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Species Differences in the Effects of TCDD on a Transcriptional Regulatory Region within the Ig Heavy Chain Gene

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SPECIES DIFFERENCES IN THE EFFECTS OF TCDD IN A TRANSCRIPTIONAL REGULATORY REGION WITHIN THE IG HEAVY CHAIN GENE

A thesis submitted in partial fulfillment

Of the requirements for the degree of

Master of Science

By

ZAHRA ALFAHEEDA

B.S., Riyadh Colleges for Dentistry and Pharmacy, 2010

2016

Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION
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Transcriptional Regulatory Region within the Ig Heavy Chain Gene BE ACCEPTED IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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Abstract

Alfaheeda, Zahra. M.S., Microbiology and Immunology Graduate Program. Wright State University, 2016. Species differences in the effects of TCDD on a transcriptional regulatory region within the Ig heavy chain gene.

The immunoglobulin heavy chain gene \((Igh)\) is regulated by numerous regulatory elements including the \(3’Igh\) regulatory region \((3’IghRR)\). Several transcription factors are involved in modulating the \(3’IghRR\) including NF-κB, AP-1, and the aryl hydrocarbon receptor \((AhR)\). The AhR is a ligand-activated transcription factor that mediates the transcription of genes involved in the metabolism of environmental toxicants such as TCDD. TCDD binds AhR and regulates immunoglobulin \((Ig)\) expression in B cells of animal models. This modulation appears to be directly mediated by binding of the AhR to dioxin response elements \((DRE)\) within the \(3’IghRR\). Structural differences have been found between the human \(IGH\) and mouse \(Igh\) genes, including a duplication of the \(3’IGHRR\) in humans and a polymorphic region in the human \(hs1.2\) enhancer. This region is sensitive to TCDD-mediated modulation in mouse models whereas in humans, several polymorphisms have been correlated with several autoimmune diseases. The objective of this study was to determine the transcriptional influence of human \(3’IGHRR\) enhancer and sensitivity to TCDD. Using luciferase reporters controlled by the \(3’IGHRR\) enhancers and cellular models including two well characterized B-cell lines: mouse CH12.LX and human CL-01. These cell lines can be induced to secrete Ig and can undergo class switch recombination. Our results suggest that, unlike mouse \(3’IghRR\), human \(3’IGHRR\) is not sensitive to TCDD. Therefore, the role of this region remains to be determined.
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List of Abbreviations

AhR: Aryl hydrocarbon Receptor

ARNT: AhR Nuclear Translocator

AHRR: AhR Repressor

BHLH/PAS: Basic Helix-Loop-Helix/Per-ARNT-Sim

BCR: B-Cell Receptor

CSR: Class Switch Recombination

Cyp1A1: Cytochrome P4501A1

C_{H}: Heavy Chain Constant Region Gene

DMSO: Dimethyl Sulfoxide

DRE: Dioxin Responsive Elements

E_{μ}: Intronic Enhancer

hs: DNase I Hypersensitivity site

IS: Invariant Sequence

_Igh_: Mouse Immunoglobulin Heavy Chain

_IgH_: Human Immunoglobulin Heavy Chain

_IgL_: Immunoglobulin Light Chain

_IgHRR_: Ig Heavy Chain Regulatory Region
Ig: Immunoglobulin

LPS: Lipopolysaccharide

MCS: Multiple Cloning Site

MHC: Major Histocompatibility Complex

NF-κB: Nuclear Factor Kappa Beta

Oct: Octamer Transcription Factor

PCDD: Polychlorinated Dibenzo-p-Dioxins

PCDF: Polychlorinated Dibenzofuran

PCB: Polychlorinated Biphenyl

Pax5: Paired Box Protein

PCR: Polymerase Chain Reaction

pVH: V_H promoter

pIγ: Intronic Gamma3 Promoter

pIε: Intronic Epsilon Promoter

SHM: Somatic Hypermutation

SNPs: Single Nucleotide Polymorphisms

SP1: Transcription Factor SP1

TCDD: 2, 3, 7, 8-Tetrachlorodibenzo-p-Dioxin
TLR: Toll-Like Receptors

$V_H$: Variable Heavy Chain Promoter
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I. Introduction

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

Dioxin is a term that describes a group of chemicals sharing similar chemical structures and mechanisms of toxicity. They are classified as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs). They bioaccumulate in adipose tissues, with PCDD and PCDF being the most resistant compounds to metabolism, which makes them a major cause of dioxin long-term effects. The most highly toxic dioxin is the polychlorinated dibenzodioxin 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) (Van den Berg et al. 1998). TCDD has been a primary contaminant in several high profile environmental exposures and it has high affinity for the nuclear aryl hydrocarbon receptor (AhR), which make it a widely studied chemical.

TCDD is not intentionally produced but is a by-product of manufacturing processes such as herbicide and plastic production and it can be transmitted through air, soil, and food (Gilpin et al., 2003). It is a colorless to white solid at room temperature and is a water insoluble compound with a chemical formula of $C_{12}H_4C_{14}O_2$ (Fig. 1). Though TCDD is considered a carcinogenic agent in humans, most of the toxicity studies have been done on animal models, (IARC, 1998). In animals TCDD causes several biological and immunological effects such as tumorigenesis, immunological dysfunction, and teratogenesis. Biological responses to TCDD are varied based on the age, dose, exposure duration, and species type (Mandal 2005). Humans exposed to TCDD appear to be less sensitive, however biological responses including carcinogenesis, liver dysfunction,
chloracne, cardiovascular diseases have been recorded (Pesatori et al., 2003, 2009; Marinkovic et al., 2010; Calvert et al., 1992; McGregor et al., 1998; Yu et al., 1997; Zober et al., 1994). In mouse models, TCDD-induced immunosuppression is mediated in AhR-dependent manner (Denison et al., 2002, 2003). In humans the effect of TCDD and the AhR on the immune system is unclear.

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

Humans can be exposed by inhalation, ingestion or dermal exposure to directly or through its by-products in food, dust, air, or smoke (Braune, 2010; Malisch 2014). In 1961-1971 during Vietnam War U.S military forces sprayed the herbicide Agent Orange, which was contaminated with TCDD causing many health problems. Another incident happened in 1976 where TCDD was released by mistake in Seveso, Italy impacting public health from minor disturbances like changes in hormones to diabetes, cardiovascular complications, and elevated incidence of certain cancers (Baccarelli 2008; Eskenazi 2010; Mocarelli 2008; Pesator 2003, 2009). In 2004 during the Ukrainian election, Viktor Yushchenko was poisoned with the second highest known dose of TCDD (108 ng/g of fat) causing
chloracne, a severe skin rash, gastritis, colitis with multiple ulcers, hepatitis, and pancreatitis (Sorg et al. 2009; Holt, 2005; Schecter et al., 2006; Sterling and Hanke, 2005). In humans, acute effects of TCDD have been reported, including chloracne, vision difficulties, nausea, porphyria, neurotoxicity, transient hepatotoxicity. Chronic exposure for TCDD results in chloracne, nail problems, vascular ocular changes, signs of neural system damage, and cancer. (McGregor 1998; Marinkovic et al., 2010).

The Aryl Hydrocarbon Receptor Signaling Pathway

The AhR is a ligand-activated transcription factor which under basal conditions is expressed as an inactive protein complex in the cytosol (Noakes, 2015). AhR belongs to the basic-helix-loop/Per-ARNT-Sim protein (bHLH-PAS) and is encoded by the AhR gene. AhR protein has been detected in high levels in several human tissues including lung, placenta and liver while the lowest expression levels were found in brain, kidney, and skeletal muscle (Manchester et al. 1987; Dolwick et al. 1993a; Shmueli et al. 2003). AhR has also been found in mouse and rabbit placentae (Tscheudschilsuren et al. 1999; Kitajima et al. 2004), as well as in liver, lung, and kidney of the mouse embryos between days 12–16 (Abbott et al. 1995). Activated AhR induced xenobiotic/drug metabolizing enzymes such as cytochrome P450 (CYP1A1), which can be used as a biomarker or as a surrogate of AhR action (Yueh et al., 2003; Bittinger et al., 2003). AhR binds either synthetic ligands, such as halogenated or aromatic to naturally produced chemicals (Schecter et al. 2006). TCDD is the most potent AhR ligand known so far, possessing the highest affinity for the receptor compared with any other ligands (Vogel et al., 1997). Beside its role in drug metabolism, AhR plays an important role in other physiological process such as apoptosis,
proliferation, differentiation and cell growth (Hu et al. 2007; Abel and Haarmann-Stemmann, 2010).

In the cytosol the inactive AhR associates with several proteins such as heat shock protein 90 (HSP90), p23 and AhR-associated 9 (also known as XAP2). HSP90 maintains the AhR in its inactive state. p23 blocks AhR from non-specific binding to ARNT (the AhR nuclear translocator) in the absence of ligand and keeps AhR in the cytosol. XAP2 binds to both AhR and HSP90 and p23 binds to HSP90 (Kazlauska et al., 1999, 2001). These protein-interactions help keeping the AhR stable (Dolwick et al., 1993; Coumailleau et al., 1995; Kazlauskas et al., 2000). The current model of AhR actions include the following steps: once TCDD binds the AhR in the cytoplasm, the XAP2 and P23 dissociate promoting a conformational change in the AhR that promote its translocation to the nucleus (McGuire J, et al., 1994). In the nucleus, the AhR dimerizes with ARNT and the complex TCDD-AhR-ARNT binds to specific DNA sequences located in target genes These sequences are called dioxin response elements (DRE, 5’-GCGTG-3’), they are located within the promoter regions of many genes (Furue 2014; Schnekenburger 2007) (Fig.2).
Figure 2. Aryl hydrocarbon receptor signaling pathway. The AhR-ligand, TCDD binds to the receptor and the AhR tranlocates from cytosol to the nucleus where it pairs with ARNT. The complex of AhR-TCDD-ARNT binds to DRE sequences within either the promoter or enhancer region of targeted genes such as CYP1A1.
The AhR signaling pathway is controlled by at least two regulatory pathways; the AhR is either degraded in the cytoplasm through the 26S proteasome or through a negative feedback loop. The negative feedback loop involves an AhR transcriptional repressor (AHRR) that competes with AhR for dimerizing with ARNT and binding to the DRE sequence. The affinity of AhRR for ARNT is higher than that for AhR thus AhRR displaces AhR from the complex AhR-ARNT (Mimura 1999; Evans et al., 2008). The AhR signaling pathway cross-talks with steroid receptors. In fact, the AhR modulates androgen, estrogen, or progesterone gene expression (Ohtake, 2008). In addition, the AhR plays important roles in mediating immunological response indirectly, such as that mediated by induction of interleukin-22 (Monteleone et al., 2011). AhR physically interacts with NF-KB contributing to in regulating immunological and inflammatory responses (Vogel, 2014; Sulentic et al., 2004a; Sulentic et al., 2004b).

AhR KO mice have variable phenotypes including in spite of being resistant to TCCD actions. These decreased count of T and B cells in spleen and skin inflammation. Deficient in liver development, portal fibrosis, defective reproductive system and vascular abnormalities (Schmidt, 1996; Fernandez-Salguero, 1995, 1997). Interestingly, mouse studies in vitro and in vivo generally show a higher response to TCDD than human cellular models likely because the mouse AhR has a 10-fold higher affinity for TCDD than the human AhR (Denison et al., 1986). The difference in responsiveness and sensitivity for TCDD between human and mouse may be attributed to the difference in AhR affinity. In humans, TCDD toxic effects are evidenced by chloracne (Connor 2006) whereas in rodents TCDD appears hepatotoxic and impacts the reproductive and immune systems in addition to promoting tumor growth and exhibiting some teratogenicity (Abbott 1999).
The Immune System

The immune system is a functional network composed of cells, organs and tissues. This network protects humans and animals from infectious organisms such as bacteria, viruses and fungi by distinguishing foreign invaders from self-tissue. There are two major branches of the immune system which, work together in a concerted manner innate immune system or non-specific immunity and adaptive or specific immunity (Parham, 2005). Innate immunity serves as a first line of defense. It consist of physical barriers (e.g. epithelial layer covering the respiratory and gastrointestinal tracts) protect from pathogen invasion; chemical barriers (e.g. the killing enzymes in sweat, mucus, tears, and saliva) help in pathogen neutralization; effector cells (phagocytes and natural killer cells); and proteins (complement system and cytokines). Once the pathogen invades the host body, the innate effector cells recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRR) expressed on the surface of innate effector cells. Pathogen recognition induces innate effector cells to secrete inflammatory mediators such as cytokines and chemokines. Chemokines secreted by macrophages recruit more effector cells such as neutrophils and monocytes from the bloodstream. If a pathogen or any foreign substance evades the innate immune system, the second line of immunity, adaptive immunity, is activated. Adaptive immunity (aka acquired immunity) is slower and more complex than the innate system. Adaptive immunity is considered antigen-specific and provides long-lasting protection. Adaptive immunity can be subdivided into cell-mediated immunity and antibody-mediated immunity. Humoral immunity is carried out by B cells, which produce immunoglobulins (Ig) while cell-mediated immunity is governed by T cells. T lymphocytes are subdivided into two major categories: cytotoxic-T cells and helper-T
cells. Naïve CD4+ T helper cells bind to a specific antigen that is presented via major histocompatibility complex class II (MHCII) on the antigen-presenting cell surface (macrophages, dendritic cells and B cells). Upon activation, CD4+ T helper cells (T_H) further proliferate and differentiate into memory, effector or regulatory T_H cells. Cytotoxic-T cells express CD8 receptors on their surface and bind to MHCI, which are found on all nucleated cell surfaces as well as as part of viral or tumor antigens. Cytotoxic-T cells recognize antigens on MHCI and destroy the cells bearing them. Generally, MHCI molecules present intracellular pathogens such as viruses whereas MHCII molecules present epitopes from extracellular pathogens such as bacteria (Parham, 2005). Overall, cell-mediated immunity and humoral-mediated immunity are linked as antigen-activated T_H cells are needed to activate B cells.

**B cells And Immunoglobulins**

Ig-secreting B lymphocytes are the major effector cells of the humoral immune system, which is responsible for antibody-mediated immunity in the adaptive immune response. They express transmembrane Igs, which are a major components of the B-cell receptors (BCR). The immature B cell stage is found in the bone marrow. B cells continue their maturation in the bone marrow or other secondary lymphoid tissues. Mature B cells that have not been exposed to antigens are called naïve. They express IgM and IgD on their surface and circulate in the bloodstream and lymph as inactive B cells. Once naïve B cells recognize specific antigens, they become activated and differentiate into antibody-secreting (plasma cells).

Naïve B cells are first activated initially through direct interactions between the BCR and antigens, then by receiving signals from T helper cells. The antigen involved in B cell
activation binds the BCR promoting its internalization. Once digested in its presented on MHC II to T cells. TCR on T helper cells interact with antigen-complexed MHCII on B cells, leading to T cell activation. Activated T-cells help activating and differentiating more B cells into plasma or memory B cells by providing co-stimulatory signals and cytokines. B cells can also be activated in a T cell-independent manner through bacteria epitopes directly interacting with the IgM on B cells. There are two types of T-independent B cell activation: Type 1 (polyclonal) B cells interact with an antigen and receive secondary signals by Toll-like receptors whereas in type 2 activation, antigens presenting multiple repeating epitopes simultaneously cross-link B cells receptors.

The B cell response to T cell signals and antigens results in clonal expansion and B-cell differentiation into antibody-secreting plasma cells that produce large amounts of soluble Ig. However, memory B cells are also produced, which mediate secondary and subsequent immune responses to the same antigen, which are immediate and far more potent than the first response due to the expression of a high affinity antibodies. (Parham, 2005). Immunoglobulins (Ig) or antibodies secreted by plasma cells are the soluble effector proteins of humoral immunity that ultimately recognize and neutralize antigens. There are five isotypes of immunoglobulins: IgM, IgD, IgG, IgE and IgA. Structurally each isotype is made up of two identical heavy chains (H) and two identical light chains (L) connected together by disulfide bonds (Fig. 3). H and L chains are composed of constant and variable regions. The variable regions are responsible for antigen-binding whereas the constant regions determine Ig isotype and function. There are two light chain isotypes: kappa (κ) or lambda (λ), which have no known function. The immunoglobulin heavy chain gene encodes the five constant region isotypes: Cμ, Cd, Cγ, Cε, and Cα for IgM, IgD, IgG, IgE,
and IgA respectively. Sub-isotypes of human IgG (IgG1, IgG2, IgG3 and IgG4) and IgA (Cα1 and Cα2 for IgA1 and IgA2) have been identified (Parham, 2005).

The immunoglobulin genes are found in encoded by three different chromosomes. In humans the heavy chain locus is located on chromosome 14 while light chain isotype loci are located on chromosome 2 (κ light chain) and 22 (λ light chain). During early lymphocyte development a process called somatic recombination or VDJ recombination happens in the bone marrow, which leads to the generation of antigen-binding sites by joining one of the V (variable) segments with one of D (diversity) and J(jointing) heavy

Figure 3: Immunoglobulin (Ig) general structure. Four polypeptide chains; (two identical heavy chains and two identical light chains connected) together by disulfide bonds. Variable regions of both heavy and light chains form the antigen binding sites and the constant regions determine the Ig function such as activating complement proteins or interacting with Fc receptors on other immune cells.
chain segments and one of V and J gene segments of light chain. This process is initiated by recombination activating genes 1 and 2 (RAG-1 and RAG-2). The first segments to recombine in the heavy chain are a D segment to a J segment and the intervening DNA sequences (other possible D and J segments) between these two segments are spliced and removed. Recombined D-J eventually joins to one of the possible V segments ending with fully rearranged VDJ DNA. The recombined DNA encodes for the variable region of the Ig heavy chain. Therefore, there are two recombination events that must occur for the heavy chain. Only one recombination (V J) is needed for the light chain. Successful transcription of the rearranged heavy chain and light chain loci results in expression of surface IgM and IgD BCR receptors. The VDJ recombination leads to producing Ig with high diversity of the antigen-binding site, allowing the Ig to interact with a wide diversity of antigens (Parham, 2005). Mature B cell interaction with an antigen eventually results in two more genetic mechanisms called isotype class switch recombination (CSR) and somatic hypermutation. CSR results in changing the B cells from IgM and IgD expression to expression of one of the five major isotypes. CSR changes the constant region of the Ig and therefore its properties, but not the variable region, whereas somatic hypermutation changes Ig affinity for its antigen (Manis et al., 2002; Rogozin et al., 1992).

**TCCD-Induced Immunological defects**

The mouse immune system is sensitive to dioxins such as TCDD. TCDD-induced immunological dysfunctions have been well-studied in rodents. Low (acute or chronic) exposure to TCDD results in thymus involution, suppression of cell- and humoral-mediated immunity and increased susceptibility to many infectious diseases (Mandal 2005; Vos
1989; Kerkvliet 2002, 2009, 2012; Holsapple et al., 1991). In mouse models TCDD suppresses humoral and cell-mediated immunity, increases tumor growth and metastasis, and causes teratogenesis, endocrine disruption, thymic atrophy, hepatic dysfunction, tumor transplant rejection, and an increase in the susceptibility to infections (Kerkvliet, 2012). Moreover, it is been found that TCDD suppress B-cell maturation differentiation and activation, therefore inhibiting Ig production (Sulentic & Kaminski, 2011).

As mentioned humans are less sensitive to TCDD immunotoxicity than rodents. This difference may be attributed to TCDD binding affinity to the AhR. Sufficient evidence supports a primary role of the AhR pathway in TCDD immunotoxicity but the mechanisms involved is remain unclear (Hansen et al., 2014). Some mice strains express high affinity AhR, and therefore are more sensitive to TCDD-immune-suppressive effects than other strains with lower AhR affinity. AhR-knockout mice (AhR-/-) resistant to TCDD immunomodulation suggesting that TCDD mediates its immunosuppressive effects through AhR (Vorderstrasse at el., 2001). However, LPS-stimulated mouse CH12.LX AhR-expressing cells and BCL-1 AhR-deficient cells showed that TCDD only inhibited IgM secretion in CH12.LX but not in BCL-1 (Sulentic 1998; Salisbury 2014; Henseler 2009). Moreover, in AhR knockdown or antagonist inhibited, TCDD had no effect on IgA-secreting mouse B cells (Wourms and Sulentic, 2015). These studies support a potential role of the AhR in mediating the inhibitory effects of TCDD on Ig and B cell development.

**The AhR and B Cells Dysfunction**

Several studies using mouse B cells suggested that TCDD-induced immunomodulation might be mediated by the AhR (Sulentic et al., 1998; Vorderstrasse at el., 2001). Mouse *Igh* locus mediates TCDD-induced suppression of mouse B cell
differentiation and maturation (Fernando et al., 2012; Sulentic et al., 1998). Within the mouse 3’IgHRR, precisely in hs1.2 and hs4, there are DRE binding sites where AhR-ARNT complex binds and TCDD induces AhR-binding (Sulentic, 2000). Mouse 3’IgHRR is a sensitive target to numerous AhR agonists including TCDD (Henseler, 2009). TCDD inhibited LPS stimulated-IgM in CH12.LX mouse B cell-line (Holsapple et al., 1986; Morris et al., 1993; Sulentic et al., 1998). Another study conducted using the CH12.LX mouse cell line expressing IgA showed the involvement of AhR in mediating immunosuppression of TCDD. In these cells, the AhR was knocked down by shRNA or inhibited by an AhR antagonist and both methods reversed the inhibitory effect of TCDD on the 3’IgHRR and on IgA secretion (Wourms and Sulentic, 2015). In addition, AhR knockdown or AhR antagonist reduced TCDD-induced inhibitory effect in IgA secretion (Sulentic and Wourms, 2015). Moreover, AhR−/− mice responded normally to TCDD or blood sheep stimulation (Vorderstrasse, at el., 2001). Thus the AhR mediates TCDD-induced inhibition of Ig expression in mouse studies.

The response of human B-cells to TCDD is variable. For instance, 9 donors had IgM suppression, 2 donors had no effect on IgM secretion and one donor showed an increase in IgM secretion (Lu et al., 2010). Interestingly, humanized mice models expressing the human AhR, CYP1A1 was induced but its level of induction differed between the human AhR and mouse AhR. These suggest that the human AhR may regulate gene expression by a different mechanism (Flaveny, 2009; Moriguchi 2003). Another study showed that the human AhR was activated selectively by omeprazole but by an unknown mechanism (Lesca 1995). Overall, the AhR role in mediating TCDD effects on mouse B
cell is relatively well defined whereas the involvement of the AhR on the human Ig remains elusive yet.

**Immunoglobulin Heavy Chain (IgH) Locus**

Immunoglobulin heavy chain locus (IGH) is located on chromosome 14 in human and chromosome 12 in mouse and in both cases it is located near the telomere on the respective short arms of the chromosomes. As mentioned before, during early B cell development and differentiation, numerous DNA assemblies and rearrangements such as V (D) J recombination, somatic hypermutation (SHM) and class switch recombination (CSR) occur to the IGH (Pinaud et al. 2011). The human IGH gene contains several regulatory regions including the variable heavy chain promoter (VH), the V D J region, the intronic enhancer (Eμ) and the heavy chain constant regions Cμ, Cδ, Cγ3, Cγ1, CΨε and Cα1, which are regulated by the α1 3’IGHRR. Another regulatory region the α2 3’IGHRR regulates Cγ2, Cγ4, Cε and Cα2 (Mills et al. 1997; Cogne and Brishtein 2004).

Transcription of the IGH gene is initiated by the VH promoter and the Eμ enhancer regulates V D J joining and enhances Cμ expression. (Cogne et al. 1994). The IGH gene is also under the control of the 3’IGHRR, which is located downstream the Eμ enhancer. This region is involved in CSR but not in V (D) J recombination (in mouse studies) (Cogne et al. 1994). Mouse IgH gene is composed of one regulatory region 3’IGHRR which regulates the constant region genes; Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε and Cα. Little is known about the role of the regulatory region in humans. Mouse and human heavy chain locus are 90% homologous; however, there are differences (Mills et al. 1997; Sepulveda et al., 2004, 2005). Mouse 3’Igh regulatory region is composed of four separate regions (hs3a; hs1.2;
hs3b; hs4) while the human 3′IGHRR contains only three regions (hs3; hs1.2; hs4). In addition, the human IgH locus contains a duplication of the 3′IGHRR and constant regions, resulting in α1 3′IgHRR and α2 3′IgHRR (Chauveau and Cogne 1996, Mills et al. 1997, Sepulveda et al., 2004, 2005) (Fig. 5).

**Human IGH locus**

**Mouse Igh locus**

**Figure 4. Comparison of the human and mouse Igh locus.** VDJ represent variable regions; μ, δ, γ, ε, α the constant regions and the transcriptional regulators are V_{H} promoter, intronic enhancer (E_μ), intronic promoters upstream of each constant region, and the 3′IgHRR including its enhancers.

**Mouse Heavy Chain (Igh) Locus**

Mouse 3′Igh regulatory region contains four regulatory elements located within DNase hypersensitivity I sites (hs): hs3b, hs1.2, hs3a, and hs4. These enhancers regulate class switch of isotypes and are B-cell specific regulators (Giannini 1993; Madisen 1994; Michaelson 1995). The hs4 enhancer shows transcriptional activity throughout B-cell development, the hs1.2 enhancer is highly active in mature and plasma B cells, and the hs3 is slightly active in activated B cells (Madisen and Groudine, 1994; Matthias and Baltimore 1993; Saleque et al., 1997; Chauveau et al., 1998). Together, the four enhancers cooperatively boost the activity of the regulatory region at all B-cell development stages.
Within the 3’IghRR there are numerous transcription factor binding sites including DRE, NFκB, Octamer, NFαP, AP-1/Ets, and Pax5. Therefore the murine 3’IghRR could be a sensitive target of several exogenous and endogenous toxic chemicals such as the AhR-ligand TCDD. TCDD induced AhR/ARNT-DRE binding within hs1.2 and hs4 enhancers. Moreover, TCDD inhibited μ heavy chain expression and IgM secretion in an AhR-dependent manner (Sulentic et al. 2000; Sulentic et al. 2004; Salisbury 2014; Wourms and Sulentic 2015). Overall, mouse 3’IghRR controls class switch recombination and modulates Ig transcription and appears to be a sensitive target of exogenous chemicals (Cogné et al., 1994; Lieberson 1995; Vincent-Fabert 2010).

A study on Eμ enhancer elucidated that deletion of this enhancer does not decrease IgH production but Eμ enhancer boosts germline V (D) J rearrangement in myeloma cell-line (Aguilera, 1985; Eckhardt and Birshtein, 1985; Perlot et al., 2005). Replacement of mouse hs1.2 enhancer with neomycin resistance gene resulted in impacting CSR from μ to γ3a, γ2b, γ3 and ε and affected germline transcription and deletion of neomycin restored CSR (Cogne 1994; Manis 1998; Bebin 2010). However, individual deletion of one of the mouse 3’IghRR enhancers exhibited minor effect on germline transcription and CSR to all isotypes except μ and γ1, while combined deletion of hs4 and hs3b decreased CSR and germline transcription, suggesting that 3’IghRR enhancers compensate for each other’s function (Pinaud 2001; Vincent-Fabert, 2009).

**Human Heavy Chain (IGH) Locus**

Human IGH gene has two regulatory regions, the α1 and α2 3’IGHRRs. Each regulatory region is composed of three distinct enhancers: hs3, hs1.2 and hs4. Compared
to the mouse 3’IghRR, the human 3’IGHRR shares 74%, 90% and 76% similarity respectively (fig4). Moreover, the human hs1.2 enhancer is polymorphic; it contains repeats of a 55 bp invariant sequences (IS) that can be repeated up to four times (alleles $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1C}$, $\alpha_{1D}$). Within the IS there are several transcription factor binding sites including DRE, NFκB, NF1, and AP-1. Unlike the mouse hs1.2 and mouse hs4, the human hs1.2 and human hs4 have no Pax5 transcription factor binding sites (Fernando et al. 2012; Denizot et al. 2001; Chen and Birshtein 1997). With increasing IS number the basal transcriptional activity of hu-hs1.2 enhancer increases (Cogné et al., 1994). The Polymorphic hs1.2 enhancer has been correlated with many autoimmune diseases such as plaque psoriasis, psoriatic arthritis, rheumatoid arthritis, coeliac disease, dermatitis herpetiformis, systemic sclerosis lupus, and IgA nephropathy. The $\alpha_{1B}$ allele (two IS repeats) has been most strongly correlated with incidence and/or severity of the above diseases (Aupetit et al. 2000; Cianci et al. 2008; Frezza et al. 2004; Giambra et al. 2009; Tolusso et al. 2009). In addition, the human 3’IGHRR is involved in some B cell malignancies such as lymphomas where a chromosomal translocation occurs between c-myc or bcl-2 and the 3’IGHRR (Heckman et al., 2003). The human hs1.2 enhancer is activated by TCDD while the mouse hs1.2 enhancer is inhibited (Fernando et al., 2012), therefore the effect of TCDD on the human 3’IGHRR role to date is not yet identified.
Hypothesis and Objectives:

TCDD is an immunosuppressant which it interferes with Ig production and B cell differentiation (reviewed by Sulentic and Kaminski 2011). Our lab has demonstrated species difference in the effect of TCDD on the hs1.2 enhancer. TCDD inhibited mouse hs1.2 enhancer activity but enhanced human hs1.2 enhancer activity. This apparent discrepancy appears related to the gene sequence rather than the species differences in the cellular model since human hs1.2 was enhanced by TCDD in both mouse B-cell line and human models (i.e. CH12.LX vs. IM-9 and CL-01, respectively data not published) (Wourms and Sulentic 2015; Fernando et al., 2012; Sulentic et al., 2004a). In the human gene, the hs1.2 enhancer is polymorphic and lacks a Pax5 binding site, which is an important inhibitory transcription factor binding sites in the mouse hs1.2 enhancer. In addition, there are two 3'IGHRRs in human IGH but there is only one in mouse. It is possible that human 3'IGHRR enhancers are functionally not related to the mouse 3'IghRR. Since the mouse 3'IghRR enhancers showed cooperative interaction of the enhancers to regulate Igh expression and CSR, we hypothesized that the human 3'IGHRR enhancers cooperatively regulate Ig transcription by regulating the V_H or intronic promoter activity and TCDD targets the transcriptional activity of one or more of these regulatory elements. Therefore, the aim of this study was to determine: 1) the cooperative effect of the human 3'IGHRR enhancers on the V_H and intronic promoters (γ3, ε) in stimulated and unstimulated B cells and 2) the effect of TCDD and the AhR on the activity of the VH and intronic promoters and these promoters regulated by the 3'IGHRR enhancers.

To test these hypotheses, we used human CL-01 and mouse CH12.LX B-cell lines. Luciferase reporter constructs driven by one of the following promoters (V_H, Iγ3, or Iε) and
one or more of the enhancers (hs4, hs3-1.2 or hs3-1.2,4) were analyzed after stimulating the cells with (TLR ligands or CD40+IL4) and treated with TCDD in the presence of absence of an AhR antagonist.

II. MATERIALS AND METHODS

Chemicals and Reagents

99.1% purified TCDD dissolved in 100% dimethyl sulfoxide (DMSO) was purchased from Accustandard Inc. (New Haven, CT). DMSO was purchased from Sigma-Aldrich (St. Louis, MO). Lipopolysaccharide (LPS, Escherichia coli) was purchased from Sigma-Aldrich (St. Louis, MO). The R848 a ligand for TLR 7 and 8 was purchased from Enzo Life Sciences and dissolved in pure DMSO. Human interleukin 4 (hIL-4) suspended in 1x sterile PBS with 10% bovine calf serum was purchased from Cell Signaling (Danvers, MA). Human Mega CD40 ligand was diluted in 100 μl sterile water and in fresh media with 10% bovine calf serum and purchased from Enzo Life Sciences (San Diego, CA). AhR antagonist (CH-223191) was dissolved in 100% DMSO and purchased from Calbiochem (Carlsbad, CA)

Cell Line Models and Tissue Culture:

The CH12LX mouse erythroleukemia derived from a B-cell lymphoma that arose in B10.H-2aH-4bp/Wts mice was used for some transfection experiments. CH12LX mouse B cell expresses IgA and 3′IghRR-regulated γ2b-transgene mini-locus. Lipopolysaccharide was used to activate this cell line. The CL-01 human B cell derived from a Burkitt’s lymphoma was used also for some transfection experiments. CL-01 human
B cell expresses surface IgM and IgD and may be specific by going through somatic hypermutation and class switch recombination processes. These features allow CL-01 B cell to be a model for in vitro studies for Human B cell differentiation and maturation (Cerutti et al. 1998).

**Reporter Plasmid Constructs**

pGL3 basic luciferase reporter plasmid (Promega, Madison, WI) was used and modified to construct luciferase reporter plasmids with different human enhancers: hs3,1.2, hs4 and hs3,1.2,4. These luciferase reporter plasmids an ampicillin resistance cassette and the luciferase gene and either variable heavy chain promoter (V\text{H}), gamma3 (γ\text{3}) or epsilon (ε) intronic promoters. (Fig 5).

![Diagram of reporter plasmid constructs](image)

*Figure 5. Human 3'IGHRR reporter plasmid constructs with either the Iγ3, Iε or V\text{H} promoters. Each plasmid contains a luciferase gene regulated by the enhancers of the 3'IGHRR and including the intervening sequence between hs3 and hs1.2.*
Electroporation Transient Transfection:

1.0 x 10^7 cell/ml of CL-01 or CH12LX cells were harvested by centrifugation at 500 x g for 5 minutes at 4°C. The pellet was resuspended with 10 μg of plasmid and fresh complete media to bring the final volume to 200 μl. The 200 μl mixture was added to a 2mm electroporation cuvette. The cells were electroporated using two different conditions corresponding to CL-01 and CH12LX: 150 V, 1500 μF, and 75 ohms, 250 V, 150 μF, and 75 ohms respectively. More than one cuvette were used and the cells were mixed, pooled and diluted to seeding concentrations of 1 x 10^5 cells/ml or 1.5 x 10^5 cells/ml for CL-01 or CH12LX correspondingly. Depending on the objective of the experiment the cells were aliquoted into 7 ml of complete media and subjected to different treatments: (naïve, NA), 0.01% DMSO vehicle (0 nM TCDD), or TCDD (30 nM) in the absence or presence of R848 (1 μg/mL) or LPS (10 nM). 2 ml of cells were aliquoted into 12-well plates. The plate(s) was/were incubated for 24, 48, 72 hours at 37°C in 5% CO₂. In the AhR antagonist experiments (AHRA), we treated as follows: (naïve, NA), 0.05% DMSO vehicle (0 nM AhR), or AhR (30 μM) in the absence or presence of R848 (1 μg/mL). Cells were pre-treated with 30 μM AhRA and incubated at 37°C in 5% CO₂ for 1 hr before adding other treatments. 24 , 48 or 72 hours later , the cells were collected by centrifugation at 500 x g for 5 minutes at 4°C and 1x reporter lysis buffer (Promega) was used to lyse the pellet. The lysates were frozen at -80°C for at least an hour. The samples were prepared for Luciferase enzyme activity reading by thawing the samples on ice or at 4°C. After the samples were thawed they were centrifuged at 14000 x g for 5 minutes at 4°C. In a glass tube 20 μl of lysate was mixed with 100 μl of luciferase substrate (Promega) and luciferase enzyme
activity was detected by a Sirius luminometer (Berthold Detection Systems, Oak Ridge, TN) and read as relative light units (RLU).

**Statistical Analysis of Data**

Data were analyzed by a one way ANOVA and Dunnett post hoc test. Data were compared to vehicle control (DMSO) and significant differences were calculated for each treatment group (n=3). “*”, “**”, “***” represent significance at p<0.05, p<0.01 and p<0.001, respectively. All results are expressed as relative light units (RLU).
III. RESULTS

Differential effects of TCDD on human 3’IGHRR enhancers in LPS-stimulated mouse CH12.LX B cells line.

Previous studies have found that TCDD inhibited LPS-induced activation of the mouse hs1.2 enhancer in the mouse CH12.LX B-cell line, while it activated the human hs1.2 enhancer in the CH12.LX B-cell line (Fernando et al. 2012). Similar results were obtained in a human B-cell line (CL-01) stimulated with R848 in that TCDD enhanced activation of each allele of the polymorphic human hs1.2 enhancer activity (Freiwat 2013, data not published). The different effects of TCDD on human and mouse hs1.2 enhancer might be due to genetic differences between the human and mouse IgH genes. Because our previous studies utilized the mouse CH12.LX B-cell line, which is a well-characterized cell line in terms of its AhR signaling pathway and similarity to primary B cells, we used these cells to allow for comparisons between mouse and human (Sulentic et al., 1998, 2004, 2000; Schneider, 2008; Williams 1996). Therefore an initial objective was to use the mouse CH12.LX cells to determine the effect of TCDD on the human 3’IGHRR and its individual enhancers (i.e. hs3, hs1.2, hs4) as well as to determine the role of the individual 3’IGHRR enhancers in influencing different IgH promoters (i.e. VH and intronic) and their sensitivity to TCDD.

One or more of the human 3’IGHRR enhancers were inserted 3’ of the luciferase gene in a pGL3 luciferase plasmid containing the VH promoter 5’ of the luciferase gene (i.e. pVH hs4, pVH hs3-1.2, pVH hs3-1.2,4) (Fig. 5). Each plasmid was transiently transfected into the CH12.LX cells by electroporation. Moreover, we inserted the
intervening sequences between the hs3 and hs1.2 enhancers but not the intervening sequences between hs1.2 and hs4 due to the size of this fragment (about 10kb, NCBI). Transfected cells were then treated with 30 nM TCDD in the absence or presence of 0.1 μg/ml LPS stimulation. LPS, a ligand for Toll-like receptor 4 (TLR-4), is a murine polyclonal B-cell activator, which drives B cells to differentiate into IgM-secreting B cells (Bucala 1992). Therefore, the effect of TCDD on different activation states was evaluated.

Similar to the effects on the mouse hs4 enhancer (Sulentic et al., 2004), after 24 hours, LPS induced hs4 enhancer activity, which was enhanced by TCDD. TCDD trended toward an increase in reporter activity in unstimulated cells (Sulentic et al., 2004) (Fig.6). Similar to hs4, LPS induced the activity of both the hs3-1.2 and hs3-1.2,4 reporters (Figs. 7 and 8). TCDD inhibited both the hs3-1.2 and hs3-1.2,4 reporters (Fig. 9), which corresponds to previous results with the mouse 3'IghRR reporters in that TCDD induced mouse hs4 activity but inhibited the activity of hs1.2 and all of the 3'IghRR enhancers in combination (Fernando et al., 2012). Our results showed time-dependent effect on hs3-1.2,4 reporter because the effect of TCDD appear after 48 hrs while after 24 hrs TCDD showed no consistency (data no shown).
Figure 6. TCDD activates the human hs4 enhancer. (A) Human pVH hs4 luciferase reporter construct. (B, C) CH12.LX cells were transiently transfected with the human pVH hs4 reporter. Transfected cells were either cultured for 24 hrs in the absence of any additional treatment (naïve) or treated with 0.01% DMSO vehicle (0 nM TCDD) or 30nM TCDD in the presence or absence of 1.0 μg/ml LPS stimulation. “C” represents the naïve or LPS control. Luciferase enzyme activity is represented on the y-axis as relative light units. (C) Represents fold change relative to vehicle control (0 nM TCDD) of (SEM ≥3). Comparisons between the treatment groups were analyzed using a one-way ANOVA. Asterisk, “**”, denotes significance compared to the corresponding vehicle control and (†) denotes significance compared to the corresponding naïve control at p<0.05 followed by a Dunnett’s Multiple Comparison post test
Figure 7. **TCDD inhibits the human hs3-1.2 enhancer.** (A) Human pV_H hs3-1.2 luciferase reporter construct. (B, C) CH12.LX cells were transiently transfected with the human pV_H hs3-1.2 reporter. Transfected cells were either cultured for 24 hrs. in the absence of any additional treatment (naïve) or treated with 0.01% DMSO vehicle (0 nM TCDD) and 30nM TCDD in the presence or absence of 1.0 μg/ml LPS stimulation. C represents the naïve or LPS control. Luciferase enzyme activity is represented on the y-axis as relative light units. (C) Represents fold change relative to vehicle control (0 nM TCDD) of (SEM ≥3). Comparison between the treatment groups were analyzed using a one-way ANOVA. Asterisk, “**”, denotes significance compared to the corresponding vehicle control and (†) denotes significance compared to the corresponding naïve control at p<0.05 followed by a Dunnett’s Multiple Comparison post test.
Figure 8. Effect of TCDD on human pVH hs3-1.2,4 enhancer activity. (A) Human pVH hs3-1.2,4 luciferase construct. (B) CH12.LX cells were transiently transfected with human pVH hs3-1.2-4 construct. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr or 48 hr. with 0.01% DMSO vehicle (0 nM TCDD) and 30nM of TCDD in the presence of 1.0 μg/ml LPS stimulation. C represents the naïve or LPS control. Luciferase enzyme activity is represented on the y-axis as relative light units. (C) Represents fold change relative to vehicle control (0 nM TCDD) of (SEM ≥2). Luciferase enzyme activity is represented on the y-axis as relative light units. Comparisons between the treatment groups were analyzed using a one-way ANOVA. Asterisk, “**”, denotes significance compared to the corresponding vehicle control and (†) denotes significance compared to the corresponding naïve control at p<0.05 followed by a Dunnett’s Multiple Comparison post test.
Differential effects of TCDD on the human 3'IGHRR enhancers in the human CL-01 B-cell line

In humans the polymorphic hs1.2 enhancer lacks Pax5 binding sites, which are present in the mouse hs1.2 enhancer and Pax5 binding strongly inhibits the mouse hs1.2 enhancer (Schneider et al., 2008). In mice, Pax5 has been identified as an essential regulator of B-cell development and a negative regulator of B-cell differentiation into antibody secretion. Moreover, transcriptional regulation of the human IGH gene or the role of the 3'IGHRR enhancers or the AhR signaling pathway is unknown. In previous studies TCDD activated the human hs1.2 enhancer in CL-01 and in IM-9 human B-cell lines but the human 3’IGHRR needs further evaluation and studies (Fernando et al., 2012). Using CL-01 human B-cell line, the effect of TCDD and cellular stimulation on the human 3’IGHRR enhancers was evaluated.

To determine the effect of TCDD on the human 3’IGHRR or its individual enhancers (hs4, hs3-1.2, or hs3-1.2, 4), luciferase reporters driven by pVH as identified above were transfected into human CL-01 B-cell. Additionally, we evaluated the ability of the human intronic enhancers pIε or pIγ3 to interact with the human 3’IGHRR. We used 6.25 µg/ml CD40L plus 50 µg/ml IL4 or 1 µg/ml R848 to stimulate CL-01, TCDD and stimulation (R848) had no significant effect on the human hs4 enhancer transfected into human CL-01 B-cell regardless of the time point (Fig. 9 and Data not shown). Our previous study demonstrated that R848 induced IgG induction but not IgM, which means R848 activates the CL-01 B cells (Brooke Johnson, data unpublished). R848 stimulation tended to inhibit hs3-1.2 enhancer activity at 48 hour incubation (data not shown, Fig 10). Co-treatment of TCDD and R848 or TCDD alone had no effect on hs3-1.2 activity at both time
points (24, 48 hours) (Fig 10). Due to a general lack of effect of TCDD and stimulation on the human hs4 alone and human hs3-1.2 enhancers, we hypothesized that the individual enhancers may work more effectively together as seen in mouse 3’IghRR. However, R848, CD40L plus IL4 or TCDD showed variable effect on the human 3’IGHRR in both time points (i.e. increase, inhibit or no effect) (Fig 11 A, B).

Because of the lack of stimulation, we decided to construct plasmids with Iγ or Iε (i.e. human intronic promoters), which are involved in promoting CSR and may more readily interact with 3’IGHRR rather than with the VH promoter (Bottaro 1997, Stavnezer 1996). In addition, the gamma3 promoter has a DRE core motif that might bind the AhR and mediate a TCDD effect. Furthermore the epsilon promoter has IL-4 binding sites, which may make the epsilon promoter more sensitive to CD40L and IL4 stimulation. No consistent effect of the stimulation (R848 or CD40L plus IL4) on hs3-1.2, 4 driven by Iγ however, an increase under stimulation has been observed in hs3-1.2, 4 driven by Iε. Additionally TCDD had no effect on on hs3-1.2, 4 driven by Iγ but hs3-1.2, 4 driven by Iε showed refractory effect (Fig 13 A, B).
Figure 9. Effect of TCDD on human hs4 enhancer. (A) CL-01 cells were transiently transfected with human pVH hs4 construct. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr or 48 hr with 0.01% DMSO vehicle (0 nM TCDD) and 30nM of TCDD in the presence of 1.0 μg/ml R848 stimulation. C represents the naïve or R848 control. Luciferase enzyme activity is represented on the y-axis as relative light units. B represents fold change relative to vehicle control (0 nM TCDD) of (SEM ≥3). Luciferase enzyme activity is represented on the y-axis as relative light units. Comparisons between the treatment groups were analyzed using a one-way ANOVA.
Figure 10. Effect of TCDD on human pVH hs3-1.2 enhancer activity. (A) CL-01 cells were transiently transfected with human pVH hs3-1.2 construct. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr or 48 hr. with 0.01% DMSO vehicle (0 nM TCDD) and 30nM of TCDD in the presence of 1.0 μg/ml R848 stimulation. C represents the naïve or R848 control. Luciferase enzyme activity is represented on the y-axis as relative light units. B represents fold change relative to vehicle control (0 nM TCDD) of (SEM ≥3). Luciferase enzyme activity is represented on the y-axis as relative light units. Comparisons between the treatment groups were analyzed using a one-way ANOVA. Asterisk, “***”, denotes significance compared to the corresponding vehicle control and (†) denotes significance compared to the corresponding naïve control at p<0.05 followed by a Dunnett’s Multiple Comparison post test.
Figure 11. Effect of TCDD on human \( \text{pV}_{\text{H}} \text{hs3-1.2,4 enhancer activity} \). (A) CL-01 cells were transiently transfected with human \( \text{pV}_{\text{H}} \) hs3-1.2-4 construct. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr or 48 hr. with 0.01% DMSO vehicle (0 nM TCDD) and 30nM of TCDD in the presence of 1.0 \( \mu \)g/ml R848 stimulation. C represents the naïve or R848 control. Luciferase enzyme activity is represented on the y-axis as relative light units. (B) Represents fold change relative to vehicle control (0 nM TCDD) of (SEM ≥3). Luciferase enzyme activity is represented on the y-axis as relative light units. Comparisons between the treatment groups were analyzed using a one-way ANOVA.
Figure 12. Effect of TCDD on human pIγ3 hs3-1,2,4 pGL3 luciferase construct. (A) Transfected CL-01 cells were stimulated with either 1.0 μg/ml R848 or 6.25 μg/ml CD40L plus 50 μg/ml IL4 and incubated 24, 48 hours. (B) Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr or 48 hr. with 0.01% DMSO vehicle (0 nM TCDD) and 30nM of TCDD in the presence of 1.0 μg/ml R848 or 6.25 μg/ml CD40L plus 50 μg/ml IL4 stimulation. C represents the naïve, R848 or CD40L plus IL4 control. Luciferase enzyme activity is represented on the y-axis as relative light units. (C) Represents fold change relative to vehicle control (0 nM TCDD) of (SEM ≥3).Luciferase enzyme activity is represented on the y-axis as relative light units. Comparisons between the treatment groups were analyzed using a one-way ANOVA. Asterisk, “***”, denotes significance compared to the corresponding vehicle control and (†) denotes significance compared to the corresponding naïve control at $p<0.05$ followed by a Dunnett’s Multiple Comparison post test.
Figure 13. Effect of TCDD on human pIε hs3-1.2,4 pGL3 luciferase construct. (A) CL-01 cells were transiently transfected with human pIε hs3-1.2-4 construct. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 48 hr or 72 hr. with 0.01% DMSO vehicle (0 nM TCDD) and 30nM of TCDD in the presence of stimulation 6.25 µg/ml CD40L plus 50 µg/ml IL4. C represents the naïve or CD40L plus IL4 control. Luciferase enzyme activity is represented on the y-axis as relative light units. (B) Represents fold change relative to vehicle control (0 nM TCDD) of (SEM ≥3). Luciferase enzyme activity is represented on the y-axis as relative light units. Comparisons between the treatment groups were analyzed using a one-way ANOVA. Asterisk, “***”, denotes significance compared to the corresponding vehicle control and (†) denotes significance compared to the corresponding naïve control at p<0.05 followed by a Dunnett’s Multiple Comparison post test.
Effect of AhR antagonist on 3'IGHRR in CL-01 human B-cells

Since we believe that AhR mediates TCDD effects in animals, we want to elucidate its role in a human model. Experiments with AhR antagonist (AhRA) have been done to study the functionality of AhR receptor in CL-01 human B cells. Our lab found that co-treatment of stimulation (CD40L plus interleukin 4) with AhRA increased the secretion of IgG while TCDD inhibited IgG secretion (Burra, 2015 data not published). CL-01 cells transfected with reporters containing human hs3-1.2, 4 enhancers and driven by either V_H, γ3 or ε promoters were treated with AhR antagonist. The pV_H hs3-1.2-4 reporter showed no consistent effect of AhRA at any time point (Fig 14 A). Cells transfected with the pIγ3 hs3-1.2-4 plasmid showed an increase in activity with AhRA and TCDD co-treatment had no effect on the reporter and TCDD alone has no effect as well (Fig 14 B). In the plasmid driven by Iε promoter there was an increase in activity with stimulation, which is similar to what we found in our lab in pervious study (Burra, 2015, data not published). However, AhRA alone trended toward an increase in activity and TCDD co-treatment with AhRA did not affect the activity (Fig14 C). There was an increase in the activity with AhRA alone in some of the data (Fig 14 C).
Figure 14. AhR antagonist effect on the human hs3-1.2,4 luciferase construct. CL-01 cells were transiently transfected with either human (A) pVH hs3-1.2, 4, (B) pγ3 hs3-1.2, 4 or (C) pε hs3-1.2, 4 constructs. Transfected cells were either cultured in the absence of any additional treatment (naive, NA) or treated for 48 hr with 0.01% DMSO vehicle (0 nM TCDD) and 30nM of TCDD in the presence of stimulation 6.25 µg/ml CD40L plus 50 µg/ml IL 4. “C” represents the naive or CD40L plusIL 4 control. Data were normalized to DMSO (vehicle) treated with the stimulation.
To evaluate the role of the 3’IGHRR and to elucidate the transcriptional activity of each promoter, a construct with only the promoter and driven by luciferase gene was constructed for each promoter and compared to its corresponding plasmids with hs3-1.2, 4 enhancers (i.e. pVH vs pVH hs3-1.2,4, and pIε vs pIε hs3-1.2,4). Enhancerless reporter activity was lower than the reporters driven by the 3’IGHRR indicating that the 3’IGHRR mediates the effects of treatments. As shown in (Fig 15 A, B) TCDD, stimuli, or AhRA treatment did not influence enhancerless reporter activity.

**Figure 15. Treatment effect on basic pGL3 and human hs3-1.2,4 pGL3 luciferase constructs.** (A) Luciferase reporter activity driven by Vh promoter alone or with Vh promoter and 3’IGHRR. (B) Luciferase reporter activity driven by Iε promoter alone or with Iε promoter and 3’IGHRR. In both cases, transfected CL-01 cells were treated with 30nM of TCDD and 10 μM in the presence of stimulation 6. 25 μg/μl CD40L plus 50 μg/μl IL4.
Discussion

TCDD targets the heavy and light chains of mouse Igh locus and therefore suppressing mouse Ig expression (Holsapple et al., 1991b; Kerkvliet, 2002). Although in animal models AhR mediates TCDD inhibition of Ig expression the exact mechanism is not fully understood (Sulentic et al., 1998). Mouse Igh is regulated by several elements including the 3’Igh regulatory region (3’IghRR) and numerous transcription factors. The mouse 3’IghRR is composed of four enhancers (hs3b, hs1.2, hs3a, and hs4). Previous studies in our lab suggest that the mouse 3’IghRR is a sensitive target of TCDD and TCDD induced AhR binding to DRE sites within the mouse hs1.2 and hs4 enhancers (Sulentic et al., 2000; Sulentic et al., 2004; Salisbury 2015; Wourms and Sulentic 2015). Additionally, AhR antagonist and AhR-knockdown studies proved the involvement of the AhR in mediating the inhibitory effect of TCDD on Ig expression and 3’IghRR activation (Vorderstrasse, et al., 2001; Wourms and Sulentic 2015). However, due to the differences between the mouse and human Igh, it is unclear if these results in mouse models can be translated to humans. The human 3’IGHRR is duplicated (α1 3’IGHRR and α2 3’IGHRR) and contains only three enhancers (hs3, hs1.2 and hs4). Moreover, the human α1 hs1.2 enhancer is polymorphic in human, which means a 55 bp invariant sequence (IS) can be repeated up to four times (α1A, α1B, α1C, α1D). Within this invariant sequence there are several essential transcription factors including DRE, NF-κB, SP-1, NF-1 and AP-1. Additionally, the hs1.2 polymorphism is associated with many autoimmune disorders such as IgA nephropathy, celiac disease, systemic sclerosis, plaque psoriasis, psoriatic arthritis, dermatitis herpetiformis, and rheumatoid arthritis.
Our lab has identified functional differences between the human and mouse hs1.2 enhancer. TCDD inhibited the mouse hs1.2 enhancer activity while it induced the human hs1.2 enhancer in the mouse CH12.LX B cell-line. This dichotomy appeared specific to the gene sequence rather than the species differences in the cellular model since the human hs1.2 was enhanced by TCDD in both mouse and human models (i.e. CH12.LX vs. IM-9 and CL-01, respectively). Beside the polymorphism in the human hs1.2 enhancer, which is not present in the mouse, the mouse hs1.2 enhancer, but not human, has Pax5 binding sites, which play an important role in mouse B cell development and negatively regulate the hs1.2 enhancer (Fernando et al. 2012; Denizot et al. 2001; Chen and Birshtein 1997). A previous study in our lab found that the invariant sequences contribute to the human hs1.2 activation and the AhR mediates TCDD-induced activation in mouse cell line (Salisbury 2015). Additionally, our lab has identified that the NF-1 binding site, which exists in human gene but not in mouse, may mediate the effect of TCDD on the human hs1.2 enhancer (Figure 16). Mutation in the NF-1 binding site in the human hs1.2 enhancer decreased TCDD-induced activation. Also, other studies have shown a direct interaction between Sp1, NF-κB, or AP-1 and the AhR/ARNT complex (Salisbury 2015; Kobayashi et al., 1996). Moreover, a previous study on the different alleles of the human hs1.2 enhancer suggested this enhancer may work as a negative regulator of the human 3’IGHRR and Ig expression (Freiwan 2014). A previous study in our lab showed that inserting the IS had no influence on the transcriptional activity induced by TCDD, which may support the hypothesis that TCDD effect may be mediated by not only the 3’IGHRR enhancers but with the help of other transcription factors (Fernando 2012, Freiwan 2014 data not published).
Taken together we suggest that the human hs1.2 enhancer may effectively work in cooperation with other enhancers (hs3, and hs4). Therefore the aim of this study was to elucidate the role of each enhancer of the 3’IGHRR alone or together in enhancing the V\textsubscript{H} or intronic promoters. Results in this study showed different responses of human enhancers to TCDD between human and mouse cell-lines. The CH12.LX showed more sensitivity to TCDD in all enhancers as well as the stimulation where LPS activated all the enhancers. By contrast in the human CL-01 cell-line all enhancers with either the V\textsubscript{H}, I\gamma\textsubscript{3} or I\varepsilon exhibited less responses to TCDD or stimulation. However, other results in our lab on CL-01 cell-line showed that CD40L plus IL4 stimulated the cells and TCDD had different effects on IgM, IgG, and IgE secretion. Moreover, CD40L plus IL4 and TCDD had variable effects on \mu transcript levels, while CD40L plus IL4 increased \gamma\textsubscript{1-4} and C\varepsilon germline and functional transcripts. TCDD co-treatment with CD40L plus IL4 inhibited \gamma\textsubscript{1-4} and C\varepsilon germline and functional transcripts (Kaulin Burra 2015 data not published). In a study, an

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**Figure 16.** Showing the differences in transcription binding sites within the polymorphic human hs1.2 enhancer and mouse hs1.2 enhancer.

Adapted from Fernando, 2012
inhibition of the human hs1.2 enhancer when CL-01 cells were treated with a combination of CD40L and IL4+ IL10, same effect found with mouse hs1.2 enhancer transfected into human cell-line. Mutations of some of the critical transcriptional motifs within the human hs1.2 enhancer did not reverse the inhibitory effect of the CD40L alone or IL4+IL10; however these mutations lowered the enhancer activity. (Bernstein 2004). Additionally, CD40L and IL-4 increased mouse hs1.2 enhancer activity in mouse splenic B cell (Grant et al., 1996; Stevens et al., 2000). There is a possibility that an indirect mechanism or pathway is mediating the inhibitory effect of CD40L, IL4 and IL10.

In addition, our constructs lack the intervening sequences (this sequence is about 10kb, which makes it difficult to include it in our reporter, NCBI) between the human hs1.2 and hs4 enhancers, which may play a critical role in 3’IghRR function. In 3’IghRR-deficient-mice SHM was strongly affected but no effect on light chain. Deleting the intervening sequences between mouse hs3a and hs1.2 in the presence of hs3b and hs4 resulted in reducing SHM rate by four fold comparing to wild type mouse, and presence of hs3a and hs1.2, while left part of intervening sequences were deleted, sustained SHM at intermediate level. Presence of all enhancers, regardless of the palindromic structure, was enough for CSR to occur. However, SHM and CSR do not need the 3’IgHRR presence in plasma cell, which means that the 3’IgHRR controls SHM and CSR by two different regulatory mechanisms. This suggests that 3’IghRR enhancers cooperatively regulate SHM and the intervening sequences contribute to regulating IgH expression. This study may imply the importance of including the intervening sequences to maintain SHM as well as to keep 3’IghRR in 3D transcriptional configuration (Saintamand, 2016). In addition, human and mouse hs1.2 enhancers show high sequence conservation of most functional
motifs, however, differences in some binding sites (Pax5 in mouse hs1.2 enhancer) and the polymorphism in human hs1.2 enhancer may explain the contrary results between both models (Mills et al., 1997). Perhaps there is an involvement of other factors and pathways in regulating Ig expression. An ongoing effort by our lab using CRISPR, which is a gene-editing technique, to delete one or more of the transcription factor binding sites within the polymorphic human hs1.2 enhancer may provide insight understanding the mechanism of Ig expression inhibition (Snyder, unpublished data). Since many studies have been done on mouse and the role of the 3’IgHRR is well known, focusing on the human 3’IGHRR intervening sequences is needed to elucidate the mechanism of 3’IGHRR in regulating human Ig expression.

The AhR mediates TCDD toxicity on the mouse 3’IghRR, however, the mechanism is unclear in humans. For more investigation, AhR antagonist (AhRA) has been used in our lab to test if TCDD has an effect on the human 3’IGHRR enhancers and whether the AhR is involved in mediating this effect. AhR antagonist competes with dioxin binding to the AhR binding site, therefore antagonist blocks AhR activation and translocation (Kim et al., 2006). A previous study in our lab on CL-01 cell-line showed that IgM response to AhRA was refractory to TCDD and AhRA (data not published). Surprisingly, AhRA increased IgG secretion in human CL-01 B cell by two fold and TCDD co-treatment antagonized the AhRA effect. These results suggest differences in sensitivity of the IgM and IgG to TCDD and AhRA (Burra 2015, data not published). Additionally, AhRA induced γ1-4 germline and functional transcripts. Moreover, using shAhR, a lentiviral vector plasmids, which targets AhR mRNA, we knockdown the AhR. The AhR-knockdown cells showed opposite result from AhRA on IgG secretions these results suggest that AhR is indirectly affecting
IgG expression and secretion (Kashgari 2015, unpublished data). This increase in IgG secretion may be mediated through AhRA by activating signaling proteins in the cytosol, which are associated with the AhR. Since AhR is kept in the cytosol as inactive transcription factor by binding to several proteins, targeting AhR may release AhR-associated proteins therefore making them free to interact through other signaling pathways. In previous study in our lab, AhRA increased human IgG transcript. AhRA alone increased Cε germline transcripts while TCDD co-treatment reversed TCDD-induced inhibition to Cε transcripts (Burra 2015 unpublished data). In our current study AhRA trended to stimulate the human-pIε-hs3-1.2,4, however co-treatment with TCDD had no effect or it increased the activity of the hu-pIε-hs3-1.2,4.

Our lab has found (Kashgari, 2015 data not published) that our CL-01 cells are AhR heterozygous for two SNPs (P517S and V570I) within the AhR transactivation domain on exon-10 (Rowlands et al. 2010). Sequencing analysis showed that one allele of CL-01 cells, isolated from a Burkitt’s lymphoma patient from African-American population, has non-functional transactivation domain at nucleotides positions 2367 and 2274. Exon-10 encodes the transactivation domain that directly regulates expression of other genes (Harper et al. 2002). Those two SNPs (P517S and V570I) are associated with only African-American population (Rowlands et al. 2010). These SNPs have been found to abrogate CYP1A1 induction by TCDD (Wong et al., 2001). Taken together, our result shows general lack of sensitivity to TCDD, which may reflect AhR polymorphism, or the lack of the 3D conformational shape of the gene. Another cell-line or modified CL-01 cell with functional AhR must be used for further investigation and more understanding of AhR mechanism. In addition more studies with AhRA would give more knowledge about the role of AhR in
humans especially on the human 3’IGHRR. Focusing more on AhR signaling pathway in human may provide insight to pharmaceutical development. Indeed, revealing the role of AhR in Ig expression and TCDD immunomodulation effect would expand our knowledge on some autoimmune diseases related to the polymorphic human hs1.2 enhancer and will give more understanding toward autoimmune diseases cure. In addition, knowing the exact AhR signaling pathway would provide insight to where environmental toxicants may target. Moreover, understanding the role of the human 3’IGHRR on CSR and SHM in human cellular model is important for more understanding of many immune-related diseases such as lymphomas since the human 3’IGHRR is involved in protoncogenes chromosomal translocation. However, our cell line is expressing one functional AhR allele, which limits our work. A cell line with two transcriptionally active AhR allele must be used to test the effect of TCDD on human enhancers. In addition, using different human B cell line with specific characteristics representing normal human B cells may provide better understanding for the human 3’IGHRR and the effect of TCDD on it. Testing the effect of TCDD on different B cell lines would reveal the actual effect of this dioxin on B cell development. An ongoing work in our lab on U266 B cell line, which is a human B cell line isolated from myeloma may add more knowledge about B cells sensitivity to dioxin in different stages of B cell development. Since both CL-01 and U266 are isolated from lymphomas, using normal cell line is necessary to elucidate involvement of TCDD in promoting cancer and to confirm if TCDD affect healthy cells and cancerous cells in the same way. Generally, more effort in using a characterized B cell line and constructing reporters that mimic the human 3’IGHRR must be done for better understanding.


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