhR pathway (Wourms & Sulentic, 2015; Vorderstrasse et al., 2001) perhaps by the AhR binding DRE sites within the hs1.2 enhancer of the mouse 3'*Igh*RR (Sulentic et al., 2000; Salisbury & Sulentic, 2015). Moreover, the AhRA (CH223191) inhibits the AhR signaling pathway and decreases the inhibition of Ig production in response to TCDD (Wourms & Sulentic, 2015; Salisbury & Sulentic, 2015). In a human B-cell line, IgG secretion is inhibited in response to TCDD but antagonism of the AhR [CH223191 or 6,2',4',-trimethoxyflavone (TMF)] increased IgG secretion about two-fold (Burra 2015; Panstingel 2017, unpublished data). In addition, AhR knockdown by shRNA showed that IgG secretion was nearly eliminated (Kashgari 2015, unpublished data). Other studies have shown that the AhR can interact directly or indirectly with different transcription factors like SP1, NF-κB and AP-1 (Kobayashi et al., 1996; Suh et al., 2002; Tian et al., 1999). The AhR signaling pathway has also been shown to modulate a number of signaling pathways such as Src and Akt/PKB (Marlowe & Puga, 2005). These data suggested that the AhR antagonist could cause a conformational change in the cytosolic AhR resulting in activation of other cytoplasmic proteins that leads to an increase in IgG secretion. In addition, the human AhR has SNPs that decrease CYP1A1 induction by TCDD (Flaveny et al. 2008; Wong, et al., 2001). In CL-01 human B cells, AhR is heterozygous with a non-functional transactivation domain in one of its alleles due to three SNPs in exon-10 (Kashgari 2015, unpublished data). These SNPs could explain why TCDD has variable effects on human 3'*IGH*RR reporters in the CL-01 cells (Alfaheeda 2016, unpublished data). Moreover, knockout of the AhR will help

to elucidate the role of the AhR in Ig expression and 3'*IGH*RR activity in response to TCDD or AhR antagonism. Therefore, we hypothesized that in human cells the AhR regulates *IGH* expression by altering 3'*IGH*RR activation through both genomic and non-genomic mediated mechanisms. To test this hypothesis, three main experimental objectives were developed utilizing the human CL-01 B-cell line. Objective one was to evaluate the potential non-genomic effects of the AhR on the activation of cytosolic signaling proteins (i.e. phosphorylation and activation of downstream transcription factors). Objective two was to determine the role of the AhR transactivation domain in the regulation and activity of the 3'*IGH*RR enhancers by using CL-01 cells expressing an AhR with a functional transactivation domain. Objective three was to directly evaluate the role of the AhR in the effects of the AhRA and TCDD on Ig expression and 3'*IGH*RR activity using CRISPR/Cas 9 gene editing to knockout the AhR. Significantly, the human 3'*IGH*RR has a polymorphism in the hs1.2 enhancer that has been associated with Burkitt's lymphoma and a number of autoimmune disorders such as celiac disease, psoriatic arthritis, IgA nephropathy, rheumatoid arthritis, systemic sclerosis, and plaque psoriasis. Consequently, determining the role of the 3'*IGH*RR and its potential sensitivity to the AhR could provide a greater understanding of *IGH* regulation in general and new insight into potential environmental triggers of immune disorders, perhaps providing new targets for drug development.

II MATRIALS AND METHODS

Chemical and Reagents

TCDD with 99.1% purity was purchased from AccuStandard, Inc and was dissolved in 100% DMSO. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO). R848, a TLR 7/8 ligand, was dissolved in 100% DMSO and was purchased from Enzo Life Sciences. The AhR antagonist (CH223191) was purchased from Calbiochem (Carlsbad, CA) and was dissolved in 100% DMSO. Human interleukin 4 (hIL-4) was purchased from Cell Signaling and dissolved in 1x phosphate buffer saline (PBS) with 10% bovine calf serum (BCS). Human Mega CD40 ligand (CD40L) was purchased from Enzo Life Sciences (San Diego, CA) and was reconstituted in 100 µl sterile H_2O and further dilution in fresh media with 10% BCS.

Cell Line Models

The Novus CL-01 human cell line was isolated from a Burkitt's lymphoma patient. It is a human monoclonal B-cell line that expresses surface IgM and IgD. It was purchased from Novus Biologicals (Littleton, CO). Cells were cultured at 37°C in a 5% CO_2 atmosphere and grown in RPMI-1640 (Mediatech, Herndon, VA) enhanced with 2 mM L-glutamine, 10% BCS (Hyclone, Logan, UT), 13.5 mM HEPES, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol and 23.8 mM sodium bicarbonate. The medium was changed every 2-3 days by simply removing some supernatant and adding some fresh medium. CL-01 cell line is EBV transformed and the cells can activate each other easily by cell-cell contact.

The cells were kept in density within the range between 0.1-0.2 x 10^6 cell/ml. they would be hyper-activated if too crowded, and die if too diluted. The cells were discarded after 4-5 generations or after one month of thawing the cells unless requiring linger culture to do growing up a clone from a single cell.

Reporter Plasmid Constructs

The plasmids were constructed using a pGL3 basic luciferase reporter backbone (Promega, Madison, WI) containing ampicillin resistance and the luciferase gene. The DNA sequence of the variable heavy chain (V_H) (179 bp, AJ851868.3 from 562798 to 562976, NCBI), gamma3 ($I\gamma_3$) (176 bp, NG_001019.6 from 1104579 to 1104754, NCBI) or epsilon (Iε) (291 bp, X56797.1 from 239 to 529, NCBI) promoters was inserted into the 5' multiple cloning site within the pGL3 luciferase reporter, and the human 3'*IGH*RR enhancers hs3, hs1.2, hs4 were inserted into 3' of the luciferase gene in the pGL3 reporter plasmid containing one of the above promoters (Fig. 7). The plasmid constructs have the intervening sequences between hs3 and hs1.2 but lack the intervening sequences between hs1.2 and hs4 enhancers because this part is about 10 kb that makes it difficult to include it in plasmid construct.

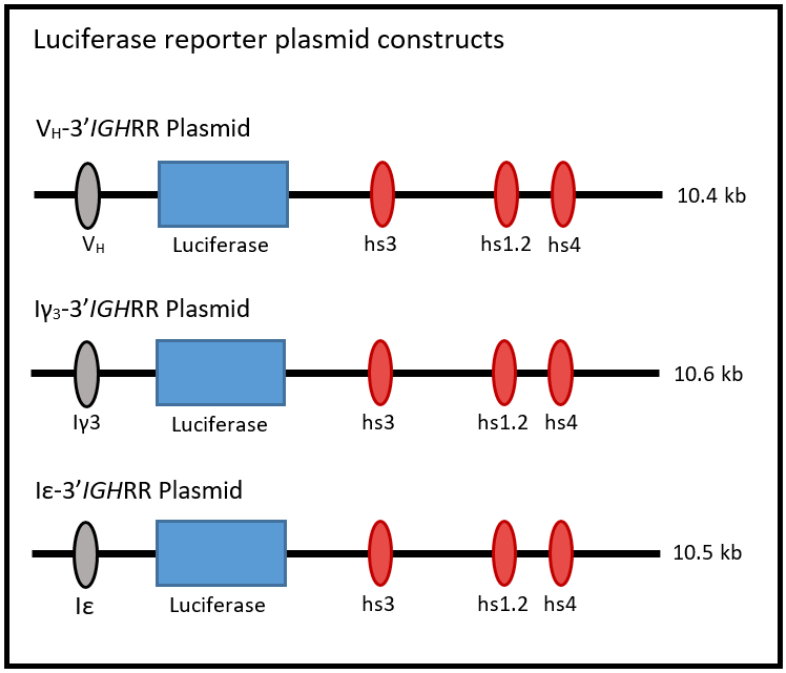


Figure 7. Human 3'*IGH*RR reporter plasmid constructs with V_H, Iγ3 or Iε promoters. Each plasmid contains a luciferase gene regulated by hs3, hs1.2, hs4 enhancers of the human 3'*IGH*RR. They include the intervening sequence between hs3 and hs1.2 enhancers and lack it between hs1.2 and hs4 enhancers.

The DRE luciferase reporter has six DRE binding sequences inserted into a pGL3 luciferase reporter with a SV40 promoter (Kress et al., 1998). NFκB luciferase reporter contains three consensus κB DNA binding sequences derived from the Ig κ light chain conjugated within the conalbumin promoter (Arenzana-Seisdedos et al., 1993). For the AP-1 luciferase reporter plasmid, three AP-1 sequences (Vasanwala et al., 2002) were inserted into a pGL3 luciferase reporter with a SV40 promoter (Fig. 8).

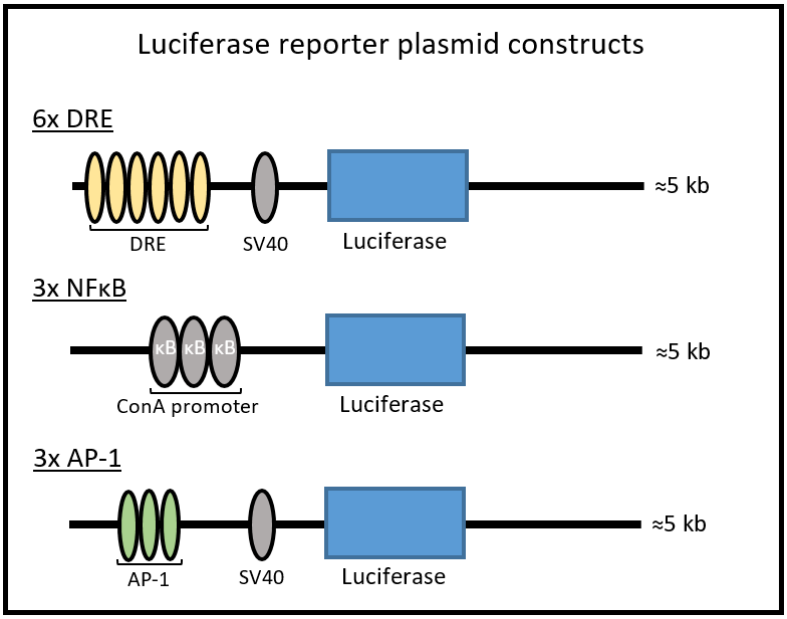


Figure 8. Luciferase reporters evaluating the activity of the transcription factors AhR, NFκB, and AP-1. 6x DRE plasmid contains six repeats of DRE motifs upstream of the SV40 promoter. 3x NFκB plasmid contains three repeats of κB sequence conjugated with Conalbumin (ConA) promoter. 3x AP-1 plasmid has three AP-1 binding motifs upstream of the SV40 promoter.

Transient Transfection

CL-01 cells ($1x10^7$) were pelleted by centrifugation at 500 x g for 5 minutes at 4°C. The pellet was re-constituted with 10 µg of plasmid and fresh complete media to a total volume of 200 µl then transferred to a 2mm electroporation cuvette. The cells were electroporated at 150 V, 1500 µF, and 75 ohms. Multiple transfections were performed for each plasmid and transfected cells were pooled together then diluted in culture tubes to obtain 1.5 x 10^5 cell/ml. Cells were cultured without any treatment [naïve (NA)], or treated with 0.01% DMSO [vehicle control (VH)] or 30 nM TCDD in absent or presence of cellular stimulation (1 µg/ml R848 or 6.25 ng/ml CD40L with 50 ng/ml IL-4). In AhR antagonist (AhRA) experiments, transfected cells in culture tubes were treated with 10 µM AhRA and incubated for 1 hour at 37ºC prior to additional treatments. 2 ml of treated cells (1.5 x 10^5 cell/ml) were aliquoted into a 12-well plate. The plates were incubated at 37ºC in 5% CO_2 for 24 or 48 hours.

Following the incubation period, the cells were centrifuged for 5 min at 500 x g at 4ºC to collect the pellets. Supernatant was removed and cells were lysed with 1x reporter lysis buffer (Promega). Lysates were immediately frozen at -80ºC. To measure the luciferase enzyme activity, samples were prepared by thawing on ice then centrifugation at 14,000 x g for 5 minutes at 4ºC. In a glass tube, 20 µl of supernatant was mixed with 100 µl of luciferase substrate reagent (Promega, Madison, WI), then luciferase activity, measured as relative light units (RLU), was determined using a luminometer (Berthold Detection Systems, Oak Ridge, TN).

Transfection Efficiency

When evaluating multiple plasmids or different clonal populations of the CL-01 cells (i.e. AhR knockdown clones), the transfection efficiency was determined by measuring the amount of luciferase gene within the transfected cells by real-time polymerase chain reaction (RT-PCR) and normalizing the RLU to the transfection efficiency relative to either a control plasmid or the WT CL-01 cells. Briefly, DNA was isolated using a genomic DNA miniprep kit (Sigma) at 2-4 hours post-transfection. Real time polymerase chain reaction (RT-PCR) was performed on the extracted DNA using luciferase forward and reverse primers (Table 1) as previously described (Fernando et al., 2012; Sulentic et al., 2004b). RT-PCR results were compared to a standard curve ranging from 1 to $1x10^{-6}$ ng/µl of a standard luciferase reporter to determine the lucifererase reporter concentration (ng/µl). The amount of transfected plasmid (ng) was calculated using the following equation: DNA concentration (ng/µl) x the volume of sample added (µl) x the sample fold dilution. Number of plasmids/cell was measured using the equation: [ng of plasmid DNA x (plasmid/ng)] / total cells used in DNA isolation. To normalize the RLU to transfection efficiency relative to a control (i.e. WT CL-01 cells or a control plasmid), the calculated plasmid/cell of each transfected cell population was divided by the calculated plasmid/cell of the control to generate a transfection efficiency factor and the RLUs of each transfected population was multiplied by the efficiency factor to normalize the luciferase activity to transfection efficiency relative to a control plasmid or the WT CL-01 cells.

Primer	Primer Sequence
Luciferase forward primer	5’-ACTGGGACGAAGACGAACACTT-3’
Luciferase reverse primer	5’-TCAGAGACTTCAGGCGGTCAA-3’

Table 1. Primers for the luciferase gene to measure transfection efficiency. Forward and reverse primers specific for the luciferase gene. Transfection efficiency is determined by measuring using RT-PCR the copies of the luciferase gene in transfected cell populations.

Protein Isolation and Western Blot

Proteins were isolated by centrifuging cells for 5 minutes at 500 x g then adding 50 µl of mild lysis buffer (1% NP40, 150 mM NaCl, 2 mM EDTA, 10 mM $NaPO_4$) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail; Roche) to the cell pellet. For cytoplasmic and nuclear proteins, cells were centrifuged for 5 minutes at 500 x g then proteins were extracted using nuclear and cytoplasmic extraction kit (Thermo). Samples were frozen and stored at -80^{o}C. For Western blot analysis, lysates were thawed on ice then centrifuged at 14,000 x g for 5 minutes. The supernatants were collected and protein concentration was quantified by Bradford protein assay. Equal amounts of protein were denatured by heating at 95^{o}C for 5 minutes, and separated by 10% polyacrylamide gel at 100 volts for about one hour. Proteins were transferred to a polyvinylidene fluoride membrane (PVDF) at 300 mAMPs for 2 hours. Membranes were blocked by 1x tris buffered saline (TBS) with 5% non-fat milk for 1 to 3 hours at room temperature. After that, membranes were probed with primary antibody [rabbit anti-AhR (Santa Cruz, cat#

5579) at 1:1000 dilution; mouse anti-β-actin (Cell Signaling, cat# 3700S) at 1:10,000 dilution; rabbit anti-phospho-Akt (Ser473) (Cell Signaling, cat#4060) at 1:1000 dilution; rabbit anti-Akt (pan) (Cell Signaling, cat#4691) at 1:1000; rabbit anti-phospho-Src family (Tyr416) (Cell Signaling, cat#2101) at 1:1000; mouse anti-Src (L4A1) (Cell Signaling, cat#2110) at 1:1000; mouse anti-GAPDH (Gene Tex, cat#627408) at 1:1000 or mouse anti-p84 (Gene Tex, cat#70220) at 1:1000], and incubated overnight at 4^{o}C. The membranes were washed three times for 5 minutes in TBS with 0.05% tween 20 (TBS-T) and then incubated with a secondary antibody conjugated to horseradish peroxidase (HRP); [donkey anti-rabbit-HRP 1:20,000 (Promega) or goat anti-mouse-HRP 1:20,000 (Thermo-scientific)], and incubated on a rotator for one hour at room temperature. Both primary and secondary antibodies were diluted in 1x TBS/5% non-fat milk/0.05% tween-20. The probed membranes were washed again four times in TBS-T and then exposed to a Super Signal West Femto chemiluminescent substrate (Thermoscientific) and analyzed on a Chemidoc MP Imager (Biorad).

RNA Extraction and polymerase Chain Reaction (PCR)

RNA was extracted from the cells using Tri-Reagent (Sigma) according the manufacturer's protocol. The RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the instructions of manufacturer. PCR was performed on 100 ng of cDNA using the following: 10x buffer, dNTPs, Taq polymerase (New England Biolabs), forward and reverse primers (Table 2) and volume was made up to 25 µl with nuclease-free water. PCR products were run on 1% agarose gel and analyzed on a Chemidoc MP Imager (Biorad).

Primer	Primer Sequence	Annealing temperature
β-actin	FP: 5'-GCGGATGTCCACGTCACACTTCA-3' RP: 5'-CACCATTGGCAATGAGCGGTTC-3'	58^{o}C
AhR	FP: 5'- GCACCGATGGGAAATGATAC -3' RP: 5'- TTGACTGATCCCATGTAAGTCTG -3'	59^{o}C

Table 2. β-actin and AhR primers for PCR. Forward and reverse primers specific for *β-actin* and *AHR* gene with the annealing temperature. FP- forward primer, RP- reverse primer.

Enzyme-Linked Immunosorbent Assay (ELISA)

CL-01 cells (1.25 x10^5 cells/ml) were cultured for 96 hours and then centrifuged at 500 x g for 5 minutes at 4^0C. The supernatants were transfered into 1.5 ml tubes and stored at -80^{o}C for later analysis of secreted Ig. 96-well plates for ELISA were coated at 4^{o}C overnight with poly-Ig goat anti-human (GAH) (Southern Biotech, Birmingham, AL) diluted to 1:1500 in 0.1M sodium carbonate bicarbonate buffer. Plates were washed three times with 1xPBS with 0.05% Tween-20 then four times with dH_2O, then blocked with 1x PBS with 3% bovine serum albumin (BSA) for 1.5 hours at room temperature. Different standard concentrations to generate a standard curve and cell supernatant samples were added and incubated in a dry incubator for 1.5 hours at 37^{o}C. Purified IgG (Bethyl Laboratories, Montgomery, TX) was used for the standard curve for the IgG ELISA. Plates were washed three times with 1xPBS with 0.05% Tween-20 then four times with dH_2O and then incubated with goat anti-human IgG HRP-conjugated antibody (Bethyl Laboratories, Montgomery, TX) at 1:10,000 diluted with 1X PBS with 0.05% Tween 20

and 3% BSA for 1.5 hours in a dry incubator at 37°C. after the incubation period, plates were washed three times with 1xPBS with 0.05% Tween-20 then four times with dH_2O then Tetramethylbenzidine (TMB) substrate was added to develop the enzyme reaction. The reaction was stopped by adding 4N H_2SO_4 and the color changed to yellow. Absorbance was read with a SpectramaxPlus microplate Spectrophotometer at 450 nM. The SOFTmax PRO software was used to calculate the sample Ig concentrations using the standard curve.

CRISPR/Cas9 Gene Editing

The CRISPR plasmid (pCas-Guide-EF1a-GFP) was purchased from Origene (Rockville, MD). The plasmid was cut using the restriction enzymes BamHI and Esp3I (an isoschizomer of BsmBI) and the cut plasmid was purified from a 1% agarose gel in preparation for cloning the targeting sequences. The targeting sequences specific for the human *AHR* gene (Fig. 9 and table 3) were inserted into the cut plasmid using T4 ligase (Promega) and the ligated plasmids were transformed into chemically competent E. coli (Zymo). Isolated colonies were plucked from an LB agar plate, grown in a 3 ml culture of LB broth, and the plasmids extracted via miniprep (Zymo). The minipreps were screened for successful insertion of the targeting sequence by DNA sequencing (Retrogen). When a successfully cloned plasmid was identified a transfection-quality preparation of the plasmid was created using an endotoxin-free maxiprep kit (Qiagen). The CRISPR plasmid was transfected into CL-01 cells and the transfected cells were allowed to incubate for 72 hours at a concentration of 1×10^5 cells/ml and were then sorted for GFP expression by the Research Flow Cytometry Core at Cincinnati Children's Hospital. Cells expressing GFP were seeded into a 96-well plate at 1 cell/well. When the cells outgrew the 96-well plate,

they were transferred to a 24-well plate. When sufficient cell numbers were achieved, a portion of the cells were collected for protein, DNA and RNA analysis.

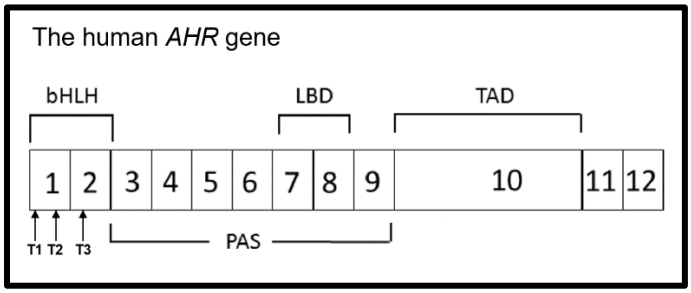


Figure 9. The human *AHR* gene with CRISPR targets. The targeting sequence for CRISPR plasmid 1 (T1) and 2 (T2) are in exon 1, and CRISPR plasmid 3 (T3) is in exon 2 of the *AHR* gene. Basic helix–loop–helix domain (bHLH); ligand binding domain (LBD); Per/Arnt/Sim domain (PAS) and transactivation domain (TAD).

Target #	CRISPR Target	Targeting Sequence
T1	AhR exon 1, 24-43F	AGTAGCCGCCGCCGTCGGCT
T2	AhR exon 1, 73-92F	TCACCTACGCCAGTCGCAAG
T3	AhR exon 2, 45-64RC	AAGTCGGTCTCTATGCCGCT

Table 3. CRISPR/Cas9 targeting sequences. These targeting sequences were cloned into the CRISPR plasmids following the manufacturer's (Origene) instructions.

Statistical Analysis

Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison test. GraphPad Prism software was used to determine significant differences between treatment groups (mean ± SEM) and the corresponding vehicle control as represented by "*", "**", "***" at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Significant differences between clones and WT clone were denoted by †, †† and †††, at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Comparisons between treatment groups and VH of each time point were analyzed using a two-way ANOVA followed by a Bonferroni post-tests, significant differences were denoted by "*", "**", "***" which represents significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Significant differences between AhRA groups and AhRA plus TCDD were denoted by "#", "##", "###", at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

III RESULTS

The role of the AhR on the activation of cytosolic signaling proteins.

Previous studies in a mouse B-cell line stimulated with LPS demonstrated a concentration-dependent inhibition of IgM secretion (Sulentic et al., 1998). In another study using human primary B cells, TCDD reduced IgM expression in nine of twelve human donors while having no effect in two donors and enhancing IgM expression in one donor (Lu et al., 2010). In a previous study in our lab, using a human B-cell line that can be induced to secrete antibodies, IgM secretion was fairly unresponsive to treatment with TCDD or the AhRAs CH223191 or 6,2',4',-trimethoxyflavone (TMF) (Panstingel 2017, unpublished data). In contrast, IgG secretion was inhibited by TCDD and surprisingly increased by AhRA. Moreover, TCDD inhibited the induction of $C\gamma_{1\text{-}4}$ germline/functional transcripts while the AhRA alone increased all of the $C\gamma_{1\text{-}4}$ germline/functional transcripts (Burra 2015, unpublished data). Our lab has also used shRNA to target and degrade AhR mRNA and knockdown (KD) the AhR protein levels. IgM secretion was unaffected by AhR KD, whereas IgG levels were nearly eliminated with AhR KD. Treatment with the AhRA had little effect on IgG secretion in the AhR KD cells suggesting that the AhRA effect is AhR dependent (Kashgari 2015, unpublished data). These data suggest a physiological role of the AhR (independent of ligand) on IgG expression and that the AhRA may induce a conformational change in the cytosolic AhR that results in an activation of other signaling proteins. The AhRA prevents translocation of the AhR into the nucleus and inhibits its activation (Kim et al. 2006) but binding of the AhRA could influence interaction of the AhR with other cytosolic proteins to lead to the increase in IgG

secretion. Therefore, the objective of these studies was to determine if AhRA treatment activated cytosolic signaling pathways known to play a role in B-cell differentiation and Ig production and previously shown to interact with the AhR.

Previous studies in our lab has shown AhR binding to DRE in the mouse hs1.2 and hs4 enhancers of the 3'*Igh*RR following TCDD treatment (Sulentic et al., 2000; Salisbury & Sulentic, 2015). Interestingly, our human B-cell line model (CL-01) is heterozygous for the AhR and expresses a non-functional transactivation domain in one of its alleles due to three SNPs (Kashgari 2015, unpublished data) that could decrease the functional activity of the AhR. In this study, CL-01 cells were transfected with a DRE reporter and treated with TCDD and/or AhRA (Fig. 10). While TCDD treatment alone had no effect on DRE activity, AhRA at 10 µM nearly completely inhibited basal DRE activity, which was partially reversed by 30 nM TCDD reversed the inhibitory effect of AhRA (Fig. 10). These results suggest a high basal activity of the DRE reporter that is AhR-dependent. The AhRA prevents translocation of the AhR into the nucleus and inhibits its binding to DRE motifs while TCDD competes with the AhRA for binding to the AhR in the cytosol and increases AhR translocation into nucleus. The unexpectedly high basal activity of the DRE suggests either basal translocation of the AhR perhaps via an unknown endogenous ligand or increased activity of the SV40 promoter. To determine if either was responsible for the high basal activity of the DRE reporter, first a luciferase reporter containing only the SV40 promoter (without DRE motifs) was used to compare its basal activity with the DRE reporter. The result showed that the SV40 reporter plasmid had very low luciferase activity compared to the DRE reporter activity (Fig. 11), indicating that the increase in DRE reporter activity was mediated by the transcriptional activity of the DRE binding sites and

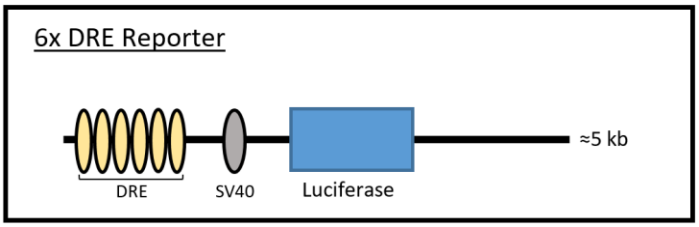
not from high basal activity of the SV40 promoter. This suggest that the basal activity of the DRE reporter could be from nuclear AhR. Therefore, the cytosolic and nuclear AhR was evaluated by Western blot analysis. Human CL-01 cells were treated with TCDD and AhRA. After cultured the cells for 24 hours, the cells were collected and lysed by cytosolic lysate buffer and transferred the supernatant containing the cytosolic proteins into new tubes. The nuclear pellets were lysed with nuclear lysate buffer in order to extract nuclear proteins. Both cytosolic and nuclear extractions were examined for AhR protein (Fig. 12). The Western blot identified nuclear AhR in naïve cells, which may account for the high basal activity of the DRE reporter.

Src protein kinase is associated specifically with the cytosolic AhR complex along with hsp90 and upon ligand binding to the AhR, Src is activated and released from the complex (Enan & Matsumura, 1996). Since the AhRA competes with TCDD for binding to the AhR, and then prevents the AhR from translocating to the nucleus (Kim et al. 2006), we hypothesized that AhRA binding also results in Src disassociation and activation in the human CL-01 B cells. Human CL-01 B cells were stimulated with R848 and treated with AhRA for different time points (5 min, 15 min, 30 min, 2 h and 24 h). At each time point, cells were collected and lysed in order to extract proteins and examine for Src phosphorylation. Phospho-Src (Y416) antibody has been used to detect Src phosphorylated at tyrosine 416, which is located in the activation loop and required for optimal activity of Src (Frame, 2002). Most previous studies have used phospho-Src (Y416) antibody to detect activated Src kinase in Western blot analysis (Dong et al., 2011), including human B cells (Ye et al., 2016). Unexpectedly, phospho-Src was not detected at any time point, with or without cellular stimulation and AhRA treatment (Fig. 13), suggesting that the R848

stimulation does not induce phosphorylation of Src, which may require other stimulation such as BCR activation.

The Akt signaling pathway plays a crucial role in multiple cellular processes, including cell proliferation, angiogenesis, metabolism, differentiation and survival. Basal expression of phospho-Akt was observed in a Burkitt lymphoma cell line under untreated conditions (Huang et al., 2012). Many studies have showed that AhR regulates Akt activation (Wu et al., 2007; Al-Dhfyan et al., 2017). These studies indicate that Akt kinase is activated during B-cell differentiation and class switching. As the AhRA keeps the AhR in the cytoplasm and prevents its translocation into the nucleus, we suspected the AhRA could increase Akt activity. Western blot analysis showed that Akt is basally phosphorylated in CL-01 cells (Fig. 14A) but after 30 minutes of AhRA and R848 co-treatment, Akt phosphorylation significantly increased and at 2 hours returned to basal phosphorylation level (Fig. 14B). This indicates that the cytosolic AhR could activate the Akt pathway after 30 minutes of AhRA treatment, which might lead to increase IgG production.

A.



B.

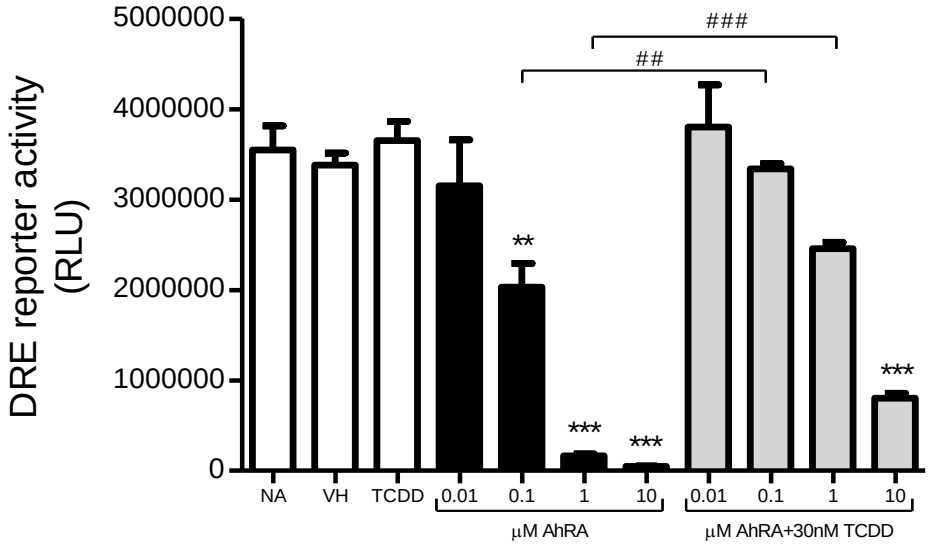


Figure 10. Concentration-dependent decrease of DRE reporter activity by AhRA. A. The DRE luciferase reporter has six DRE binding sequences inserted into a pGL3 luciferase reporter with a SV40 promoter. **B.** CL-01 cells were transiently transfected with the DRE reporter, and were either cultured in the absence of any additional treatment (naïve, NA) or with the vehicle control (VH, 0.01% DMSO), TCDD (30nM) or AhRA (0.01, 0.1, 1, 10 µM), then incubated at 37ºC for 24 hours. Luciferase enzyme activity is represented on the y-axis as relative light units (RLU). Comparisons between the treatment groups were analyzed using a two-way ANOVA followed by a Bonferroni post-tests. Significant differences between treatment groups and VH were denoted by ** and ***, which represents significance at $p<0.01$ and $p<0.001$, respectively. Significant differences between AhRA groups and AhRA+30nM TCDD were denoted by ## and ###, which represents significance at $p<0.01$ and $p<0.001$, respectively. Results are representative of three independent experiments (mean SEM, n=3).

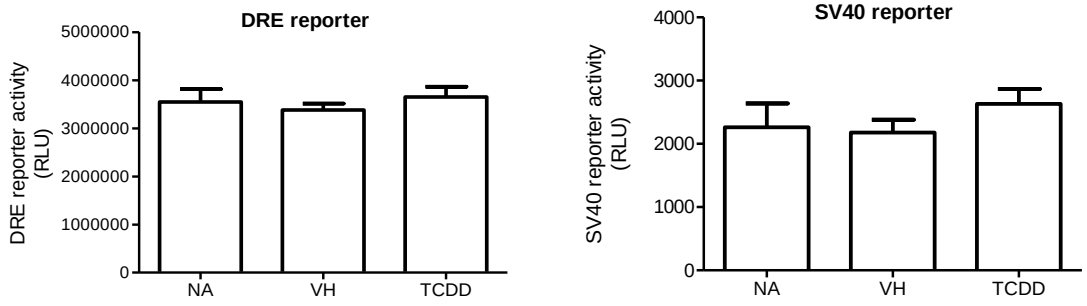


Figure 11. Low SV40 reporter activity compared to DRE reporter activity. CL-01 wild type (WT) cells were transiently transfected with the DRE reporter or SV40 reporter, and were either cultured in the absence of any additional treatment (naïve, NA) or with the vehicle control (VH, 0.01% DMSO) or TCDD (30 nM), then incubated at 37°C for 24 hours. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Results are representative of three independent experiments (mean SEM, n=3).

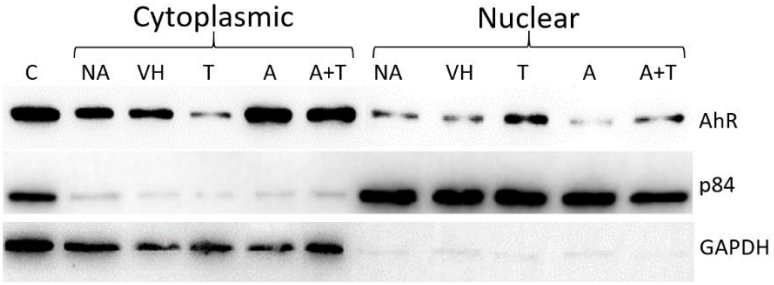


Figure 12. Nuclear AhR in naïve cells. CL-01 cells were cultured without further treatment naïve (NA) or treated with vehicle (VH, 0.01% DMSO), 10 µM AhRA (A) or both AhRA and 30 nM TCDD (A+T). Whole cells (C), cytoplasmic and nuclear proteins were isolated. 30 µg of proteins were subjected to Western blot analysis. All membranes were probed with antibodies specific for AhR (122 kDa), p84 (84 kDa, nuclear loading control) or GAPDH (37 kDa, cytoplasmic loading controls). Results are representative of three independent experiments.

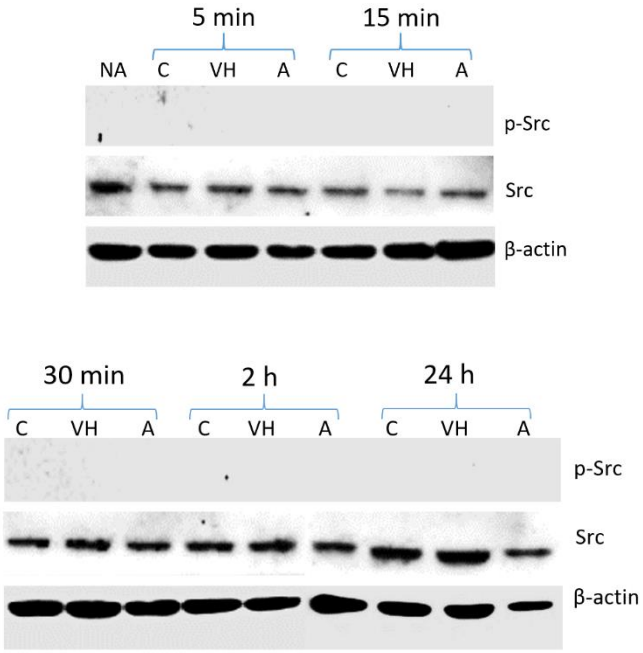
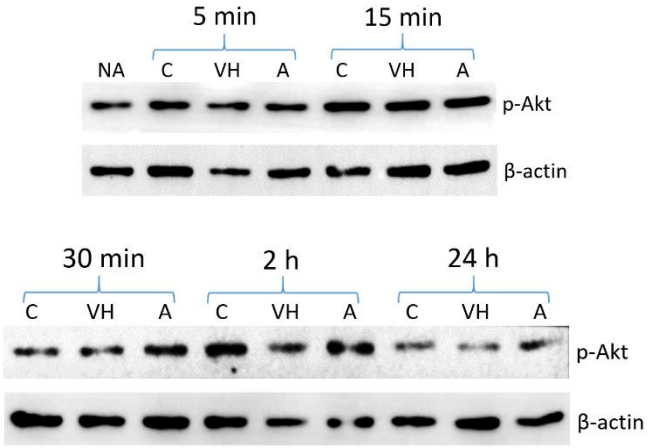


Figure 13. The AhRA has no effect on Src activation. CL-01 cells were cultured without treatment naïve (NA) or stimulated with 1µg/ml R848 (C), R848 plus vehicle 0.01% DMSO (VH) or R848 plus 10 µM AhRA (A). Cells were lysed with mild lysis buffer containing protease and phosphatase inhibitors at different time points (5 min, 15 min, 30 min, 2 h, 24 h). 30 µg of proteins were subjected to Western blot analysis. All membranes were probed with antibodies specific for phospho-Src (60 kDa), Src (60 kDa) or β-actin (42 kDa). Src and β-actin served as loading controls. Results are representative of three independent experiments.

A.



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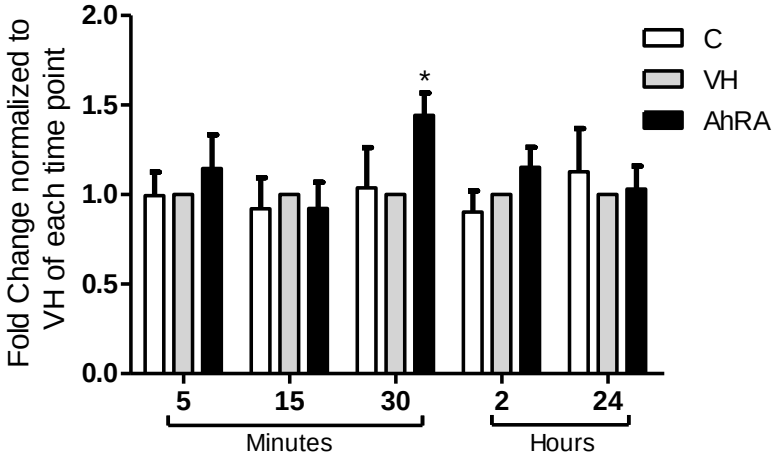
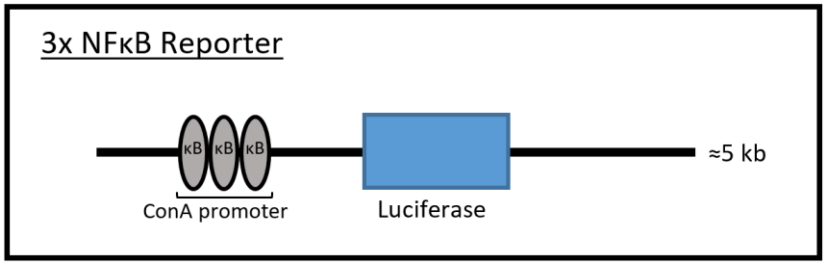


Figure 14. The AhRA increases Akt activity after 30 minutes. **A.** CL-01 cells were cultured without treatment naïve (NA) or stimulated with 1µg/ml R848 (C), R848 plus vehicle 0.01% DMSO (VH) or R848 plus 10 µM AhRA (A). Cells were lysed with mild lysis buffer containing protease and phosphatase inhibitors at different time points (5 min, 15 min, 30 min, 2 h, 24 h). 30 µg of proteins were subjected to Western blot analysis. All membranes were probed with antibodies specific for phospho-Akt (60 kDa) or β-actin (42 kDa), which served as loading controls. **B.** Densitometric estimation of expression was graphically represented for phospho-Akt. Fold change normalized to the corresponding VH in each time point. Results are representative of three independent experiments (mean SEM, n=3). Comparisons between the treatment groups were analyzed using a two-way ANOVA followed by a Bonferroni post-tests. Significant differences between treatment groups and VH were denoted by (*), which represents significance at $p<0.05$.

In B-lymphocytes, the 3'*IGH*RR enhancers contain numerous transcription factor-binding sites within hs1.2 and hs4 enhancers. The hs1.2 enhancer is polymorphic due to the potential of having one to four 55 bp invariant sequences. The invariant sequence contains a DRE, NFκB, SP-1, NF-1 and AP-1 binding site (Pinaud et al., 2011). Because of the important role of NFκB in B-cell activation and activity of the mouse 3'*Igh*RR, we measured NFκB transcriptional activity using a luciferase reporter plasmid that has three consensus NFκB binding motifs. CL-01 cells were transfected with the NFκB reporter plasmid then stimulated with R848. and treated with 30nM TCDD and 10μM AhRA. As expected of a TLR ligand, R848 stimulation significantly increased NFκB activity but TCDD and AhRA had no effect on NFκB activity (Fig. 15). The AhRA and TCDD do not appear to influence any interaction between the AhR and NFκB pathways.

AP-1 is another transcription factor binding site in the hs1.2 enhancer of the 3'*IGH*RR (Pinaud et al., 2011). Since it plays an important role in B-cell differentiation, we utilized a luciferase reporter with three AP-1 binding motifs to measure the transcriptional activity of AP-1. The CL-01 cells were transfected with the AP-1 plasmid then stimulated with 1 μg/ml R848 and treated with 30nM TCDD or 10μM AhRA. AP-1 activity was not affected by TCDD or AhRA in either stimulated nor unstimulated cells. Surprisingly, R848 stimulation decreased AP-1 transcriptional activity (Fig. 16). This result suggests that AP-1 does not mediate an increase in IgG production by R848 stimulation or AhRA and the AhRA does not disrupt the activation of AP-1 pathway proteins like fos and jun proteins.

A.



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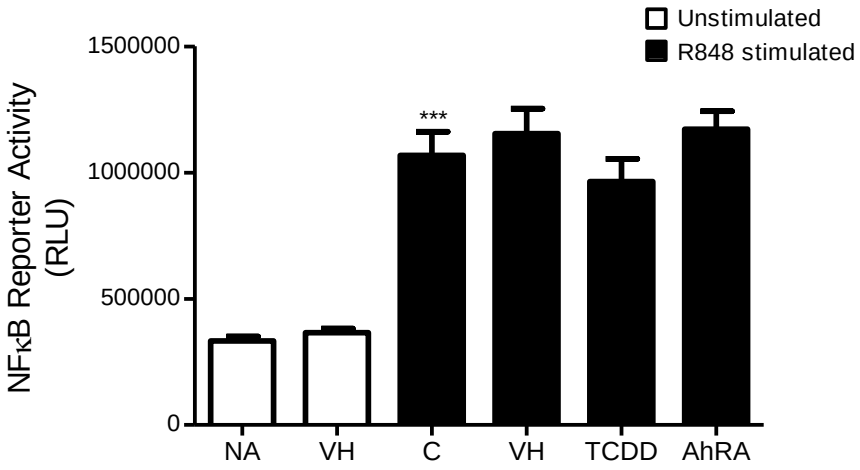
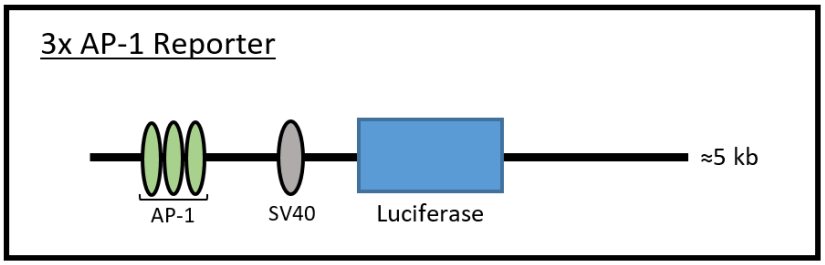


Figure 15. The AhRA and TCDD had no effect on NFκB activation. A. The NFκB luciferase reporter plasmid construct which contains three κB motifs conjugated within a Conalbumin A promoter (ConA). **B.** CL-01 cells were transiently transfected with the NFκB plasmid and incubated for 24 hours at 37^{o}C. Transfected cells were stimulated with R848 (1 μg/ml) in all groups except naïve (NA) and vehicle (VH, 0.01% DMSO). Cells were stimulated with R848 alone (C) or with the vehicle control (VH, 0.02% DMSO), TCDD (30nM), or AhRA (10μM). Luciferase enzyme activity is represented on the y-axis as relative light units (RLU). Results are representative of three independent experiments (mean SEM, n=3). Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between stimulated VH and VH alone were denoted by ***, which represents significance at $p<0.001$.

A.



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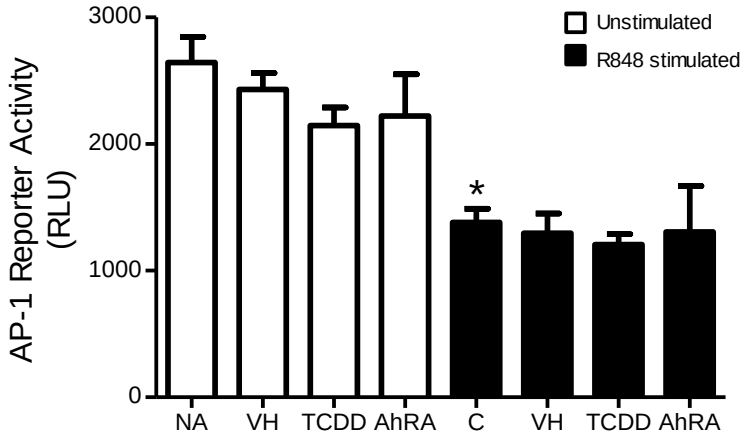


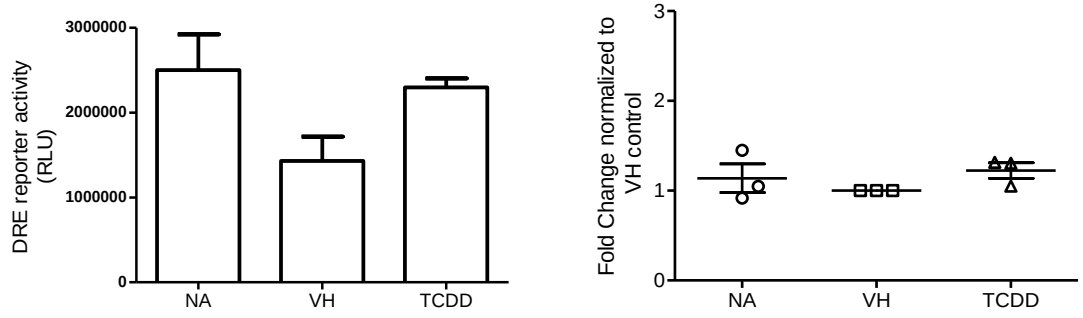
Figure 16. The AhRA and TCDD have no effect on AP-1 activity but cellular stimulation inhibits it. A. The AP-1 luciferase reporter has three AP-1 binding sequences inserted into a pGL3 luciferase reporter with a SV40 promoter. **B.** CL-01 cells were transiently transfected with the AP-1 plasmid and incubated for 24 hours at 37ºC. Transfected cells were incubated in absent of treatments naïve (NA) or treated with vehicle (VH, 0.01% DMSO), TCDD (30nM) or AhRA (10µM). Cells were stimulated with R848 alone (C) or with the vehicle control (VH, 0.02% DMSO), TCDD (30nM), or AhRA (10µM). Results are representative of three independent experiments (mean SEM, n=3). Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between C group and unstimulated VH group were denoted by (*), which represents significance at $p<0.05$.

Differential effects of TCDD and AhR antagonist on the human 3'*IGH*RR in CL-01 cells.

B-cell stimulation would be expected to increase the activity of the 3'*IGH*RR enhancers since B-cell stimulation is known to increase the transcriptional activity of the *IGH* gene, which is at least partially driven by the 3'*IGH*RR based on mouse studies. TCDD treatment was expected to result in reduced activity of the plasmids since TCDD is known to act as an immunosuppressant in both mouse and human B cells. The transcriptional activity of mouse 3'*Igh*RR in mouse CH12.LX cells was increased with LPS stimulation, and inhibited when stimulated cells were co-treated with TCDD (Sulentic et al., 2004a). The human 3'*IGH*RR activity has been evaluated in human CL-01 cells and mouse CH12.LX cells in response to B-cell stimulation or TCDD. The CH12.LX cells transfected with human 3'*IGH*RR showed more sensitivity to TCDD as well as LPS stimulation. The human 3'*IGH*RR activity was induced by LPS and inhibited by TCDD, which is similar to the effect on the mouse 3'*Igh*RR activity. By contrast, 3'*IGH*RR activity in the human CL-01 cell line with either the V_H promoter or the intronic promotors Iγ3 or Iε exhibited variable and statistically insignificant change in response to TCDD or stimulation (R848 or CD40L plus IL-4) (Alfaheeda 2016, unpublished data). In another study with CL-01 cells, there was no upregulation of CYP1A1 mRNA expression in response to TCDD (Burra 2015, unpublished data). Consequently, these results suggested that the AhR in the CL-01 cells might be not functional. As described above, sequence analysis found that the AhR is heterozygous in CL-01 cells, likely with a non-functional transactivation domain in one of its alleles due to the presence of three SNPs (P517S, R554K, and V570I) in Exon 10 (Kashgari 2015, unpublished data). Exon 10 encodes the

transactivation domain that regulates expression of other genes (Harper et al. 2002). These SNPs were demonstrated to impair the ability of CYP1A1 induction by TCDD (Wong et al., 2001). Therefore, we hypothesized that the lack of sensitivity of the 3'*IGH*RR to TCDD is due to not having two AhR alleles with functional transactivation domains. Our lab used CRISPR/Cas9 gene editing to target the AhR gene with the goal of inducing an insertion or deletion mutation to prevent AhR gene expression. After screening and characterizing many clones, a small number of clones appeared to have a monoallelic knockout of the AhR based on Western analysis and DRE reporter inducibility by TCDD. Further analysis was conducted on one clone that appeared to have a monoallelic knockout and expressed an AhR with a functional transactivation domain (AhR TA) (Panstingel 2017, unpublished data). As demonstrated above in Fig. 10, TCDD treatment did not induce the 6x DRE reporter in CL-01 wild type cells but in the monoallelic knockout clone, TCDD induced the DRE reporter (Fig. 17). This suggests that the CL-01 wild type cells may express a dominant negative AhR due to the heterozygous presence of SNPs in the transactivation domain of the AhR (Kashgari 2015, unpublished data).

A) CL-01 WT



B) CL-01 AhR TA

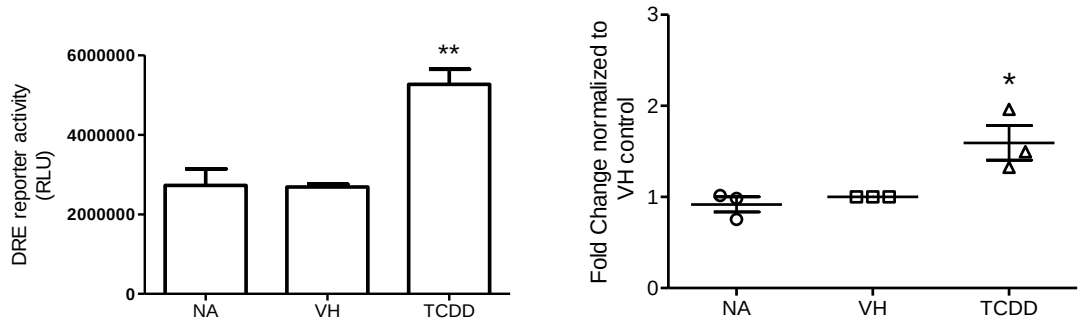


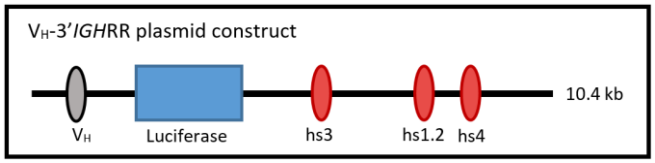
Figure 17. TCDD induced the DRE reporter in CL-01 cells targeted for AhR gene editing by CRISP/Cas9. A. CL-01 WT cells were transiently transfected with the DRE reporter plasmid and cultured for 24 hours. **B.** CL-01 AhR TA cells were transiently transfected with the DRE reporter plasmid and cultured for 24 hours. All transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated with the vehicle control (VH, 0.01% DMSO) or 30 nM TCDD. The graphs on the left represent luciferase activity as relative light units (RLU) and graphs on the rights represent the fold change in luciferase activity compared to VH control that was generated from averaging the means of three independent experiments. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and VH were denoted by *, which represents significance at $p<0.05$. (mean SEM, n=3).

To determine if the sensitivity of the 3'*IGH*RR to TCDD is dependent on the AhR transactivation domain, 3'*IGH*RR luciferase reporters were transfected into CL-01 WT cells and CL-01 AhR TA cells, which appear to express a transactivation functional AhR. There was little to no effect of CD40L plus IL-4 stimulation or TCDD treatment on 3'*IGH*RR activity with the V_H promoter in both CL-01 WT cells and CL-01 AhR TA cells (Fig. 18). Additionally, we evaluated the effect of stimulation and TCDD on the 3'*IGH*RR when linked with the intronic promoters $I\gamma_3$ or Iε. The intronic promoters are involved in promoting CSR and may interact more with the 3'*IGH*RR as compared to the V_H promoter (Bottaro 1997, Stavnezer 1996). Moreover, the $I\gamma_3$ promoter has a DRE core motif that the AhR could bind to and mediate a TCDD effect. Additionally, the Iε promoter has IL-4 binding sites that should make this promoter more sensitive to CD40L plus IL4 stimulation (Delphin, 1995). Interestingly, in both CL-01 WT and AhR TA cells, stimulation and TCDD had no effect on 3'*IGH*RR activity when linked to either intronic promoter (Fig. 19 and 20).

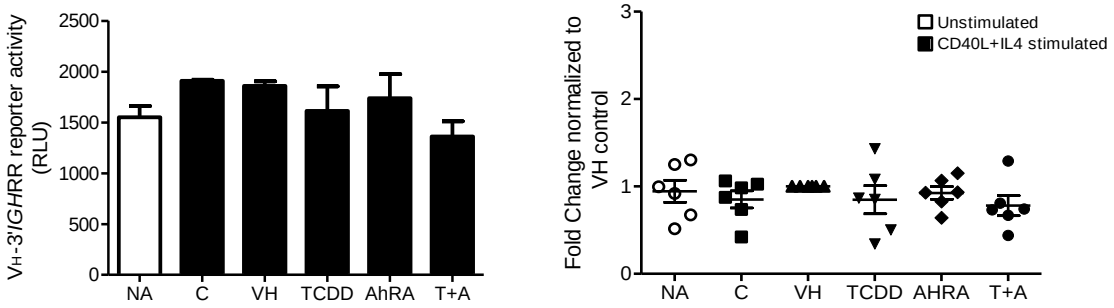
As discussed above, our lab found that co-treatment of stimulation (CD40L plus IL-4) and AhRA increased the secretion of IgG while TCDD inhibited IgG secretion (Burra 2015, unpublished data). Therefore, the effect of the AhRA on each of the 3'*IGH*RR reporter was also analyzed. The V_H-3'*IGH*RR reporter showed no consistent effect of AhRA treatment in both CL-01 WT and CL-01 AhR TA cells (Fig. 18). In addition, both CL-01 Cells transfected with the $I\gamma_3$-3'*IGH*RR reporter showed an increase in activity with AhRA (Fig. 19 and 20), which is compatible with previous results that AhRA increased the secretion of IgG (Burra 2015, unpublished data). In the plasmid driven by Iε-3'*IGH*RR there was an increase in activity in both CL-01 WT and AhR TA cells with AhRA.

Therefore, AhRA have the same effect on 3'*IGH*RR activity in both CL-01 wild type and CL-01 AhR TA. These results indicate that the dysfunction of the AhR transactivation domain in the CL-01 wild type has no effect on the sensitivity of the 3'*IGH*RR to TCDD or AhRA since it has the same effects as in CL-01 cells expressing only an AhR with a functional transactivation domain.

A)



B) CL-01 WT



C) CL-01 AhR TA

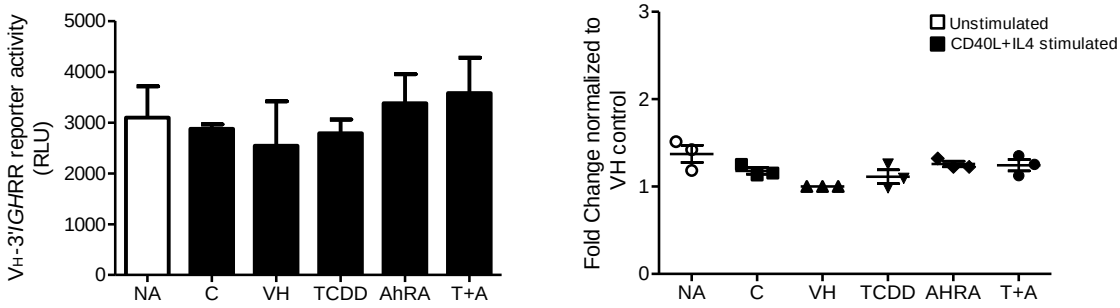
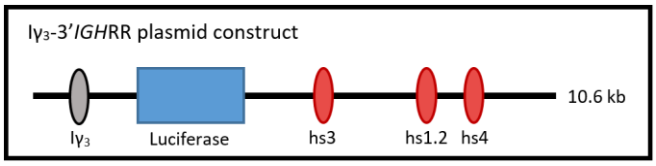
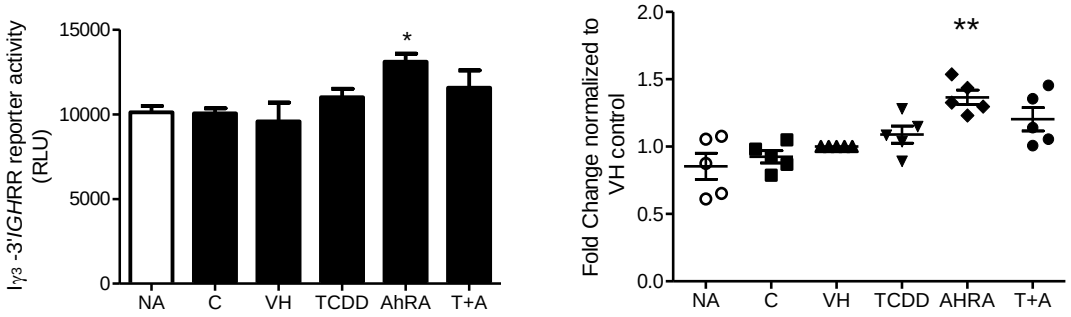


Figure 18. TCDD and AhRA have no effect on V_H-3'*IGH*RR reporter activity in CL-01 WT and AhR TA cells. A. V_H-3'*IGH*RR luciferase plasmid construct. **B.** CL-01 WT cells. **C.** CL-01 AhR TA cells. All cells were transiently transfected with V_H-3'*IGH*RR plasmid and cultured for 48 hours. Cells were either cultured in the absence of any treatment (naïve, NA) or treated with CD40L plus IL-4 stimulation alone (C) or co-treatment stimulation with vehicle control (VH, 0.01% DMSO), 30 nM TCDD, 10 µM AhRA or TCDD plus AhRA (T+A). The graphs on the left represent luciferase activity as relative light units (RLU) and graphs on the rights represent the fold change in luciferase activity compared to VH control that was generated from averaging the means of at least three independent experiments. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test.

A)



B) CL-01 WT



C) CL-01 AhR TA

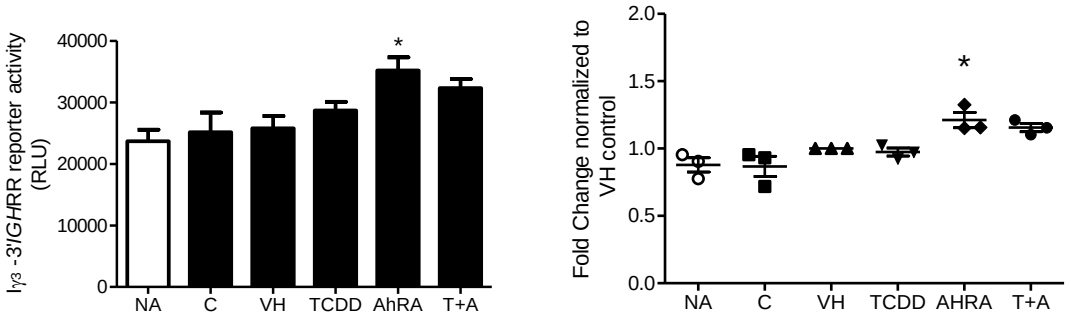
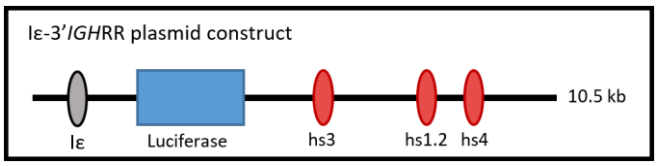
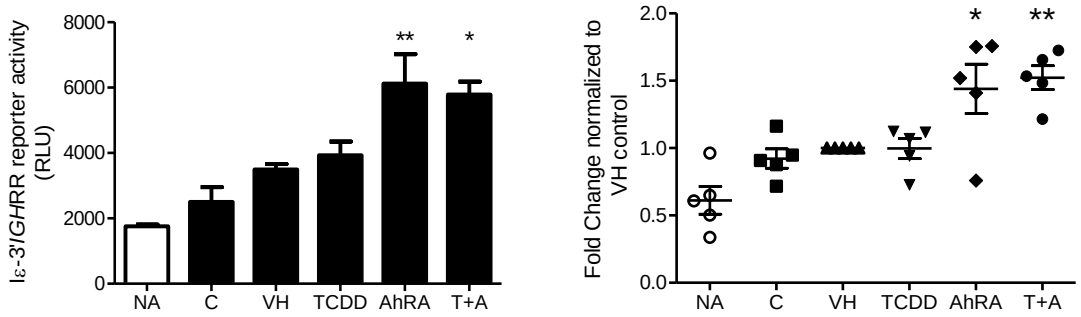


Figure 19. Iγ3-3'*IGH*RR reporter activity is not effected by TCDD but increased by AhRA in both CL-01 WT and AhR TA cells. A. Iγ3-3'*IGH*RR luciferase plasmid construct. **B.** CL-01 WT cells. **C.** CL-01 AhR TA cells. All cells were transiently transfected with Iγ3-3'*IGH*RR plasmid and cultured for 48 hours. Cells were either cultured in the absence of any treatment (naïve, NA) or treated with CD40L plus IL-4 stimulation alone (C) or co-treatment stimulation with vehicle control (VH, 0.01% DMSO), 30 nM TCDD, 10 μM AhRA or TCDD plus AhRA (T+A). The graphs on the left represent luciferase activity as relative light units (RLU) and graphs on the rights represent the fold change in luciferase activity compared to VH control that was generated from averaging the means of at least three independent experiments. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and VH were denoted by * and **, which represents significance at $p<0.05$ and $p<0.01$, respectively.

A)



B) CL-01 WT



C) CL-01 AhR TA

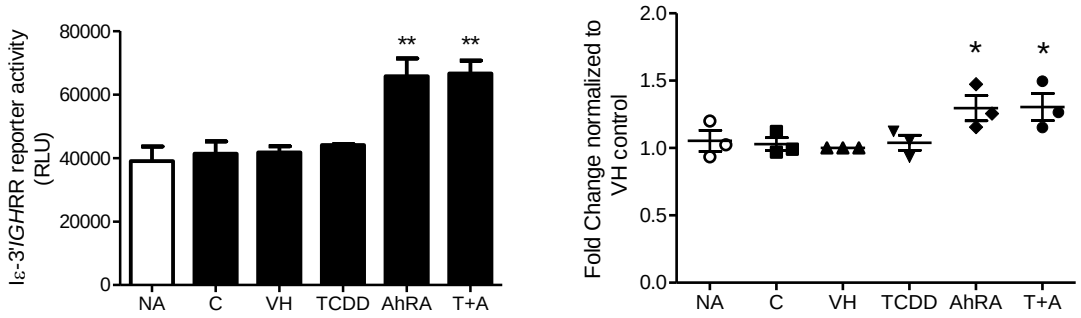


Figure 20. Iε-3'*IGH*RR reporter activity is not effected by TCDD but increased by AhRA in both CL-01 WT and AhR TA cells. A. Iε-3'*IGH*RR luciferase plasmid construct. **B.** CL-01 WT cells. **C.** CL-01 AhR TA cells. All cells were transiently transfected with Iε-3'*IGH*RR plasmid and cultured for 48 hours. Cells were either cultured in the absence of any treatment (naïve, NA) or treated with CD40L plus IL-4 stimulation alone (C) or co-treatment stimulation with vehicle control (VH, 0.01% DMSO), 30 nM TCDD, 10 μM AhRA or TCDD plus AhRA (T+A). The graphs on the left represent luciferase activity as relative light units (RLU) and graphs on the rights represent the fold change in luciferase activity compared to VH control that was generated from averaging the means of at least three independent experiments. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and VH were denoted by * and **, which represents significance at $p<0.05$ and $p<0.01$, respectively.

AhR knockout abrogates IgG secretion

In a mouse B-cell line (CH12.LX) stimulated with LPS, TCDD decreased IgM secretion, whereas it did not affect IgM secretion in an AhR-deficient B-cell line (BCL-1) suggesting that the AhR plays an important role in inhibition of IgM secretion by TCDD (Sulentic et al., 1998). Moreover, AhR knockdown by shRNA or inhibition by an AhR antagonist reversed TCDD-induced inhibition of IgA secretion but did not alter basal or stimulation-induced IgA secretion in CH12.LX cells (Wourms & Sulentic, 2015). Another study with $AhR^{-/-}$ mice showed normal immune function and Ig production after treatment with TCDD (Vorderstrasse et al., 2001). These provide evidence that the AhR mediates TCDD-induced inhibition of Ig and B-cell differentiation but it is not required for normal immune function (Wourms & Sulentic, 2015; Vorderstrasse et al., 2001). However, in a human B-cell line, our lab previously used shRNA to knockdown the AhR and found a loss of IgG secretion with knockdown of the AhR. In contrast, TCDD inhibited IgG secretion in wild type cells and the AhR antagonist increased IgG expression (Kashgari 2015, unpublished data). Therefore, the objective of the current studies was to confirm the shAhR knockdown results by generating a stable knockout of the AhR in the CL-01 cells using CRISPR-Cas9 gene editing. The CL-01 cells were transfected with CRISPR vectors expressing GFP and guide RNA targeting sequences in exon 1 or in exon 2 of the AhR gene (Table 3). After sorting GFP^{+} cells and seeding one cell per well, 284 clones were grown and screened for AhR expression by Western blot (data not shown). Only three clones (10F10, 7C8, 8F7) demonstrated low expression of the AhR by Western analysis (Fig. 21), and they targeted exon 2. These three clones were tested by PCR for AhR mRNA

expression. Two clones (10F10, 8F7) showed decreased AhR mRNA expression while one clone (7C8) showed very low expression (Fig. 22).

Since IgG levels were nearly eliminated with AhR KD (Kashgari 2015, unpublished data) as mentioned above, IgG secretion was evaluated by ELISA in the CRISPR/Cas9 clones. Cells were stimulated with R848 and treated with TCDD then incubated for 96 hours. As previously seen with AhR knockdown by shRNA IgG secretion in the CRISPR/Cas9 clones was not induced with stimulation (Fig. 23). These results suggest a physiological role of the AhR in IgG expression.

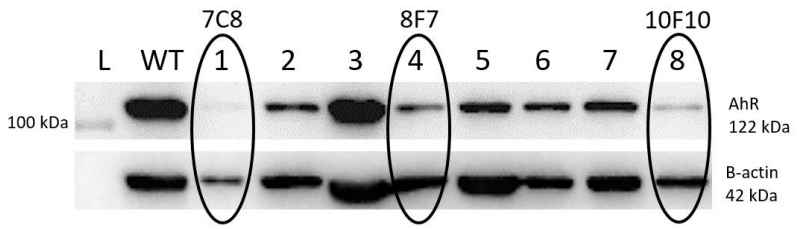


Figure 21. AhR expression in clones transfected with CRISPR-Cas9 targeting the exon 2 in the *AHR* gene. CL-01 wild type (WT) and 8 CRISPR-Cas9 clones were lysed with mild lysis buffer and 40 μg of protein was subjected to Western blot analysis. All membranes were probed with AhR antibody (122 kDa) and anti-β-actin (42 kDa), which served as the loading control. Result is representative of three independent experiments.

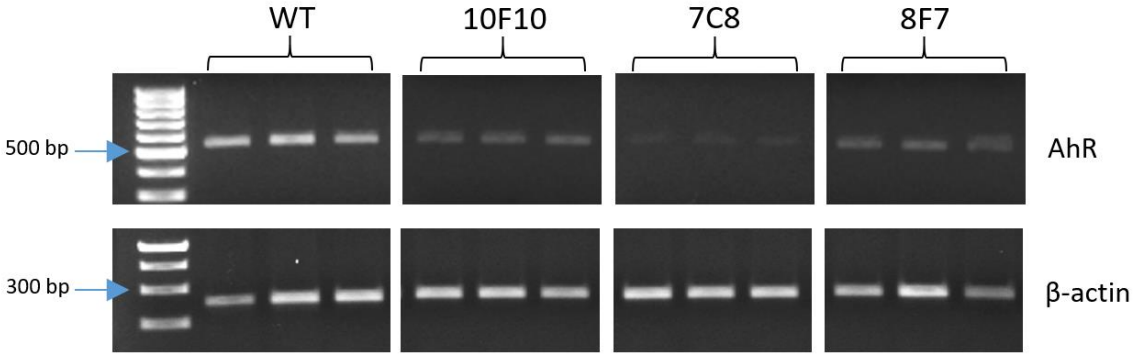


Figure 22. AhR mRNA expression in CRISPR-Cas9 clones showing decreased AhR protein levels. RNA was isolated from CL-01 wild type (WT) and the clones 10F10, 7C8 and 8F7. RNA was converted by reverse transcription into cDNA and 100 ng of cDNA was amplified using AhR and β-actin primers (Table2). Product size for AhR is 549 bp and β-actin is 224 bp. Results are representative of three independent experiments.

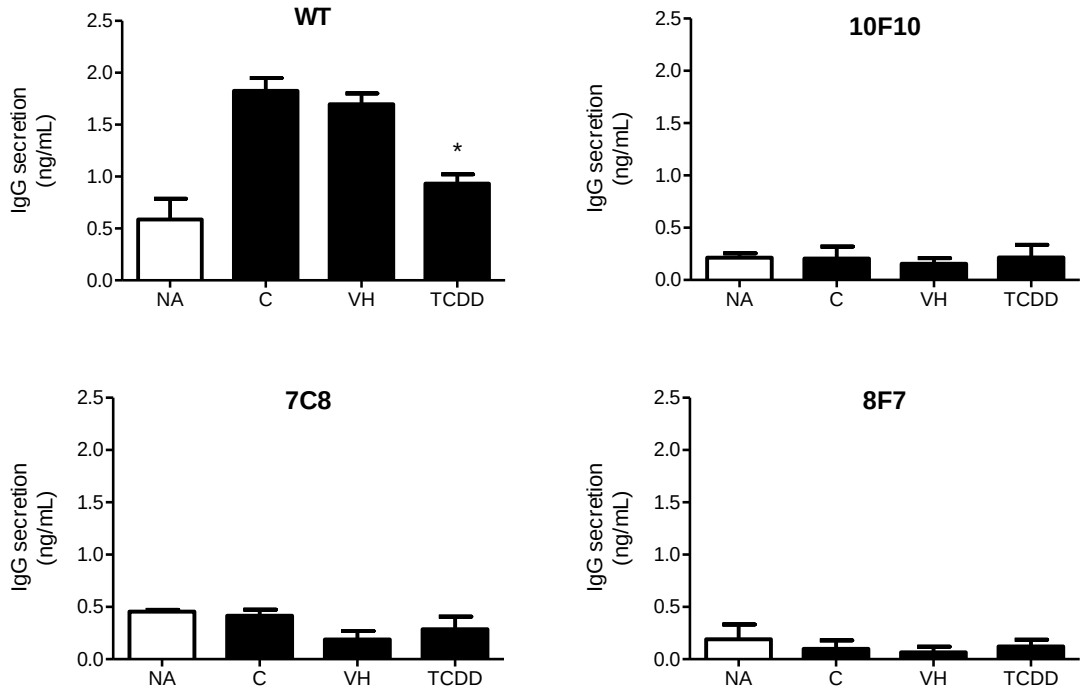


Figure 23. Low IgG secretion in CRISPR/Cas9 clones compared to WT. WT and CRISP/Cas9 clones (10F10, 7C8 and 8F7) were cultured without treatment naïve (NA) or stimulated with 1 µg/ml R848 (C) and treated in addition to R848 with the vehicle control 0.02% DMSO (VH) or 30nM TCDD. Cells were incubated for 96 hours, then supernatant collected and analyzed for IgG secretion by ELISA. Results are representative of three independent experiments (mean SEM, n=3). Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and VH were denoted by *, which represents significance at $p<0.05$, respectively.

Interestingly, the 10F10 and 8F7 clones appeared to change in the expression level of the AhR over time. Within about 5 months between the initial analysis and the last analysis and after about 6 new thaws, Western analysis showed an increased expression of the AhR in 10F10 and 8F7 while the 7C8 clone still had low AhR expression (Fig. 24). In addition, AhR mRNA expression was increased in the 10F10 and 8F7 clones while the 7C8 clone still had low expression (Fig. 25). The next step was to evaluate the TCDD effects on DRE reporter in these clones. TCDD induced the DRE reporter in the 10F10 and 8F7 clones while it did not affect the DRE activity in 7C8 clone (Fig. 26). These data suggest a monoallelic edit in the 10F10 and 8F7 clones disrupting the AhR allele that has a non-functional transactivation domain, resulting in decreased AhR expression. Over time, the functional AhR allele appears to compensate for the decrease in AhR expression and returns it to normal expression level. The 7C8 clone showed stable AhR knockdown suggesting that CRISPR/Cas9 edited and disrupted both AhR alleles.

To evaluate the role of the AhR in the effects of the TCDD and AhRA on 3'*IGH*RR activity, CRISPR/Cas9 clones were transfected with the Iγ_3-3'*IGH*RR reporter plasmid. Transfected cells were stimulated with CD40L plus IL4 and treated with TCDD and AhRA. TCDD had no effect on the CRISPR/Cas9 clones while the AhRA increased Iγ_3-3'*IGH*RR reporter activity in the high AhR expression clones, 10F10 and 8F7. In contrast, the AhR knockdown clone 7C8 was not effected by AhRA (Fig. 27). These results are compatible with previous results that AhRA increased the secretion of IgG (Burra 2015, unpublished data) and that IgG expression is dependent on the AhR (Fig. 26 and Kashgari 2015, unpublished data). To determine if the basal activity of the 3'*IGH*RR reporters is affected

by loss of the AhR, the RLUs were normalized to transfection efficiency. The clones (10F10 and 8F7) with high AhR expression demonstrated significantly increased basal activity of both the V_H-3'*IGH*RR and Iγ_3-3'*IGH*RR reporter plasmids compared to the WT while the 7C8 clone had similar basal activity as the WT cells (Fig. 28).

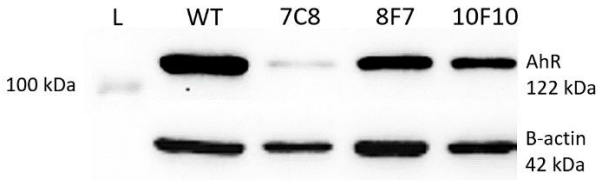


Figure 24. Increased AhR expression in the 10F10 and 8F7 clones. CL-01 wild type (WT) and CRISPR/Cas9 clones were lysed with mild lysis buffer and 40 µg of protein was subjected to Western blot analysis. All membranes were probed with antibodies specific for AhR (122 kDa) and anti-β-actin (42 kDa), which served as the loading control. Results are representative of three independent experiments

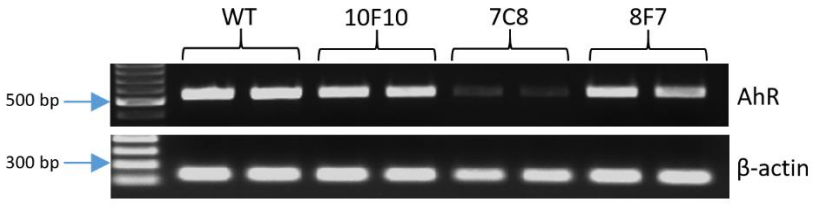


Figure 25. AhR mRNA expression was increased in 10F10 and 8F7 clones. RNA was isolated from CL-01 wild type (WT) and the clones 10F10, 7C8 and 8F7. RNA was converted by reverse transcription into cDNA and 100 ng of cDNA was amplified using AhR and β-actin primers (Table2). Product size for AhR is 549 bp and β-actin is 224 bp. Results are representative of three independent experiments

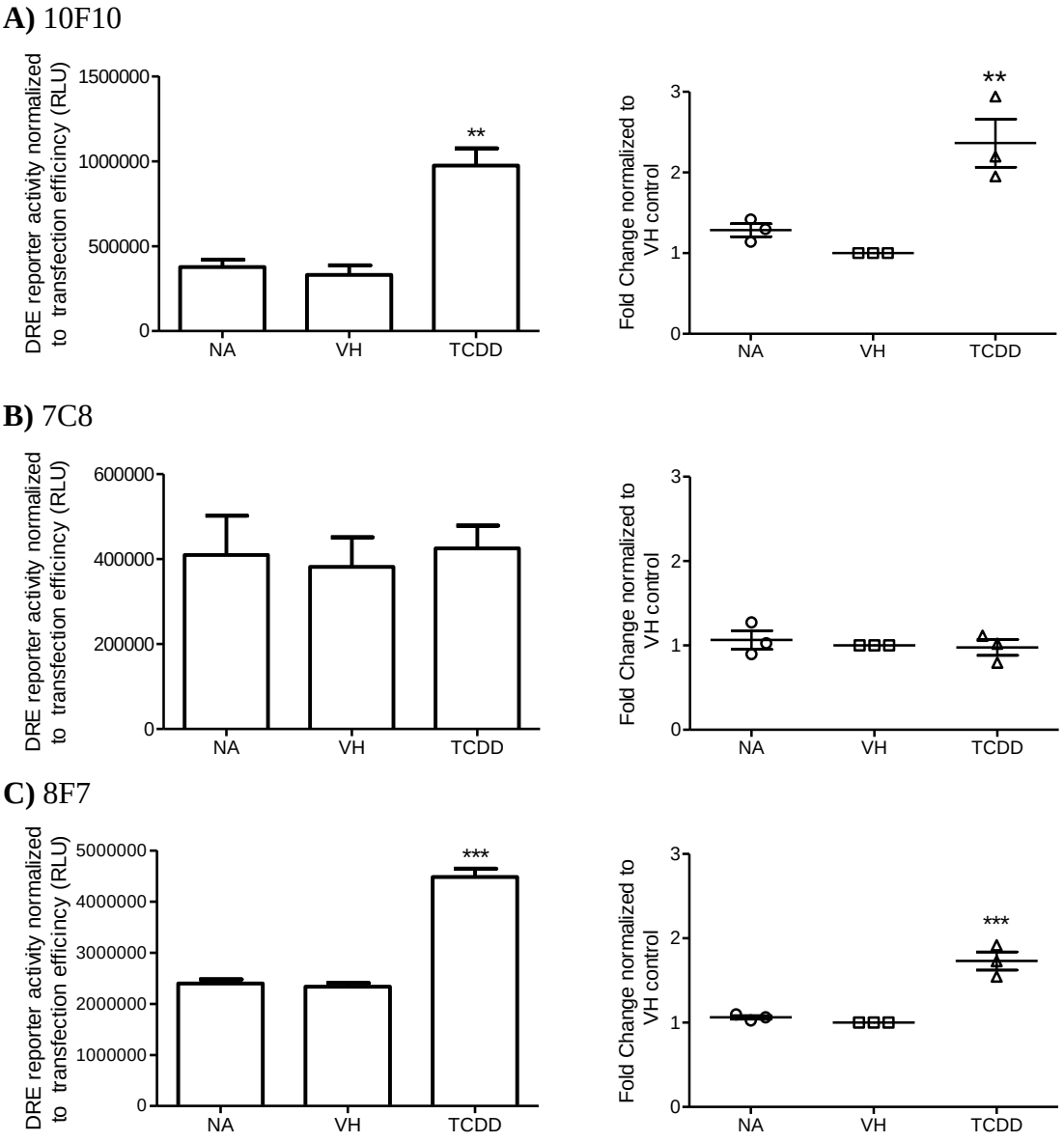


Figure 26. TCDD increased the DRE reporter activity in 10F10 and 8F7 clones but not in the 7C8 clone. (A) 10F10 clone. (B) 7C8 clone. (C) 8F7 clone. Cells were transiently transfected with DRE plasmid then cells were either cultured in the absence of any additional treatment (naïve, NA) or with 0.01% DMSO vehicle control (VH) and 30nM of TCDD, and then incubated at 37ºC for 24 hours. The graphs on the left represent luciferase activity as relative light units (RLU) normalized to transfection efficiency and graphs on the rights represent the fold change in luciferase activity compared to VH control that was generated from averaging the means of at least three independent experiments. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and VH were denoted by ** and ***, which represents significance at $p<0.01$ and $p<0.001$, respectively. (mean SEM, n=3)

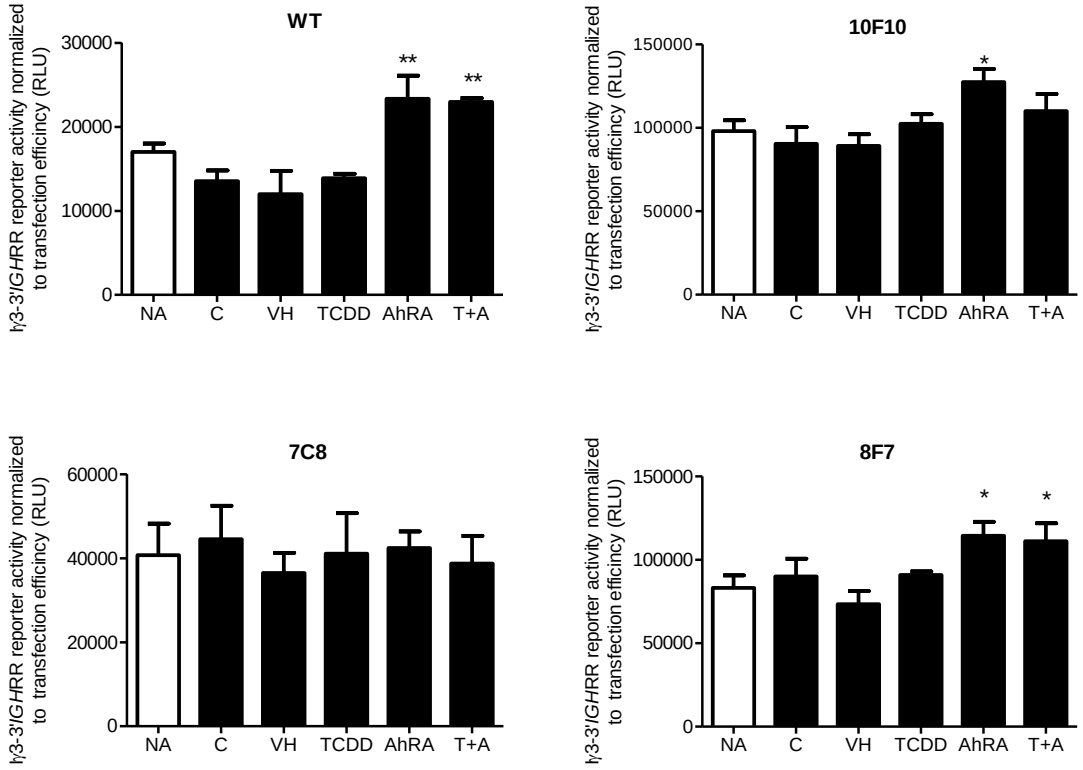


Figure 27. Iγ$_3$-3'*IGH*RR reporter activity in the AhR knockdown clone 7C8 was not effected by AhRA. CL-01 WT and CRISPR/Cas9 clones were transiently transfected with Iγ$_3$-3'*IGH*RR plasmid and cultured for 48 hours. Cells were either cultured in the absence of any treatment (naïve, NA) or treated with CD40L plus IL-4 stimulation alone (C) or co-treatment stimulation with vehicle control (VH, 0.01% DMSO), 30 nM TCDD, 10 μM AhRA or TCDD plus AhRA (T+A). Results are representative of three independent experiments (mean SEM, n=3). Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and VH were denoted by * and **, which represents significance at $p<0.05$ and $p<0.01$, respectively.

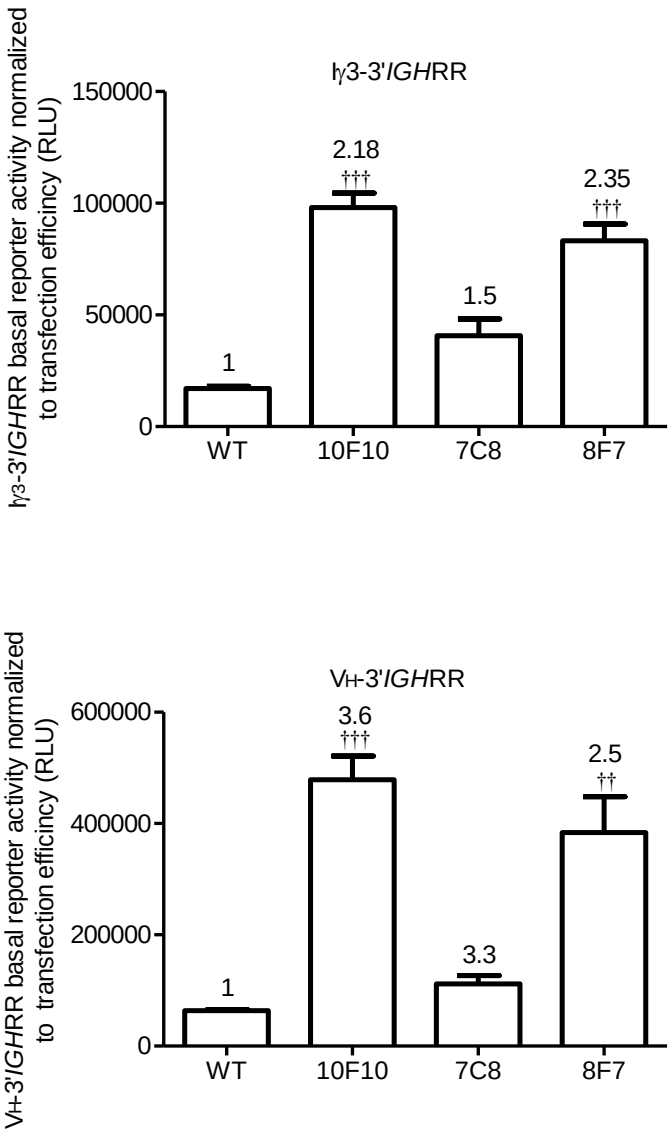


Figure 28. The 3'*IGH*RR basal reporter activities are high in 10F10 and 8F7 clones but not in 7C8 clone. CL-01 WT and CRISPR/Cas9 (10F10, 7C8 and 8F7) clones were transiently transfected with $I\gamma_3$-3'*IGH*RR or V_H-3'*IGH*RR plasmid and cultured for 48 hours. Results are representative of three independent experiments (mean SEM, n=3). Numbers above bars represented the fold-change to the WT clone. Comparisons between clones were analyzed using a one-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between clones and WT clone were denoted by †† and †††, which represents significance at $p<0.01$ and $p<0.001$, respectively.

IV DISCUSSION

The TCDD and AhR mechanisms in inhibition of Ig expression in humans are not clear yet. In a human B cell line, IgG secretion is inhibited in response to TCDD but increased about two-fold when the B cells are treated with an AhRA [CH223191 or 6,2',4',-trimethoxyflavone (TMF)] (Burra 2015; Panstingel 2017, unpublished data). In addition, AhR knockdown by shRNA eliminated IgG secretion (Kashgari 2015, unpublished data). Other studies have shown that AhR can bind to other transcription factors like SP1, NF-κB and AP-1 (Suh et al., 2002; Tian et al., 1999; Kobayashi et al., 1996). These data suggest a physiological role of the AhR on IgG expression and that the AhRA may induce non-genomic effects mediated by the cytosolic AhR that result in activation of other signaling proteins.

In this study, we determined the activity of the AhR using the DRE reporter. The DRE reporter plasmid showed highly basal activity and AhRA nearly completely inhibited this activity, while TCDD reversed the inhibitory effect of AhRA. This suggest a high basal activity of the DRE reporter that is AhR-dependent. The basal activity of the DRE reporter is mediated by the transcriptional activity of the DRE binding sites and not from high basal activity of the SV40 promoter. In addition, Western analysis showed the presence of nuclear AhR in naive cells that could be responsible for the increased basal activity of the DRE reporter. A portion of the AhR could be localized in the nucleus independent of ligand activation or an endogenous ligand may promote a low level of basal AhR activation.

Additionally, TCDD treatment alone had no effect on DRE reporter activity in the human CL-01 cells that is very different from mouse B cells in which TCDD enhanced the AhR binding to DRE sites (Wourms & Sulentic, 2015). In CL-01 human B cells, it was found that the AhR is heterozygous, likely with a non-functional transactivation domain in one of its alleles due to the identification of three SNPs (P517S, R554K, and V570I) present in Exon 10 (Fig. 29) (Kashgari 2015, unpublished data). Exon 10 is a region encoding the transactivation domain that regulates expression of genes sensitive to the AhR (Harper et al. 2002). Moreover, These SNPs were demonstrated to impair the ability of CYP1A1 induction by TCDD (Wong et al., 2001). Therefore, the low AhR activity could be due to a nonfunctional transactivation domain in one of the AhR alleles (Kashgari 2015, unpublished data). Notably, these exon 10 SNPs do not affect the ligand binding affinity to the AhR (Harper et al., 2002). And should not affect the potential non-genomic function of the AhR. Non-genomic functions could be mediated by protein-protein interactions between the AhR and regulatory proteins such as Src protein kinase and NFκB (Enan and Matsumura, 1996; Tian et al., 1999). Moreover, the AhR signaling pathway has been shown to modulate tyrosine kinases associated with the epidermal growth factor receptor (EGFR) and Akt/PKB (Marlowe & Puga, 2005). Therefore, the biological effects of the AhR could occur via at least two general mechanisms: direct influence on gene transcription by binding to DRE motifs and/or modulation of signaling pathways by protein–protein interaction (Suh et al., 2002).

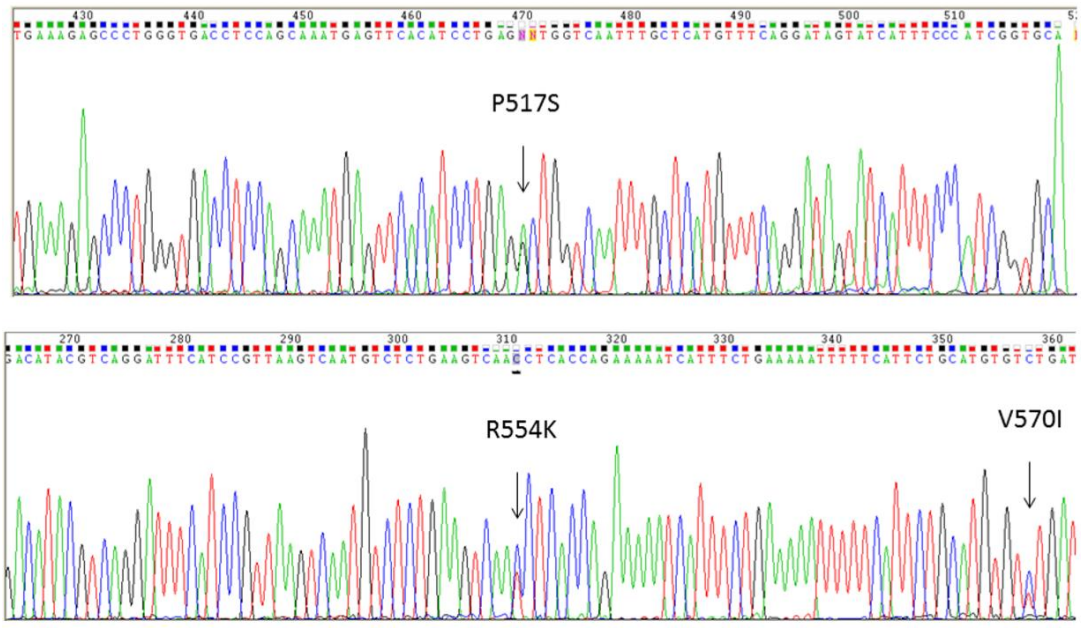


Figure 29. Chromatograph showing the three SNPs location in the transactivation domain of the CL-01 AhR exon 10. The heterozygous SNPs are shown in three positions with two overlap peaks. Each peak represents a nucleotide with a coded color: green for Adenine; red for Thymine; black for Guanine and blue for Cytosine (Kashgari 2015, unpublished data).

Although the Src protein kinase is associated with the AhR complex in the cytosol (Enan & Matsumura, 1996), phospho-Src was not in the CL-01 cells with or without stimulation. However, Src kinases are initially activated after BCR ligation by antigen. Therefore, signaling through the BCR could be the only pathway to activate Src; whereas, in these studies R848 stimulation was used, which activates the TLRs 7 and 8. Src activation may need a BCR ligand to increase B-cell activation and Ig production. Alternatively, the Src protein associated with the cytosolic AhR complex may only be released and activated by TCDD binding (Enan & Matsumura, 1996). In other words, binding of the AhRA may not induce the proper conformational change to release Src. Moreover, Western blot analysis may not be the most sensitive technique to detect phospho-Src. In a previous Src study, they used fluorescence resonance energy transfer (FRET) to visualize and quantify phosph-Src (Tyr416) (Dong et al., 2011).

The Akt protein kinase was also activated by the AhR signaling pathway in several human cells (Wu et al., 2007; Al-Dhfyan et al., 2017). The results showed that Akt is basally phosphorylated in CL-01 cells but after 30 minutes of AhRA treatment, Akt phosphorylation significantly increased and at 2 hours returned to basal phosphorylation level. In another study using the mouse CH12.LX cell line, the most abundance of p-Akt was observed within the first 60 minutes after treating R848-stimulated cells with 0.3 nM TCDD but 30 nM TCDD significantly suppressed R848-activated phosphorylation of Akt protein, which is compatible with the suppression of Ig production by TCDD (North et al., 2010). Additionally, Loss of the AhR lead to impaired activation of Akt and enhanced sensitivity to apoptosis in a hepatoma cell line (LA1) (Wu et al., 2007). Another study showed that the AhR signaling pathway controls proliferation and development of breast

cancer cells through activation of Akt pathways (Al-Dhfyan et al., 2017). These studies indicate that the cytosolic AhR can modulate the Akt pathway. Chemical antagonism of the AhR in the CL-01 cells appears to induce Akt phosphorylation, which may contribute to the increase in IgG secretion. Upon phosphorylation of Akt, the Akt signaling pathway mediates multiple downstream events. Phosphorylated Akt activates the mTOR complex, causing increased mRNA translation, protein synthesis and cellular proliferation (Agata et al., 2010). Phosphorylated Akt enters the nucleus to inactivate forkhead box O (FOXO) protein, which blocks cell cycle progression and promotes stress resistance, which help in B-cell proliferation (Limon & Fruman, 2012). Moreover, Akt phosphorylation induces activation of IKKα leading to dissociate NFκB from IκB and increase the NFκB transcription activity (Liou et al., 1994).

The NFκB has an important role in B-cell activation, activity of the mouse 3'*Igh*RR, and the AhR physically interacts with NFκB (Tian et al., 1999). In human, the invariant sequence in the hs1.2 enhancer of the 3'*IHG*RR contains DRE, NFκB, SP-1, NF-1 and AP-1 binding sites (Pinaud et al., 2011). Two separate pathways can activate NFκB; the "canonical" pathway is triggered by TLR, TNFα and IL-1, and the "alternative" pathway is activated by CD40L, BAFF and lymphotoxin (Lawrence, 2009). The AhRA could influence interaction of AhR with any protein in these two pathways that lead to increase Ig expression. Previous studies in our lab has shown that stimulation with R848, a synthetic molecule that activates B cells via the TLR7/8, or with CD40L plus IL4 increased IgG production (Burra 2015, unpublished data). Both methods of stimulation activate either canonical or alternative NFκB activation pathways (Hanten et al., 2008; Lawrence, 2009), which suggests that activation of NFκB transcription factors could influence IgG

production. Our results showed that the R848 stimulation significantly increased the NFκB plasmid activity but TCDD and AhRA have no effect on NFκB activity. As mentioned, Akt signaling pathway activates NFκB and this activity should be increased with cells treated with AhRA because AhRA increase the Akt activity. But the result showed that NFκB reporter activity was not affected by AhRA. The Akt activates other signaling pathway like mTOR or FOXO but not NFκB pathway, or Akt may increase the translation of NFκB but not transcription activity. In addition, the NFκB reporter may not reflect the complexity of protein-protein interactions and transcriptional regulation that may vary from gene to gene.

The AP-1 is another transcription factor binding site in the hs1.2 enhancer of the 3'*IGH*RR (Pinaud et al., 2011). The results suggests that AP-1 has no role in increased IgG production by AhRA and therefore, the AhRA may not influence an AhR interaction with AP-1 pathway proteins like c-Fos or c-Jun. Furthermore, R848 stimulation lowered AP-1 suggesting that activation of the TLR7/8 signaling pathway inhibits AP-1 transcriptional activity. Another possibility is that the CL-01 cells were isolated from a Burkett's lymphoma patient. In Birkett's lymphoma, the B cell lymphoma-6 (BCL-6) gene is involved in chromosomal translocation that could lead to sustained expression of BCL-6. The BCL-6 encodes a transcriptional repressor protein that is expressed in B cells in the germinal center reaction and is down-regulated upon B cell differentiation into plasma cells (Vasanwala et al.,2002). TLR stimulation of CL-01 cells may activate the BCL-6 that binds to c-Jun and disrupts dimerization of c-Jun leading to repression of AP-1 activity and inhibit B cell differentiation (Vasanwala et al.,2002). Previous studies in our lab showed that both R848 and CD40L plus IL-4 stimulation inhibited the basal activity of the hs1.2

and hs3-1.2 enhancers, which has the intervening sequences between hs3 and hs1.2, reporters activity (Freiwan, 2014; Alfaheeda, 2016 unpublished data) suggesting that AP-1 may have a positive regulator role in the transcriptional activity of the 3'*IGH*RR enhancers in human CL-01 cells stimulated with either R848 or CD40L plus IL-4.

The human and mouse AhR gene have single nucleotide polymorphisms (SNPs) in different locations and they only share about 58% amino acid sequence in the C-terminal of the gene that includes the transactivation domain (Flaveny et al. 2008). As mentioned, CL-01 human B cells have a heterozygous AhR, likely with a non-functional transactivation domain in one of its alleles due to the identification of three SNPs (Fig. 29) (Kashgari 2015, unpublished data). These SNPs could be the reason why the human 3'*IGH*RR is more sensitive to TCDD in a mouse B-cell line that expresses a highly functional AhR signaling pathway compared to the human (Alfaheeda 2016, unpublished data). Using an AhR with a functional transactivation domain (AhR TA) (Panstingel 2017, unpublished data), TCDD induced the DRE reporter activity unlike the CL-01 WT cells suggesting that these CL-01 WT cells may express a dominant negative AhR. The dysfunction of the AhR transactivation domain in the CL-01 wild type had no effect on the sensitivity of the 3'*IGH*RR to TCDD or AhRA since it has the same effects as in CL-01 cells expressing only an AhR with a functional transactivation domain. Moreover, Increases in the $I\gamma_3$-3'*IGH*RR reporter activity is compatible with previous results that AhRA increased the secretion of IgG (Burra 2015, unpublished data) supporting that the cytosolic AhR induces a non-genomic effect perhaps by interacting with signaling proteins leading to an increase in IgG secretion and $I\gamma_3$-3'*IGH*RR activity. In addition, TCDD has no effect on the 3'*IGH*RR reporter activity may results of the TCDD induces multiple

interaction of the AhR with different proteins. TCDD induces the crosstalk between AhR and many signaling pathways like Src, Akt/PKB, NFκB and Wnt/β-catenin (Suh et al., 2002; Wu et al., 2007; Enan and Matsumura, 1996; Tian et al., 1999) therefore, the non-genomic interaction of the AhR with other proteins may consume the AhR proteins and decrease nuclear translocation of the AhR.

Previous studies on mouse B cells showed that the AhR mediates TCDD-induced inhibition of Ig and B-cell differentiation (Wourms & Sulentic, 2015; Vorderstrasse et al., 2001). Moreover, AhR knockdown by shRNA or inhibition by an AhR antagonist reversed TCDD-induced inhibition of IgA secretion but did not alter basal or stimulation-induced IgA secretion in CH12.LX cells (Wourms & Sulentic, 2015). Another study with AhR-/- mice showed normal immune function and Ig production after treatment with TCDD (Vorderstrasse et al., 2001). These provide evidence that the AhR mediates TCDD-induced inhibition of Ig and B-cell differentiation but it is not required for normal immune function (Wourms & Sulentic, 2015; Vorderstrasse et al., 2001). However, in a human B-cell line, IgG secretion was inhibited by TCDD and surprisingly increased by AhRA. Moreover, TCDD inhibited the induction of Cγ1-4 germline/functional transcripts while the AhRA alone increased all of the Cγ1-4 germline/functional transcripts (Burra 2015, unpublished data).Our lab has also used shRNA to target and degrade AhR mRNA and knockdown the AhR protein levels. IgM secretion was unaffected by AhR knockdown, whereas IgG levels were nearly eliminated with AhR knockdown (Kashgari 2015, unpublished data). These observations match with our results for AhR knockdown (KD) using the CRISPR/Cas9 gene editing. The AhR KD clone inhibited IgG secretion as previously seen with AhR knockdown by shRNA suggesting a physiological role of the AhR in IgG expression. In

addition, the result suggests that the non-functional transactivation domain of the AhR has no effect on IgG production. CRISPR/Cas9 clones that have edited the nonfunctional AhR transactivation domain (TA) allele appeared to change in the expression level of the AhR over time. Suggesting the functional AhR TA allele, which not edited by CRISPR/Cas9, appears to compensate for the decrease in AhR expression and returns it to normal expression level. While the knockdown clone showed stable AhR knockdown suggesting, that CRISPR/Cas9 edited and disrupted both AhR alleles.

Although TCDD had no effect on Iγ$_3$-3'*IGH*RR reporter activity in CL-01 WT, AhR TA and AhR KD clones, the AhRA increased Iγ$_3$-3'*IGH*RR activity in CL-01 WT and AhR TA clones while it has no effect in the AhR KD clone. Indicating that TCDD has no effect on 3'*IGH*RR regardless of AhR expression or function while the AhRA could influence the interaction of the cytosolic AhR with signaling proteins leading to an increase in Iγ$_3$-3'*IGH*RR activity that could increase IgG secretion supporting an AhR non-genomic effect and a physiological role of the AhR in the expression of IgG. Furthermore, the basal activity of the 3'*IGH*RR reporters in the AhR knockdown clone was lower than in the AhR-expressing cells indicates the AhR mediated the basal activity of the 3'*IGH*RR reporters.

A limitation of using reporter plasmids is that they may not actually reflect enhancer activity in the context of chromatin. Additionally, the 3'*IGH*RR reporter constructs lack the intervening sequence between hs1.2 and hs4 enhancers because it is difficult to include in the reporter because of size. The current 3'*IGH*RR reporter contains about 10 kb while the intervening sequence between the hs1.2 and hs4 enhancers is about 10 kb. This intervening sequence may play a critical role in 3'*IGH*RR function since it contains transcription factor binding sites and has a unique repetitive nucleotide sequences that may

contribute to the secondary structures of the 3'*IGH*RR and may affect its function (Giambra et al., 2005). In addition, the 3'*IGH*RR reporter plasmids behave differently depending on if they are transfected into mouse or human B-cell lines. Previous work has shown that a plasmid containing just the human hs1.2 enhancer will show a decrease in activity in response to B-cell stimulation when transfected into human B cells, whereas in mouse B cells stimulation will increase its activity (Fernando et al., 2012; Freiwan 2014, unpublished data). Moreover, the 3'*IGH*RR reporters have only one of the two 3'*IGH*RRs and perhaps both work in concert for expressing some of the C_H isotypes. We also had difficulty sequencing exon 1 and exon 2 of the AhR to determine the edits induced by CRISPR/Cas9. We have tried to amplify and sequence the exon 1 and exon 2 in order to confirm the CRISPR/Cas9 edit. We suspect that these areas are polymorphic, preventing primers based on a reference AhR gene from binding within the CL-01 AhR gene. Ongoing studies in our lab are tring to address this peoblem by using a method called rapid amplification of 5′ complementary DNA ends (5′ RACE) in order to sequence the exons 1 and 2 of the AhR gene. This method is used to extend partial cDNA clones by amplifying the 5′ sequences of the corresponding mRNA.

In future studies, we should expand the signaling analysis to cover most of the B-cell signaling pathways such as Ca^{2+}, Syk pathways or transcription factors like SP1 and NFAT that could interact with AhR in order to understand the AhR role in IgG production. Studies should also focus on analysis of all Akt signaling pathways such as mTOR and FOXO pathways to define if AhRA has an effect on one of these pathways to mediate the increase IgG secretion. Measuring the Akt in the AhR knockdown clone could confirm the interaction of AhR with Akt protein. In addition, using gene editing like CRISPR/Cas9

technique to mutate or delete specific 3'*IGH*RR enhancers in order to determine the role of this enhancer in TCDD and AhRA effects. Moreover, a cell line with two transcriptionally function AhR alleles must be used to test the effect of TCDD on human 3'*IGH*RR enhancers. Additionally, using different human B cell line with specific characteristics representing normal human B cells may provide better understanding for the human 3'*IGH*RR and the effect of TCDD on it. An ongoing work in our lab on another B cell line (U266), which is a human B cell line isolated from myeloma may add more knowledge about B cells sensitivity to TCDD in different stages of B cell development.

In conclusion, our results suggest that the AhRA nearly completely inhibits AhR translocation into the nucleus and TCDD reverses this inhibition. B cells have nuclear AhR that could be localized in the nucleus independent of ligand activation or an endogenous ligand may promote a low level of basal AhR activation. The AhR has non-genomic effects by activating cytosolic signaling protein like Akt and increasing the $I\gamma_3$-3'*IGH*RR reporter activity in response to AhRA that could lead to an increase in IgG secretion. Interestingly, the dysfunction of the AhR transactivation domain in the CL-01 wild type has no effect on the sensitivity of the 3'*IGH*RR to TCDD or AhRA suggesting that the AhRA is mediating non-genomic effects through the AhR. AhR knockdown by gene editing markedly inhibited IgG secretion, both basal and induced, and AhRA has no effect on $I\gamma_3$-3'*IGH*RR activity supporting an AhR non-genomic effect and a physiological role of the AhR in the expression of IgG. Significantly, understanding how the AhR-ligands modulate Ig expression and CSR and its relation to the 3'*IGH*RR will provide insight into how chemically-induced immunotoxic effects are mediated. The human 3'*IGH*RR has a polymorphism in the hs1.2 enhancer that has been associated with Burkitt's lymphoma and

a number of autoimmune disorders such as celiac disease, psoriatic arthritis, IgA nephropathy, rheumatoid arthritis, systemic sclerosis, and plaque psoriasis. Consequently, determining the role of the 3'*IGH*RR and its potential difference in sensitivity to the AhR could provide new insight into potential environmental triggers of immune disorders and provide new targets for drug development.

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