TCDD-Induced Modulation of the HS1,2 Enhancer within the 3'Immunoglobulin Heavy Chain Regulatory Region

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TCDD-INDUCED MODULATION OF THE HS1,2 ENHANCER WITHIN THE 3’ IMMUNOGLOBULIN HEAVY CHAIN REGULATORY REGION

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

By

THARU M. FERNANDO
B.S., Wright State University, 2007

2009
Wright State University
WRIGHT STATE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

June 26, 2009

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Tharu M. Fernando ENTITLED TCDD-induced modulation of the hs1,2 enhancer within the 3’ immunoglobulin heavy chain regulatory region BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Fernando, Tharu M. M.S., Department of Pharmacology & Toxicology, Wright State University, 2009. TCDD-induced modulation of the hs1,2 enhancer within the 3’ immunoglobulin heavy chain regulatory region.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent environmental toxin that inhibits immunoglobulin (Ig) gene expression in mice. Transcriptional regulation of the Ig heavy chain (IgH) involves the 3’IgH regulatory region (3’IgH RR). The murine 3’IgH RR consists of four enhancers (hs3A, hs1,2, hs3B, and hs4), which are homologous to the human 3’IgH RR enhancers (hs3, hs1,2, and hs4). In humans, a polymorphism of the hs1,2 enhancer, resulting in a varying number of tandem repeats of a 53 bp sequence, has been correlated with autoimmune diseases like IgA nephropathy and Celiac disease. The repeated sequence contains a κB and DRE binding site. Previous studies have shown that TCDD inhibits the murine 3’IgH RR but activates the hs4 enhancer in a well-characterized mouse B-cell line, CH12.LX. Therefore, the objective of the current study was to determine if TCDD inhibits the murine 3’IgH RR by repressing hs1,2 enhancer activity and if this effect will be mirrored by the human polymorphic hs1,2 enhancer in the CH12.LX model. Using transient luciferase studies and CH12.LX cells that stably express a transgene under the regulation of the hs1,2/hs3A enhancer pair, we have found that indeed the mouse hs1,2 enhancer is inhibited by TCDD in LPS-induced B cells. However the human hs1,2 undergoes a striking activation after TCDD treatment, much
like the murine hs4 enhancer. These results suggest a difference in transcriptional regulation between the mouse and human hs1,2 sequence. Mutational analyses determined that DRE, κB, AP-1, and Oct binding motifs found within the human hs1,2 enhancer act in concert to mediate TCDD-induced activation of the human polymorphic hs1,2 enhancer. Since TCDD represents a large class of chemicals found in the environment, diet, and pharmaceuticals, understanding chemical-induced modulation of the 3’IgH RR enhancers may provide a clue to the etiology of certain autoimmune diseases.
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I. INTRODUCTION

2,3,7,8-tetrachlorodibenzo-\(\rho\)-dioxin

The term dioxin encompasses a host of toxic chemicals that are bioaccumulative and share similar structure and mechanism of action. These halogenated aromatic hydrocarbons include polychlorinated dibenzo dioxins (PCDDs), polychlorinated dibenzo furans (PCDFs), and polychlorinated biphenyls (PCBs). All of these chemicals are persistent environmental contaminants, which are formed as a by-product during many industrial processes and the combustion of organic materials in the presence of chlorine (i.e. waste incineration, herbicide/pesticide manufacturing, and paper bleaching). The general population is exposed to dioxins through food, drinking water, soil, dust, smoke, and air (for review, Mandal, 2005). 2,3,7,8-tetrachlorodibenzo-\(\rho\)-dioxin (TCDD) is the most potent and intensively studied of all the dioxins (Fig. 1). TCDD was the primary toxic contaminant found in Agent Orange, a pesticide used in the Vietnam War, in addition to being the basis for evacuations of the Love Canal at Niagara Falls, NY, Times Beach, MO, and Seveso, Italy. Due to these well-publicized events of human exposure, dioxins have initiated over thirty years of research. In animal studies, toxicities that have resulted from TCDD exposure include thymic atrophy, immune dysfunction, hepatic damage and steatosis, embryonic teratogenesis, endocrine disruption, and tumor promotion (for review, Mandal, 2005). With the exception of chloracne and the induction of xenobiotic metabolizing enzymes, the effects of TCDD in humans are still
unclear. However, many of the effects produced by TCDD and other dioxins are thought to be mediated via binding to a high affinity cellular protein, the aryl hydrocarbon receptor (AhR).

Figure 1. Chemical structure of 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin.
Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) is a ubiquitously expressed, cytosolic, ligand-activated receptor, which belongs to the basic helix-loop-helix/Per/ARNT/Sim (PAS) family of heterodimeric transcriptional regulators. The PAS protein family plays a significant role in the regulation of development and controls a number of physiological processes including circadian rhythm, neurogenesis, metabolism and the hypoxic stress response (for review, Ma et al., 2009). The AhR is the only member of the PAS family that is known to be activated by xenobiotics (i.e. TCDD, PCBs, and other polycyclic aromatic hydrocarbons). However, recent discoveries suggest that natural chemicals, dietary metabolites, and synthetic drugs are also AhR ligands (Denison and Nagy, 2003). In the absence of ligand, the AhR is complexed in the cytosol with two HSP 90 chaperone molecules, p23, and XAP2. Upon ligand binding, the AhR becomes activated, translocates to the nucleus where it then dissociates from HSP90/p23/XAP2, and forms a heterodimer with the AhR nuclear translocator (ARNT). This AhR/ARNT complex is now able to bind to specific consensus regulatory sequences (5’-A/TNGCGTG-3’) referred to as dioxin response elements (DREs) and modulate gene transcription (Fig. 2) (for review, Ma et al., 2009; Tian, 2009). The AhR signaling pathway has been primarily characterized in the induction of the Phase I xenobiotic-metabolizing enzyme, cytochrome P450 1A1 (CYP1A1). CYP1A1 induction does not directly correlate with TCDD-induced toxicity. However, DRE-like sites are present in the promoter regions of several other genes, including a number of cytokines and transcription factors (Lai et al., 1996), supporting the possibility of AhR-mediated transcriptional regulation of these genes. In addition, the AhR has also been shown to interact with other transcriptional...
regulators, including NF-κB, Sp1, transcription factor IIB, TATA binding protein, SRC-1, and the retinoblastoma protein (for review, Tian, 2009; Hankinson, 2005), potentially regulating gene transcription through a DRE-independent pathway.

**Figure 2.** The AhR signaling pathway.
The Immune System and B cells

The immune system is a collection of biological processes that act in concert to protect the body against foreign substances. It can detect a number of non-self agents referred to as antigens which include bacteria, viruses, parasites, and any non-self proteins. The immune system is divided into two arms: innate immunity and adaptive immunity. Innate immunity is the body’s first line of defense against invading organisms and infection, providing an immediate, nonspecific response with anatomical, humoral, and cellular barriers to infection. In addition to mounting an initial immune response, the cellular components of the innate immune system can also activate the machinery of the adaptive immune response. Adaptive immunity is an antigen-specific defense mechanism that allows the body to recognize and remember specific pathogens and mount a stronger, quicker response each time the pathogen is encountered.

Adaptive immunity is divided into two branches according to which cell type mediates the response: cell-mediated immunity or humoral immunity. Cell-mediated immunity is primarily mediated by the activation of antigen-specific T cells and is most effective at defending against fungi, protozoans, cancers, and intracellular bacteria. On the other hand, humoral immunity is mediated by the production of antigen-specific antibodies or immunoglobulins (Ig), which are secreted by differentiated B cells (plasma cells). In the adaptive immune response, circulating antigens are recognized by T cells, B cells, and dendritic cells in the secondary lymphoid organs. Dendritic cells engulf antigen and present it on surface major histocompatibility complex II (MHC-II) molecules, which are then recognized by a T cell through its T-cell receptor (TCR) that is specific to that particular antigen. Upon recognition, the T cell is stimulated by self-
produced interleukins to proliferate, creating thousands of clones specific to the original antigen (clonal expansion) and then differentiating into effector cells. A B cell can recognize a T-cell dependent antigen by its respective antigen-specific receptor, the B-cell receptor (BCR), which consists of a membrane-bound Ig molecule complexed with Igα and Igβ chains. Once bound, the antigen is engulfed and presented by MHC-II molecules on the B-cell surface. B-cell activation can only occur after interaction with a T cell that has undergone processing of the same antigen (as described above). B cells can also recognize T-cell independent antigens like lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, through other cell surface receptors (i.e. toll-like receptors) or BCR cross-linking. Once T-cell independent antigens are bound, B cells can undergo activation without T-cell interaction. After B-cell activation occurs, B cells can then differentiate into Ig-secreting plasma cells. Circulating Ig can react with antigens and mediate their clearance by activating the complement pathway and recruiting nonspecific immune cells to destroy and remove the antigen.

Ig molecules have two identical heavy chains and two identical light chains, which are connected to each other through disulfide bonds. Each chain can be further divided into variable and constant regions. The variable region determines antigen specificity through a recombination event that links different variable, diversity, and joining regions (VDJ). The constant region determines the Ig class or isotype. There are five major isotypes of Ig, IgM, IgD, IgG, IgE, IgA, which are defined by their respective heavy chain constant regions ($C_H$, $C_\mu$, $C_\delta$, $C_\gamma$, $C_\varepsilon$, $C_\alpha$ (Fig. 3). Naïve B cells express surface IgM and IgD, which act as BCRs. After encountering antigen, some B cells can
migrate to secondary lymphoid tissues and form germinal centers, i.e. clusters of rapidly dividing activated B cells. While undergoing proliferation, their Ig genes undergo somatic hypermutation (SHM), and clonal selection occurs to subsequently select clones with the highest affinity BCR to the original antigen. In addition to affinity maturation, activated B cells can undergo class switch recombination (CSR), which allows the B cell to change the Ig isotype it is expressing. During CSR, the variable region (and antigen specificity) of the Ig gene is kept constant. However certain constant regions in the heavy chain locus are removed from the chromosome at switch regions located upstream of each constant region with the exception of Cδ. DNA flanking the deleted regions are then rejoined to allow for a functional Ig gene. Therefore a B cell expressing IgG will not have Cμ and Cδ; a B cell expressing IgE will not have Cμ, Cδ, and Cγ, and so forth.

**Regulation of Murine Immunoglobulin Heavy Chain Production**

The V\textsubscript{H} promoter, upstream of the variable region, initiates transcriptional regulation of the Ig heavy chain (IgH) gene. The intronic enhancer, E\textsubscript{μ}, which lies between the variable region and Cμ, plays an essential role in VDJ recombination and can also contribute to increased IgH transcription. However, a number of mouse myeloma cell lines lacking E\textsubscript{μ} demonstrated normal levels of functional heavy chain mRNA (Klein et al., 1984; Aguilera et al., 1985; Eckhardt and Birshtein, 1985). Moreover, a natural deletion of a downstream sequence of the C\textalpha gene resulted in a drastic decrease in IgH transcription in a mutant myeloma cell line that still retained E\textsubscript{μ} (Gregor and Morrison, 1986). These studies led to the discovery of a regulatory region downstream of the IgH locus: the 3’IgH RR.
The 3′IgH RR was first discovered 25 kb downstream of the rat IgH locus (Pettersson et al., 1990) and then later found 13 kb downstream of the mouse IgH locus (Dariavach et al., 1991). The mouse 3′IgH RR has at least four DNase I hypersensitive sites (hs), hs1,2; hs3A; hs3B; and hs4, contained in a 40 kb DNA segment and thought to act as a locus control region (Madison and Groudine, 1994). The mouse hs3A and hs3B are virtually identical and flank the hs1,2 enhancer, making up a very large (25 kb) palindromic sequence (Saleque et al., 1997; Chauveau and Cogne, 1996). The fourth and most distal enhancer is the hs4, which lies 4 kb further downstream of the hs3B enhancer (Michaelson et al., 1995). The murine hs1,2, hs3A, and hs3B enhancers are DNase I hypersensitive and together transcriptionally active only in mature B cells and plasma cells (Dariavach et al., 1991; Madisen and Groudine, 1994; Saleque et al., 1997). Of the three enhancers, the hs1,2 is the most transcriptionally active in mature B cells and plasma cells while the hs3 enhancers individually have no activity in pre-B cells, B cells, or plasma cells (Matthias and Baltimore, 1993; Madison and Groudine, 1994; Saleque et al., 1997; Chauveau et al., 1998). On the other hand, the mouse hs4 enhancer appears to function throughout B-cell development, being active in pre-B cells and plasma cells (Madisen and Groudine, 1994). However maximal synergistic activation occurred when all four enhancers were together in all stages of B-cell development (Madisen and Groudine, 1994; Ong et al, 1998; Stevens et al, 2000). Additionally, Eμ enhanced the activity of the four murine enhancers in combination in pre-B cells and B cells (Chauveau et al., 1998).

Because the four mouse enhancers are transcriptionally active at different stages of B-cell lineage, it has been suggested that they may have distinct functions at the
various stages of B-cell differentiation. The hs4 may have a greater influence in pre-B cells, being the only active enhancer element of the 3’IgH RR at that stage, and play a significant role in VDJ recombination. The hs1,2 enhancer may have the greatest influence in plasma cells, having the highest activity levels of all the 3’IgH RR enhancer domains, and possibly influencing increased IgH production and class switch recombination. In addition, the hs1,2 and hs4 enhancers in the mouse appear to be differentially regulated by the same transcription factors. B-cell specific activator protein (BSAP, Pax5), nuclear factor κB (NF-κB), and Octamer (Oct) binding result in positive regulation of hs4 activity in mature B cells (Michaelson et al., 1996). In contrast, BSAP prevents NF-αP (PU.1) binding to the αP site and is thought to cause the NF-κB and Oct motifs to exert a repressive influence on mouse hs1,2 activity (Michaelson et al., 1996; Singh and Birshtein, 1996). BSAP maintains B-cell lineage at the earlier stages of B-cell development (i.e. pro-B cells, pre-B cells, mature B cells) while repressing B-cell differentiation into plasma cells. Downregulation of BSAP expression levels are imperative for increased Ig secretion and further B-cell differentiation (Neurath et al., 1994; Reimold et al, 1996; Rinkenberger et al, 1996; Roque et al, 1996; Usui et al, 1997). This correlates well with the increase in mouse hs1,2 enhancer activity in plasma cells and the role reversal of NF-κB and Oct, which can function has activators of hs1,2 enhancer activity (Michaelson et al., 1996; Singh and Birshtein, 1996).

While the dichotomy between the murine hs1,2 and hs4 enhancers support differential functions during the course of B-cell differentiation, several studies (Manis et al., 1998; Morvan et al., 2003; Zhang et al., 2007) have implied a possible functional redundancy for each individual enhancer. While initial studies have shown that the
hs3B/hs4 enhancer pair was essential for SHM of an associated transgene (Terauchi et al. 2001), Morvan and colleagues (2003) determined that the hs4 enhancer was dispensable for VDJ recombination of the endogenous IgH locus. In addition, initial studies implicated the murine hs1,2 enhancer in CSR when its replacement with a Neomycin resistance gene drastically inhibited CSR to certain Ig isotypes (Cogne et al., 1994). However, later studies with a cleaner deletion of the hs1,2 showed that it too was dispensable for IgH transcription and CSR, and Neomycin replacement likely interfered with the interaction of the other enhancer elements (Manis et al., 1998). However, collectively the 3’IgH RR seems to be critical for IgH transcription and CSR (Lieberson et al., 1995; Pinaud et al., 2001; Gregor and Madison, 1986; Shi and Eckhardt, 2001; Cogne et al., 1994). In addition, while the functional effect of an individual enhancer deletion in the 3’IgH RR can be overcome with the presence of the other enhancers, it is still possible for the greatest transcriptionally active enhancer to have the most influence at a particular stage of B-cell development.

The Human IgH Locus

Because the murine 3’IgH RR has been implicated in IgH transcription and CSR, there has been considerable interest in finding how homologous regions in humans might regulate Ig expression. The human IgH locus appears to have greater complexity when compared to the mouse IgH locus. Humans have more subclasses of human IgG (IgG1, IgG2, IgG3, IgG4) and IgA (IgA1, IgA2) due to increased C_H regions (Fig. 3). In addition, some kind of duplication event occurred in the evolution of the human IgH locus resulting in two γ−γ−ε−α segments, and therefore two 3’IgH RRs are located
downstream of $C_{\alpha 1}$ and $C_{\alpha 2}$ (Fig. 3). The human 3’IgH RR is comprised of 3 enhancer elements (hs3, hs1,2, and hs4), which are respectively 74%, 90%, and 76% homologous to core segments within each mouse 3’IgH RR enhancer. In both $\alpha 1$ and $\alpha 2$ 3’IgH RRs, the hs1,2 enhancer element resides near the center of a smaller palindromic sequence (10 kb) with only one hs3 enhancer located upstream of the hs1,2 and the hs4 domain lying 4 kb downstream (Mills et al, 1997; Chen and Birshstein, 1997; Pinaud et al, 1997). The human 3’IgH RR enhancers share the same expression pattern and synergistic activation as seen in the mouse (Chen and Birshtein, 1997; Mills et al, 1997; Pinaud et al, 1997; Chauveau et al, 1998). However, some notable sequence differences do exist. The human hs1,2 enhancer lacks both BSAP binding sites and one NF-$\alpha$P motif (Mills et al., 1997; Chen and Birshstein, 1997), both of which are important to mouse hs1,2 regulation (Michaelson et al, 1996; Singh and Birshtein, 1996). The human hs4 enhancer lacks one of the two Oct motifs and the only BSAP binding site in the mouse enhancer (Mills et al., 1997; Chen and Birshtein, 1997), which are also significant to mouse hs4 regulation (Michaelson et al., 1996). It appears the murine hs1,2 and hs4 enhancers share a dichotomous relationship, due in part to the diverging effect of BSAP regulation (Michaelson et al, 1996; Singh and Birshtein, 1996). However if a similar dichotomy exists between the human hs1,2 and hs4 enhancer is still unclear given the absence of BSAP and other central binding motifs in both enhancers.

Interestingly in humans, a polymorphism of the hs1,2 enhancer in the $\alpha 1$ 3’IgH RR has been correlated with several autoimmune diseases including IgA nephropathy, Celiac disease, systemic sclerosis, and psoriasis (Aupetit et al., 2000; Frezza et al., 2004, 2007; Cianci et al., 2008). This polymorphism results in a varying number of 53 bp
sequences and yields three alleles (α1A, α1B, and α1C) which contain one, two, or three 53 bp sequences, respectively. Increased frequency of the α1B allele has been correlated with an increased severity of disease progression (Aupetit et al., 2000; Frezza et al., 2004, 2007; Cianci et al., 2008). Moreover, increases in serum Ig were found to be significantly higher in IgA nephropathy and Celiac disease patients carrying the α1B allele (Denizot et al., 2001; Frezza et al., 2004). Using transient luciferase reporters, Denizot et al. (2001) has shown increases in basal transcriptional activity with each additional 53 bp sequence. Included in this 53 bp sequence are a number of transcriptional regulatory binding sites (i.e. DRE, κB, and AP-1), which closely resemble the binding sites found in the mouse hs1,2 enhancer (Fig. 4). Additionally, these polymorphisms are expressed at a considerably high frequency in the general population (Giambra et al., 2005; Giambra et al., 2006). In addition to the human polymorphic hs1,2 enhancer, chromosomal translocations involving the human 3’IgH RR and the proto-oncogenes, c-myc and bcl-2, have been implicated in a number of B-cell lymphomas (van Ooteghem et al., 1994; Wang and Boxer, 2005).
**Figure 3.** Schematic of the IgH gene locus of the human and mouse. \( V_H \), variable heavy chain promoter; \( E_\mu \), intronic or \( \mu \) enhancer; open rectangles, germline promoters upstream of each heavy chain constant region.
Figure 4. Binding motifs present in the human polymorphic hs1,2 and mouse hs1,2 enhancer.
TCDD-Induced Immunological Effects

The immune system is one of the most sensitive targets of TCDD, yielding immunotoxicity at low levels of exposure in animal models (Holsapple et al., 1991; Kerkvliet, 2002). TCDD suppresses both the innate and adaptive immune response and inhibits cell-mediated and humoral immunity. Animals exposed to TCDD underwent thymic atrophy, were more susceptible to infection, were unable to reject transplanted tumors, and had an increased incidence of tumor growth and metastasis (Holsapple et al., 1991; Kerkvliet, 1995, 2002, 2009). T-cell suppression is also thought to occur after TCDD exposure, possibly by the induction of T-regulatory cells, which has a negative influence on T-helper cells (Quintana et al., 2008). In addition to suppressing T-cell function, reconstitution-separation studies have shown that TCDD directly targets the B cell (Dooley and Holsapple, 1988). Due to a definite structure activity relationship (SAR), the use of AhR nonresponsive/nonexpressing mouse cell-lines (BCL-1), and the use of AhR nonresponsive (DBA/2) and knock-out mice, many of TCDD’s immunotoxic effects are thought to be mediated by the AhR (Poland and Glover, 1980; Holsapple et al., 1991; Kerkvliet, 1995, 2002, 2009, Sulentic et al., 1998, 2004b). Interestingly, T cells, B cells, and macrophages have been shown to increase AhR expression after activation (Kerkvliet, 2002). These results are consistent with the fact that many of the immunotoxic effects by TCDD can only be detected after stimulation with antigen (Kerkvliet, 2002). However TCDD-induced suppression of the human immune system remains uncertain.
AhR and B-cell Dysfunction

The murine B cell is a sensitive and direct target of TCDD. TCDD inhibits mouse B-cell proliferation, differentiation, and Ig secretion (Dooley and Holsapple, 1988). Studies with AhR knock-out mice have also determined a role for the AhR in TCDD-induced suppression of B-cell differentiation and the sheep red blood cell (sRBC) antibody response (Vorderstrasse et al., 2001). Additionally, using SAR, a mouse B-cell line that expressed high levels of AhR (CH12.LX), and a mouse B-cell line that was AhR-deficient (BCL-1), Sulentic and coworkers (1998, 2000) demonstrated an AhR-dependent inhibition of LPS-induced IgM secretion by TCDD. TCDD has been shown to inhibit both Ig heavy chain and light chain mRNA expression (Yoo et al., 2004), and Sulentic et al. (2000) demonstrated that TCDD-induced inhibition of μ heavy chain expression was AhR-dependent. Moreover, TCDD-induced binding of the AhR nuclear complex to DRE-like sites in the two most transcriptionally active murine 3’IgH RR enhancer elements, the hs1,2 and hs4. These results suggested that the 3’IgH RR, a regulatory region critical for IgH transcription and CSR, might be a possible transcriptional target of the AhR signaling pathway, and the 3’IgH RR may mediate the inhibitory effects on heavy chain expression by TCDD. Indeed, the murine 3’IgH RR was markedly inhibited by TCDD in LPS stimulated CH12.LX cells (Sulentic et al., 2004a). However, the exact mechanism behind TCDD-induced inhibition of 3’IgH RR was still unclear. Transient luciferase studies have shown that the mouse hs4 was profoundly activated by TCDD, contrasting the TCDD-induced inhibition of the 3’IgH RR activity (Sulentic et al., 2004a). Activation of the mouse hs4 by TCDD appeared to be mediated by an overlapping DRE and κB binding motif found within the enhancer
(Sulentic et al., 2004a/b). It has been postulated that the murine hs1,2 may then mediate the inhibitory effect of TCDD on 3’IgH RR activity although this has yet to be confirmed. More importantly, TCDD-induced modulation of the human immune system remains unclear.

**Significance and Objectives**

Previous research has deemed the murine 3’IgH RR a sensitive target of TCDD. However, the exact mechanism remains unclear. TCDD has been demonstrated to activate the mouse hs4 enhancer, which contradicts the inhibitory effect of TCDD on LPS-induced murine 3’IgH RR activity. Therefore, we hypothesize that TCDD-induced inhibition of mouse 3’IgH RR activity is mediated by the hs1,2 enhancer, and due to the similarities between the mouse and human hs1,2 enhancers, TCDD also modulates human polymorphic hs1,2 activity. Therefore, one objective of the current study was to determine if TCDD inhibits the murine 3’IgH RR by repressing hs1,2 enhancer activity using transient luciferase studies and a transgenic cell line. Another objective of the study was to determine if this effect will be mirrored by the human polymorphic hs1,2 enhancer in the mouse CH12.LX B-cell line model. The current study will provide the foundation for future studies aimed at identifying the molecular components (i.e. role of the AhR signaling pathway) involved in TCDD-mediated 3’IgH RR modulation. The 3’IgH RR not only plays a major role in Ig expression, but it is also associated with many diseases including Burkitt’s lymphoma, IgA nephropathy, and Celiac disease. Since TCDD represents a large class of chemicals found in the environment, diet, and pharmaceuticals, understanding chemical-induced modulation of the 3’IgH RR enhancers
may provide a clue to the etiology of certain diseases and lead to the discovery of novel therapeutic targets.
MATERIALS AND METHODS

Chemicals and Reagents

TCDD, in 100% DMSO, was purchased from AccuStandard Inc. (New Haven, CT). The certificate of product analysis stated the purity of TCDD to be 99.1%, as determined by AccuStandard using gas chromatography/mass spectrometry. Dimethyl sulfoxide (DMSO) and lipopolysaccharide (LPS, *Escherichia coli*) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Line Model

The CH12.LX murine B-cell line and/or variants of this line were utilized in all experiments. The CH12.LX cell line was derived from the murine CH12 B-cell lymphoma (Arnold et al., 1983), which arose in B10.H-2*b*H-4*b*/p/Wts mice (B10.A x B10.129). CH12.LX cells have been characterized by Bishop and Haughton (1986) and were generously donated by Dr. Geoffrey Haughton (University of North Carolina, Chapel Hill, NC).

Modified versions of the CH12.LX cell line were generated by stably transfecting a γ2b mini-locus, which was developed and generously provided by Dr. Laurel Eckhardt (Hunter College, New York, NY). The CH12.γ2b-3*IgH* cell line, generated and characterized by our lab (Wright State University, Dayton, OH), is a variant of the CH12.LX cell line that stably expresses one copy of the γ2b mini-locus under the
regulation of the 3'IgH RR. This mini-locus also has loxP sites that flank the hs3B/hs4 enhancer pair. The enhancer deletion derivative cell line, CH12.γ2b-Δhs3B4, was generated by transiently transfecting the CH12.γ2b-3'IgH cell line with a GFP-CRE recombinase expression vector (also provided by Dr. Eckhardt). 18 hr after transfection, GFP-expressing cells were sorted and isolated by Fluorescent Activated Cell Sorting in the Core Flow Cytometry Facility at Cincinnati Children’s Hospital Medical Center (Cincinnati, OH). Cells were subcloned by limiting dilution into 96-well culture plates and evaluated for CRE-mediated deletion of the hs3B/hs4 enhancer pair by PCR.

All cell lines were grown in RPMI 1640 medium (Mediatech, Inc., Manassas, VA) supplemented with heat-inactivated 10% bovine calf serum (HyClone Laboratories, Logan, UT), 13.5 mM HEPES, 23.8 mM sodium bicarbonate, 100 units/mL penicillin, 100 µg/ml streptomycin, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol. Cells were maintained at 37 °C in an atmosphere of 5% CO₂.

**PCR Analysis of CRE-Mediated Enhancer Deletion**

For determining CRE-mediated deletion of the hs3B/hs4 enhancer pair, DNA was isolated from GFP-CRE transfected clones, using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich). Primers were generated using Primer3 Software (http://fokker.wi.mit.edu/primer3/input.htm) and were specific for the following: primer set 1 consisted of a forward primer that bound within the 3’ region of the hs3B and a reverse primer that bound within the 5’ region of the hs4; primer set 2 consisted of a forward primer that bound upstream of the loxP site flanking the hs3B and a reverse
primer that bound within the 5’ region of the hs3A (Fig. 6). PCR reactions were performed with HotMaster Taq DNA polymerase kit (5 Prime, Gaithersburg, MD). Briefly, 100 ng of DNA was mixed with 10x HotMaster Taq buffer with Mg²⁺, 10 mmol of dNTP mix, 10 pmol of both forward and reverse primers, and 0.5 µL Hot Master Taq to a final volume of 50 µL in thin-walled PCR tube. The samples were heated to 94 °C for 2 min and cycled 40 times; each cycle consisted of 94 °C for 20 s, 55 °C for 15 s, and 65 °C for 30 s. PCR products were visualized by electrophoresis in a 2% agarose gel stained with ethidium bromide.

**Protein Isolation for γ2b Analysis**

CH12.γ2b-3’IgH and CH12.γ2b-Δhs3B4 cell lines were divided into treatments at 3.13 x 10⁴ cells/mL. Cells were then treated with LPS, TCDD, and/or vehicle (0.01% DMSO) and then aliquoted in triplicate into a 12-well plate for 48 hr. After the incubation period, cells were centrifuged at 3,000 rpm, washed with 1x PBS, and then lysed with mild lysis buffer (NP-40, 1M NaCl, 0.1 NaPO₄, 0.5M EDTA) containing freshly added protease inhibitors (Complete Mini Pro tease Inhibitor Cocktail, Roche Applied Sciences, Indianapolis, IN). Samples were immediately frozen at -80°C. Cell lysates were thawed on ice, centrifuged at 14,000 rpm, and supernatants were collected for analysis. Protein concentrations were determined by a Bradford assay (Bio-Rad Laboratories, Hercules, CA), and samples were then diluted to the lowest sample concentration and 1 µg of total protein was analyzed for γ2b by ELISA.
**Enzyme Linked Immunosorbent Assay (ELISA)**

Cell lysates were analyzed for γ2b by sandwich ELISA, as described previously (Sulentic et al., 2000). Colorimetric detection was performed every minute over a 1 hr period using a Spectramax plus 384 automated microplate reader with a 405-nm filter (Molecular Devices, Sunnyvale, CA). The SOFTmax PRO analysis software (Molecular Devices) calculated the concentration of γ2b in each sample from a standard curve generated from the kinetic rate of absorption for known γ2b concentrations.

**Reporter Plasmids**

Human luciferase reporter plasmids were generously provided by Dr. Michel Cogné (Laboratoire d’ImmunoLogic, Limoges, France) and included the enhancerless variable heavy chain promoter (V_{H}) and the α1A, α1B, and α1C human polymorphic hs1,2 plasmids. The human hs1,2 plasmids contained the V_{H} promoter 5’ of the luciferase gene and the human hs1,2 enhancer with either one (α1A), two (α1B), or three (α1C) 53 bp repeats 3’ of the luciferase gene, as described previously (Denizot et al., 2001) (Fig. 5). Mouse luciferase reporter plasmids were kindly provided by Dr. Robert Roeder (Rockefeller University, New York, NY) and consisted of the enhancerless mouse V_{H} and the hs1,2 plasmids. The mouse hs1,2 consisted of the V_{H} promoter upstream and the mouse hs1,2 enhancer downstream of the luciferase gene, as previously described (Ong et al., 1999; Stevens et al., 2000) (Fig. 5). All reporter plasmids were constructed using a pGL3 basic luciferase reporter construct (Promega, Madison, WI).
Figure 5. Schematic of the human and mouse luciferase reporter constructs. All luciferase reporter plasmids originated from pGL3 basic (Promega) and contained a variable heavy chain (VH) promoter. Similar to the IgH locus, the human and mouse hs1,2 enhancers were inserted 3' of the luciferase gene. Asterisk, "*", denotes the number of a 53 bp sequence repeated in the polymorphic alleles of the human hs1,2 enhancer.
Site-Directed Mutagenesis

Site-directed mutagenesis was performed on the α1A human hs1,2 luciferase reporter construct according to the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Oligonucleotide primers (Table 1) were designed to delete the DRE, κB, Oct, and 53 bp sequence (containing the DRE, κB, and AP-1 binding motifs) using the QuikChange Primer Design Program (http://www.stratagene.com/sdmdesigner). Briefly, 10 ng of the α1A human hs1,2 luciferase reporter was mixed with 5 μL 10x reaction buffer, 125 ng of each oligonucleotide primer, 1 μL of dNTP mix, 3 μL of Quik Solution, and 1 μL of PfuTurbo DNA polymerase to a final volume of 50 μL in thin-walled PCR tubes. The samples were heated to 95 °C for 1 min and cycled 18 times; each cycle consisted of 95 °C for 50 s, 60 °C for 50 s, and 68 °C for 6 min (1 min/kb). After 18 cycles, a final extension step occurred at 68 °C for 7 min. Following temperature cycling, the samples were treated with Dpn I to digest the parental DNA template. The resulting DNA plasmid containing the desired deletion was then transformed into XL10-Gold Ultracompetent cells, isolated, and validated by sequencing (Retrogen, Inc., San Diego, CA).
<table>
<thead>
<tr>
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<th>PRIMER 1</th>
<th>PRIMER 2</th>
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<td>5'-GAG CAG CTT GCC</td>
<td>5'-GGG GGG GGG GGC</td>
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<tr>
<td></td>
<td>GGA CTC GTC GGA CCG GTCGACG GCC GCC TCC GCC GGC</td>
<td>GGCC GGC GGG GGG GGG GGG GGG GGG</td>
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<tr>
<td>u3 deletion</td>
<td>5'-GGG GGG GGG GGC</td>
<td>5'-GGG GGG GGG GGC</td>
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<tr>
<td></td>
<td>AGA CCC CTT CCC CCC CTC ACCCT GTG GCC GGC</td>
<td>TCT GGG GGG GGG GGG GGG GGG GGG</td>
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<td></td>
<td>TGT GGG GGG GGG GGG GGG GGG GGG</td>
<td>ACT GGG GAG GGG GGG GGG GGG</td>
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<tr>
<td>25bp deletion</td>
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<td>TGT GGG GGG GGG GGG GGG GGG GGG</td>
<td>ACT GGG GAG GGG GGG GGG GGG</td>
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<td>Cys deletion</td>
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<td>5'-GGG GGG GGG GGC</td>
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<td></td>
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<td>TCT GGG GGG GGG GGG GGG GGG GGG</td>
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<td>TGT GGG GGG GGG GGG GGG GGG GGG</td>
<td>ACT GGG GAG GGG GGG GGG GGG</td>
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**Table 1.** Oligonucleotide primers for site-directed mutagenesis. Primers are italicized, and the nucleotides in bold are deleted.
**Mutated α1A human hs1,2 reporters**

*Figure 6.* Schematic of the mutated α1A human hs1,2 luciferase reporter. Sp1 binding motifs overlap the 5’ and 3’ portions of the 53 bp sequence while κB, AP-1, and DRE binding sites are found within the 53 bp sequence. Oct and AP-1/Ets binding sites are also found outside the 53 bp sequence. Site-directed mutagenesis was performed to delete the DRE (DREdelα1A), κB (κBdelα1A), DRE and κB (DRE-κBdelα1A), 53 bp sequence (53bpdelα1A), Oct (Octdelα1A), or 53 bp sequence and Oct (53bp-Octdelα1A) binding motifs.
**Transient Transfection**

CH12.LX cells (1.0 x 10^7) were resuspended in 200 µl of culture media with 10 µg of plasmid and transferred to a 2-mm gap electroporation cuvette. Cells were electroporated using an electro cell manipulator (ECM 630, BTX, San Diego, CA) with the voltage at 250 Volts, the capacitance at 150 µF, and the resistance at 75 ohms. For each plasmid, multiple transfections were pooled and divided into treatment groups at 2 x 10^5 cells/mL.

**Luciferase Assay**

Immediately after transfection, CH12.LX cells were treated with LPS, TCDD, and/or vehicle (0.01% DMSO) and then aliquoted in triplicate into a 12-well plate for 24 hr. After the 24 hr incubation period, cells were washed with 1x PBS and then lysed with 1x reporter lysis buffer (Promega). Samples were immediately frozen at -80°C. To measure luciferase enzyme activity, samples were thawed at room temperature, and then 20 µL of sample lysate was mixed with 100 µl of luciferase assay reagent (Promega). Luciferase activity or luminescence was measured by a Sirius luminometer (Berthold Detection Systems, Oak Ridge, TN) and represented as relative light units.

To determine transfection efficiency, plasmid per cell was directly measured by quantitative real-time PCR rather than cotransfecting a control plasmid such as β-gal. Common stimulants such as LPS can activate the promoters and enhancers of control plasmids, making it difficult to accurately standardize transfection results based on the activity of the control plasmid. A time course was performed to determine the optimal time point when plasmid quantification would be stable, i.e. less variation in the plasmid
quantification between replicates. Therefore, DNA was isolated from untreated samples 2 hr post transfection. DNA isolation was performed using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich). Purified DNA was diluted 10-fold and then analyzed by quantitative real-time PCR for the luciferase gene. Primers were generated using Primer3 Software (http://fokker.wi.mit.edu/primer3/input.htm) and were specific for the luciferase gene encoded within the pGL3 luciferase vector series.

Briefly, 2 µl of diluted DNA was mixed with 10 pmol of both forward and reverse primers and 1x SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) to a final volume of 25 µL. SYBR Green incorporation was measured for 40 cycles by a 7500 real time PCR system (Applied Biosystems). The concentration (nanograms per µL) of plasmid DNA was calculated from a standard curve using known quantities of plasmid DNA. The total amount of plasmid (nanograms of plasmid) was determined by multiplying the concentration (nanograms per µL), the volume of DNA added (2 µL), and the fold dilution (10). The plasmid number per cell was calculated using the following equation: [(nanograms of plasmid) x (molecules of plasmid/nanogram of plasmid)/cell number], as previously described (Sulentic et al., 2004). While transfection efficiency varied slightly, luciferase activity was normalized to plasmid number per cell when making direct comparisons between plasmids. Consistent with previous studies (Sulentic et al., 2004), transfection efficiency for each plasmid was not affected by LPS and/or TCDD treatment (data not shown).
Statistical Analysis of Data

The mean ± S.E. (n=3) was determined for each treatment group of a given experiment. To determine significance between treatment groups and vehicle controls within one plasmid, the data were analyzed by a one-way ANOVA followed by a Dunnett's two-tailed t test. Comparisons between plasmids were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. Fold-change of TCDD-induced activation was determined by dividing the mean luciferase activity of the TCDD treatment by the mean luciferase activity of its appropriate vehicle in a given experiment. Comparisons of the fold-changes of TCDD-induced activation between plasmids were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. The mean fold-change of TCDD activation ± S.E. was determined for each treatment group by analyzing three to five separate experiments.
III. RESULTS

**TCDD inhibits LPS-induced mouse hs1,2 enhancer activity in transiently transfected mouse CH12.LX B cells**

Previous transient luciferase studies have demonstrated a marked inhibition of the mouse 3’IgH RR by TCDD in CH12.LX B cells stimulated with LPS, a common B-cell stimulant (Sulentic et al., 2004). In contrast, LPS and TCDD cotreatment resulted in a striking activation of the mouse hs4 enhancer in CH12.LX cells, perhaps suggesting that TCDD-induced inhibition of LPS stimulated 3’IgH RR activity is mediated by the hs1,2 enhancer (Sulentic et al., 2004). In previous studies, the activity of a mouse hs1,2 luciferase reporter regulated by a variable heavy chain (V_{H}) promoter 5’ of a luciferase gene and the mouse hs1,2 enhancer inserted 3’ of the luciferase gene was very low, impeding the ability to analyze the effects of LPS and TCDD on hs1,2 enhancer activity (Sulentic et al., 2004). However, in the current study, there was greater success with the hs1,2 reporter perhaps due to improved transfection efficiency in the CH12.LX cell line. We chose to maintain the use of the CH12.LX cell line to remain consistent with previous research regarding TCDD-induced modulation of B-cell function and 3’IgH RR activity. Additionally, CH12.LX cells have been extensively used due to their high AhR expression and functional AhR signaling pathway, and many of the results obtained from CH12.LX cells have been validated in primary mouse B cells and in vivo mouse models. Consistent with previous results (Arulampalam et al., 1994), the mouse hs1,2 reporter
plasmid demonstrated low but detectable basal activity, which was significantly activated following LPS stimulation (Fig. 7). Additionally, as hypothesized, TCDD significantly inhibited LPS-induced activation of the mouse hs1,2 enhancer at concentrations as low as 0.1 nM (Fig. 7). These results suggest that the hs1,2 enhancer may mediate the inhibition by TCDD of LPS-induced 3’IgH RR activation.
Figure 7. TCDD inhibits LPS-induced mouse hs1,2 enhancer activity. CH12.LX cells were transiently transfected with the mouse hs1,2 reporter constructs. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr with 0.01% DMSO vehicle (0 nM TCDD) or varying concentrations of TCDD (0.1-10.0 nM) in the presence of 1.0 μg/ml LPS stimulation. C represents the LPS alone control. 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 followed by a Dunnett’s two-tailed t-test. Asterisk, “**”, denotes significance compared to the corresponding vehicle control at $p<0.01$. 
**TCDD inhibits LPS-induced hs1,2/hs3A enhancer activity in CH12.LX cells stably expressing a transgene under the regulation of the hs1,2/hs3A enhancer pair**

The above mentioned results validate the hypothesis that the hs1,2 enhancer may mediate TCDD-induced inhibition of mouse 3’IgH RR activity; however, further investigation is warranted. Although transient luciferase studies provide a convenient method of analyzing chemical-induced modulation of the 3’IgH RR and its enhancers, high experimental variation due to low transfection efficiencies and differences between transfection experiments are often problematic. Utilizing an experimental model that decreases these variations and more accurately reflects endogenous IgH regulation was highly advantageous. Therefore, one objective of the current study was to stably express the 3’IgH RR and to evaluate the role of individual enhancer elements in mediating TCDD-induced modulation of the 3’IgH RR.

For these studies, we utilized a γ2b mini-locus, developed and provided by Dr. Laurel Eckhardt (Hunter College, City University of New York City, NY). This mini-locus is a reporter construct with an inducible γ2b transgene under the regulation of the 3’IgH RR with loxP sites flanking the hs3B/hs4 enhancer pair (Fig. 8). CRE-loxP technology allows for the deletion of the hs3B/hs4 enhancer pair, leaving the γ2b transgene under the regulation of only the hs3A/hs1,2 enhancer pair and allowing a unique opportunity to evaluate the enhancers in mediating the effect of TCDD on the 3’IgH RR. Using the CH12.LX cell line, our laboratory generated and characterized a transgenic cell line, CH12.γ2b-3’IgHRR, that expresses one copy of the γ2b mini-locus. The CH12.LX and CH12.γ2b-3’IgHRR B-cell lines express IgA and have therefore undergone CSR, which leaves them unable to endogenously express γ2b or IgG2b. With
CRE-loxP technology, we created an enhancer deletion derivative cell line, CH12.γ2b-Δhs3B4, which expressed the γ2b transgene under the regulation of the hs3A/hs1,2 enhancer pair. The CRE-mediated deletion of the hs3B/hs4 enhancer pair was evaluated using PCR and two sets of primers (Fig. 8A). Primer set 1 consisted of a forward primer that bound within the 3’ region of the hs3B and a reverse primer that bound within the 5’ region of the hs4, and the deletion was verified by the absence of a PCR product (Fig. 8B, left). Endogenous product from primer set 1 would be too large (2 kb) for amplification with standard PCR. Primer set 2 consisted of primers that bound outside of the deleted site, and the deletion was verified by the presence of a PCR product (Fig. 8B, right).

After verifying CRE-mediated deletion of the hs3B/hs4 enhancer pair in the CH12.γ2b-Δhs3B4 cell line, it was necessary to evaluate the effect of LPS and TCDD cotreatment on γ2b expression in the parent, CH12.γ2b-3’IgHRR, and deletion derivative cell lines. Consistent with transient luciferase studies, LPS significantly activated γ2b expression in a concentration-dependent manner in both CH12.γ2b-3’IgHRR and CH12.γ2b-Δhs3B4 cells at concentrations as low as 0.01 μg/mL LPS, with the highest γ2b expression at 1.0 μg/mL as determined by sandwich ELISA (Fig. 10). As expected, TCDD markedly inhibited LPS-induced γ2b expression in the parent cell line as well as the enhancer deletion derivative cell line at concentrations as low as 0.1 nM (Fig. 9). The percent inhibition of γ2b expression by TCDD increased slightly with higher concentrations of LPS concentration in both the CH12.γ2b-3’IgHRR and CH12.γ2b-Δhs3B4 cells. Our results suggest the hs1,2 enhancer (and possibly the hs3A) may mediate TCDD-induced inhibition LPS stimulated 3’IgH RR activity.
A. $\gamma_2b$ transgene

![Diagram of genomic analysis of IgH minilocus before and after CRE-mediated deletion.](image)

CH12.$\gamma_2b$-3'IgHRR  \quad CH12.$\gamma_2b$-$\Delta$hs3b4

B. Verifying CRE-mediated deletion

![PCR analysis of DNA from CH12.$\gamma_2b$-3'IgHRR, CH12.$\gamma_2b$-$\Delta$hs3b4, and negative controls.](image)

**Figure 8.** Genomic analysis of IgH minilocus before and after CRE-mediated deletion. (A) Schematic of the $\gamma_2b$ minilocus before and after CRE-mediated deletion. LoxP sites are designated by small arrows flanking the hs3B/hs4 enhancer pair. Primers are designated by larger arrows above the minilocus. Primer set 1 encompasses a forward primer that binds within the hs3B enhancer and a reverse primer that binds within the hs4 enhancer. Primer set 2 includes a forward primer that binds a region flanking the loxP site upstream of the hs3B enhancer and a reverse primer that binds within the hs3a enhancer region. (B) CRE-mediated deletion of the hs3B/hs4 enhancer was verified by PCR analysis of CH12.LX (negative control), CH12.$\gamma_2b$-3'IgHRR (3'IgHRR), and CH12.$\gamma_2b$-$\Delta$hs3B4 ($\Delta$hs3B4) DNA using primer set 1 and 2. Deletion of the hs3B/hs4 enhancer was verified by the absence of a PCR product with primer set 1 and a presence of a PCR produce with primer set 2, as seen in DNA from CH12.$\gamma_2b$-$\Delta$hs3B4 ($\Delta$hs3B4) cells. First and last lanes are DNA ladders.
Figure 9. Concentration-dependent inhibition of the 3’IgH RR and hs3a/hs1,2 enhancer pair by TCDD. Cells from the CH12.γ2b-3’IgH cell line and the deletion derivative, CH12.γ2b-Δhs3B4, were either cultured for 48 hr in the absence of any additional treatment (naïve, NA) or in the presence of LPS (1.0 μg/ml) stimulation and treated with 0.01% DMSO vehicle (0 nM TCDD) or varying concentrations of TCDD (0.01-10.0 nM). C represents the LPS alone control. γ2b assayed by ELISA is normalized to 1.0 μg total protein. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett’s two-tailed t-test. Asterisk, “**”, denotes significance compared to the corresponding vehicle control at p<0.01.
Figure 10. The 3′IgH RR and hs3a/hs1,2 enhancer pair are equally sensitive to LPS and TCDD modulation. Cells from the CH12.γ2b-3′IgH cell line and the deletion derivative, CH12.γ2b-Δhs3B4, were cultured for 48 hr in the absence of additional treatment (0 μg/mL LPS control) or stimulated with varying concentrations of LPS (0.001-1.0 μg/mL) and simultaneously treated with 0.01% DMSO vehicle or 10.0 nM TCDD. γ2b assayed by ELISA is normalized for 1.0 μg total protein. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett’s two-tailed t-test. Asterisks, “*” and “**”, denote significance compared to the corresponding vehicle control at \( p<0.05 \) and \( p<0.01 \), respectively.
LPS activates the human polymorphic hs1,2 enhancer in the CH12.LX mouse B-cell line

Interestingly in humans, a polymorphism of the hs1,2 enhancer, which results in a varying number of tandem repeats of a 53 bp sequence, has been correlated with several autoimmune diseases. Included in this 53 bp sequence are DRE and κB binding sites, which closely resemble the binding sites found in the mouse hs1,2 enhancer (Fig. 4), and we have previously identified TCDD-induced AhR binding to the DRE site within the mouse hs1,2 enhancer (Sulentic et al., 2000). Furthermore, these polymorphisms are expressed at a considerably high frequency in the general population (Giambra et al., 2005; Giambra et al., 2006). Previous luciferase reporter studies have shown increases in basal transcriptional activity by the human polymorphic hs1,2 enhancer that correlated with an increased number of these 53 bp sequences (Denizot et al., 2001). Since the above results demonstrate sensitivity of the mouse hs1,2 enhancer to TCDD-induced modulation, the human polymorphic hs1,2 enhancer could also be chemically modulated, and the increase in DRE and κB sites with each repeat may lead to a greater sensitivity to TCDD. Therefore, the effect of both LPS and TCDD was evaluated on the human polymorphic hs1,2 enhancer by utilizing reporter plasmids obtained from Denizot et al. (2001) containing a V_{H} promoter upstream of a luciferase reporter gene and the human hs1,2 enhancer containing one, two, or three of the 53 bp sequences (α1A, α1B, or α1C alleles, respectively) inserted downstream of the luciferase gene (Fig. 5). These human reporters were evaluated in the CH12.LX mature B-cell line model for several reasons. As previously mentioned, the CH12.LX cell line is the model of choice in our lab because it has been extensively utilized to study a variety of cellular processes specific to
B cells and therefore is relatively well-characterized. Due to a functional AhR signaling pathway and high AhR expression (Sulentic et al., 1998), the CH12.LX cells have also provided a useful model in studying the effects of TCDD on B-cell differentiation, most of which have been validated in mouse primary B cells or in vivo mouse models. Furthermore, the CH12.LX model has already been used in the current study to evaluate the role of the mouse hs1,2 enhancer in TCDD-induced inhibition of mouse 3’IgH RR activity. Therefore, the well-characterized CH12.LX cell line provides a suitable model to initiate an evaluation of sequence differences between the mouse and human hs1,2 enhancers.

LPS has been shown to effectively activate the mouse 3’IgH RR, the hs1,2 enhancer, and, more modestly, the hs4 enhancer in CH12.LX cells and mouse splenic B lymphocytes (Sulentic et al., 2004; Arulampalam et al., 1994). In addition, previous characterization studies involving TCDD have also utilized LPS as a B-cell stimulant. However, it was unclear if LPS could induce human hs1,2 activity since only TGF-β or IL-4/anti-CD40/phorbol dibutyrate inducibility has been reported (Hu et al., 2000; Pan et al., 2000). Therefore, the CH12.LX cells were transiently transfected with the human hs1,2 luciferase reporters (α1A-luc, α1B-luc, α1C-luc) to determine if LPS could induce human hs1,2 activity and to evaluate the potential impact of the repeated sequence on LPS activation. Luciferase reporter results were normalized to transfection efficiency as determined by quantitative real-time PCR for the luciferase gene.

As seen previously in the RPMI human plasma cell line (Denizot et al., 2001), the basal transcriptional activity in CH12.LX B cells increased with increasing number of the 53 bp sequences (Fig. 11). However this effect was less consistent in our mouse mature
B-cell line, resulting in very similar basal activities between the α1A and the α1B alleles while the α1C allele always yielded the greatest basal transcriptional activity. This difference may relate to the difference in maturation state between the RPMI cells (plasma cell) and the CH12.LX cells (mature B cell) or species differences in signaling pathways. Correlating with the increase in basal transcription, there was also an increase in LPS-induced reporter activity with increased repeats (Fig. 11). This is a novel observation since the effect of B-cell stimulation on the polymorphic human hs1,2 enhancer has not been previously reported. Interestingly, when LPS-inducibility was analyzed as fold-change from basal activity using multiple luciferase experiments, a different pattern compared to overall reporter activity emerged. The V_{H} promoter, α1A and α1B human hs1,2 reporters all had very similar fold-changes of LPS activation (Fig. 12). However, the α1C reporter construct, which had the highest basal and LPS-induced reporter activity, had a significantly lower fold-change in reporter activity following 1.0 μg/mL LPS activation (1.9 fold-change) when compared to the V_{H} promoter alone (3.0 fold-change). Although there seems to be no difference in fold-changes of LPS-induced activity with the exception of the α1C reporter, our results demonstrate increases in human hs1,2 reporter activity levels with a greater number of 53 bp sequences, which may be due to increased κB binding sites.
Figure 11. Basal activity of human hs1,2 enhancer activity increases with increasing number of 53 bp sequences. CH12.LX cells were transiently transfected with either the human $V_H$ promoter alone or the human hs1,2 constructs of $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1C}$. LPS enhances the activity of the $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1C}$ reporter constructs of the human hs1,2 enhancer. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr with 1.0 $\mu$g/mL LPS. Luciferase enzyme activity is represented on the y-axis as relative light units relative to the $V_H$ naïve control. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett’s two-tailed $t$-test. Asterisk, “**”, denotes significance compared to the corresponding naïve control at $p<0.01$. Comparisons between the $V_H$ promoter alone and the $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1C}$ human hs1,2 plasmids were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed $t$ test. “†” and “‡‡” denote significance compared with the $V_H$ naïve at $p<0.05$ and $p<0.01$, respectively. Transfection efficiency, as measured by quantitative real-time PCR for the luciferase gene, did not differ between the human reporter constructs and was used to normalize the results.
Figure 12. Fold-changes of LPS-induced activation remain the same between the human polymorphic alleles and the $V_H$ promoter, with the exception of the $\alpha 1C$ allele. The CH12.LX cells were transiently transfected with the $V_H$ (solid line), $\alpha 1A$ (dotted line), $\alpha 1B$ (dashed-dotted line), and $\alpha 1C$ (dashed line) reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated with varying concentrations of LPS (0.001-1.0 $\mu$g/mL) for 24 hr. LPS-induced activation is represented on the y-axis as fold change relative to the appropriate naïve control. Comparisons of the fold-changes of LPS-induced activation between the $V_H$ promoter and human hs1,2 plasmids were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. Asterisk, “*”, denotes significance compared to the $V_H$ fold-change at the corresponding LPS concentration at $p<0.05$. 
**TCDD activates the human polymorphic hs1,2 enhancer in mouse CH12.LX cells**

We have demonstrated that the hs1,2 enhancer likely mediates TCDD’s inhibitory effect on mouse 3’IgH RR activity. In humans, it is possible that the hs1,2 enhancer plays an identical role in human 3’IgH RR regulation. In addition, the polymorphisms of the hs1,2 enhancer could lead to an even more striking effect due to the increased number of κB and DRE binding sites. Therefore, we utilized the hs1,2 luciferase reporters to evaluate TCDD’s effect on the human polymorphic hs1,2 enhancer. Surprisingly, TCDD had a striking but divergent effect on the human hs1,2 enhancer alleles as compared to the mouse hs1,2 enhancer. In the absence of LPS stimulation, TCDD significantly activated the human polymorphic hs1,2 enhancer in a concentration-dependent fashion at concentrations as low as 0.1 nM TCDD (Fig. 13). In the presence of LPS stimulation, cotreatment with TCDD led to an enhanced activation of the human polymorphic hs1,2 enhancer (Fig. 13 and 14). The magnitude of induction after TCDD and LPS cotreatment depended on the concentration of TCDD and LPS in addition to the number of 53 bp sequences (Fig. 13 and 14). TCDD-induced activation of the human hs1,2 reporters plateaued at higher concentrations of TCDD in unstimulated and stimulated CH12.LX cells (Fig. 13 and 14). Interestingly, the luciferase reporter regulated by only the V_{H} promoter was maximally activated by LPS and TCDD cotreatment, meaning TCDD did not further enhance LPS-induced activation of the promoter at higher concentrations of LPS (0.01, 0.1, and 1.0 μg/mL) (Fig. 13A and 14A). This effect was seldom seen with the human hs1,2 luciferase reporters and only occurred if LPS-induced activity was exceptionally high (7-8 fold activation) (unpublished observation).
Figure 13. The polymorphic human hs1,2 enhancer is modulated by TCDD in the absence and presence of LPS. The CH12.LX cells were transiently transfected with the V_H (A), α1A (B), α1B (C), and α1C (D) reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr with 0.01% DMSO vehicle (0 nM TCDD) or varying concentrations of TCDD (0.01-10.0 nM) in the absence or presence of LPS (1.0 μg/ml) stimulation. C represents the LPS alone control. Luciferase enzyme activity is represented on the y-axis as relative light units normalized to the naïve control of each plasmid. Comparisons between the unstimulated and stimulated treatment groups were analyzed using a one-way ANOVA followed by a Dunnett’s two-tailed t-test. Asterisk, “***”, denotes significance compared to the corresponding vehicle control at p<0.01. Transfection efficiency, as measured by quantitative real-time PCR for the luciferase gene, did not differ between the human reporter constructs and was used to normalize the results.
Figure 14. LPS enhances the activity of the \(\alpha_1A\), \(\alpha_1B\), and \(\alpha_1C\) reporter constructs of the human hs1,2 enhancer. The CH12.LX cells were transiently transfected with the \(V_H\) (A), \(\alpha_1A\) (B), \(\alpha_1B\) (C), and \(\alpha_1C\) (D) reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (0 \(\mu\)g/mL LPS control) or activated with varying concentrations of LPS (0.001-1.0 \(\mu\)g/mL), or simultaneously treated with 0.01\% DMSO vehicle or 10.0 nM TCDD for 24 h. Luciferase enzyme activity is represented on the y-axis as relative light units normalized to the naïve control of each plasmid. Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett’s two-tailed \(t\)-test. Asterisks, “*” and “**”, denote significance compared to the corresponding vehicle control at \(p<0.05\) and \(p<0.01\), respectively. Transfection efficiency, as measured by quantitative real-time PCR for the luciferase gene, did not differ between the human reporter constructs and was used to normalize the results.
Multiple luciferase human hs1,2 reporter experiments (n ≥ 3) were analyzed and averaged for the fold-change of reporter activity following TCDD treatment compared to the DMSO vehicle control to evaluate the impact of an increase in 53 bp sequences on promoter-enhancer interactions. This analysis indicates that the fold-change in luciferase activity (TCDD compared to DMSO vehicle) did not increase with increasing number of repeats in unstimulated CH12.LX cells (Fig. 15). In fact, in unstimulated cells, the greatest fold-change of TCDD to vehicle occurred in the VH promoter (solid line) at 1.0 and 10.0 nM concentrations of TCDD though there was some variability (Fig. 15A). However with LPS stimulation, the presence of two 53 bp sequences (α1B, dash-dot line) resulted in a significant (p<0.05) increase in fold-change of TCDD-induced activation compared to the VH promoter alone at lower concentrations of TCDD (0.1 nM) while all three alleles reached a plateau in activation at 10.0 nM TCDD (Fig. 15A). The fold-change of TCDD-induced activation did not increase in the VH promoter in stimulated CH12.LX cells (solid line, Fig. 15A). In addition, varying the LPS concentration did not produce vast differences in the fold-activation induced by TCDD for any of the human hs1,2 reporter constructs (Fig. 15B). However, at 0.01, 0.1, and 1.0 μg/mL concentrations of LPS, the fold-effect of TCDD on the VH promoter was markedly decreased compared to that with TCDD alone (Fig. 15B), which was concordant with previously stated results showing that the VH promoter is maximally activated following LPS and TCDD cotreatment (Fig. 13A and 14A). These results suggest the VH promoter alone reporter and the human hs1,2 reporters may be differentially regulated after LPS and TCDD cotreatment in CH12.LX cells.
Figure 15. An increase in the number of 53 bp repeats may result in an increase in sensitivity to TCDD. The CH12.LX cells were transiently transfected with the $V_H$ (solid line), $\alpha_{1A}$ (dotted line), $\alpha_{1B}$ (dashed-dotted line), and $\alpha_{1C}$ (dashed line) reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment or treated with 0.01% DMSO vehicle or (A) varying concentrations of TCDD (0.001-10.0 nM) in the absence or presence of 1 $\mu$g/ml LPS stimulation or (B) varying concentrations of LPS (0.001-1.0 $\mu$g/mL) in the absence or presence of 10 nM TCDD for 24 hr. TCDD-induced activation is represented on the y-axis as fold change relative to the appropriate vehicle control. Comparisons of the fold-changes of TCDD-induced activation between the $V_H$ promoter and human hs1,2 plasmids were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed $t$ test. Asterisks, “*” and “**”, denote significance compared to the $V_H$ fold-change at the corresponding TCDD concentration at $p<0.05$ and $p<0.01$, respectively.
Concerted roles of DRE, κB, AP-1, and Oct binding sites in TCDD-induced activation of the human polymorphic hs1,2 enhancer in the CH12.LX mouse B-cell line

We have demonstrated that TCDD activates the human polymorphic enhancer in mouse CH12.LX B cells. In addition, TCDD enhancement of human hs1,2 reporter activity increases with an increasing number of 53 bp sequences within the hs1,2 enhancer, with the α1C-luc reporter (three 53 bp sequences) having the greatest overall activity levels but the α1B-luc reporter (two 53 bp sequences) having the greatest fold-change of TCDD-induced activation in stimulated CH12.LX cells (Fig. 15). DRE, κB, AP-1 binding sites are present in the 53 bp sequence while Sp1 flanks the repeat and Oct and AP-1/Ets sites exist outside the repeat but still within the hs1,2 enhancer (Fig. 6). Therefore, the α1A human hs1,2 reporter has one DRE, κB, and AP-1 binding site; the α1B human hs1,2 reporter has two DREs, κB, and AP-1 binding sites. However, the α1C reporter has only two DRE and AP-1 binding sites and three κB binding sites, which may have resulted from an incomplete recombination event that occurred during the duplication of the third 53 bp sequence. Because these reporter constructs were made by performing PCR on DNA samples from individuals (Denizot et al., 2001), this inconsistency may be limited to one individual and may not be present in the entire population that carries the α1C allele. Nevertheless, the lack of a third DRE binding site and the resulting interplay from a disproportionate number of transcriptional factors (i.e. AhR and NF-κB/Rel proteins) may have influenced the low fold-change in TCDD-induced activation of the α1C reporter. This would support the hypothesis that the DRE binding site, at least partially, mediates TCDD-induced activation of the human
polymorphic hs1,2. This hypothesis is in line with a large body of work supporting the role of the AhR/DRE as a transcriptional regulator of a number of genes involved in the metabolism of xenobiotics, inflammation, and cell cycle regulation (Whitlock, 1999; Vogel, 2007; Singh et al., 2007; Ito et al., 2004; Bock and Khole, 2006). Additionally, κB sequences have also been suggested to play a role in TCDD-induced regulation of a number of inflammatory genes (Singh et al., 2007; Vogel, 2007). Furthermore, previous studies have demonstrated a role for the DRE and the κB binding site in TCDD-induced activation of the mouse hs4 enhancer (Sulentic et al., 2004a).

To evaluate the functional consequence of DRE and κB binding within the human polymorphic hs1,2 enhancer, primers (Table 1) were designed to delete the five-nucleotide core sequence of the DRE (5’-GCGTG-3’) and the seven-nucleotide κB sequence (5’-GGGACACCCG-3’) from the α1A luciferase reporter construct by site-directed mutagenesis. Unlike past results with the mouse hs4 enhancer (Sulentic et al., 2004a), deletion of the DRE in the α1A luciferase reporter resulted in no difference in basal luciferase activity (Fig. 16B). In addition, the DRE deletion also resulted in a significantly higher fold-change of TCDD-induced activation at 1.0 and 10.0 nM TCDD in unstimulated and LPS stimulated mouse CH12.LX cells when compared to the wild type α1A control (Fig. 17). Conversely, the κB deletion produced a significant (p<0.01) and striking decrease in overall luciferase activity levels of the α1A reporter (Fig. 16C). However, the fold-change of LPS activation was higher in the κB-deleted α1A compared to the wild-type, and the fold-changes in TCDD-induced activation were not affected except at the highest concentration of TCDD (10.0 nM) when the κB mutant had a fold-effect similar to that of the DRE-deleted construct (Fig. 17). Double deletion of both the
DRE and κB also resulted in lower overall luciferase activity although significantly higher than the κB deleted α1A reporter (Fig. 16D). TCDD-induced activation of the DRE/κB double-deleted α1A reporter construct was not affected in unstimulated CH12.LX cells (Fig. 17). However fold-changes of TCDD activation appeared greater in the DRE/κB double-deleted α1A when compared to the wild type in LPS stimulated CH12.LX cells. This may suggest that something else in the 53 bp sequence is influencing TCDD-induced activation in stimulated CH12.LX cells, albeit modestly.

The deletion of both the DRE and κB from the α1A luciferase reporter leaves the AP-1 binding site intact. TCDD has been shown to induce c-fos and c-jun mRNA expression and AP-1 binding in mouse Hepa-1c1c7 cells (Hoffer et al., 1996; Puga et al., 2000). Therefore, to evaluate the significance of all three binding sites (DRE, κB, and AP-1) present in the entire 53 bp sequence, primers (Table 1) were designed to delete the 53 bp segment from the α1A-luc. While the 53 bp deletion resulted in a significant decrease in basal luciferase activity (Fig. 18C), which was lower than the wild type and the double deletion of the DRE and κB, the fold-effect of TCDD-induced activation was the same or below the wild type whereas the fold-effects of the double-deleted DRE/κB construct appeared to be higher than the wild type in stimulated CH12.LX cells (Fig. 19). These results suggest that indeed another binding motif (possibly AP-1) present in the 53 bp sequence other than the DRE and κB may have a minor influence on fold-effect of TCDD activation. Because TCDD-induced activation was not completely diminished by the deletion of the 53 bp sequence, this led us to the conclusion that a binding site outside of the 53 bp sequence contributes to the activation of the α1A-luc reporter by TCDD.
Figure 16. Deletion of the DRE and κB motifs result in diverging effects of TCDD on α1A human hs1,2 activity. The CH12.LX cells were transiently transfected with the A) wild type (α1A), B) DRE-deleted (DREdelα1A), C) κB-deleted (κBdelα1A), or D) double-deleted DRE and κB (DRE-κBdelα1A) α1A human hs1,2 reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr with 0.01% DMSO vehicle (0 nM TCDD) or varying concentrations of TCDD (0.01-10.0 nM) in the absence or presence of LPS (1.0 μg/ml) stimulation. C represents the LPS alone control. Luciferase enzyme activity is represented on the y-axis as relative light units relative to α1A naïve. Comparisons between the unstimulated and stimulated treatment groups were analyzed using a one-way ANOVA followed by a Dunnett’s two-tailed t-test. Asterisks, “*” and “**”, denote significance compared to the corresponding vehicle control at p<0.05 and p<0.01, respectively. Comparisons between the α1A human hs1,2 plasmids containing a deleted DRE and /or κB were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. “††” denotes significance compared with the α1A NA at p<0.01. Transfection efficiency, as measured by quantitative real-time PCR for the luciferase gene, did not differ between the human reporter constructs and was used to normalize the results.
Figure 17. Deletion of the DRE motif increases fold-changes of TCDD-induced activation in the α1A human hs1,2. The CH12.LX cells were transiently transfected with the wild type (α1A, solid line), DRE-deleted (DREdelα1A, dotted line), κB-deleted (κBdelα1A, dashed line), or double-deleted DRE and κB (DRE-κBdelα1A, dashed-dotted line) α1A human hs1,2 reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment or treated with 0.01% DMSO vehicle or varying concentrations of TCDD (0.001-10.0 nM) in the absence or presence of 1.0 μg/ml LPS stimulation for 24 hr. TCDD-induced activation is represented on the y-axis as fold-change relative to the appropriate vehicle control. Comparisons of the fold-changes of TCDD-induced activation between the α1A human hs1,2 plasmids containing a deleted DRE and/or κB were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. Asterisks, "*" and "**", denote significance compared to the α1A fold-change at the corresponding TCDD concentration at \( p<0.05 \) and \( p<0.01 \), respectively.
Figure 18. Deletion of the 53 bp sequence diminishes the effect of TCDD on α1A human hs1,2 activity. The CH12.LX cells were transiently transfected with the wild type (α1A), B) double-deleted DRE and κB (DRE-κBdelα1A), or C) 53 bp sequence deleted (53bpdelα1A) α1A human hs1,2 reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr with 0.01% DMSO vehicle (0 nM TCDD) or varying concentrations of TCDD (0.01-10.0 nM) in the absence or presence of LPS (1.0 μg/ml) stimulation. C represents the LPS alone control. Luciferase enzyme activity is represented on the y-axis as relative light units relative to α1A naïve. Comparisons between the unstimulated and stimulated treatment groups were analyzed using a one-way ANOVA followed by a Dunnett’s two-tailed t-test. Asterisks, “*” and “**”, denote significance compared to the corresponding vehicle control at p<0.05 and p<0.01, respectively. Comparisons between the α1A human hs1,2 plasmids containing the 53 bp sequence deletion or the double deletion of the DRE and κB were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. “†” and “††” denote significance compared with the α1A NA at p<0.05 and p<0.01, respectively. Transfection efficiency, as measured by quantitative real-time PCR for the luciferase gene, did not differ between the human reporter constructs and was used to normalize the results.
Figure 19. The 53 bp deletion does not affect fold-changes of TCDD-induced activation in the α1A human hs1,2. The CH12.LX cells were transiently transfected with the wild type (α1A, solid line), double-deleted DRE and κB (DRE-κBdelα1A, dash-dot line), or 53 bp sequence deleted (53bpdelα1A, dash-dot-dot line) α1A human hs1,2 reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment or treated with 0.01% DMSO vehicle or varying concentrations of TCDD (0.001-10.0 nM) in the absence or presence of 1 μg/ml LPS stimulation for 24 hr. TCDD-induced activation is represented on the y-axis as fold-change relative to the appropriate vehicle control. Comparisons of the fold-changes of TCDD-induced activation between the α1A human hs1,2 plasmids containing the 53 bp sequence deletion or the double deletion of the DRE and κB were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. Asterisk, “*”, denotes significance compared to the α1A fold-change at the corresponding TCDD concentration at \( p<0.05 \).
While a number of transcription factor binding sites exist outside of the 53 bp sequence, an Oct site lies closest to the segment (Fig. 6). Oct has been shown to play a significant role in the regulation of the mouse hs1,2 enhancer (Singh and Birshstein, 1996). In addition, TCDD has been shown to induce Oct binding in mouse Hepa-1c1c7 cells (Puga et al., 2000). Consequently, primers (Table 1) were designed to delete the eight nucleotide Oct site (5’-ATTTGCAT-3’) from the α1A-luc reporter construct by site-directed mutagenesis. Upon deletion of the Oct site, basal luciferase activity of the α1A reporter decreased modestly (Fig. 20). However, LPS-induced activation remained the same between the Oct-deleted and wild type α1A reporters (Fig. 20). In addition, TCDD still significantly activated the Oct-deleted α1A construct in unstimulated and stimulated CH12.LX cells (Fig. 20), and the fold-effect of TCDD activation did not appear to differ between the Oct-deleted and wild type α1A-luc (Fig. 21). To assess the combined roles of the DRE, κB, AP-1, and Oct binding sites, the Oct site and the entire 53 bp segment was deleted from α1A reporter construct. Once again, this resulted in a marked drop in basal activity that was even lower than the 53 bp sequence deleted construct (Fig. 22). Surprisingly, the fold-effect of LPS activation was the highest in the Oct/53 bp sequence double deletion when compared with the wild type and the deletion of the 53 bp sequence by itself. However, TCDD-induced activation was completely abolished (Fig. 23). These results suggest a concerted activation of the α1A luciferase reporter by the DRE, κB, AP-1, and Oct binding sites.
Figure 20. Deletion of the Oct motif diminishes basal activity without affecting LPS or TCDD activation of the α1A human hs1,2. The CH12.LX cells were transiently transfected with the A) wild type (α1A) or B) Oct-deleted (Octdelα1A) α1A human hs1,2 reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr with 0.01% DMSO vehicle (0 nM TCDD) or varying concentrations of TCDD (0.01-10.0 nM) in the absence or presence of LPS (1.0 μg/ml) stimulation. C represents the LPS alone control. Luciferase enzyme activity is represented on the y-axis as relative light units relative to α1A naïve. Comparisons between the unstimulated and stimulated treatment groups were analyzed using a one-way ANOVA followed by a Dunnett’s two-tailed t-test. Asterisks, “*” and “**”, denote significance compared to the corresponding vehicle control at p<0.05 and p<0.01, respectively. Comparisons between the α1A human hs1,2 plasmids containing a deleted Oct were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. “††” denotes significance compared with the α1A NA at p<0.01. Transfection efficiency, as measured by quantitative real-time PCR for the luciferase gene, did not differ between the human reporter constructs and was used to normalize the results.
Figure 21. The deletion of the Oct motif does not affect fold-changes of TCDD-induced activation in the α1A human hs1,2. The CH12.LX cells were transiently transfected with the wild type (α1A, solid line) or Oct deleted (Octdelα1A, dashed line) α1A human hs1,2 reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment or treated with 0.01% DMSO vehicle or varying concentrations of TCDD (0.001-10.0 nM) in the absence or presence of 1 μg/ml LPS stimulation for 24 hr. TCDD-induced activation is represented on the y-axis as fold-change relative to the appropriate vehicle control. Comparisons of the fold-changes of TCDD-induced activation between the α1A human hs1,2 plasmid and the Oct-deleted construct were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. There was no significant difference between the fold-effects of α1A and Octdelα1A.
Figure 22. Deletion of the 53bp sequence and Oct motif diminishes basal activity and the effect of TCDD on α1A human hs1,2 activity. The CH12.LX cells were transiently transfected with the A) wild type (α1A), B) 53 bp sequence deleted (53bpdelα1A), or C) 53 bp sequence and Oct deleted (53bp-Octdelα1A) α1A human hs1,2 reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr with 0.01% DMSO vehicle (0 nM TCDD) or varying concentrations of TCDD (0.01-10.0 nM) in the absence or presence of LPS (1.0 μg/ml) stimulation. C represents the LPS alone control. Luciferase enzyme activity is represented on the y-axis as relative light units relative to α1A naïve. Comparisons between the unstimulated and stimulated treatment groups were analyzed using a one-way ANOVA followed by a Dunnett’s two-tailed t-test. Asterisks, “*” and “**”, denote significance compared to the corresponding vehicle control at p<0.05 and p<0.01, respectively. Comparisons between the α1A human hs1,2 plasmids containing a deleted Oct were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. “†” and “††” denote significance compared with the α1A NA at p<0.05 and p<0.01, respectively. Transfection efficiency, as measured by quantitative real-time PCR for the luciferase gene, did not differ between the human reporter constructs and was used to normalize the results.
**Figure 23.** The deletion of the 53 bp sequence and the Oct motif completely abrogates TCDD-induced activation of the α1A human hs1,2. The CH12.LX cells were transiently transfected with the wild type (α1A, solid line), 53 bp sequence deleted (53bpdelα1A, dotted line), or 53 bp sequence and Oct-deleted (53bp-Octdelα1A, dashed) α1A human hs1,2 reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment or treated with 0.01% DMSO vehicle or varying concentrations of TCDD (0.001-10.0 nM) in the absence or presence of 1 μg/ml LPS stimulation for 24 hr. TCDD-induced activation is represented on the y-axis as fold-change relative to the appropriate vehicle control. Comparisons of the fold-changes of TCDD-induced activation between the α1A human hs1,2 plasmids containing a deleted 53 bp sequence and/or Oct were analyzed using a two-way ANOVA followed by a Bonferroni’s two-tailed t test. Asterisk, “***”, denotes significance compared to the α1A fold-change at the corresponding TCDD concentration at p<0.01.
DISCUSSION

The hs1,2 enhancer mediates TCDD-induced inhibition of 3'IgH RR activity

The 3’IgH RR has been implicated in a number of processes critical for B-cell function, including IgH transcription, (Madisen and Groudine, 1994, Lieberson et al., 1995), somatic hypermutation (Terauchi et al., 2001; Komori et al., 2006), and class switch recombination (Cogne et al., 1994, Manis et al., 1998, Saleque et al., 1999; Pinaud et al., 2001, Laurencikiene et al., 2007). We have previously demonstrated that the mouse 3’IgH RR is a sensitive target of TCDD, leading to a marked inhibition of 3’IgH RR activity, which may ultimately be the cause behind TCDD-induced inhibition of IgM secretion in mouse CH12.LX mature B cells (Sulentic et al., 1998; Sulentic et al., 2004a). While TCDD induced AhR binding to DREs in both the mouse hs1,2 and hs4 enhancers, AhR’s role in the inhibition of 3’IgH RR activity by TCDD is still unclear (Sulentic et al., 2000). In addition to TCDD, other nondioxin AhR ligands, including dietary metabolites (ICZ), pesticides (carbaryl), and drugs (primaquine and omeprazole), have also been shown to modulate mouse 3’IgH RR activity in CH12.LX cells (Henseler et al., 2009).

While it is unclear which enhancer within the 3’IgH RR mediates chemical-induced modulation, there appears to be a dichotomous relationship between the two most transcriptionally active enhancers, the hs1,2 and hs4. The hs1,2 enhancer is primarily active in activated B cells and plasma cells whereas the hs4 enhancer appears
to be active even at early stages of B-cell development (i.e. pro- and pre-B cells) (Madisen and Groudine, 1994; Michaelson et al., 1995). In addition, Singh and colleagues reported a concerted repression of the mouse hs1,2 enhancer by NF-κB, Oct, and BSAP transcription factors (1996); however Michaelson et al. showed that these same transcription factors work together to enhance mouse hs4 activity (1996). Furthermore, we have previously shown that TCDD activates the mouse hs4 enhancer in a DRE- and κB-dependent manner, which contrasts with TCDD-induced inhibition of 3’IgH RR activity (Sulentic et al., 2004a). These results suggest that perhaps the hs1,2 mediates TCDD-induced inhibition of mouse 3’IgH RR activity. To test this hypothesis, we used transient studies with a mouse hs1,2 luciferase reporter and a mature mouse B-cell line that stably expressed a transgene under the regulation of only the hs3A/hs1,2 enhancer pair. Transient luciferase studies showed that indeed TCDD inhibits LPS-induced activity of mouse hs1,2 enhancer activity. These results were then verified with our mouse CH12.γ2b-Δhs3B4 B-cell line, which more closely represents endogenous Ig regulation. Although the CH12.γ2b-Δhs3B4 cell line contains the γ2b mini-locus under the regulation of both the hs1,2 and hs3A enhancers, several studies have found that the hs3A has no activity on its own although it may augment the activity of other enhancers it is paired with (Matthias and Baltimore, 1993; Madison and Groudine, 1994; Saleque et al., 1997; Chauveau et al., 1998). Therefore, it is unlikely that the hs3A is responsible for this phenotype, and our results support the premise that in fact the hs1,2 enhancer mediates inhibition of LPS-induced mouse 3’IgH RR activity by TCDD in our mature CH12.LX B-cell line.
Although a definite synergism exists with all the 3’IgH RR enhancers, it has been suggested that the individual enhancer elements may play different roles throughout B-cell development. Concordantly, past studies have shown that the mouse hs1,2 enhancer has the highest activity levels in activated B cells and plasma cells while the mouse hs4 enhancer is active throughout B-cell lineage (Madison and Groudine, 1994; Michaelson et al., 1995). Therefore, our results suggest that chemical-induced modulation of the 3’IgH RR at different stages of B-cell development could result in drastically different outcomes. Nonetheless, our results imply that the hs1,2 mediates TCDD-induced inhibition of mouse 3’IgH RR activity in our mature mouse B-cell line. It is tempting to speculate that the hs1,2 may very well mediate TCDD-induced modulation of human 3’IgH RR activity as well.

**TCDD-induced activation of the human polymorphic hs1,2 enhancer by DRE, κB, AP-1, and Oct binding sites.**

While many of the studies concerning IgH regulation have been done with the mouse IgH locus, fairly little is known about the transcriptional regulation of the human IgH locus, which appears to be more complex. As mentioned previously, the murine 3’IgH RR is sensitive to chemical-induced modulation (Sulentic et al., 2004a; Henseler et al., 2009), and the hs1,2 has been shown to mediate inhibition of mouse 3’IgH RR activity by TCDD. Because the human 3’IgH RR and polymorphic hs1,2 enhancer have been implicated in several diseases (i.e. Burkitt’s lymphoma, diffuse large cell lymphoma, IgA nephropathy, and Celiac disease), chemicals could in fact also modulate these various disease states. Therefore, another objective of this study was to
characterize LPS and TCDD’s effect on the human polymorphic hs1,2 enhancer in the mouse CH12.LX B-cell line. The CH12.LX cell line has been extensively utilized to study a variety of cellular processes specific to B cells and therefore is relatively well-characterized. The CH12.LX has also been often used in studying the effects of TCDD on B-cell differentiation due to a well-characterized and functional AhR signaling pathway and high AhR expression (Sulentic et al., 1998). Additionally many of these results with the CH12.LX model have been validated using primary B cells or in vivo mouse models. Furthermore, the well-characterized CH12.LX cell line provides a suitable model to initiate an evaluation of sequence differences between the mouse and human hs1,2 enhancers.

LPS activated the human hs1,2 reporter constructs, and the fold-change in activation was fairly consistent between the $V_H$ promoter, $\alpha_{1A}$ and $\alpha_{1B}$ alleles, with the $\alpha_{1C}$ allele having the lowest fold-change of LPS induction. With the exception of the effect on the $\alpha_{1C}$ allele, these results are concordant with previous studies showing no difference in fold-change activation between human (Hu et al., 2000; Pan et al., 2000) and mouse hs1,2 (Arulampalam et al., 1994) enhancer reporter constructs and promoter reporter constructs after various stimuli. LPS has been shown to induce DNA binding of NF-κB (for review, Ghosh et al., 1998), AP-1 (Suh et al., 2002), and Oct (Lu et al., 2009), and LPS activation of the $V_H$ promoter and human hs1,2 enhancer constructs may be mediated by any or a combination of these transcription factors binding within these regulatory sites ($V_H$ promoter: AP-1 and Oct; human hs1,2 enhancer: κB, AP-1, Oct). As previously mentioned in the Results section, the $\alpha_{1C}$-luc lacks a third AP-1 binding site in the third 53 bp sequence. Many of the genes regulated by AP-1 are also regulated by
NF-κB, and the protein complexes recruited to these binding sites have been previously shown to act together to synergistically activate IL-8 mRNA production (Roebuck, 1999). If AP-1 and NF-κB cooperatively activate the human hs1,2 enhancer following LPS stimulation, the lack of a third AP-1 binding site leaves the NF-κB/Rel proteins at the third κB binding motif without protein partners, and the resulting disproportionate number of interacting proteins may have led to the lower fold-effect of LPS activation in the α1C-luc reporter.

Surprisingly, TCDD activated the human polymorphic hs1,2 luciferase reporter constructs, which contrasts with the inhibitory effect of TCDD on the mouse hs1,2 enhancer. TCDD enhanced overall activity levels that increased with increasing number of 53 bp sequences in the human polymorphic hs1,2 enhancer (V_H < α1A < α1B < α1C). However it appears the fold-changes of TCDD-induced activation were greater in the α1B-luc reporter at lower concentrations of TCDD in stimulated CH12.LX cells, perhaps suggesting that the α1B allele has a greater sensitivity to TCDD-induced modulation. Additionally, there was a clear difference in fold-effect of TCDD activation between the human hs1,2 alleles and the V_H promoter. Like the minimal fold-changes in activity following LPS treatment alone, TCDD treatment alone also resulted in no difference in fold-change of activity between the V_H promoter and the human hs1,2 reporters in unstimulated CH12.LX cells. At first glance, one may think similar fold-changes in LPS or TCDD activation may solely be due to an effect on V_H promoter activity. However there are unique binding motifs present in the human hs1,2 enhancer (i.e. DRE, κB), which are not shared by the V_H promoter, and chemical treatment with either LPS or TCDD has been previously shown to induce DNA binding of NF-κB and AhR (Ghosh et
Therefore, it is highly likely that the molecular profile of proteins recruited to the construct will differ in the presence of the enhancer. In addition, basal transcriptional activity was clearly higher in the human hs1,2 enhancer reporter in comparison to the $V_H$-luc reporter, further supporting a role for obvious enhancer-promoter interactions. Interestingly, LPS and TCDD cotreatment resulted in greater fold-effects of activation in the human hs1,2 reporter constructs compared to the $V_H$ promoter, which was hardly activated at all by TCDD in LPS stimulated CH12.LX cells. This leads one to believe that differential protein-protein interactions between the $V_H$ promoter and human hs1,2 enhancer exist in the presence of both LPS and TCDD cotreatment in comparison to treatment with either chemical by itself, suggesting that two different pathways are involved. As previously mentioned, the $V_H$ promoter contains an Oct and potential AP-1 binding sites. Although TCDD has been demonstrated to increase Oct and AP-1 binding in Hepa-1 liver cells (Puga et al., 2000), Suh and colleagues showed that TCDD inhibited LPS-induced consensus AP-1 binding in CH12.LX B cells (Suh et al., 2002), which may be the reason behind the low fold-change of TCDD-induced activation of the $V_H$ promoter in LPS stimulated CH12.LX cells. The human hs1,2 enhancer contains a plethora of potential binding sites, including DRE, κB, AP-1, Oct, and Sp1 (Denizot et al., 2001), all of which can be modulated by TCDD (Safe, 1995; Puga et al., 2000; Hankinson, 2005; Tian, 2009). TCDD-induced inhibition of AP-1 binding in the human hs1,2 enhancer could be overcome by the presence of the other binding sites located in the human hs1,2 enhancer. Alternatively, because Suh et al. only evaluated consensus AP-1 binding, TCDD may not even inhibit binding to the AP-1 site in the $V_H$ promoter.
and/or human polymorphic hs1,2. If that were the case, the low fold-effect of \( V_{11} \) promoter activity following LPS and TCDD cotreatment could possibly be due to a competition between the pathways activated after LPS and TCDD treatment although the exact mechanism remains unclear and difficult to predict.

To better evaluate the impact of potential binding sites involved in TCDD-induced activation of the human polymorphic hs1,2 enhancer, mutational analysis of the \( \alpha_{1A} \) human hs1,2 reporter construct was performed by first targeting the sequences within the 53 bp sequence (i.e. DRE, \( \kappa B \), AP-1). While Sulentic et al. (2004a) demonstrated a role for the DRE and \( \kappa B \) sites in TCDD-induced activation of the mouse hs4 enhancer in LPS stimulated CH12.LX cells, our results also suggest a significant role for the \( \kappa B \) binding site in not only TCDD-induced activation but also basal activity of the \( \alpha_{1A} \) human hs1,2 reporter, but a less important role for the DRE. However a different pattern emerged in the fold-changes of TCDD-induced activation after deletion of the \( \kappa B \), DRE, or both. While deletions of \( \kappa B \), DRE, or both resulted in slight increases in fold-effect of TCDD-induced activation in stimulated CH12.LX cells, the DRE-deleted \( \alpha_{1A} \)-luc reporter also had an increase in TCDD-induced activation in unstimulated CH12.LX cells and the greatest increase in TCDD-induced activation in stimulated CH12.LX cells. These results imply that the deletion of the DRE produces higher fold-effects in TCDD activation perhaps by being an inherent negative regulator of human hs1,2 activity or by altering protein-protein interactions and DNA binding within the hs1,2 enhancer. In fact, there are a number of studies demonstrating crosstalk between the AhR and proteins from other signaling pathways, including Sp1 and NF-\( \kappa B \)/Rel proteins. In breast cancer cells, AhR disrupts interactions of ER\( \alpha \) and Sp1, resulting in an inhibition of ER\( \alpha \)/Sp1 action.
(Khan et al., 2006). In addition, there is a large body of work that suggests AhR physically interacts with RelA (p65) and RelB, and some of these interactions may even result in an inhibition of TCDD-induced CYP1A1 (for review, Tian, 2009). Because the deletion of the entire 53 bp sequence did not abrogate TCDD-induced activation of the α1A-luc reporter, sites outside of the 53 bp sequence but still within the human hs1,2 enhancer were targeted for mutational analyses. Oct (Singh and Birshtein, 1996) and its coactivator OCA-B (Stevens et al., 2000) have been implicated in mouse hs1,2 and 3’IgH RR activity, respectively. In addition, TCDD has been shown to induce and sustain Oct binding in Hepa-l liver cells (Puga et al., 2000). Our results demonstrated a moderate decrease in basal activity of the α1A-luc reporter with TCDD-induced activation unaffected after the Oct binding motif was deleted. However, when the Oct and 53 bp sequence were deleted from the α1A-luc reporter, TCDD-induced activation was completely abrogated in unstimulated and stimulated CH12.LX cells. These results suggest a concerted role for the Oct and 53 bp sequence binding sites in TCDD-induced activation of the α1A human hs1,2 enhancer although there may be competition in DNA binding to the 53 bp sequence due to the presence of many regulatory motifs.

**Differences between TCDD-induced regulation of the mouse hs1,2 enhancer and human polymorphic hs1,2 enhancer in CH12.LX cells.**

TCDD-induced modulation of the mouse and human hs1,2 enhancers produce clearly diverging effects in the same CH12.LX cell line. We have demonstrated that TCDD activates the human polymorphic hs1,2 enhancer by modulating a number of the binding sites located within the enhancer, including DRE, κB, AP-1, and Oct. However,
the mechanism behind inhibition of LPS-induced mouse hs1,2 enhancer activity by TCDD is still unclear. Although both the murine and human hs1,2 enhancers share similar expression pattern and much of the sequences are highly conserved (Mills et al., 1997; Chen and Birshtein, 1997), we suspect sequence differences could account for the divergent responses of the mouse and human hs1,2 after TCDD treatment. Others have shown that the human hs1,2 lacks specific binding sites important to the regulation of the mouse hs1,2, particularly NF-αP (PU.1) and BSAP binding sites (Mills et al., 1997; Chen and Birshtein, 1997). We speculate that BSAP in particular may be responsible for TCDD-induced inhibition of the mouse hs1,2. BSAP, a repressor in B-cell differentiation, has been shown to influence and sometimes even physically interact with key transcription factors involved in the B cell’s response to antigen, i.e. NF-κB, Oct, and PU.1 (Maitra and Atchison, 2000; Linderson et al, 2000; Sepulveda et al, 2004). In addition, BSAP is a negative regulator of the mouse hs1,2 enhancer (Michaelson et al, 1996; Singh and Birshtein, 1996). In fact, BSAP has been shown to inhibit PU.1/NF-κB (p50/cRel) driven activation of the mouse hs1,2 (Maitra and Atchison, 2000). In addition, Michaelson, Singh, and colleagues demonstrated that BSAP, κB, and Oct binding sites all participate in the concerted repression of mouse hs1,2 transcriptional activity in mature B cells (Michaelson et al, 1996; Singh and Birshtein, 1996). While the concerted repression by BSAP, κB, and Oct could be responsible for the low basal transcriptional activity of the mouse hs1,2 enhancer, the hs1,2 is markedly activated after LPS stimulation. LPS has been shown to downregulate BSAP expression levels, which is imperative for increased Ig secretion and further B-cell differentiation (Neurath et al, 1994; Reimold et al, 1996; Rinkenberger et al, 1996; Roque et al, 1996; Usui et al, 1997).
This correlates well with the role reversal of NF-κB and Oct, which both seem to function as activators of mouse hs1,2 activity in plasma cells (Michaelson et al., 1996; Singh and Birshtein, 1996). However, TCDD has been shown to maintain levels of BSAP (Yoo et al, 2004; Schneider et al, 2008). Because BSAP expression essentially stops in plasma cells while NF-κB and Oct are thought to be constitutively expressed but awaiting activation, BSAP could potentially modulate the binding of κB and Oct motifs, or influence the composition of the subunits that bind. In addition to modulating NF-κB and Oct, BSAP could also influence AhR/DRE binding to elicit TCDD’s repression of the mouse hs1,2 enhancer and perhaps the entire mouse 3’IgH RR. We suspect that the lack of BSAP in the human hs1,2 enhancer allows for TCDD-induced activation of the human hs1,2 enhancer.

**Conclusion**

Past research with animal in vitro models has deemed the 3’IgH RR a sensitive target of TCDD and other chemicals (Sulentic et al., 2004; Henseler et al., 2009). While it was originally unclear as to how TCDD inhibits LPS-induced mouse 3’IgH RR activity, the current study demonstrates that the hs1,2 enhancer most likely mediates TCDD-induced inhibition of the 3’IgH RR. Interestingly in humans, a polymorphism of the hs1,2 enhancer, which results in a varying number of tandem repeats of a 53 bp sequence, has been correlated with several autoimmune diseases including IgA nephropathy, Celiac’s disease, system sclerosis, and psoriasis (Aupetit et al., 2000; Frezza et al., 2004; 2007; 2009; Cianci et al., 2008). DRE, κB, and AP-1 sites are present in the 53 bp sequence while Sp1, Oct, and AP-1/Ets sites lie outside the 53 bp sequence.
but still within the enhancer. TCDD produced a striking activation in the human polymorphic hs1,2 enhancer in mouse CH12.LX B cells, which increased with a greater number of the 53 bp sequence, and we showed that TCDD-induced activation of the α1A human hs1,2 was dependent on DRE, κB, AP-1, and Oct sites present in the enhancer. Mutational analyses are currently underway to evaluate the impact of each individual binding site and the entire 53 bp sequence in the α1B human hs1,2 allele, which harbors two 53 bp sequences.

One limitation of this study was the use of human reporters in a mouse model. Although the well-characterized mouse CH12.LX B-cell line has been used in many of the studies concerning TCDD-induced modulation of B cells and contains a functional AhR signaling pathway that became very useful to initiate an evaluation of the human polymorphic hs1,2 reporters, species differences between murine and human signaling pathways could very well have produced the diverging effects seen between the mouse and human hs1,2 enhancers after TCDD treatment. There is a growing concern that the human AhR may respond differently to TCDD because of lower affinity for the ligand, approximately 10-fold lower than other laboratory animal strains with a KD comparable to DBA/2 mice (TCDD nonresponsive) (for review, Connor and Aylward, 2006). However ligand binding to AhR is only one step in an intricate web of pathways involved in eliciting TCDD’s toxic effects. Because we have shown that the DRE may play a less significant role in comparison to the κB binding site in TCDD-induced activation of human hs1,2 enhancer activity, a low-affinity human AhR may be less important. Nonetheless, the development of a human B-cell model is necessary for a more thorough evaluation of TCDD-induced modulation of the human polymorphic hs1,2 enhancer.
Characterization studies are currently underway to evaluate a number of human B-cell lines for functional AhR signaling pathways. However, one cannot ignore the value of the current study as it is the first of its kind to comparatively evaluate chemical-induced modulation of both mouse and human polymorphic hs1,2 enhancer. The clear differences between the mouse and human hs1,2 is just another example of the difficulty involved in extrapolating mouse toxicology studies to human risk assessment, giving yet another reason why it is important to understand the differences between the two species and the mechanism of action for chemical-induced modulation.

While TCDD is the prototype and most toxic of AhR ligands, it is not the only AhR ligand. Recent studies have shown that a variety of chemicals from environmental, dietary, and therapeutic origins can activate the AhR without necessarily producing dioxin-like toxicities (Denison and Nagy, 2003; Nguyen and Bradfield, 2008). In addition, the 3’IgH RR has also been shown to be sensitive to chemical modulation by TCDD and other nondioxin AhR ligands (Sulentic et al., 2004a; Henseler et al., 2009). The 3’IgH RR not only plays a major role in Ig expression, but it is also associated with many diseases including Burkitt’s lymphoma, IgA nephropathy, and Celiac disease. Since TCDD represents a large class of chemicals found in the environment, diet, and pharmaceuticals, understanding chemical-induced modulation of the 3’IgH RR enhancers may provide a clue to the etiology of certain diseases and lead to the discovery of novel therapeutic targets.


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