

2015

Differential Effects of The AhR on Immunoglobulin Gene Expression in Human B Cells

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DIFFERENTIAL EFFECTS OF THE AhR ON IMMUNOGLOBULIN
GENE EXPRESSION IN HUMAN B CELLS

A thesis submitted in partial fulfillments
of the requirements for the degree of
Master of Science

By

NAGA LAKSHMI KAULINI BURRA
B.S., Osmania University, 2011

2015
Wright State University

**WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL**

August 26th, 2015

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Naga Lakshmi Kaulini Burra ENTITLED Differential Effects of The AhR on Immunoglobulin Gene Expression in Human B Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Burra, Naga Lakshmi Kaulini. M.S., Department of Pharmacology and Toxicology, Wright State University, 2015. Differential effects of The AhR on Immunoglobulin Gene Expression in Human B Cells.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent and persistent environmental toxin known to inhibit immunoglobulin (Ig) gene expression in various animal models. TCDD is thought to modulate gene expression through the aryl hydrocarbon receptor (AhR) and thereby a subsequent alteration in gene expression. The AhR is a ligand-activated transcription factor that regulates xenobiotic-metabolizing enzymes. The mouse 3'*Ig* heavy chain regulatory region (3'*IghRR*) is a sensitive transcriptional target of TCDD that may mediate, in an AhR-dependent manner, the inhibitory effect of TCDD on Ig expression. Human B cells could also be a sensitive target of TCDD. The current study focuses on determining the effects of TCDD and the AhR on human Ig expression utilizing a human Burkitt lymphoma cell line (CL-01) model that can be activated to secrete Ig and undergo class switch recombination (CSR) from IgM to IgA, IgG or IgE antibody isotypes. Our results suggest that TCDD has a variable effect on IgM secretion, but significantly inhibits IgG secretion, an effect reversed by addition of the AhR antagonist. Surprisingly, the AhR antagonist alone markedly increased IgG secretion above stimulation. At transcript level,

TCDD has variable effects on μ *IGH* functional transcripts but significantly inhibits γ_{1-4} germline/functional transcripts and C_ϵ germline transcripts. Additionally, CD40L and IL-4 stimulation induced de novo synthesis of C_ϵ germline transcripts, a precursor to CSR. However, α_{1-2} germline/functional transcripts increased in response to TCDD. Notably, TCDD and stimulation had no effect on *CYP1A1* expression. Additionally, in CL-01 cells, we recently discovered SNPs in Exon-10 of the AhR, which encodes the transactivation domain that regulates expression of other genes but does not affect ligand binding. The AhR is heterozygous with one non-functional transactivation domain. Results also indicate that a small proportion of the cells have undergone spontaneous class switch to all of the γ and α isotypes rather than being induced to CSR.

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ACKNOWLEDGEMENTS

I am profoundly grateful to Dr. Courtney Sulentic for giving me an opportunity to work in her lab. She has been a great friend, mentor and wonderful support and encouraged me throughout the time it took me to complete my thesis work. I am also grateful to the members of my thesis committee Dr. Nancy Bigley, Dr. David Cool and Dr. Mauricio Di Fulvio for their generously given time and expertise to better my work. I would like to specially thank my lab members Andrew Snyder, Bassam Kashgari and Zahra Alfaheeda. I will cherish the memories I shared with them and also their valuable friendship. I would also like to thank my other lab members Siham Abdulla, Nicole Pastingel, Gabriel Crabb, Abdullah Freiwan and Brooke Johnson for their support during my thesis work. I might not remember everything I had done one day, but will remember all the lab meetings, SOT meeting, fun-filled lab lunches, Cincinnati Escape Room, Dick's Last Resort (particularly), friendship, lab work at ungodly hours, and all the laughter and this would not have been possible without Dr. Sulentic! I would also like to thank the Department of Pharmacology and Toxicology for giving me this wonderful opportunity to my pursue higher education, the staff of Pharmacology and Toxicology, my family, and friends back home (The Zany's) and also at Wright State University.

DEDICATION

I dedicate my thesis work to my parents, Vijaya Lakshmi and Sesa Sai Burra, my brother, Nrupendra Sai Burra and my grandmother, Srilakshmi Devasena Burra, for their unconditional love, encouragement and support throughout my life. Thank you very much, I wouldn't have been here this day without you all. I hope I made you proud.

I INTRODUCTION

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

Dioxin(s) belong to a class of chemicals very similar in structure and toxic in nature. They include polyhalogenated aromatic hydrocarbons (PAH) like polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF) and polychlorinated biphenyls (PCB) (Mandal 2005). Since all of them exhibit hydrophobic properties and long half-lives, dioxins tend to bio-accumulate and persist as environmental contaminants. 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) (Fig. 1) is the most studied PCDD as it is the most potent and toxic amongst all dioxins.

TCDD is a planar molecule ($C_{12}H_4Cl_4O_2$), colorless and odorless at room temperature. TCDD is not produced intentionally but as by-product of many industrial processes such as paper and textile bleaching manufacture of chlorophenoxy herbicides and pesticides, and metal smelting (McGregor et al. 1998). In fact, TCDD is produced during the combustion of substances in the presence of chlorine, for example backyard burning, forest fires, improper disposal of medical waste (Schechter et al. 2001). General exposure to TCDD could be through soil, dust and/or smoke by inhalation or consumption (Mandal 2005; Marinkovic et al. 2010). During the Vietnam War (1961-1971), TCDD was discovered to be a contaminant in the herbicide Agent Orange, which was sprayed on foliage to deliberately expose Vietnamese soldiers (Schechter et al. 2006). In Seveso, Italy 1976, several kilograms of TCDD were released from a pressure tank and inadvertently

thousands of inhabitants were exposed to TCDD (Mandal 2005). In Vienna 1997, two women were exposed to the highest ever concentration of TCDD at their workplace 144ng/g of fat (Geusau et al. 1999). In 2004, Ukrainian President Viktor Yushchenko was exposed to TCDD after a failed assassination attempt. TCDD was present at a concentration of as high as 108 ng/g of fat (Sorg et al. 2009). *In vivo* animal studies have shown that exposure to TCDD affects brain function, reproduction, hormone signaling and immunity (Mandal 2005). Besides, TCDD has been shown to promote tumor growth and is a classified human carcinogen (McGregor et al. 1998).

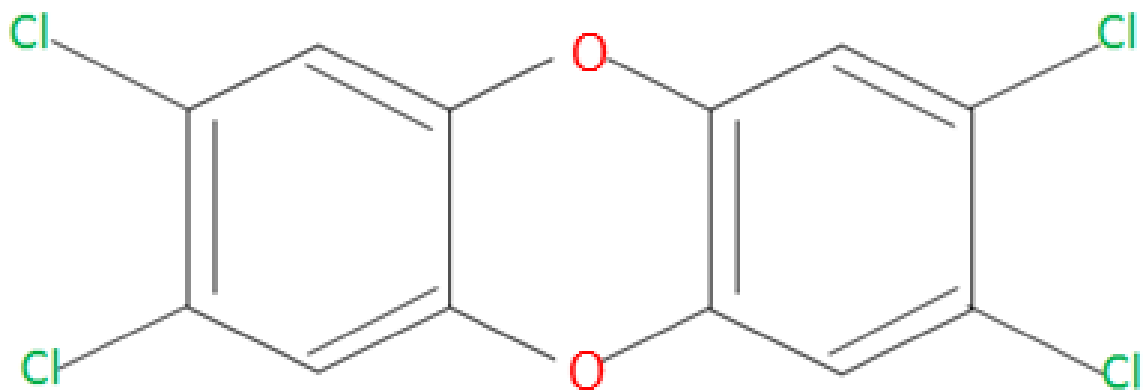


Figure 1: Chemical structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

The Aryl Hydrocarbon Receptor Signaling Pathway

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the basic-helix-loop-helix/Per-ARNT-Sim family (PAS), encoded by the *Ahr* gene. AhR, in its inactivated state in the cytosol is coupled with several proteins: two heat-shock protein 90 molecules (HSP90), co-chaperone p23, and the hepatitis B virus X-associated protein, which is an AhR-interacting protein (previously known as XAP2) (Abel and Haarmann-Stemann 2010; Nebert and Karp 2008). XAP2 plays an essential role in AhR activation, binds to the AhR and HSP90, while p23 directly binds to HSP90 (Endler, Chen, and Shibasaki 2014; Meyer and Perdew 1999). The AhR is known to play an important role in xenobiotic metabolism and also the gene expression of many drug metabolizing enzymes that belong to the cytochrome p450 family. Amongst them, the induction of *CYP1A1* is used as a biomarker to study the AhR signaling pathway (Hu et al. 2007; Hansen et al. 2014). Additionally, the AhR is well-known to cross-talk with steroid receptors like the estrogen and androgen receptors and alter their gene expression (Wormke et al. 2003), suggesting that the AhR may have regulatory roles beyond drug metabolism. AhR is best characterized by studying its ligand TCDD (Fig. 1). The AhR undergoes a conformational change upon binding to TCDD and dissociates from its protective cytosolic protein complex, translocates into the nucleus to form a heterodimer with the AhR nuclear translocator (ARNT). The AhR-ARNT heterodimer interacts with chromatin remodeling factors and histone acetyltransferases to induce specific chromatin changes (Schnekenburger, Peng, and Puga 2007) to bind to the dioxin responsive element (DRE; 5'-TNGCGTG-3'). DRE is present within the promoter or enhancer regions of target genes and consequently alters gene expression (Fig. 2) (Abel and Haarmann-Stemann 2010;

Landers and Bunce 1991). When the ligand is no longer present to activate the AhR, it undergoes negative feedback inhibition by the AhR repressor (Mimura and Fujii-Kuriyama 2003) or released into the cytosol and degraded by the proteasome pathway (Pollenz 2002).

The AhR is expressed in liver, lungs, skin, gastrointestinal tract and other tissues (Carlstedt-Duke 1979). Apart from its conventional role in metabolizing environmental ligands, the AhR is involved in development, cellular oxidation/antioxidation, epidermal barrier function (Noakes 2015), cell survival and proliferation, and in the regulation of immune system (Vogel et al. 2014). Natural or artificial chemicals, pharmaceuticals, and dietary constituents have also been identified as AhR ligands (e.g. aromatic hydrocarbons, omeprazole, carbaryl, flavonoids, indolocarbazoles, etc.).

The human AhR has relatively low affinity for TCDD compared to mouse and requires 10-fold higher concentrations of TCDD to induce a particular effect (Connor and Aylward 2006; Okey, Riddick, and Harper 1994). This could explain why animals are more sensitive to TCDD as compared to humans. Moreover, single nucleotide polymorphisms (SNPs) have been described in both mouse and human AhR genes. The human and mouse AhR proteins share 58% amino acid sequence in the C-terminal, which contains the transactivation domain (Flaveny et al. 2008). SNPs in the transactivation domain of the human AhR impairs the ability of the AhR to induce *CYP1A1* gene expression in response to TCDD (Wong, Okey, and Harper 2001). Interestingly, SNPs located in the ligand binding domain of the human AhR did not affect ligand-induced *CYP1A1* expression. SNPs occurring in Exon-10 of the human AhR gene, which encodes the transactivation domain involved in gene regulation, does not impact the ligand binding affinity (Harper et al. 2002).

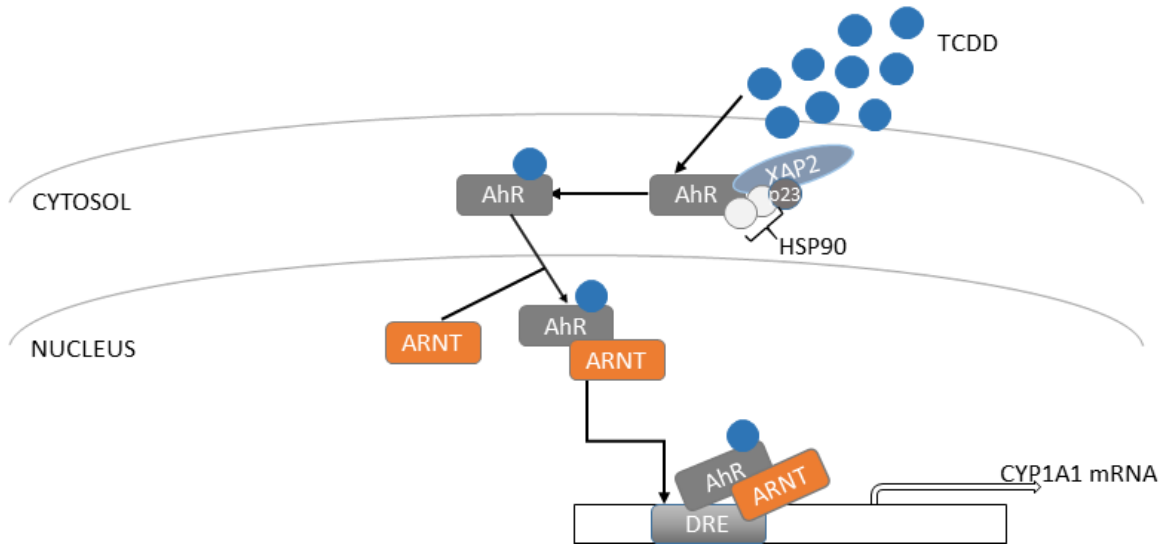


Figure 2: Aryl Hydrocarbon Receptor signaling pathway showing the translocation of AhR into the nucleus, dimerization with the ARNT, binding to the DRE region and induction of CYP1A1 mRNA expression.

The Immune System and its functions

The human body has a built-in, intricate and interesting protection mechanism against microbes including bacteria, viruses, fungi and other pathogens that invade our body. The body's defense mechanism is multi-layered and the first line of defense, the innate immune system, produces an immediate but non-specific and antigen-independent response at the site of infection. It includes physical barriers (skin and epithelial surfaces lining the gut and lung, and chemical barriers like saliva and tears), phagocytes (neutrophils, macrophages), dendritic cells, natural killer cells and also circulating plasma proteins. The innate immune system is activated when it recognizes particular types of molecules present on the pathogens that are not present in the host. Even if the body has never been exposed to a particular kind of pathogen, the pathogen-associated molecules stimulate the inflammatory responses. The innate immune system is the body's most dominant defense mechanism (Litman, Cannon, and Dishaw 2005). If the innate immune system is unsuccessful in clearing the pathogen from the body, then the adaptive immune system is activated by components of the innate immune system, is activated. The adaptive immune system improves recognition of the pathogen by retaining it as immunological memory even after the pathogen has been eliminated from the body, resulting in specificity of the response to that pathogen (Pancer and Cooper 2006). This immunological memory produces a more robust immune responses upon future exposure to the same pathogen. Any foreign substance that is able to elicit an adaptive immune response is called an antigen (*antibody generator*).

Adaptive immunity is divided into cell-mediated and humoral immunity. Cell-mediated immunity is principally orchestrated by T cells by releasing cytokines in response

to pathogens. Humoral immunity, on the other hand is primarily mediated by B cells which release antibodies (secreted form of immunoglobulins) in response to pathogens. The secreted antibodies circulate in the bloodstream and other body fluids, binding specifically to antigens that stimulated their production in the first place. Antibody binding neutralizes and destroys the antigens by involving the components of the innate immune system such as phagocytes, to ingest them. In cell-mediated immunity, some activated T cells (T helper cells) produce cytokines that help direct the immune response and activate B cells (Brian 1988; Grewal and Flavell 1998) or some T cells (T cytotoxic cells) produce powerful enzymes that induce the death of pathogen-infected cells, thereby successfully clearing the pathogen from the body.

B Cells and Antibody production

B cells are the major effectors of the humoral immune response. B cells are continuously produced in the bone marrow and as immature B cells, they circulate through blood and lymph and compete for survival signals in the secondary lymphoid tissue until they are activated by recognition of specific antigen. Immature naïve B cells have receptors for antigens that are present in the plasma membrane. The B-cell receptor (BCR) is a transmembrane protein present on the surface of B cells with a unique antigen binding site. Each B-cell receptor has a membrane-bound immunoglobulin of one isotype (IgM/IgD). After leaving the bone marrow, immature naïve B cells also start producing IgD molecules on their surface that have the same antigen binding site as the IgM. B cells mature when they co-expresses both IgM and IgD on their surface. In response to antigenic challenge and interaction with helper T cells, B cells are activated to differentiate into plasma or memory B cells. B cells are also activated in a T-cell independent manner through toll-like

receptors and BCR crosslinking. Plasma cells produce huge amounts of antibodies continuously in response to antigens, whereas memory B cells help induce a more robust and quicker immune response to antigens upon re-exposure (Brian 1988). A primary immune response, mediated by IgM, occurs when immune cells encounter antigen for the first time. Mature B cells with IgM on their surface differentiate to produce memory cells and plasma cells. A secondary immune response is elicited upon re-exposure to the same antigen, resulting in high-affinity antibodies such as IgG to eliminate the pathogen from the body.

The structural unit of an antibody consists of four chains, of which two are identical light (L) chains (each containing about 220 amino acids) and two identical heavy (H) chains (each containing about 440 amino acids). Heavy and light chain of the antibody are held together by covalent disulfide bonds (Fig. 3). Heavy and light chains together form two identical antigen binding sites at the tip of each arm of the molecule. Because of two binding sites, an antibody can be bivalent. Both heavy and light chains have a variable sequence at their N-terminal ends and a constant sequence at their C-terminal ends. The N-terminal ends of both heavy and light chains link up to form the antigen-binding site and variability in their amino acid sequences results in the diversity of antigen binding sites. In mammals, there are five different classes or isotypes of antibodies (IgA, IgD, IgE, IgG and IgM), each encoded by its own constant region (α , δ , ϵ , γ and μ) within the immunoglobulin heavy chain gene, respectively. Also, there are subclasses of IgG and IgA immunoglobulins in humans, i.e., four IgG subclasses-IgG1, IgG2, IgG3, and IgG4 with γ_1 , γ_2 , γ_3 and γ_4 heavy chain genes, respectively and two IgA subclasses- IgA1 and IgA2 with α_1 and α_2 heavy chain genes, respectively. Differences in heavy chains gives different conformation to the

hinge and tail regions of antibodies, so that each class (and subclass) has a characteristic property of its own. The two types of light chains are kappa (κ) and lambda (λ), which have no significant difference in structure. Both isotypes of the light chain can be associated with any of the heavy chain isotypes, but in an individual B cell the two light chains and the two heavy chains will always be identical. In IgA₁ for instance, the two light chains could either be κ or λ with two α_1 heavy chains but not one λ light chain and one κ light chain with two α_1 heavy chains.

Properties and functions of different antibody classes

IgM-

IgM with the heavy chain μ , is the first class of antibody to be made during the development of a B cell and also the first antibody secreted upon B-cell activation and differentiation into antibody-secreting cells or plasma cells. IgM is present as a monomer on the surface of the B cell or as a pentamer when secreted. Pentameric IgM antibodies are linked together by a J chain, having a total of 10 antigen binding sites. IgM eliminates pathogens during the early stages of a humoral immune response. Mature B cells with IgM on their surface can class switch to different antibody isotypes like IgA, IgG and IgE through DNA recombination mechanisms involving somatic hypermutation and class switch recombination, depending on the type of the antigen the cells encounter.

IgD-

IgD is secreted in very small amounts and is mostly attached to the surface of immature and mature naive the B cells. Surface IgD appears to function as a co-receptor along with IgM. IgD has also been shown to activate cells basophils and mast cells, which secrete antimicrobial factors (Chen et al. 2009).

IgG-

IgG is a monomer (on the surface of a B cell and when secreted). There are four subclasses of IgG (IgG₁₋₄) in humans and is the major class of immunoglobulin present in the blood. It is produced in large quantities during secondary immune responses and is the only class of antibody capable of crossing the placental barrier to give passive immunity to the fetus (Saji et al. 1999).

IgA-

IgA is the main class of antibody present in secretions like saliva, tears, milk, and respiratory and intestinal secretions that guard entrances in the body. IgA is a monomer on the surface of a B cell and both monomer and dimer in secretions.

IgE-

IgE protects against parasitic infections but is also responsible for binding to allergens and triggering the release of histamine from mast cells and basophils. It is involved in the symptoms of allergies like hay fever, asthma and hives. IgE is a monomer on the surface of the B cell and also when secreted.

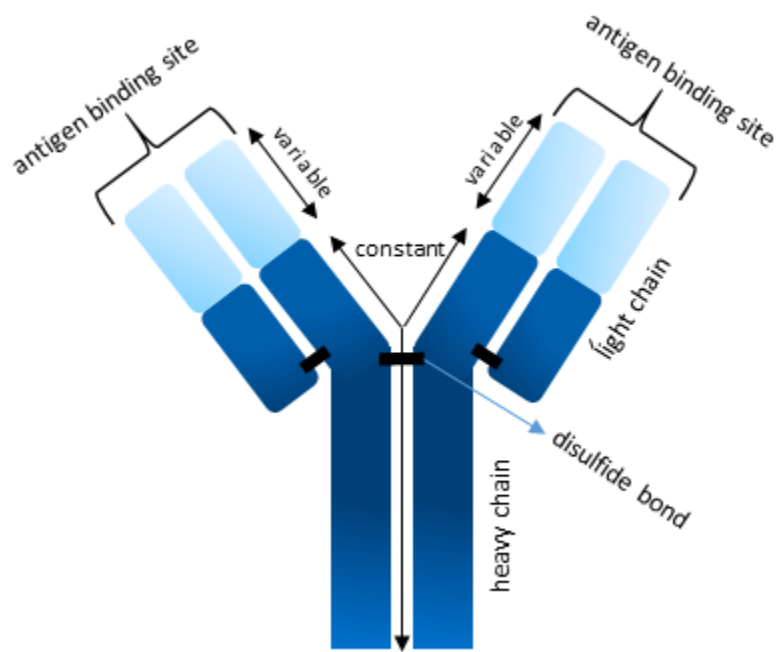


Figure 3: Structure of Immunoglobulin (Ig). Antibody (secreted immunoglobulin) contains both heavy and light chains linked through disulfide bonds that forms the antigen binding/ recognition site that is specific for a particular antigen.

The gene segments that encode the light chain variable region are the variable (V) and the joining (J) segments. The heavy chain variable region has the diversity (D) region along with the variable (V) and joining (J) segments. In V(D)J recombination, a somatic assembly of the variable, diversity and joining gene segments gives the variable region exons of the antigen receptors (Borghesi and Milcarek 2006; Market and Papavasiliou 2003). V(D)J recombination occurs during the developmental stages of the lymphocyte (Mombaerts et al. 1992) and requires recombination associated genes (RAG-1 and RAG-2) (van Gent et al. 1995; Oettinger et al. 1990). The end point of V(D)J recombination is the surface expression of IgM in developing B cells. When B cells come into contact with the antigen, they are capable of undergoing two additional forms of genetic alterations (somatic hypermutation and class switch recombination) that enhance the response to its cognate antigen by the antigen-specific B cell. During somatic hypermutation (SHM), high rates of mutations are introduced into the germline DNA sequences of the assembled exons of the immunoglobulin heavy and light chain variable regions. And it is through this process that B-cell receptors express increased affinity for a particular antigen, a necessary step for clonal selection. In the heavy chain of the immunoglobulin gene, class switch recombination (CSR) occurs adjoining a rearranged variable region exon and downstream C_H (i.e. C_γ , C_α or C_ϵ) exon through deletion of the intervening germline DNA. This allows for the expression of an antibody with the same antigen-binding specificity but with an altered C_H effector function (Stavnezer and Kang 2008).

TCDD-induced immunological defects

The immune system has been identified as a sensitive and early target of TCDD. Even at low levels of TCDD exposure (acute and chronic), rodents display innate and acquired immune-related disturbances (Kerkvliet 2009, 1995; Kerkvliet, Shepherd, and Baecher-Steppan 2002; Roman, Pollenz, and Peterson 1998; Vos, De Heer, and Van Loveren 1997). Upon TCDD exposure, T cell suppression has been observed through induction of T_{reg} cells (Quintana 2013; Quintana et al. 2008). Also, TCDD is known to directly target both mouse and human B cells (Lu et al. 2010; Sulentic and Kaminski 2011). Compared to laboratory animals, humans may be less sensitive to TCDD due to its lower affinity for AhR (Okey, Riddick, and Harper 1994).

TCDD, AhR and B Cells

In mouse B cells, TCDD affects B-cell maturation, activation, differentiation and gene regulation (Lu et al. 2011; Lu et al. 2010; Sulentic and Kaminski 2011). Previous reports in mouse primary and B-cell lines have shown that TCDD inhibits IgM secretion when stimulated with LPS and treated with varying concentrations (0.03-30 nM TCDD) (Marcus, Holsapple, and Kaminski 1998; Sulentic, Holsapple, and Kaminski 1998). In primary human B cells, the effect of TCDD on IgM secretion in CD40L plus IL-4 stimulated cells was inhibitory (Lu et al. 2010). Previous studies have shown that TCDD inhibited LPS-stimulated IgM secretion in the AhR-expressing mouse B-cell line CH12.LX. Further, there was no inhibition in LPS-stimulated IgM secretion in the AhR-deficient BCL-1 cells, suggesting a functional role of the AhR in TCDD-induced inhibition of IgM (Sulentic, Holsapple, and Kaminski 1998, 2000). Additionally, it has been shown that AhR and ARNT heterodimer binds to DRE binding sites within a 3' transcriptional

regulatory region of the Ig heavy chain gene, which is involved in antibody secretion and in Ig heavy chain gene expression (Sulentic, Holsapple, and Kaminski 2000). Also, there was no TCDD-dependent AhR binding to DRE in the hs1,2 and hs4 enhancers in AhR-deficient BCL-1 cells (Sulentic, Holsapple, and Kaminski 2000). Another study has confirmed reversal of TCDD-mediated inhibition of IgA secretion in a mouse cell line by an AhR antagonist or by shRNA-mediated AhR knock down (Wourms and Sulentic 2015). This further suggests that AhR is involved in TCDD-induced inhibition of Ig secretion in mouse B cells.

In addition, TCDD suppressed IgM secretion in human primary B cells in 9 among 12 donors, 2 donors had no effect on IgM secretion even at a dose as high as 100 nM. In fact, 1 donor showed enhancement of IgM secretion (Lu et al. 2010). Further, TCDD suppressed human primary B-cell differentiation by impairing B-cell activation through suppression of activation markers such as CD80, CD86 and CD69 (Lu et al. 2011). However, TCDD did not alter the levels of IgG, IgA or IgM from tonsillar mature B cells of atopic patients, but enhanced IgE levels. In non-atopic patients, TCDD did not affect IgE levels, suggesting that TCDD could aggravate allergic diseases (Kimata 2003). Although the inhibitory effect of TCDD on Ig secretion in mouse B cells is established, TCDD-mediated suppression of human B cells is still unclear.

The AhR antagonist

TCDD-induced activation of AhR is believed to be one of the key steps in the development of TCDD toxicity; therefore inhibition of AhR activation would be expected to provide protection against TCDD toxicity. High affinity ligands for the AhR include toxic compounds like halogenated aromatic hydrocarbons (HAHs) (polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls); numerous polycyclic aromatic hydrocarbons (PAHs) and PAH-like benzo(a)pyrene, 3-methylcholanthrene, and beta-naphthoflavone (BNF); few other ligands like flavones, flavanols and indirubin (Denison and Heath-Pagliuso 1998) have also been identified that are not structurally related to HAHs or PAHs . Chemicals like flavones found in food have been reported to be antagonistic to binding of TCDD to AhR. Although, the effect is dose dependent as these flavones could act as AhR agonists at higher doses (Amakura et al. 2002; Zhang, Qin, and Safe 2003). Also, some of these AhR antagonists had affinity for the estrogen receptor, resulting in estrogen-related effects. Upon analysis of a variety of compounds for AhR antagonist activity, CH223191 was found to competitively and preferentially inhibit TCDD binding to the AhR and subsequent localization to the nucleus (Fig. 4), an effect correlated with decreased TCDD-mediated *CYP1A1* expression (Kim et al. 2006). CH223191 at high doses showed neither AhR agonist-like activity nor cross-talk with the estrogen receptor, making it a suitable candidate to study AhR activation in response to TCDD (Zhao et al. 2010). Recent studies in our lab using the mouse CH12.LX cell line demonstrated that CH223191 inhibits the AhR-mediated signaling and TCDD-induced *CYP1A1* expression (Wourms and Sulentic 2015).

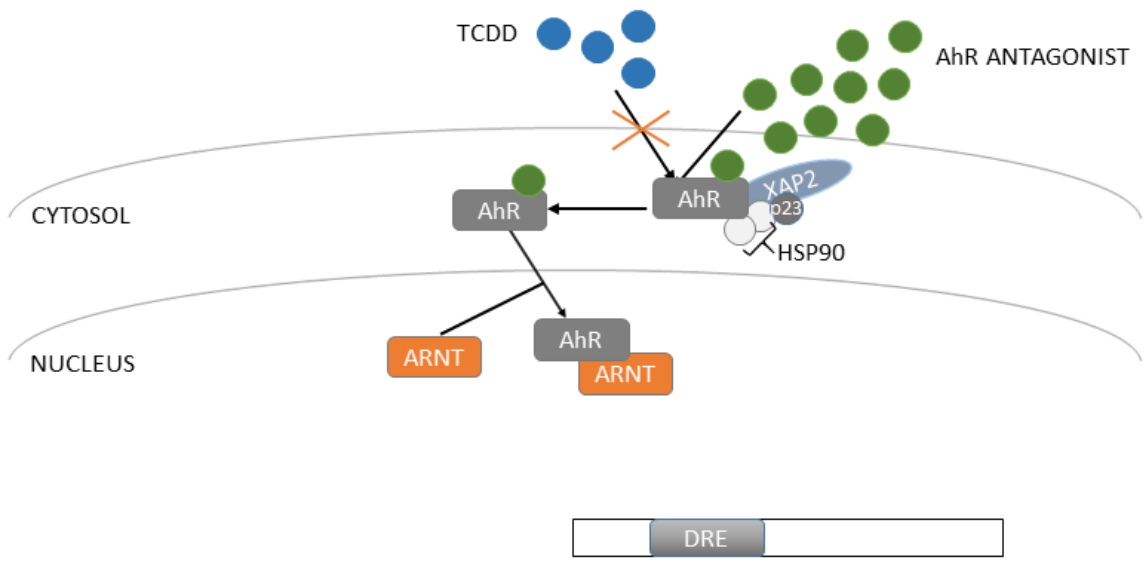


Figure 4: Inhibition of AhR translocation into the nucleus by AhR antagonist.

Immunoglobulin Heavy Chain 3' Regulatory Region (3'IGHRR)

The human immunoglobulin heavy chain (*IGH*) gene and its counterpart, the mouse immunoglobulin heavy chain (*Igh*) gene are located on chromosome 14 and 12, respectively. They encode the heavy chains of antibodies via transcription-dependent DNA remodeling events like VDJ recombination, CSR and SHM (Pinaud et al. 2011). The rearranged human *IGH* after antigen stimulation consists of the V_H promoter (variable heavy chain promoter), the VDJ region, the E_μ (heavy chain intronic enhancer), the heavy chain constant region genes for μ, δ, γ₃, γ₁, Ψ_ε and α₁, respectively, followed by the α₁ 3'IGHRR. The second set of heavy chain genes include the γ₂, γ₄, ε and α₂, followed by the α₂ 3'IGHRR (Mills et al. 1997; Sepulveda, Emelyanov, and Birshstein 2004; Sepulveda et al. 2005) in humans. The two 3'IGHRR are termed α₁ and α₂ because they are present downstream to the α₂ and α₂ heavy chain genes respectively (Fig. 5A). In mouse, the heavy chain constant region genes are μ, δ, γ₃, γ₁, γ_{2b}, γ_{2a}, ε and α respectively, followed by the mouse 3'IghRR (Madisen and Groudine 1994) (Fig. 5B). The V_H promoter, present upstream of the variable region, regulates the transcription of the *IGH* locus and gene expression, the intronic enhancer E_μ is responsible for efficient V and DJ region recombination in B-cell progenitors and *Igh* expression in mature B cells, and also the expression of C_μ (Perlot et al. 2005).

The Mouse 3'IghRR

Murine 3'IghRR contains at least four DNA hypersensitive sites (hs)- hs3b, hs1,2, hs3a and hs4. The hs1,2 enhancer is transcriptionally active in mature B cells and plasma cells; both hs3a and hs3b enhancers have no activity in pre-B cells, B cells, or plasma cells (Madisen and Groudine 1994; Matthias and Baltimore 1993; Saleque, Singh, and Birshstein

1999; Chauveau, Pinaud, and Cogne 1998) and the hs4 enhancer appears to function throughout B-cell development, mainly being active in pre-B cells and plasma cells (Madisen and Groudine 1994). Maximum effect was observed when all four enhancers were together in all stages of B-cell development. There are several transcription factor binding sites present within the 3'*IghRR* enhancers that include DRE, NF- κ B, Octamer (OCT), NF- α P, and AP-1/Ets, Pax5 that regulate 3'*IghRR* activity (Fig. 6). In the CH12.LX, mouse B-cell line, TCDD was shown to induce AhR/ARNT-DRE binding within the hs1,2 and hs4 enhancers of the 3'*IghRR*, as well as inhibition of 3'*IghRR* activity. Additionally, TCDD-induced inhibition of μ heavy chain expression and IgM secretion was observed to be AhR dependent (Sulentic, Holsapple, and Kaminski 1998, 2000). Reversal of TCDD-mediated inhibition of IgA secretion was confirmed in a mouse cell line by an AhR antagonist or via shRNA-mediated AhR knock down (Wourms and Sulentic 2015). Additionally, the murine 3'*IghRR* is sensitive to a variety of AhR and non-AhR agonists (Henseler, 2009). (Henseler, Romer, and Sulentic 2009)

The Human 3'*IGHRR*

The human 3'*IGHRR* is comprised of three enhancer elements (hs3, hs1,2 and hs4), which are 74%, 90%, and 76% homologous respectively to enhancers in the mouse 3'*IghRR*. Also, the human hs1,2 and hs4 lack the Pax5 binding sites that are significant to the murine 3'*IghRR* activity. Additionally, the human α_1 hs1,2 enhancer has a polymorphic region called the invariant sequence (IS) of approximately 55 bp that is not observed in the mouse hs1,2 enhancer (Fig. 6). The invariant sequence can be repeated one (α_{1A}), two (α_{1B}), three (α_{1C}) or four (α_{1D}) times and alter the transcriptional activity (Fernando et al. 2012; Denizot et al. 2001). The invariant sequence has several transcription factor binding sites

like AP-1, NF1, NF- κ B and also a DRE site similar to the functional DRE found in the mouse hs1,2 enhancer (Fernando et al. 2012; Chen and Birshtein 1997; Denizot et al. 2001) and an increase in the number of 55 bp repeats may result in an increase in sensitivity to TCDD. As the mouse 3'*Igh*RR is involved in class switch recombination and Ig expression, it is assumed that the human 3'*IGH*RR is also involved in CSR and Ig expression. However, there are no studies to confirm this and there are differences between the mouse 3'*Igh*RR and human 3'*IGH*RR. Additionally, varied responses to TCDD have been demonstrated on hs1,2 enhancer activity between mouse and human genes. Contrary to the observed TCDD-induced inhibition of the 3'*Igh*RR and hs1,2 activities, TCDD activates the human hs1,2 enhancer (Fernando et al. 2012).

Polymorphisms in the human hs1,2 enhancer appear to be involved in several immune disorders namely plaque psoriasis, psoriatic arthritis, rheumatoid arthritis, coeliac disease, dermatitis herpetiformis, systemic sclerosis lupus, and IgA nephropathy. Of the different hs1,2 alleles, increase in the frequency of the α_{1B} allele is shown to significantly worsen the frequency of the above mentioned diseases (Aupetit et al. 2000; Cianci et al. 2008; Frezza et al. 2004; Giambra et al. 2009; Tulusso et al. 2009). Also, some B-cell malignancies like lymphomas have chromosomal translocations between *c-myc* or *bcl-2* and the 3'*IGH*RR which can lead to deregulated gene expression (Heckman et al. 2003).

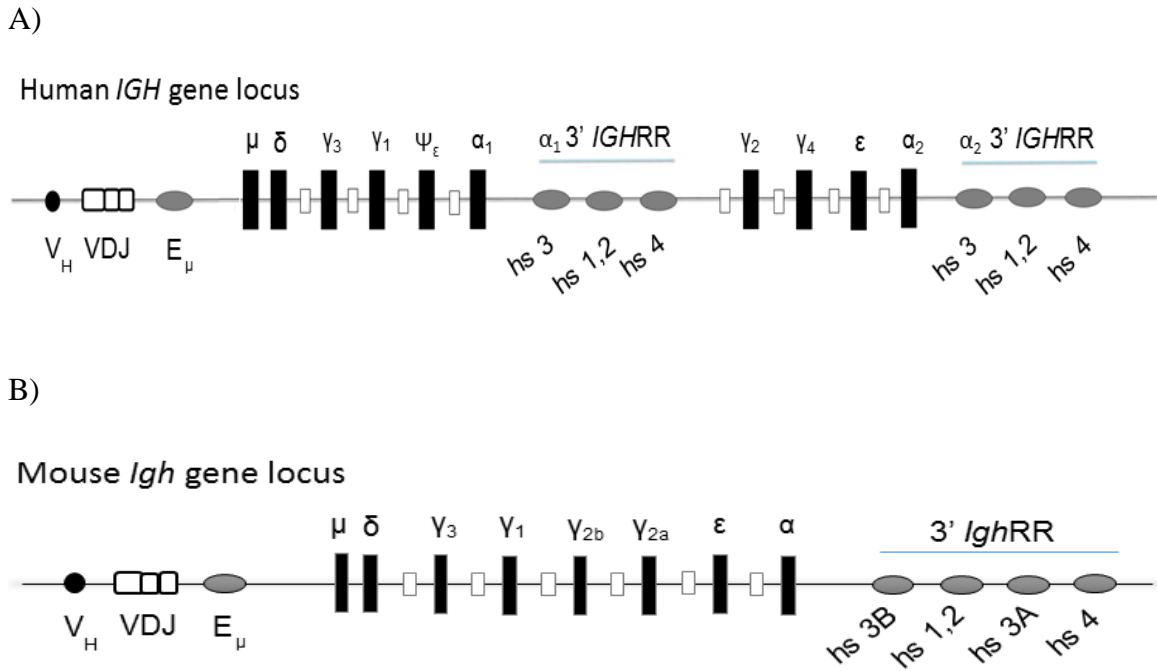


Figure 5: The Human and mouse *IGH* loci. Shown are the representations of the human *IGH* gene locus and mouse *Igh* gene locus, where black oval represents the variable heavy chain promoter (V_H); gray eclipses represent the heavy chain enhancer (E_μ) and enhancers present in the 3' *IGHRR*; open rectangles represent intronic/germline promoters (I_H); closed rectangles represent heavy constant genes (C_H); hs-hypersensitivity site.

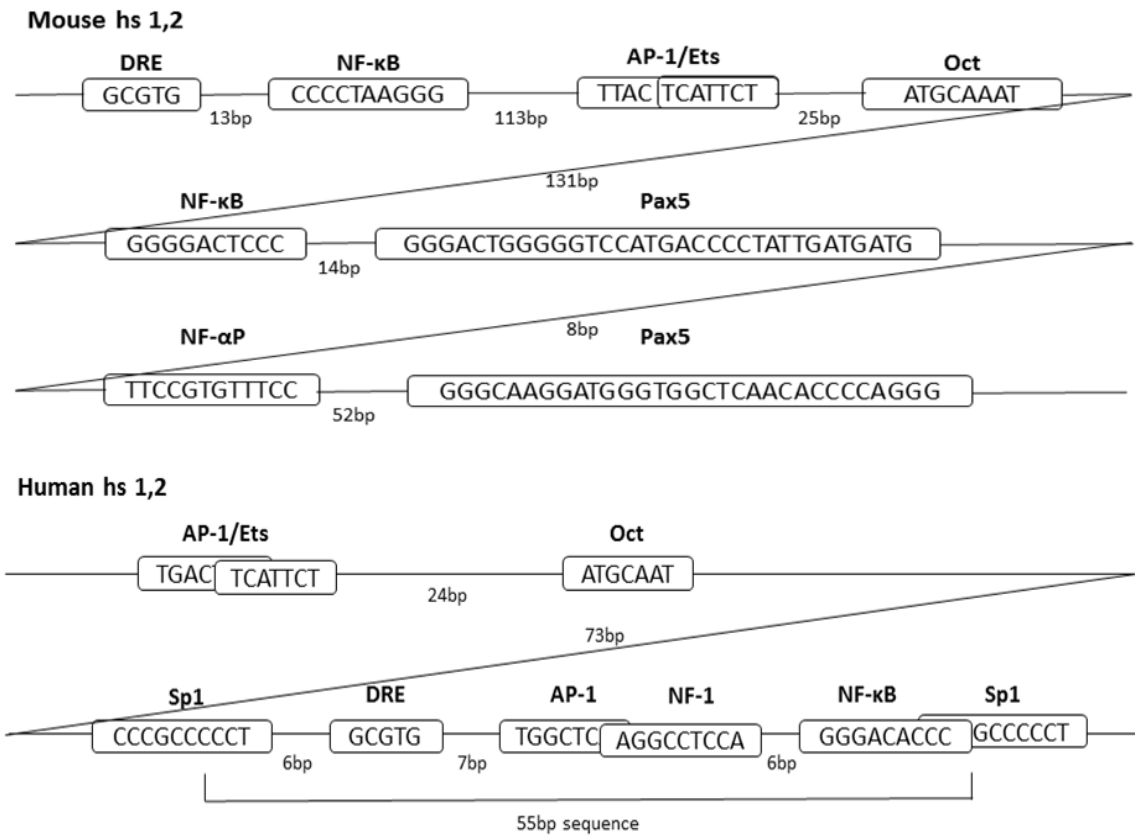


Figure 6: Differences between the mouse hs1,2 and the human hs1,2 enhancer. Shown are the hs1,2 enhancer sequences with different transcription factor binding sites that regulate the 3'RR activity. DRE- dioxin responsive element; NF-κB- nuclear factor kappa-light-chain-enhancer of activated B cells; NF-1- nuclear factor 1; Pax5- paired box 5; Oct- octamer; AP-1- activator protein 1; ETS- E26-transformation-specific; Sp1- specificity protein 1. 55bp invariant sequence contains the nucleotide sequences Sp1, DRE, AP-1, NF-1 and NF-κB.

Germline transcription and Class switch recombination

In the heavy chain of the immunoglobulin gene, class switch recombination (CSR) results in adjoining a rearranged variable region exon (VDJ) and downstream C_H (i.e. C_γ , C_α or C_ϵ) exon through deletion of the intervening DNA. CSR from the upstream C_μ (that encodes IgM) to a targeted downstream C_H in the gene is preceded by germline transcription, which occurs in or outside the germinal center. Germline transcription is an event that occurs after a few hours of antigen challenge and before the cells enter the first division in the germinal centers. Germline transcription involves the transcriptional activation of I_H (heavy chain intronic promoter), continues through the I_H exon, the S region and the C_H exon in the heavy chain gene (Stavnezer 1996; Stavnezer, Guikema, and Schrader 2008). Unlike somatic hypermutation that is precise (point mutations), switch recombination is typically imperfect and joining can occur at different positions throughout the S region. Also, switching can occur with some frequency at positions that are present outside the S regions (Mills, Brooker, and Camerini-Otero 1990). The nucleotide sequence in the S region itself is not responsible for CSR, but CSR is directed by cytokines secreted by T cells that interact with B cell via CD40/CD40L interaction (Snapper, Marcu, and Zelazowski 1997). Contrarily, studies have also demonstrated that the S region regulates isotype-specific CSR (Shanmugam et al. 2000). From the primary unspliced I_H -S- C_H transcripts, the S region is deleted to form spliced non-coding germline transcripts I_H - C_H (Bottaro et al. 1994; Gu, Zou, and Rajewsky 1993) (Fig. 7). Studies on B cells from transgenic mice have indicated that the production and splicing of mouse γ_1 switch transcripts, but not the production of the unspliced message alone, is sufficient to induce switch recombination (Lorenz, Jung, and Radbruch 1995). In mouse, deletion of the

upstream I_H exon has been found to abolish class switching to the targeted downstream isotype (Bottaro et al. 1994; Toellner et al. 1996; Zhang et al. 1993), emphasizing the functional role of the I_H in class switch recombination. Germline transcripts have very small open reading frames and are also referred to as ‘sterile transcripts’ because they cannot code for proteins. However, now it seems to be clear that the role of germline transcription is to provide the appropriate single stranded DNA as target for AID (activation-induced cytidine deaminase) (Chaudhuri and Alt 2004). Germline transcription activates the AID which activates the base excision repair pathway to create double strand breaks in the switch regions of the targeted constant region (acceptor) and the switch region present upstream of the donor constant region. Both the switch regions are joined through a nonhomologous end joining mechanism (Stavnezer 1996; Stavnezer, Guikema, and Schrader 2008). S region double strand breaks in B cells, induced during CSR, are generated and resolved during G1 phase (Schrader et al. 2007) and though S regions mostly have tandem repeats, they lack sufficient homology to undergo homologous recombination (Stavnezer, Guikema, and Schrader 2008) Another pathway that joins the two strands is mismatch repair pathway. Double strand break repair by enzymes results in juxtaposition of the rearranged VDJ region with a targeted downstream C_H exon cluster to form the functional transcript that encodes the required protein (antibody) and subsequent excision of the intervening DNA as an extrachromosomal switch circle (Figure 7). Apart from direct switching to a downstream isotype, switching to IgE could either be sequential switch μ - γ - ϵ or multi-step sequential switch μ - γ - α - ϵ (Mills et al. 1995) and a sequential switch μ - γ - α for IgA₁ and IgA₂ (Zan et al. 1998) .

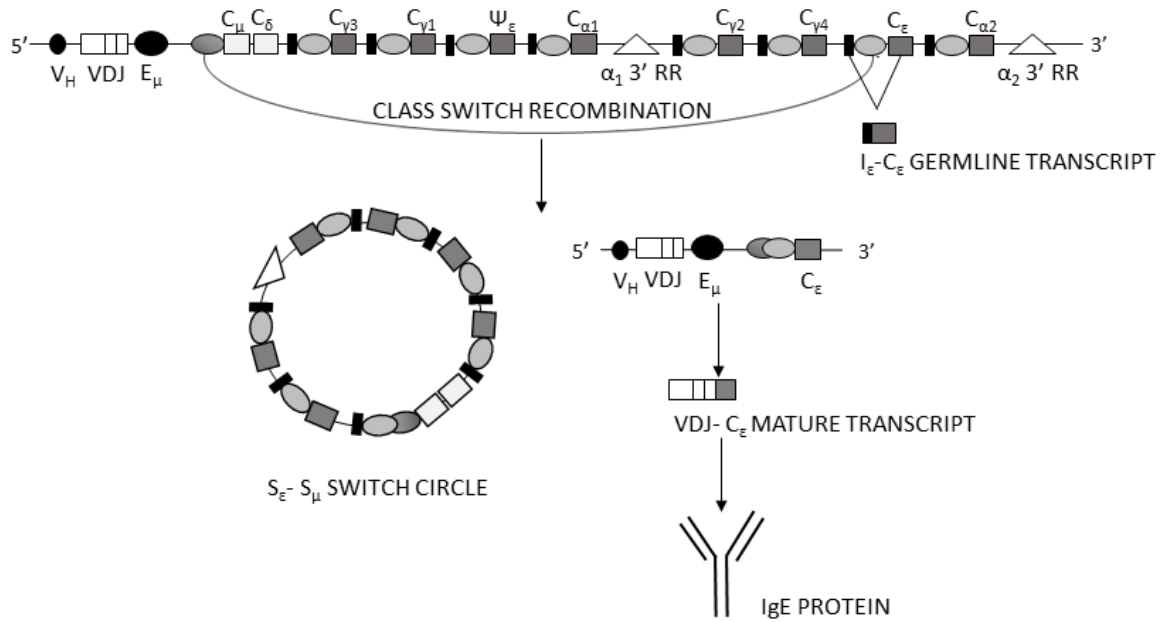


Figure 7: Class switch DNA recombination (CSR) event from IgM to IgE involving the production of germline I_ϵ - C_ϵ transcript, functional VDJ - C_ϵ transcript and S_ϵ - S_μ DNA switch circle.

Hypothesis and Objectives

The mouse 3'*Igh*RR is a sensitive and early target of TCDD. In mouse B cells, TCDD inhibits IgM secretion (Sulentic, Holsapple, and Kaminski 1998; Sulentic et al. 2004; Marcus, Holsapple, and Kaminski 1998) and in a cell line model, inhibition of Ig secretion and 3'*Igh*RR are mediated by the AhR (Wourms and Sulentic 2015). In humans, however, the effect of TCDD on 3'*IGH*RR and antibody expression is poorly understood. The current study focuses on the expression and secretion of IgM from human mature naïve B cells, and their ability to class switch to different Ig isotypes in response to TCDD. Our working hypothesis is that TCDD inhibits immunoglobulin class switching through an AhR-mediated mechanism in human B cells. To test this hypothesis, two main objectives are defined: 1) to determine the role of TCDD in germline and functional transcription. Since germline transcription is the first and key step in class switching to different downstream antibody isotypes, and functional transcripts encode the required antibody, studying the effect of TCDD on these transcripts would determine if CSR is a sensitive target of TCDD and 2) to determine the role of the AhR in class switching. The AhR is activated by its ligand, TCDD, and inhibiting this activation by using an AhR antagonist would help understand the role of the AhR in human Ig expression.

II MATERIALS AND METHODS

Chemicals and Reagents

TCDD was purchased from AccuStandard, Inc (New Haven, CT). It is supplied as 308 μ M solution in DMSO. DMSO was purchased from Sigma Aldrich (St. Louis, MO) and used to dilute TCDD and the AhR Antagonist CH-223191 (Calbiochem, Carlsbad, CA). Human Mega CD40L (soluble and recombinant) was purchased from Enzo Life Sciences (San Diego, CA) and reconstituted in 100 μ l sterile water. When needed, further dilutions were made in RPMI complete medium containing 10% Bovine Calf Serum (described later in this section), aliquoted and stored at -20°C. Human IL-4 with carrier was purchased from Cell Signaling Technology, Inc (Beverly, MA), reconstituted in sterile 1X PBS/10% BCS, aliquoted and stored at -20°C.

Cell Line Model and Culture Conditions

The cell line used in our experiments was Novus CL-01. It is a human monoclonal Burkitt's lymphoma B-cell line obtained from a patient that expresses IgM⁺ and IgD⁺ on the cell surface, and can differentiate in response to appropriate stimuli. This cell line undergoes class switch from IgM to all seven downstream isotypes (IgA₁, IgA₂, IgG₁, IgG₂, IgG₃, IgG₄, IgE) via CSR (Cerutti et al. 1998). CL-01 cells express single nucleotide polymorphisms in the transactivation domain of the *AhR*.

Cells were cultured at 37°C in a 5% CO₂ incubator and grown in complete media consisting of RPMI 1640 Medium 1X Hyclone (Thermo Scientific Laboratories, Logan, UT) supplemented with 10% bovine calf serum hyclone (Thermo Scientific Laboratories, Logan, UT), 1M HEPES, 100mM Sodium Pyruvate, 100X MEM Non- Essential Amino Acids, 100X Pencillin/ Streptomycin, 1M NaOH, 50µM 2-Mercaptoethanol. Cell viability was monitored by the addition of trypan blue dye to 1ml of the cell suspension using the Vi-cell Cell Counter (Beckman Coulter, Inc, Pasadena, CA). Cells were also checked periodically under the microscope for any abnormal growth.

Sandwich Enzyme-Linked Immunosorbent Assay (Sandwich ELISA)

After the desired time of incubation (96 hours for IgG and IgM), cells were centrifuged at 500xg for 5 minutes. Supernatants were collected, stored in separate 1.5ml Eppendorf tubes labeled appropriately and used to determine Ig expression levels by using Sandwich ELISA. The coating and detection antibody are specific for the antigen. The detection antibody is HRP-conjugated (horseradish peroxidase), an enzyme that cleaves a substrate producing color change. Colorimetric detection was performed using a Spectramax Plus 384 UV/VIS Microplate Spectrophotometer at 450nm (Molecular Devices, Sunnyvale, CA). The SOFTmax PRO software (Molecular Devices) was used to calculate the sample concentrations using a standard curve generated from the absorption of the IgG and IgM standard concentrations.

RNA Isolation

After the desired time of incubation (96 hours for IgG and IgM), cells were centrifuged at 500xg for 5 minutes. Supernatants were collected and further analyzed by ELISA as described above. The cell pellet was resuspended in Tri Reagent (Sigma Aldrich,

St. Louis, MO) and was stored at -80°C until further use. RNA was isolated following the protocol of Tri Reagent manufacturer.

cDNA Synthesis, Reverse Transcription and Real Time PCR

Quantification of RNA samples was done using NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE). One microgram of total RNA was converted into cDNA using BioRad iScript Reverse Transcription Supermix for Real Time-qPCR (Berkeley, CA) following the instructions of manufacturer. Briefly, samples were incubated initially for 5 minutes at 25°C , and reverse transcription allowed for 30 minutes at 42°C and finished by incubating samples at 85°C for 5 minutes. Total cDNA was quantified using NanoDrop ND-1000 spectrophotometer, diluted to 100ng/replicate in nuclease-free water and used for PCR. $C_{\gamma 1}$ and $C_{\alpha 1}$ germline transcripts and $\gamma 1$ *IGH* and $\alpha 1$ *IGH* functional transcripts were analyzed as follows: 10Xbuffer, dNTPs, forward and reverse primers, Taq polymerase (New England Biolabs, Ipswich, MA) and template were added and volume was made up to 25 μl with nuclease-free water. To determine μ *IGH* functional transcripts, C_{ϵ} germline transcripts, α_{1-2} and γ_{1-4} germline/functional transcripts and *CYP11A1* expression we used real time PCR (BioRad CFX 96, Berkeley, CA). The setting for real time PCR was as follows: 2X Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Logan, UT), forward and reverse primers and template were added and volume was made up to 20 μl with nuclease-free water. *β -actin* was used as loading control. The PCR primers used for amplifying the primers and their PCR cycling conditions are indicated in Table 1 and Table 2, respectively. PCR products were run on 1.5% agarose gels (GeneMate LE Agarose, Kaysville, UT) using a FB200 gel box from Fisher Scientific (Waltham, MA) and comparing with the O' Gene Ruler 100bp plus DNA ladder (Thermo

Scientific, Logan, UT). Gels were stained with Ethidium Bromide 1% solution (Fisher BioReagents, Pittsburg, PA). Real Time PCR data were analyzed using the $2^{(-\Delta\Delta CT)}$ method by comparing with the appropriate vehicle control.

| Transcripts | Primers |
|--|--|
| Control genes | |
| <i>CYP11A1</i> | FP- 5' CAGCTCCAAAGAGGTCCAAG 3' RP-5' CATGCAGAAGATGGTCAAGG 3' |
| <i>β-actin</i> | FP- 5' ATCACCATTGGCAATGAGCGGTTC 3' RP- 5' GCGGATGTCCACGTCACACTTCA 3' |
| Germline (I_H-C_H) transcripts | |
| C _{α1} | FP- 5' CAGCAGCCCTCTTGGCAGGCAGCCAG 3' RP- 3' GGGTGGCGGTTAGCGGGGTCTTGG 3' |
| C _{γ1} | FP- 5' GGGCTTCCAAGCCAACAGGGCAGGACA 3' RP- 5' GTTTTGTCACAAGATTTGGGCTC 3' |
| C _ε | FP- 5' GACGGGCCACACCATCC 3' RP- 5' CGGAGGTGGCATTGGAGG 3' |
| Functional (V_H-D_{JH}-C_H) transcripts | |
| <i>α1 IGH</i> | FP- 5' GACACGGCTGTGTATTACTGTGCG 3' RP- 5' GGGTGGCGGTTAGCGGGGTCTTGG 3' |
| <i>γ1 IGH</i> | FP- 5' GACACGGCTGTGTATTACTGTGCG 3' RP- 5' GTTTTGTCACAAGATTTGGGCTC 3' |
| <i>μ IGH</i> | FP- 5' GACACGGCTGTGTATTACTGTGCG 3' RP- 5' CCGAATTCAGACGAGGGGGAAAAGGGTT 3' |

(Continued)

| Germline (I_H-C_H) and Functional (V_H-DJ_H-C_H) transcripts | |
|---|--|
| $\alpha 1$ | FP- 5' CGCCATGACAACAGACACAT 3' RP- 5' GTATGCTGGTCACAGCGAAG 3' |
| $\alpha 2$ | FP- 5' ACATTGATGTGGGTGGGTTT 3' RP- 5' CCACCACCTACGCTGTAACC 3' |
| $\gamma 1$ | FP- 5' GATGTCGCTGGGATAGAAGC 3' RP- 5' TGTGGAGACCTTGCACTTG 3' |
| $\gamma 2$ | FP- 5' GAGATGTCGCTGGGGTAGAA 3' RP- 5' GGCAAGGAGTACAAGTGCAA 3' |
| $\gamma 3$ | FP- 5' TCACGTTGCAGGTGTAGGTC 3' RP- 5' CTA CTCC CAGA ACCGGTGA 3' |
| $\gamma 4$ | FP- 5' GGTTCTGGTCATCTCCTCCT 3' RP- 5' GAACGGCAAGGAGTACAAGTG 3' |

Table 1: Forward and reverse primers for I_H-C_H germline transcripts; V_HDJ_H-C_H functional transcripts; I_H-C_H and V_HDJ_H-C_H germline and functional transcripts; *CYP11A1* and *β -actin*. FP- forward primer, RP- reverse primer, bp- base pairs.

| Transcripts | Annealing temperature | No of cycles | Expected product size |
|---|------------------------------|---------------------|------------------------------|
| Control genes | | | |
| <i>CYP11A1</i> | 60°C for 50 seconds | 40 | 170 bp |
| <i>β-actin</i> | 63°C for 1 minute | 30 | 129 bp |
| Germline (I_H-C_H) transcripts | | | |
| C _{α1} | 63°C for 1 minute | 40 | 1194 bp |
| C _{γ1} | 61°C for 1 minute | 30 | 603 bp |
| C _ε | 63°C for 1 minute | 30 | 125 bp |
| Functional (V_H-D_{JH}-C_H) transcripts | | | |
| α1 <i>IGH</i> | 63°C for 1 minute | 40 | 804 bp |
| γ1 <i>IGH</i> | 61°C for 1 minute | 30 | 416 bp |
| μ <i>IGH</i> | 63°C for 1 minute | 30 | 152 bp |
| Germline (I_H-C_H) and Functional (V_H-D_{JH}-C_H) transcripts | | | |
| α1 | 57°C for 50 seconds | 40 | 166 bp |
| α2 | 60°C for 50 seconds | 40 | 157 bp |
| γ1 | 57°C for 50 seconds | 40 | 170 bp |
| γ2 | 60°C for 50 seconds | 40 | 188 bp |
| γ3 | 60°C for 50 seconds | 40 | 164 bp |
| γ4 | 60°C for 50 seconds | 40 | 154 bp |

Table 2: PCR cyclin conditions and expected product size for I_H-C_H germline transcripts; V_HD_{JH}-C_H functional transcripts; I_H-C_H and V_HD_{JH}-C_H germline and functional transcripts; *CYP11A1* and *β-actin*.

Statistical analysis of data

For ELISA and Real Time PCR results, a one-way ANOVA followed by Dunnett's Multiple Comparison test was used to analyze the treatments groups (mean \pm SE; n=3 for ELISA and n=3 separate RNA isolations for Routine PCR and Real Time PCR) using GraphPad Prism software for significant differences between treatment groups and appropriate vehicle control were denoted by *, **, ***; significant differences between stimulation and NA were denoted by #, ##, ### which represent significance at p<0.05, p<0.01 and p<0.001, respectively.

Effects of co-treatment of TCDD and the AhR antagonist on Ig secretion and transcripts are represented with one-way ANOVA, gray circles represent 10 and 30 nM TCDD alone, open squares represent 10 and 30 μ M of the AhR antagonist alone, green triangles represent 10 or 30 nM TCDD with 10 μ M of the AhR antagonist and blue triangles represent 10 or 30 nM TCDD with 30 μ M of the AhR antagonist. Black line represents the vehicle control at 1. Figures with TCDD and the AhR antagonist are separated from the original figure with TCDD, the AhR antagonist, TCDD and the AhR antagonist to clearly indicate the effects of TCDD and the AhR antagonist with respect to corresponding vehicle control. Only significant differences between the AhR antagonist and TCDD are represented on the two-way ANOVA graphs.

III RESULTS

Differential effects of TCDD and the AhR antagonist on immunoglobulin secretion

TCDD is known to directly target mouse B cells and inhibit IgM secretion (Sulentic, Holsapple, and Kaminski 1998). In another study with primary human B cells from donors, nine of twelve human donor primary B cells showed reduced IgM expression when treated with TCDD, while two donors showed no response and one demonstrated enhanced IgM expression (Lu et al. 2010). Expecting an inhibition with TCDD in our present study, we stimulated our cells with CD40L and IL-4. Surprisingly, stimulation and TCDD had variable effects on IgM secretion (Fig. 8), as TCDD either increased, decreased or had no effect on IgM secretion. Conversely, TCDD treatment (10 and 30 nM) inhibited the stimulation (Fig. 8). Additionally, low levels of IgG were also observed in unstimulated cells, suggesting that CL-01 cells have mixed subpopulations of cells that have undergone spontaneous CSR and express either of the IgG isotypes.

The AhR antagonist alone either increased, decreased or had no effect on IgM secretion or in co-treatment with TCDD, resulting in variable effects on IgM (Fig. 8). In contrast, the AhR antagonist alone increased IgG secretion by two-fold (10 and 30 μ M), and the AhR antagonist and TCDD antagonized each other's effects upon co-treatment (Fig. 9 and 10). Gray arrow from 30 nM TCDD (grey circle) to both green and blue triangles indicates that 10 and 30 μ M of the AhR antagonist significantly reverses the

inhibitory effect of 30 nM TCDD on IgG secretion. Green arrow from 10 μ M of the AhR antagonist (open square) to green triangles indicates that 10 and 30 nM TCDD significantly reverse the increase in IgG secretion by 10 μ M of the AhR antagonist (Fig. 10). These results demonstrate a difference in sensitivity of IgM vs. IgG to TCDD and the AhR antagonist.

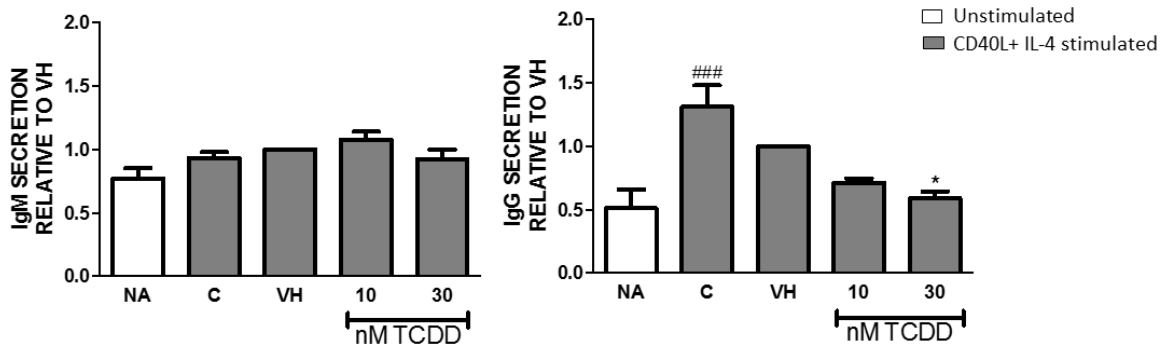


Figure 8: Differential effects of TCDD on IgM and IgG secretion. CL-01 cells were cultured for 96 hours for IgM and IgG respectively, \square without stimulation (naïve), \blacksquare with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30nM). A) IgM secretion (mean SEM, n=3), B) IgG secretion (mean SEM, n=3) is represented on y-axis as fold change relative to appropriate vehicle control (VH). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and appropriate vehicle control were denoted by one (*) asterisk, which represents significance at $p < 0.05$ and significant differences between stimulation and NA were denoted by three (###) hash, which represents significance at $p < 0.001$.

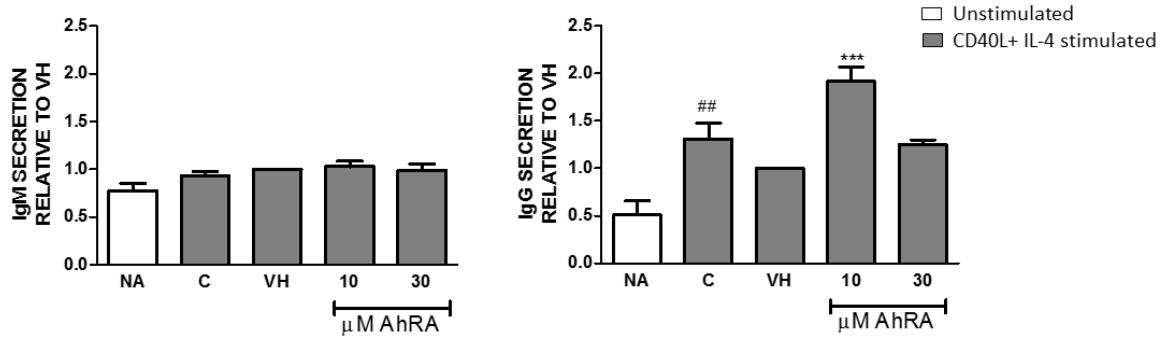


Figure 9: Differential effects of the AhR antagonist on IgM and IgG secretion. CL-01 cells were cultured for 96 hours for IgM and IgG respectively, □ without stimulation (naïve), or ■ with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or the AhR antagonist (10 and 30μM). A) IgM secretion (mean SEM, n=3), B) IgG secretion (mean SEM, n=3) is represented on y-axis as fold change relative to appropriate vehicle control (VH). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and appropriate vehicle control were denoted by three (***) asterisks, which represents significance at $p < 0.001$ and significant differences between stimulation and NA were denoted by two (##) hash, which represents significance at $p < 0.01$.

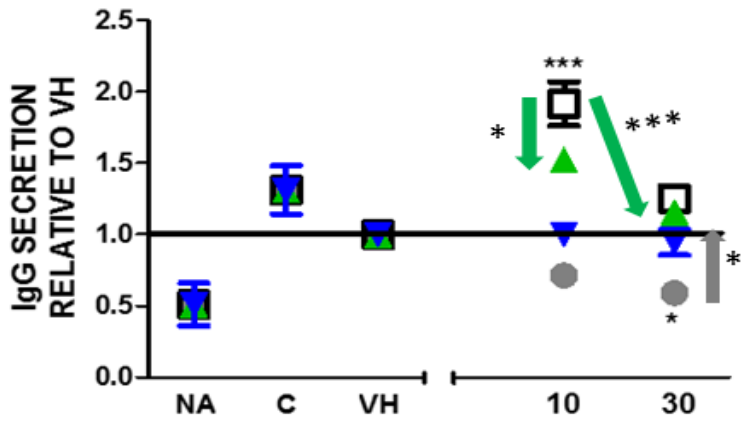
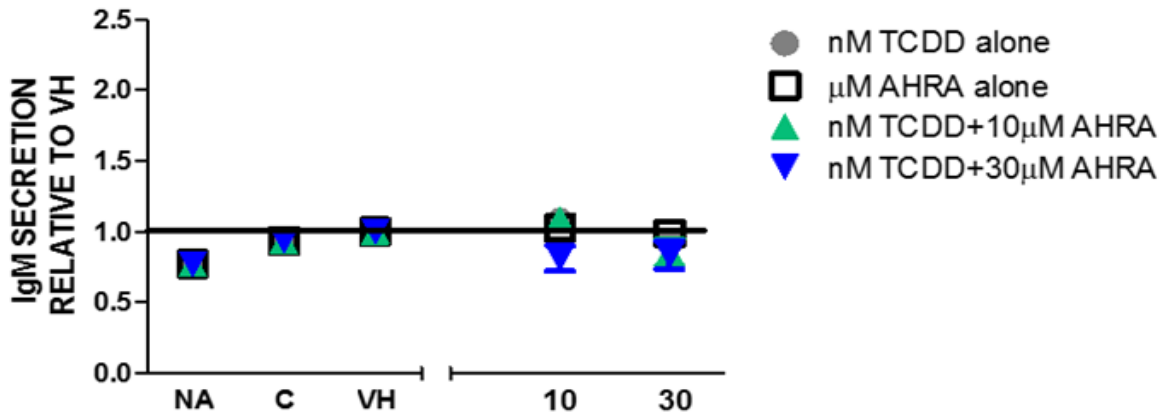


Figure 10: The AhR antagonist and TCDD antagonize each other's effects on IgG secretion and have no effect on IgM secretion. CL-01 cells were cultured 96 hours for IgM and IgG respectively, without stimulation (naïve) or with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30 nM) or the AhR antagonist (10 and 30 μ M). A) IgM secretion (mean SEM, n=3, B) IgG secretion (mean SEM, n=3) is represented on y-axis as fold change relative to appropriate vehicle control (VH). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison test. Gray circles represent 10 and 30 nM TCDD alone, open squares represent 10 and 30 μ M of the AhR antagonist alone, green triangles represent 10 or 30 nM TCDD with 10 μ M of the AhR antagonist and blue triangles represent 10 or 30 nM TCDD with 30 μ M of the AhR antagonist. The significant differences between treatment groups and appropriate vehicle control and differences between stimulation and NA are same as in figures 8 and 9.

Differential effects of TCDD and the AhR antagonist on μ *IGH* functional transcripts and γ_{1-4} germline/functional transcripts

TCDD alone and the AhR antagonist alone either increased, decreased or had no effect on μ *IGH* functional transcripts, or co-treatment of TCDD and the AhR antagonist also had variable effects on μ *IGH* functional transcripts (Fig. 11, 12 and 13). $C\gamma_1$ germline (Fig. 14) and γ_1 *IGH* functional transcripts (Fig. 14) were analyzed qualitatively by gel electrophoresis of PCR products. To quantitate the germline and functional transcript expression, real time PCR primers were designed spanning the constant regions for γ_{1-4} . Stimulation with CD40L plus IL-4 significantly increased the levels of γ_{1-4} germline/functional transcripts as expected. Addition of TCDD treatment (10 and 30 nM) inhibited γ_{1-4} germline/functional transcripts (Fig. 15). The AhR antagonist (10 and 30 μ M) alone increased γ_{1-4} germline/functional transcripts (Fig. 16). AhR antagonist and TCDD antagonized each other's effect on γ transcripts. Green arrow from 30 nM TCDD (gray circle) to 10 μ M of AhR antagonist (open square) indicates that 30 nM TCDD reversed the increase in γ_{2-4} transcripts by 10 μ M of AhR antagonist (Fig. 17).

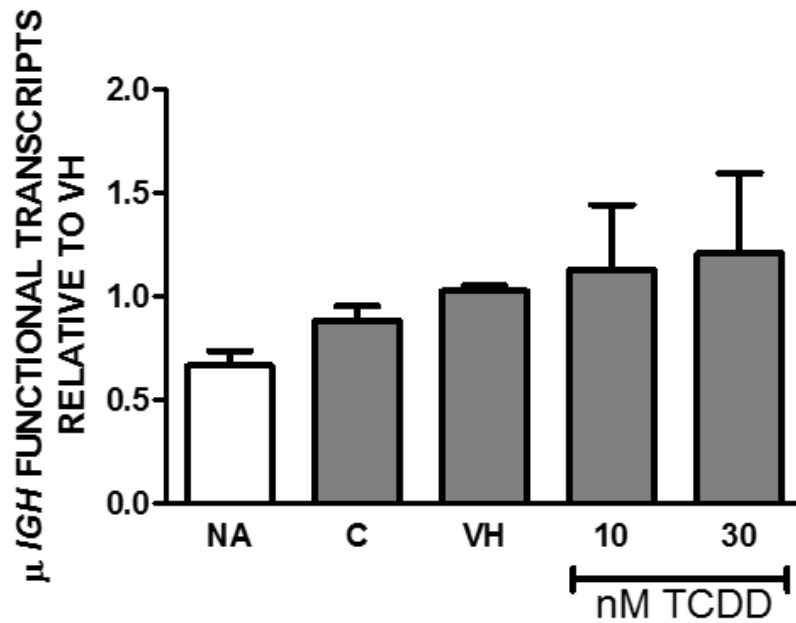


Figure 11: TCDD has variable effect on μ *IGH* functional transcripts. CL01 cells were cultured for 96 hours without stimulation (naïve), or with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30 nM). μ *IGH* transcript expression (mean SEM, n=3) was measured by real time PCR and represented on y-axis as fold change relative to appropriate vehicle control (VH). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison test.

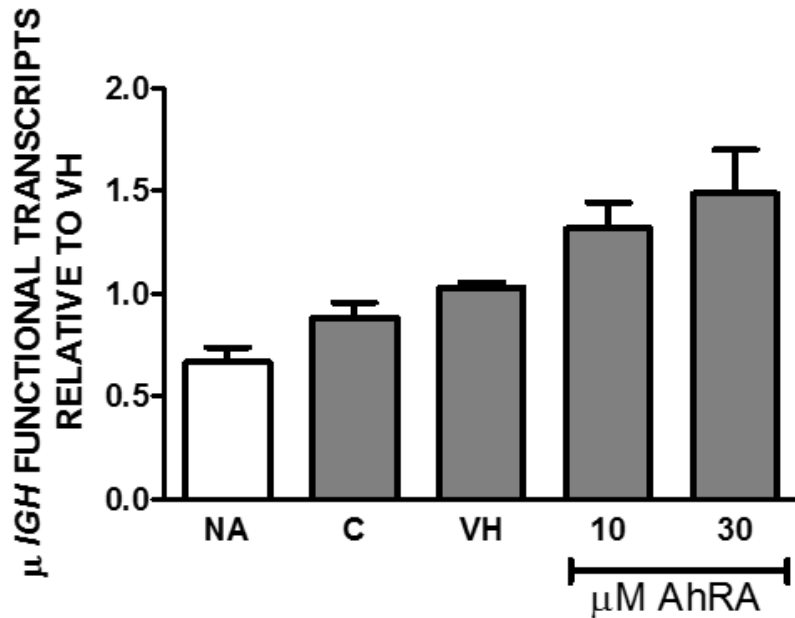


Figure 12: The AhR antagonist has variable effect on μ *IGH* functional transcripts.

CL01 cells were cultured for 96 hours without stimulation (naïve), or with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or AhR antagonist (10 and 30 μ M). μ *IGH* transcript expression (mean SEM, n=3) was measured by real time PCR and represented on y-axis as fold change relative to appropriate vehicle control (VH). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison test.

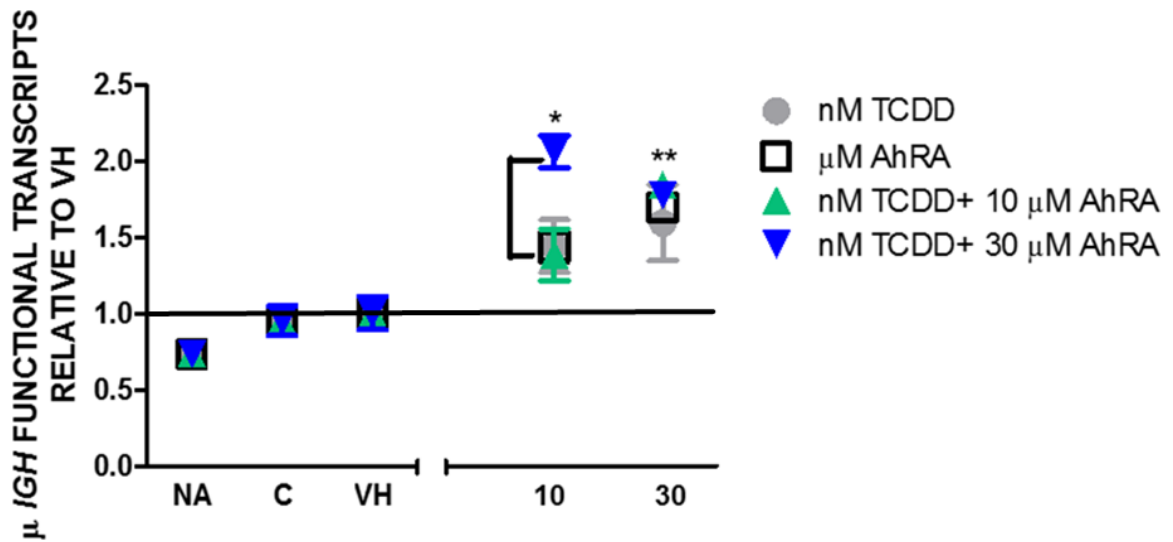


Figure 13: Co-treatment of the AhR antagonist with TCDD has variable effect on μ *IGH* functional transcripts. CL01 cells were cultured for 96 hours without stimulation (naïve), with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30 nM) or AhR antagonist (10 and 30 μ M). μ *IGH* functional transcript expression (mean SEM, n=3) was measured by real time PCR and represented on y-axis as fold change relative to appropriate vehicle control (VH). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison test. Gray circles represent 10 and 30 nM TCDD alone, open squares represent 10 and 30 μ M of the AhR antagonist alone, green triangles represent 10 or 30 nM TCDD with 10 μ M of the AhR antagonist and blue triangles represent 10 or 30 nM TCDD with 30 μ M of the AhR antagonist. The significant differences between treatment groups and appropriate vehicle control and differences between stimulation and NA are same as in figures 11 and 12.

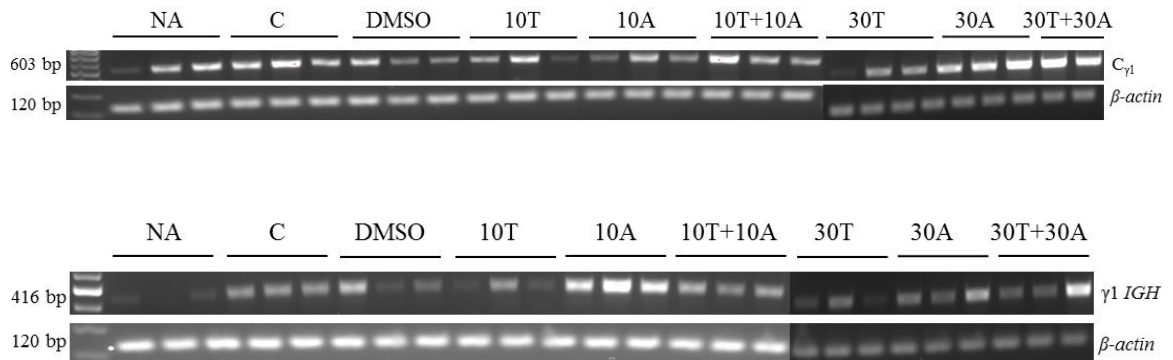


Figure 14: Spontaneous class switch from C_{μ} to $C_{\gamma 1}$. CL01 cells were cultured for 96 hours without stimulation (naïve), or with CD40L and IL-4 stimulation and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30nM) or AhR antagonist (10 and 30 μ M). ‘C’ represents CD40L and IL-4 stimulation, ‘T’ represents nM concentration of TCDD, ‘A’ represents μ M concentration of AhR antagonist and ‘T+A’ represents nM concentration of TCDD and μ M concentration of AhR antagonist respectively. $C_{\gamma 1}$ germline transcripts, $\gamma 1 IGH$ functional transcripts and β -actin could be observed at 603, 416 and 129 base pairs (bp), respectively.

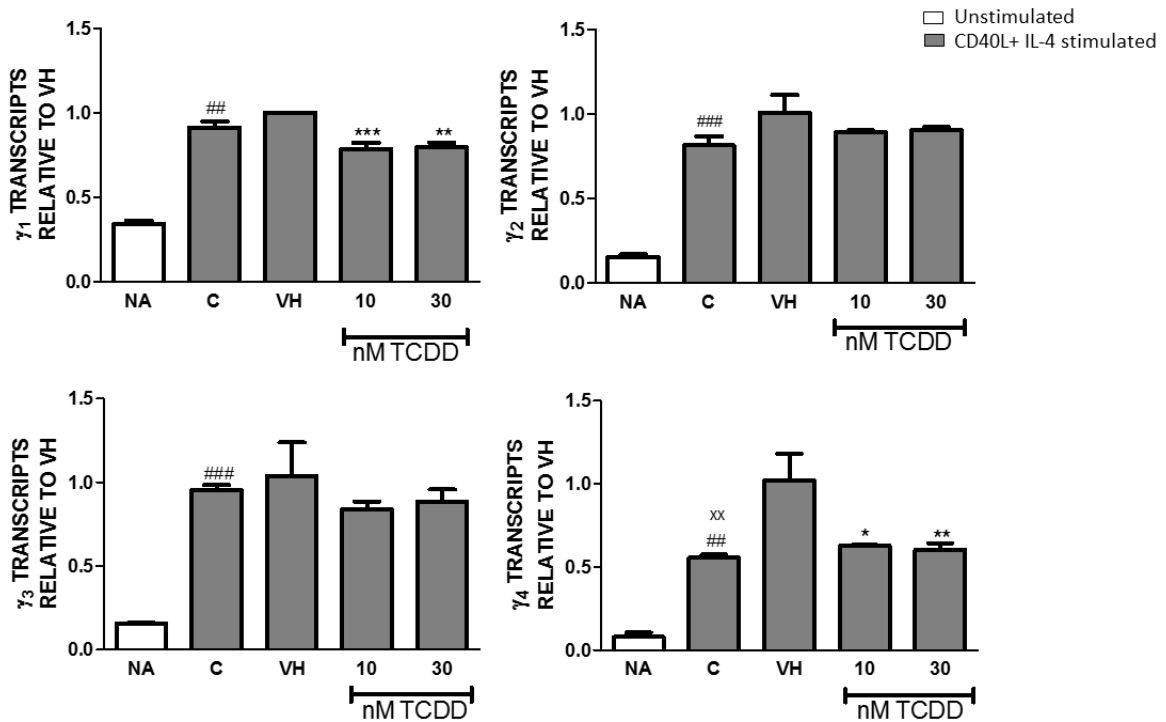


Figure 15: TCDD inhibits γ_{1-4} germline/functional transcripts. CL01 cells were cultured for 96 hours \square without stimulation (naïve), or \blacksquare with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30 nM). γ_{1-4} germline/functional transcripts were measured by real time PCR (mean SEM, n=3) and represented on y-axis as fold change relative to appropriate vehicle control (VH). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and appropriate vehicle control were denoted by one (*), two (**) and three (***) asterisks, which represents significance at p<0.05, p<0.01 and p<0.001, respectively and significant differences between stimulation and NA were denoted by two (##) and three (###) hash, which represents significance at p<0.01 and p<0.001, respectively.

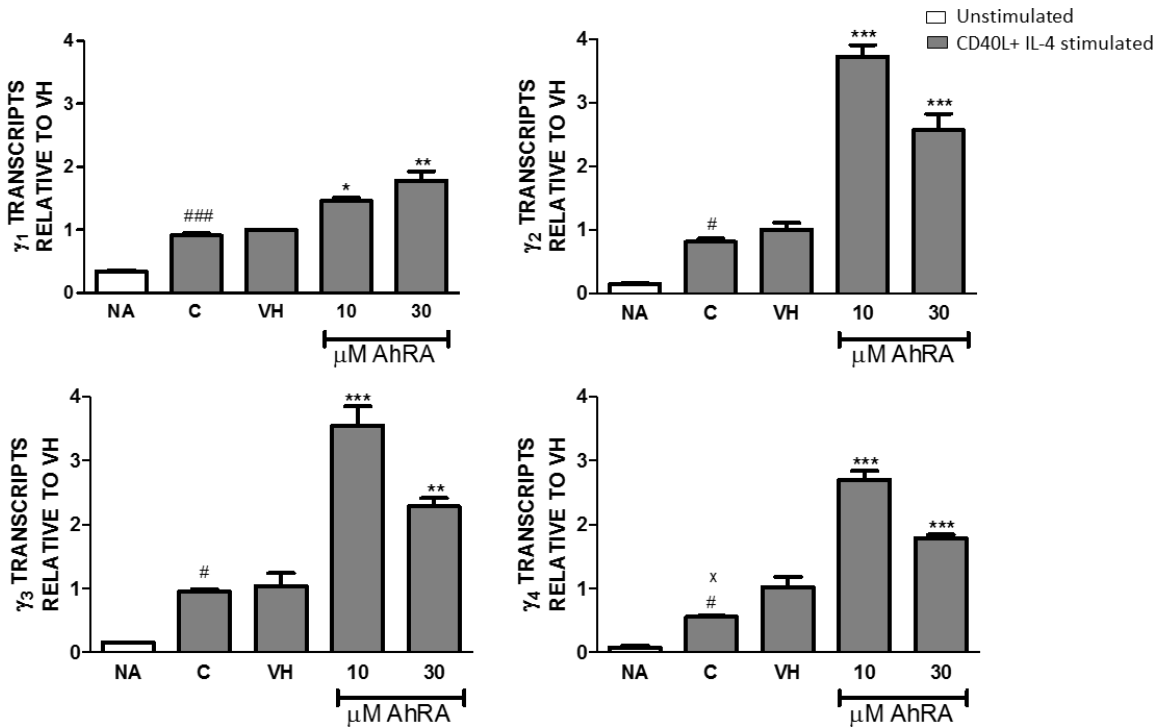


Figure 16: The AhR antagonist alone increases γ_{1-4} germline/functional transcripts.

CL01 cells were cultured for 96 hours without stimulation (naïve), or with CD40L and IL-4 stimulation and treated with 0.01% vehicle (DMSO) or AhR antagonist (10 and 30 μM). γ_{1-4} germline/functional transcripts were measured by real time PCR (mean SEM, n=3) and represented on y-axis as fold change relative to appropriate vehicle control (VH). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and appropriate vehicle control were denoted by one (*), two (**), and three (***) asterisks, which represents significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively and significant differences between stimulation and NA were denoted by one (#) and three (###) hash, which represents significance at $p < 0.05$ and $p < 0.001$, respectively.

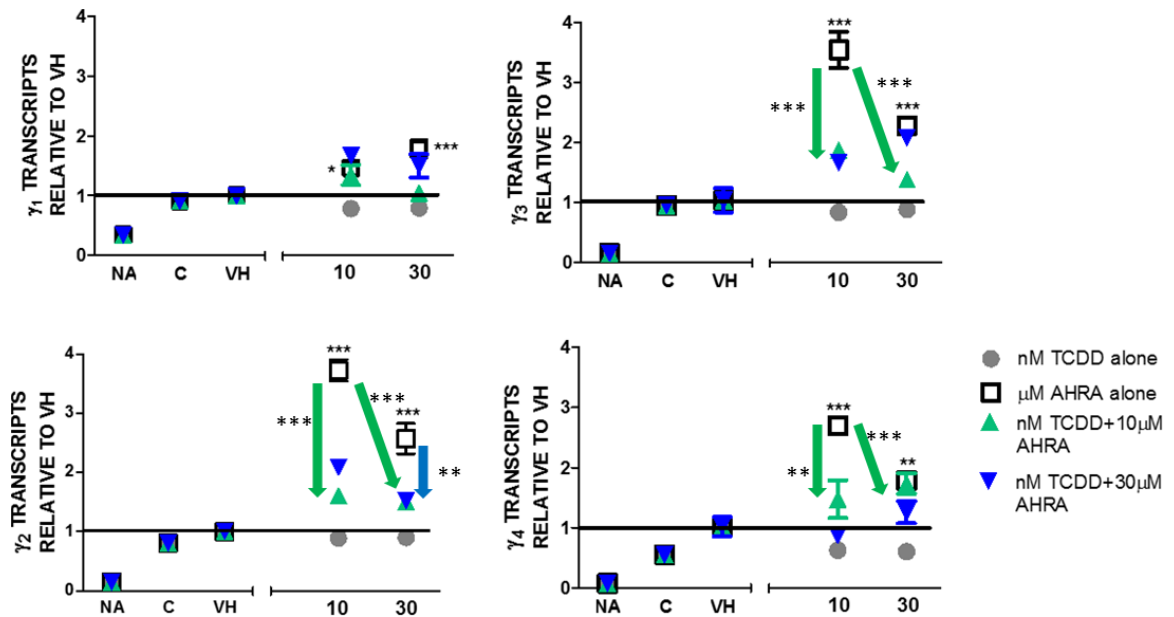


Figure 17: The AhR antagonist and TCDD antagonize each other's effects on γ_{1-4} germline/functional transcripts. CL01 cells were cultured for 96 hours without stimulation (naïve), or with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30 nM) or AhR antagonist (10 and 30 μM). γ_{1-4} germline/functional transcripts were measured by real time PCR (mean SEM, n=3) and represented on y-axis as fold change relative to appropriate vehicle control. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison test. Gray circles represent 10 and 30 nM TCDD alone, open squares represent 10 and 30 μM of the AhR antagonist alone, green triangles represent 10 or 30 nM TCDD with 10μM of the AhR antagonist and blue triangles represent 10 or 30 nM TCDD with 30 μM of the AhR antagonist. The significant differences between treatment groups and appropriate vehicle control and differences between stimulation and NA are same as in figures 15 and 16.

Differential effects of TCDD and the AhR antagonist on α_{1-2} germline/functional transcripts

α_1 *IGH* functional transcripts (Fig. 18) were analyzed by PCR and C_{α_1} germline transcripts could not be observed (Data not shown) by PCR analysis. To quantitate the germline and functional transcript expression, real time PCR primers were designed spanning the constant regions for α_{1-2} . Surprisingly, α_1 as well as α_2 germline/functional transcripts were readily expressed in unstimulated CL-01 cells.

Unexpectedly, α_1 and α_2 germline/functional transcripts are inhibited with CD40L and IL-4 stimulation, which is significantly reversed by the addition of TCDD (10 and 30 nM) (Fig. 19). Surprisingly, the AhR antagonist alone inhibited α germline/functional transcripts (Fig. 20). Co-treatment of TCDD with the AhR antagonist reversed the AhR antagonist-induced inhibition of α_1 and α_2 germline/functional transcript expression (Fig. 21).

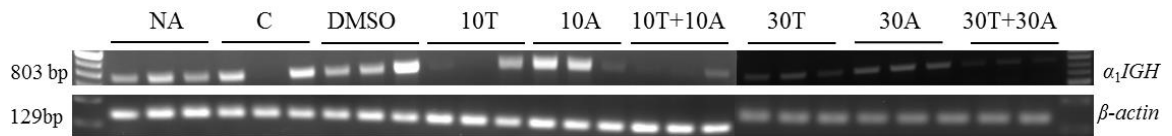


Figure 18: Spontaneous class switch from C_μ to $C_{\alpha 1}$. CL01 cells were cultured for 96 hours without stimulation (naïve), or with CD40L and IL-4 stimulation alone and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30nM) or AhR antagonist (10 and 30 μ M). ‘C’ represents CD40L and IL-4 stimulation alone, ‘T’ represents nM concentration of TCDD, ‘A’ represents μ M concentration of AhR antagonist and ‘T+A’ represents nM concentration of TCDD and μ M concentration of AhR antagonist respectively. $C_{\gamma 1}$ germline transcripts, $\gamma 1IGH$ functional transcripts and $\beta-actin$ could be observed at 603, 416 and 129 base pairs (bp), respectively.

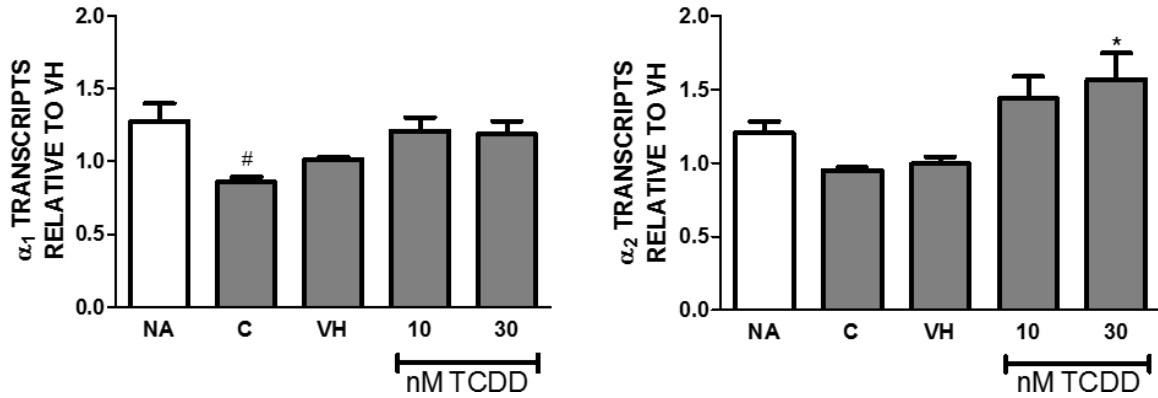


Figure 19: TCDD reverses inhibition of CD40L and IL-4 in α germline/functional transcripts. CL01 cells were cultured for 96 hours without stimulation (naïve), with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30 nM). α germline/functional transcripts were measured by real time PCR (mean SEM, n=3) and represented on y-axis as fold change relative to appropriate vehicle control. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and appropriate vehicle control were denoted by one (*) asterisk, which represents significance at $p < 0.05$ and significant differences between stimulation and NA were denoted by one (#) hash, which represents significance at $p < 0.05$.

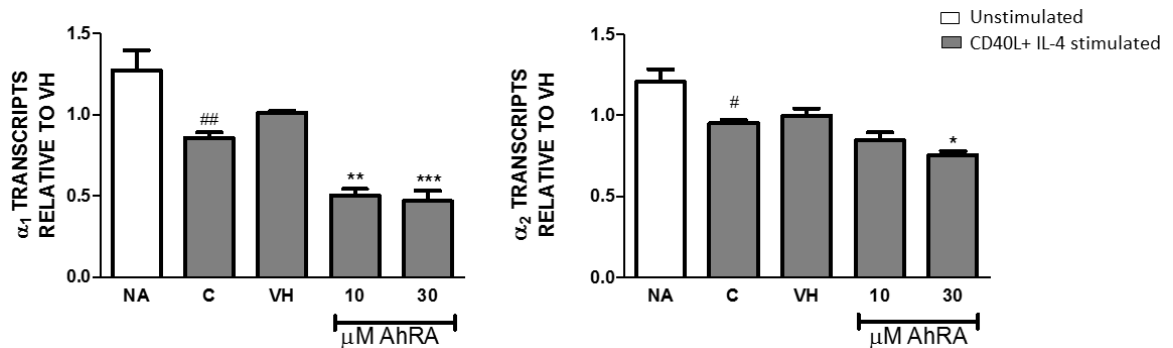


Figure 20: The AhR antagonist inhibits α germline/functional transcripts. CL01 cells were cultured for 96 hours without stimulation (naïve), or with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or AhR antagonist (10 and 30 μ M). α germline/functional transcripts were measured by real time PCR (mean SEM, n=3) and represented on y-axis as fold change relative to appropriate vehicle control. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and appropriate vehicle control were denoted by one (*), two (**), and three (***) asterisks, which represents significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively and significant differences between stimulation and NA were denoted by one (#), and two (##) hash, which represents significance at $p < 0.05$ and $p < 0.01$, respectively.

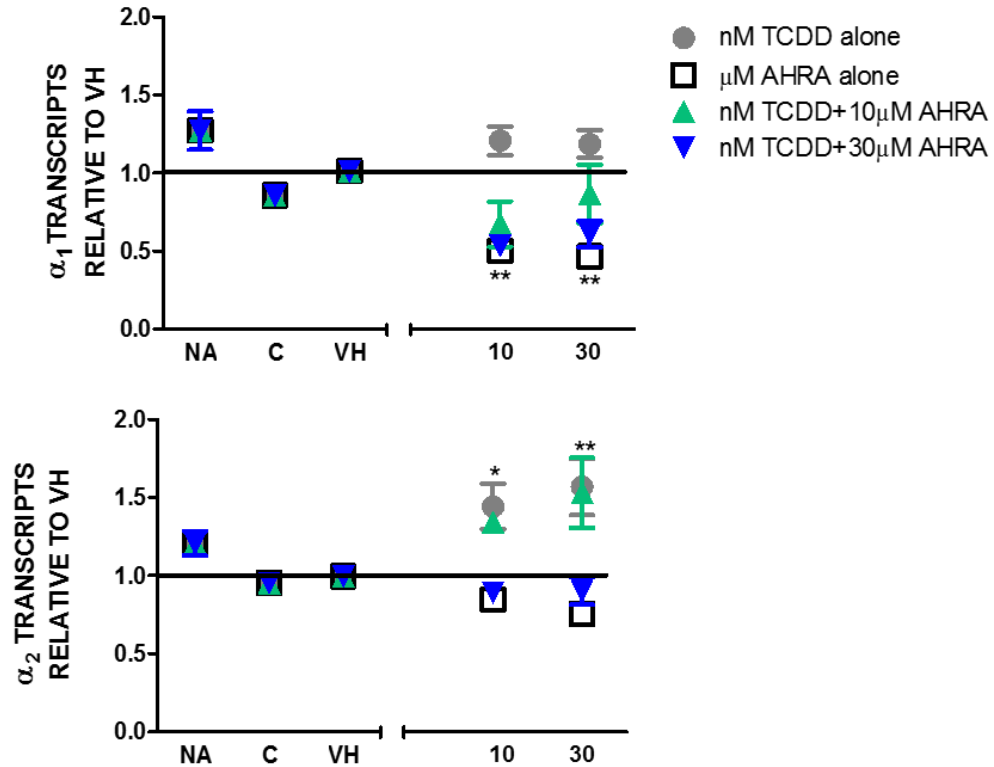


Figure 21: TCDD reverses inhibition of α germline/functional transcripts by the AhR antagonist. CL01 cells were cultured for 96 hours without stimulation (naïve), or with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30 nM) or AhR antagonist (10 and 30 μ M). α germline/functional transcripts were measured by real time PCR (mean SEM, n=3) and represented on y-axis as fold change relative to appropriate vehicle control. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison test. Gray circles represent 10 and 30 nM TCDD alone, open squares represent 10 and 30 μ M of the AhR antagonist alone, green triangles represent 10 or 30 nM TCDD with 10 μ M of the AhR antagonist and blue triangles represent 10 or 30 nM TCDD with 30 μ M of the AhR antagonist. The significant differences between treatment groups and appropriate vehicle control and differences between stimulation and NA are same as in figures 19 and 20.

TCDD inhibits CD40L and IL-4 induced de novo C_ε germline transcripts

Since we could not evaluate CSR with the above isotypes due to the fact that they are readily expressed in the absence of stimulation, we evaluated germline transcripts for C_ε and discovered no transcripts in unstimulated cells. However, CD40L plus IL-4 stimulated C_ε germline transcripts. Addition of TCDD resulted in inhibition of C_ε germline transcripts over vehicle control (Fig. 22). The AhR antagonist treatment alone increased C_ε germline transcripts levels (Fig. 23). Co-treatment of the AhR antagonist with TCDD reversed TCDD-induced inhibition but TCDD had no effect on the AhR antagonist induced increase in C_ε germline transcripts (Fig. 24).

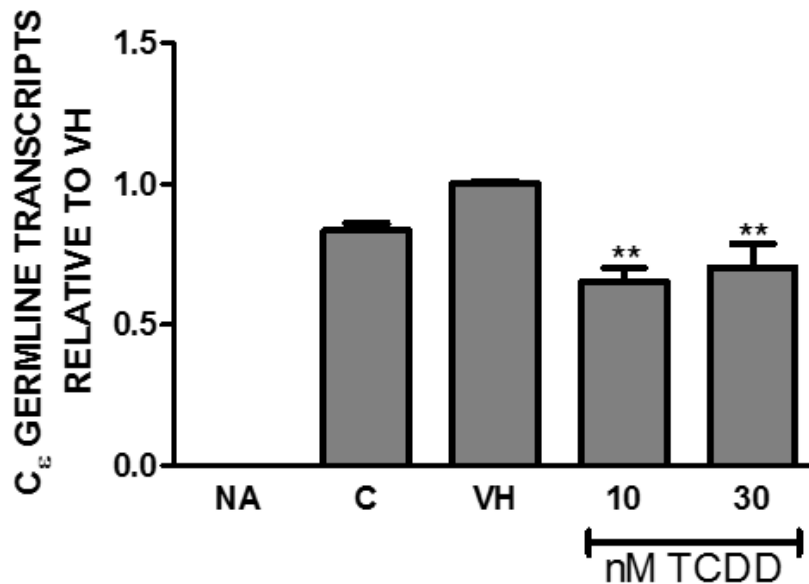


Figure 22: TCDD inhibits stimulation-induced de novo C_ε germline transcripts. CL01 cells were cultured for 96 hours □ without stimulation (naïve), ■ with CD40L and IL-4 stimulation and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30nM). C_ε germline transcripts (mean SEM, n=3) were measured by real time PCR and represented on y-axis as fold change relative to appropriate vehicle control. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and appropriate vehicle control were denoted by two (**) asterisks, which represents significance at p<0.01.

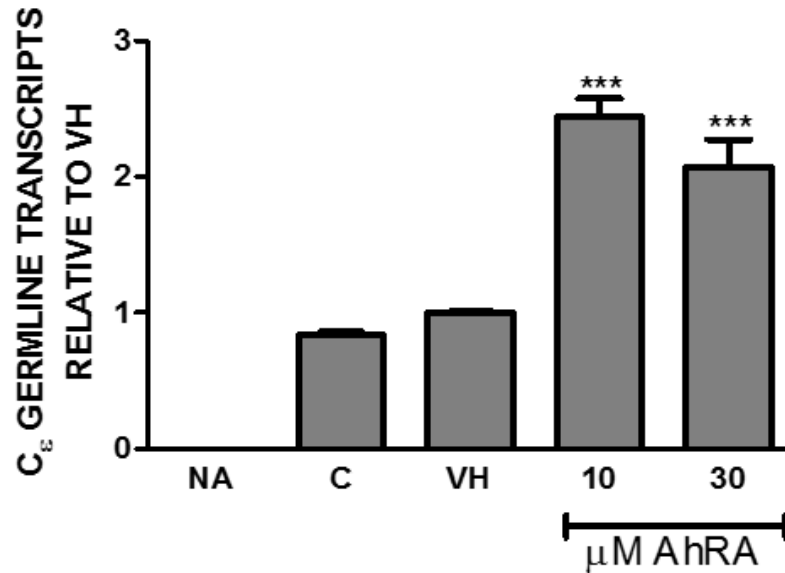


Figure 23: The AhR antagonist alone increases C_ε germline transcripts. CL01 cells were cultured for 96 hours without stimulation (naïve), with CD40L and IL-4 stimulation and treated with 0.01% vehicle (DMSO) or AhR antagonist (10 and 30μM). C_ε germline transcripts (mean SEM, n=3) were measured by real time PCR and represented on y-axis as fold change relative to appropriate vehicle control. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. Significant differences between treatment groups and appropriate vehicle control were denoted by three (***) asterisks, which represents significance at p<0.001.

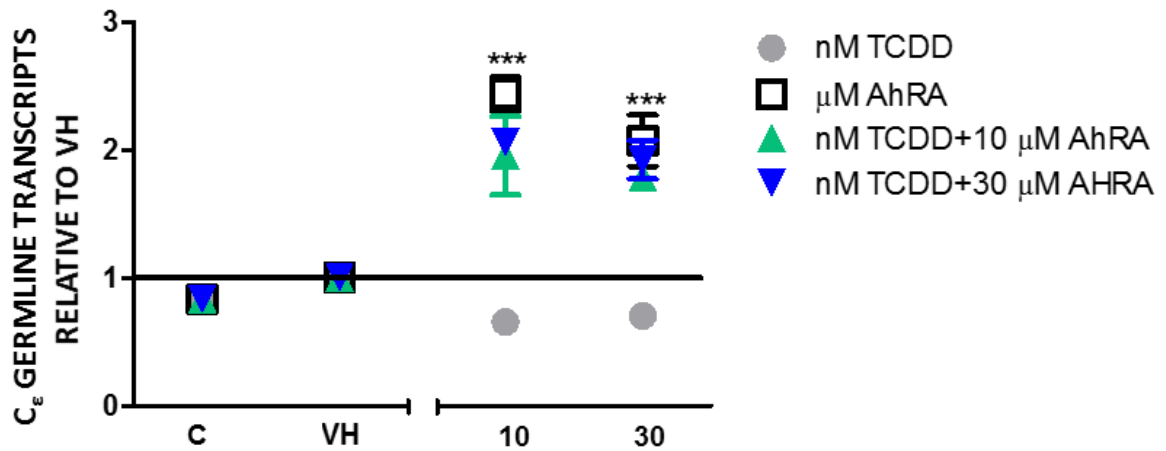


Figure 24: The AhR antagonist reverses inhibition of C_ε germline transcripts by TCDD. CL01 cells were cultured for 96 hours without stimulation (naïve), or with CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30 nM) or AhR antagonist (10 and 30 μM). C_ε germline transcripts (mean SEM, n=3) were measured by real time PCR and represented on y-axis as fold change relative to appropriate vehicle control. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison test. Gray circles represent 10 and 30 nM TCDD alone, open squares represent 10 and 30 μM of the AhR antagonist alone, green triangles represent 10 or 30 nM TCDD with 10 μM of the AhR antagonist and blue triangles represent 10 or 30 nM TCDD with 30 μM of the AhR antagonist. The significant differences between treatment groups and appropriate vehicle control and differences between stimulation and NA are same as in figures 22 and 23.

Co-treatment of stimulation with TCDD does not induce *CYP1A1* mRNA expression

A study has reported that stimulation with IL-4 increased the AhR levels and also induced low levels of *CYP1A1*. Co-treatment of IL-4 and TCDD enhanced the induction of *CYP1A1* compared to TCDD alone (Tanaka et al. 2005). In the current study, the effect of TCDD on *CYP1A1* mRNA expression with IL-4 and CD40L with IL-4 stimulation was evaluated by real time PCR and it was observed that TCDD does not induce *CYP1A1* mRNA expression (Fig. 25).

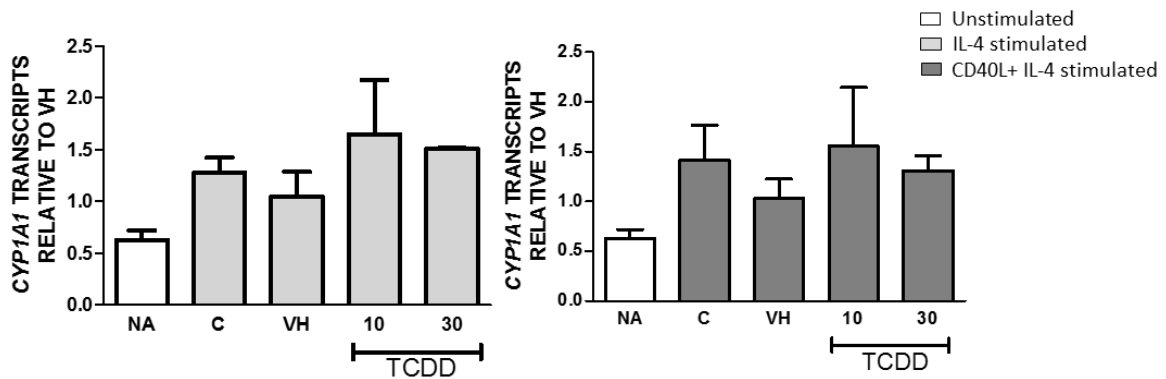




Figure 25: Lack of *CYP1A1* induction by TCDD. CL01 cells were cultured for 24 hours without stimulation (naïve),  with IL-4 alone (C) or  CD40L and IL-4 stimulation alone (C) and treated with 0.01% vehicle (DMSO) or TCDD (10 and 30 nM). *CYP1A1* transcript expression is represented on y-axis as fold change relative to appropriate vehicle control (mean SEM, n=3). Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test.

IV DISCUSSION

Previous report in mouse mature B cells has shown the inhibitory effect of TCDD on IgM secretion when stimulated with LPS and when treated with varying concentrations of TCDD (0.03-30nM) (Sulentic, Holsapple, and Kaminski 1998). In another study on primary human B cells, the effect of TCDD on CD40L plus IL-4 stimulated cells was inhibitory. Additionally, nine of twelve human donor primary B cells showed reduced IgM expression when treated with TCDD, while two donors showed no response and one demonstrated enhanced IgM expression (Lu et al. 2010). In the current study, CD40L plus IL-4, TCDD and the AhR antagonist had variable effects on IgM secretion. Similarly, R848 (TLR 7/8)-induced IgM secretion was also variable to TCDD (Data not shown). Conversely, TCDD inhibited IgG expression and in a concentration dependent manner (0.1-100nM) (Data not shown). Surprisingly, the AhR antagonist alone increased IgG expression by two-fold. Both TCDD and the AhR antagonist antagonized each other's effects on IgG secretion and also, TCDD reversed the increase observed with the addition of the AhR antagonist. These results were replicated at the transcript level supporting an effect on gene regulation.

Presence of γ_{1-4} germline/functional transcripts in unstimulated cells suggest a spontaneous class switch to IgG₁₋₄. TCDD treatment inhibited the stimulation induced γ_{1-4} germline/functional transcripts, and addition of the AhR antagonist alone significantly increased all the γ_{1-4} germline/functional transcripts. Both TCDD and the AhR antagonist

antagonized each other's effects on IgG secretion and also, TCDD reversed the increase observed with the addition of the AhR antagonist. Higher concentration (30 nM) of TCDD appears to reverse the increase observed by 10 μ M concentration of the AhR antagonist in γ transcripts. This correlates to the IgG ELISA data and confirms that the effect of TCDD on IgG secretion occurs at transcript level. Another possibility is that TCDD could decrease the stability of the transcript rather than inhibit transcription, resulting in a decrease in overall Ig expression. Additionally, NF- κ B is known to inhibit CSR to Ig $_{\gamma 1}$ in mouse B cells (Bhattacharya, Lee, and Sha 2002), and TCDD has been shown to induce the AhR binding to NF- κ B within the mouse 3'*IghRR* (Richard Salisbury, unpublished data), therefore TCDD could inhibit IgG by altering transcription factor binding within the 3'*IGHRR*. Also, differential effects of TCDD and the AhR antagonist on μ *IGH* functional transcripts and γ_{1-4} germline/functional transcripts as well as Ig expression imply a different role of the AhR in mediating TCDD-induced effects with different antibody isotypes.

In an ongoing study in our lab with shRNA-mediated AhR knockdown cells vs wild type cells with the AhR, IgM had variable effect to both TCDD and AhR antagonist in wild type and AhR knockdown cells. TCDD inhibited IgG secretion in wild type cells but AhR knockdown nearly eliminated IgG secretion in AhR knockdown cells. In contrast, when treated with AhR antagonist, both the wild type and knockdown cells expressed an increase in IgG expression (Bassam Kashgari, unpublished data). Different effects of the AhR antagonist (increase γ expression and IgG secretion) and AhR knockdown cells (decreased γ expression and IgG secretion) imply that the AhR antagonist could target signaling pathways other than the AhR. The AhR antagonist (CH223191) inhibits the AhR activation and thereby prevents its translocation into the nucleus. Since the AhR antagonist inhibits

translocation of the AhR, but potentially not the association or dissociation of the AhR with other cytosolic proteins, it is possible that the AhR antagonist influences these interactions. For example interaction of the AhR antagonist with endogenous signaling proteins like c-Src, a protein associated with the AhR activates a chain of events that lead increased Ig expression (Enan and Matsumura 1996). Alternatively, the AhR antagonist may induce off-target effects; however the concentration-dependent effect of TCDD and the AhR antagonist on γ expression suggests a competition for AhR binding.

Analysis of α_1 *IGH* functional transcripts; α_1 and α_2 germline and functional transcripts demonstrated the presence of α_1 *IGH* functional transcripts as well as α_1 and α_2 germline and functional transcripts in unstimulated cells. It is also likely that among α_1 and α_2 germline/functional transcripts, α_1 and α_2 functional transcripts are majorly being expressed compared to germline transcripts, which supports the theory that the CL-01 cells have mixed subpopulations of cells, albeit very small populations, that have undergone spontaneous CSR and express either of the IgA isotypes. Another unexpected observation was the inhibition of both α_1 and α_2 germline and functional transcript expression with stimulation, in contrast to a previous report demonstrating increased expression with stimulation (Cerutti et al. 1998). Interestingly, addition of TCDD increased α germline/functional transcripts. Also, the AhR antagonist alone inhibited both α germline/functional transcripts but α_1 transcripts were inhibited by almost 50%, and α_2 transcripts seem less sensitive to the AhR antagonist. Differences in the effects of TCDD on α_1 vs α_2 germline/functional transcripts may reflect differences in the α_1 3'*IGHRR* vs α_2 3'*IGHRR*. In CL-01 cells, the α_2 3'*IGHRR* has 4 repeats of the IS in the hs1,2 enhancer and the α_1 3'*IGHRR* has 4 repeats of the IS in the hs1,2 enhancer. This could suggest an

increase in sensitivity of the α_2 3'IGHRR to TCDD. With the presence of α_1 and α_2 heavy chain genes adjacent to the 3'IGHRR, there is a possibility that the polymorphic hs1,2 enhancer is affecting the gene transcription. In fact, higher concentrations of the AhR antagonist (30 μ M) appear to reverse the increase in α transcripts observed with TCDD (10 and 30 nM), suggesting a competitive binding to a common target (the AhR). We have previously confirmed that TCDD-mediated inhibition of IgA secretion in a mouse cell line is reversed by using an AhR antagonist or by AhR knockdown through shRNA (Wourms and Sulentic 2015). In another study, TCDD inhibited mucosal IgA secretion and this was not observed in AhR deficient mice, suggesting a role of the AhR in mediating TCDD-induced inhibition of IgA (Kinoshita et al. 2006).

Since we cannot evaluate CSR with the above isotypes due to the fact that they are readily expressed in the absence of stimulation, we evaluated germline transcripts for C_ϵ and discovered no transcripts in unstimulated cells. However, CD40L plus IL-4 stimulated C_ϵ germline transcripts. Addition of TCDD resulted in inhibition of C_ϵ germline transcripts over vehicle control. The AhR antagonist alone increased C_ϵ germline transcripts levels. The AhR antagonist reversed the TCDD-induced inhibition, but the reverse is not observed, suggesting a different mechanism is involved in mediating the effects of TCDD and the AhR antagonist. In contrast, a study on tonsillar human B cells from atopic and non-atopic patients demonstrated that TCDD elevated the levels of IgE in atopic patients, but did not alter the levels of IgE in non-atopic patients (Kimata 2003). In another ongoing study in our lab, transient transfection of the I_ϵ luciferase reporter plasmid in CL01 cells revealed basal activity of the I_ϵ promoter that was increased with CD40L and IL-4 stimulation. Addition of TCDD resulted in inhibition of the I_ϵ promoter and pre-treatment with the AhR

antagonist reversed the TCDD-induced inhibition (Zahra Alfaheeda, unpublished data). Also, evidence suggests that class switching to IgE from IgM could be an indirect switch via μ - γ - ϵ (Mills et al. 1995). This could mean that TCDD-induced inhibition of γ and ϵ could be related. But, the decreased levels of IgG in AhR knockdown cells in response to TCDD contradicts this possibility. The presence of an IL-4 response element (Schaffer et al. 2003) and DRE core motif in the I ϵ promoter region might mediate activation of the promoter by IL-4 and inhibition by TCDD, respectively, but the mechanism behind TCDD-induced inhibition on C ϵ germline transcripts is unknown.

The AhR is known to play a pivotal role in the xenobiotic metabolism through upregulation of many metabolizing enzymes, such as *CYP1A1* and the induction of *CYP1A1* mRNA is used as a biomarker for the activation of the AhR signaling pathway (Landers and Bunce 1991; Nebert and Karp 2008; Okey, Riddick, and Harper 1994; Roman, Pollenz, and Peterson 1998). The human AhR is known to have a relatively low affinity for TCDD compared to animals and requires 10-fold higher concentrations of TCDD to induce an effect (Connor and Aylward 2006; Okey, Riddick, and Harper 1994; Harper, Golas, and Okey 1988; Zeiger et al. 2001). But, human AhR has high affinity for other ligands such as indirubin [(2Z)-2,3-biindole- 2,3-(1H,1H)-dione] and quercetin [2-(3,4dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one] compared to mouse AHR (Flaveny et al. 2009) . This could explain why animals are more susceptible to TCDD compared to humans. Both human and mouse AhR are known to be polymorphic and SNPs in the transactivation domain of the human AhR have shown to result in the loss of *CYP1A1* induction by TCDD (Harper et al. 2002). A study has reported that stimulation with IL-4 increased the AhR levels and also induced low levels of *CYP1A1*. Co-treatment of IL-4

and TCDD enhanced the induction of *CYP1A1* compared to TCDD alone (Tanaka et al. 2005). In the current study, we evaluated for the effect of stimulation (IL-4 and CD40L with IL-4) and TCDD on *CYP1A1* induction in CL-01 cells. In fact, there was no upregulation of *CYP1A1* mRNA expression in response to stimulation and TCDD, suggesting that the AhR in CL-01 cells might not be functional. Upon sequence analysis of the AhR in CL-01 cells, we found that the AhR is heterozygous with a non-functional transactivation domain in one of its alleles due to 3 SNPs (P517S, R554K, and V570I) (Fig. 26 and 27) present in Exon-10. Exon-10 encodes the transactivation domain that directly regulates expression of other genes (Harper et al. 2002). Among the three SNPs mentioned before, R554K is the most common and well-studied (Harper et al. 2002). The heterozygous SNPs in the AhR of the CL-01 cells are present at nucleotides positions 2367, 2321 and 2274, respectively. The heterozygous SNPs (P517S and V570I) are primarily associated with the African-American population (Rowlands et al. 2010) and the CL-01 cells used in the current study were isolated from a Burkitt's lymphoma patient from African-American population. 3 SNPs P517S, R554K, and V570I demonstrated to impair the ability of *CYP1A1* induction by TCDD (Wong, Okey, and Harper 2001). In contrast, a study on human breast cancer cells with lower levels of AhR-554/570 expression has demonstrated that the expression of these two SNPs did not affect the transcriptional regulation of *CYP1A1* (Celius and Matthews 2010). Additionally, most polymorphisms occurring in Exon-10 don't impede the ligand binding ability of the AhR (Harper et al. 2002). Evaluation of *CYP1A1* mRNA expression in CL-01 cells in response to stimulation and TCDD demonstrated the lack of *CYP1A1* mRNA induction, suggesting that SNPs in the transactivation domain result in the loss of *CYP1A1* induction by TCDD but not affect

ligand binding ability as transactivation domain of the AhR does not regulate ligand binding. The SNPs P517S, R554K and V570I and their relation to impaired *CYP1A1* induction by TCDD could reflect the same in CL-01 cells. The ligand binding ability of the AhR is not compromised so, the effects of TCDD could either be due to the interaction of the AhR with other transcription factors or the functional allele alone could still bind to the DRE in the immunoglobulin gene to mediate TCDD's effects. Also, the number of DRE in the immunoglobulin gene are fewer compared to the DRE in the *CYP1A1* gene itself. Another possibility is that the SNP allele in the AhR acts like a dominant negative AhR in TCDD-mediated effects.

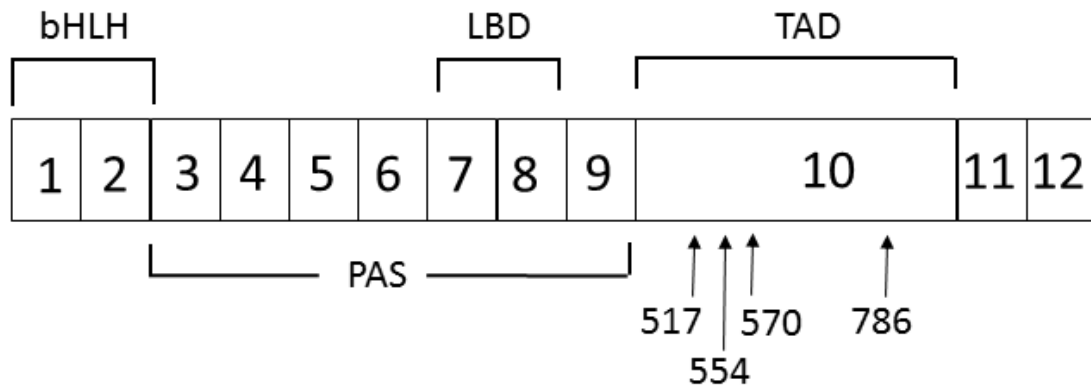


Figure 26: The human *AhR* gene with polymorphic sites. Shown is the representation of the human *AhR* gene with different domains such as basic helix–loop–helix domain (bHLH); ligand binding domain (LBD); Per/Arnt/Sim (PAS) domain and transactivation domain (TAD). 517, 554, 570 and 786 represent the SNPs in the codons in Exon-10 of the AhR.

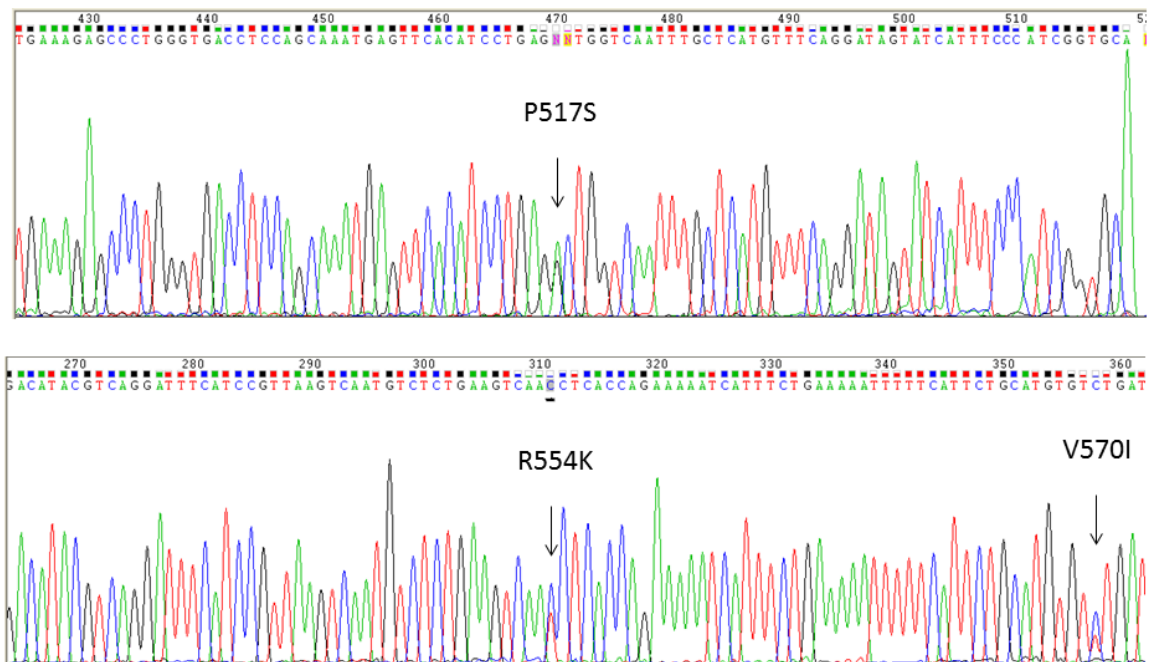


Figure 27: Chromatograph showing sequencing results of the CL-01 AhR with location of the heterozygous SNPs in the transactivation domain. Nucleotides are color coded (Adenine: green; Thymine: red; Guanine: black and Cytosine: blue) and each peak represents a nucleotide. The heterozygous SNPs are represented with double peaks in exon 10 for P517S, R554K and V570I at nucleotide positions 2367, 2321 and 2274, respectively (Bassam Kashgari).

CL-01 is a human Burkitt lymphoma cell line with *c-myc* translocated with one of the heavy chain genes. *c-myc* is generally translocated to the 3' enhancer region present downstream of the C_α gene and in all human Burkitt lymphomas the translocated *c-myc* is linked to the transcriptional regulatory regions of the *IGH* gene (Judde and Max 1992; Muller et al. 1997). However, it is unclear if the μ enhancer or one of the 3'*IGHRRs* is regulating *c-myc* expression. Animal studies strongly support the ability of 3'*IghRR* to dysregulate *c-myc*. A translocated 3'*IGHRR* will inactivate one *IGH* allele might affect the CSR and Ig expression. Though it is established that mouse 3'*IghRR* is involved in Ig expression (Sulentic, Holsapple, and Kaminski 1998), it is poorly understood if the human 3'*IGHRR* is also involved in Ig expression and CSR. Hence, translocation at any of the 3'*IGHRR* in human B cells might affect the CSR and Ig expression as well as potentially leading to an oncogenic event. Ongoing efforts in our lab to delete the entire 3'*IGHRR* (both α_1 3'*IGHRR* and the α_2 3'*IGHRR*) using the genome-editing system CRISPR could provide a permanent deletion with limited off-target effects.

We used real time PCR primers spanning the constant regions of the downstream antibody isotypes, which does not differentiate between germline and functional transcripts. Another limitation is that the results that we observed from studying the germline/functional transcripts cannot differentiate the effects of the AhR, TCDD and the AhR antagonist on post switched cells vs cells that are ready to undergo CSR. As the CL-01 cells readily express all the downstream isotypes except for ϵ , it is difficult to interpret if the AhR, TCDD or the AhR antagonist really affect CSR. For further studies, studying the AhR and ARNT binding to the DRE present in the invariant sequence of the hs1,2 enhancer in the 3'*IGHRR* through chromatin immunoprecipitation (ChIP) analysis could

help understand the mechanism of TCDD-induced effects in our cells and protein-protein interactions within the transcription factor binding sites in the 3' *IGHRR*. As the AhR in the CL-01 cells is heterozygous with one allele having a non-functional transactivation domain that regulates the gene expression but not ligand binding, there is also a possibility of other potential protein-protein interactions and cross-talk with other signaling pathways. Ongoing efforts in our lab are focused on the AhR knockout with genome-editing system, CRISPR, and this could result in the AhR with a fully functional allele in our cells. A future study involving a fully functional AhR could primarily help understand TCDD-induced effects in human Ig expression. Another possible future study could focus on studying the different AhR ligands and observe their effects on CSR as human AhR has lower affinity for TCDD, but has higher affinity for other ligands such as indirubin [(2Z)-2,3-biindole-2,3-(1H,1H)-dione] and quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one] compared to mouse AHR (Flaveny et al. 2009). This may give us a better understanding of the effect of the AhR on Ig expression and its relation to the 3' *IGHRR*.

Previous data in our lab has shown that the α_{1B} repeat of the invariant sequence in the hs1,2 enhancer is sensitive to TCDD in CL-01 cells (Abdullah Freiwan, unpublished data) and also the hs1,2 enhancer is known to be involved in many autoimmune disorders (Aupetit et al. 2000; Cianci et al. 2008; Frezza et al. 2004; Giambra et al. 2009; Toluoso et al. 2009).

In conclusion, our results suggest that TCDD has a variable effect on IgM secretion, but significantly inhibits IgG secretion, an effect reversed by addition of the AhR antagonist. Surprisingly, the AhR antagonist alone markedly increased IgG secretion above stimulation. At transcript level, TCDD has variable effects on μ *IGH* functional transcripts but significantly inhibits γ_{1-4} germline/functional transcripts and C_{ϵ} germline transcripts.

Additionally, CD40L and IL-4 stimulation induced de novo synthesis of C ϵ germline transcripts, a precursor to CSR. However, α_{1-2} germline/functional transcripts increased in response to TCDD. Notably, TCDD and stimulation had no effect on *CYP1A1* expression. Additionally, in CL-01 cells, we recently discovered SNPs in Exon-10 of the AhR, which encodes the transactivation domain that regulates expression of other genes but does not affect ligand binding. The AhR is heterozygous with one non-functional transactivation domain. Results also indicate that a small proportion of the cells have undergone spontaneous class switch to all of the γ and α isotypes rather than being induced to CSR.

As the human 3'*IGHRR* is involved in many autoimmune disorders and also in the regulation of Ig expression, the current study could elucidate the role of the AhR in Ig gene expression but also the relevance of AhR ligands and the 3'*IGHRR*.

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