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Elucidating the effects of TCDD on the polymorphic human hs1,2 enhancer

Abdullah Freiwan
Wright State University

ELUCIDATING THE EFFECTS OF TCDD ON THE POLYMORPHIC HUMAN HS1,2 ENHANCER

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By

ABDULLAH FREIWAN

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY ABDULLAH FREIWAN ENTITLED ELUCIDATING THE EFFECTS OF TCDD ON THE POLYMORPHIC HUMAN HS1,2 ENHANCER BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
Master of Science.

Courtney E.W. Sulentic, Ph.D
Thesis Director

Committee on Final Examination

Courtney E.W. Sulentic, Ph.D
Associate Professor of Pharmacology & Toxicology

Barbara E. Hull, Ph.D.
Director of Microbiology and Immunology Program, College of Science and Mathematics

Barbara E. Hull, Ph.D.
Professor of Biological Sciences

Nancy J. Bigley, Ph.D.
Professor of Neuroscience, Cell Biology, and Physiology

Robert E. W. Fyffe, Ph.D.
Vice President of Research and Dean of the Graduate School

ABSTRACT

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a potent environmental toxin known to inhibit immunoglobulin (Ig) gene expression in various animal studies. We have identified the mouse 3'Ig heavy chain regulatory region (3'*IgH* RR) as a sensitive transcriptional target of TCDD, which may mediate the inhibitory effect of TCDD on Ig expression. Interestingly, the human hs1,2 enhancer is polymorphic and has been associated with a number of autoimmune diseases. Suggesting a species difference, TCDD inhibited mouse hs1,2 enhancer activation and activated basal human hs1,2 (hs-hs1,2) enhancer activity in the mouse B-cell line. The objective of this study was to elucidate the effects of TCDD on the polymorphic human hs1,2 enhancer using a human B-cell line (CL-01) and luciferase reporter constructs regulated by each of the human hs1,2 alleles. Our results verify that TCDD alone activates each of the hu-hs1,2 alleles. Surprisingly, B-cell stimulation through the Toll-like receptors (TLR) 7, 8, and 9, and the AhR antagonist inhibited basal activity of the hu-hs1,2 alleles and TCDD co-treatment reversed TLR-induced inhibition. Contrary to this, TLR stimulates IgM secretion as well as class switching to IgG secretion. These results suggest that the hu-hs1,2 enhancer may be a negative regulator of 3'*IgH* RR activity and Ig expression.

TABLE OF CONTENTS

LIST OF FIGURES

LISTS OF TABLES

LIST OF ABBREVIATIONS

ARNT: AhR Nuclear Translocator

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

BCR: B Cell Receptors

CSR: Class Switch Recombination

Cyp1A1: Cytochrome P4501A1

C_H: Heavy Chain Constant Region

DMSO: Dimethyl Sulfoxide

DRE: Dioxin Responsive Elements

Eμ Intronic Enhancer

Hs: DNase I Hypersensitivity

IS: Invariant Sequence

IgH: Immunoglobulin Heavy Chain

IgL: Immunoglobulin Light Chain

IgH RR: Ig Heavy Chain Regulatory Region

Ig: Immunoglobulin

LPS: Lipopolysaccharide

MCS: Multiple Cloning Site

MHC: Major Histocompatibility Complex

NFkB: Nuclear Factor Kappa Beta

Oct: Octamer Transcription Factor

PCDD: Polychlorinated Dibenzo-p-Dioxins

PCDF: Polychlorinated Dibenzofurans

PCB: Polychlorinated Biphenyls

Pax5: Paired Box Protein

PCR: Polymerase Chain Reaction

RLU: Relative Light Units

SHM: Somatic Hypermutation

TLR: Toll like Receptor

TCDD: 2,3,7,8-Tetrachlorodibenzo-p-Dioxin

TLR: Toll-Like Receptors

V_H: Variable Heavy Chain Promoter

ACKNOWLEDMENT

I would like to take this opportunity to express my profound gratitude and deep regards to Dr. Courtney Sulentic for her guidance, monitoring, and constant encouragement throughout the time spent on this research. I also want to express a great sense of gratitude to Dr. Barbara Hull for believing in my abilities and giving me a chance to show my ambitions. I would also like to thank her and Dr. Nancy Bigley, as my committee members, for their cordial support, valuable information and guidance. A special thanks to my parents, family, friends, and lab members for their continuous love and support.

DEDICATION

I would like to dedicate my thesis work to my beloved wife, Chelsea Freiwan, for her unconditional love and support. I am truly thankful for having you in my life. I would like also to dedicate my thesis work to my brother, Ahmed Freiwan. He has been fully committed in supporting me throughout my journey of fulfilling my dreams.

I. INTRODUCTION

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

Dioxins or dioxin like compounds are highly toxic compounds that include polyhalogenated aromatic hydrocarbons such as polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF), and polychlorinated biphenyls (PCB) (Mandal, 2005). Dioxins have no common uses and are not intentionally produced by industry but can be formed when organic material is burned in presence of chlorine (from Cl- ion or an organochlorine compound). This compound may be released to the environment during incineration of municipal and medical waste, coal-fired utilities, metal smelting, diesel trucks, burning treated wood, misapplication of sewage sludge, and bleaching of paper fibres and textiles (Malisch & Kotz, 2014; Strucinski et al., 2011). The most potent form of dioxin is TCDD (Mandal, 2005). In animals, one high dose of TCDD causes systemic effects including tumorigenesis, immunological dysfunction, and teratogenesis. Additionally, studies of humans exposed to TCDD and supporting evidence from animal studies, suggest that TCDD induces chloracne, inhibits the immune response, metabolic disorders (porphyria), and other systemic problems as well as being a cancer promoter (Pohjanvirta & Tuomisto, 1994). Acute toxicity studies have shown marked species differences in sensitivity to TCDD. (Kociba & Schwetz, 1982).

TCDD is a polychlorinated dibenzo-p-dioxin with a chemical formula of $C_{12}H_4Cl_4O_2$ (Fig. 1). Generally, people could be exposed to TCDD through soil,

dust, and smoke by inhalation or consumption (Braune, Mallory, Butt, Mabury, & Muir, 2010). TCDD achieved notoriety when it was discovered to be a contaminant in the herbicide Agent Orange, which was used in the Vietnam War (1961-1971). In 1976, thousands of inhabitants of Seveso, Italy were exposed to TCDD after an accidental release of several kilograms of TCDD from a pressure tank (Mandal, 2005). Moreover, in 1997, two women from Vienna were poisoned with TCDD at their workplace and the measured concentration, (144 ng/g of fat), in one of them was the highest ever measured in a human being. Besides chloracne, they did not present with major clinical health effects within the first two years after TCDD intoxication (Geusau et al., 2001). In 2004, following an assassination attempt during his election campaign, Viktor Yushchenko was exposed to TCDD, at a concentration of 108 ng/g of fat. He was diagnosed him with gastritis, colitis with multiple ulcers, hepatitis, pancreatitis, and chloracne as a result of the poisoning (Sorg et al., 2009). In general, studies on the health effects in humans from exposure to TCDD show no threat to life. However, effects that are not overtly toxic or are long term consequences of exposure may occur and be difficult to measure or directly link to dioxin exposure (i.e. increased infections, cancer etc.) (Pelclova et al., 2006). The biological effects of TCDD are thought to be mediated by the aryl hydrocarbon receptor (AhR).

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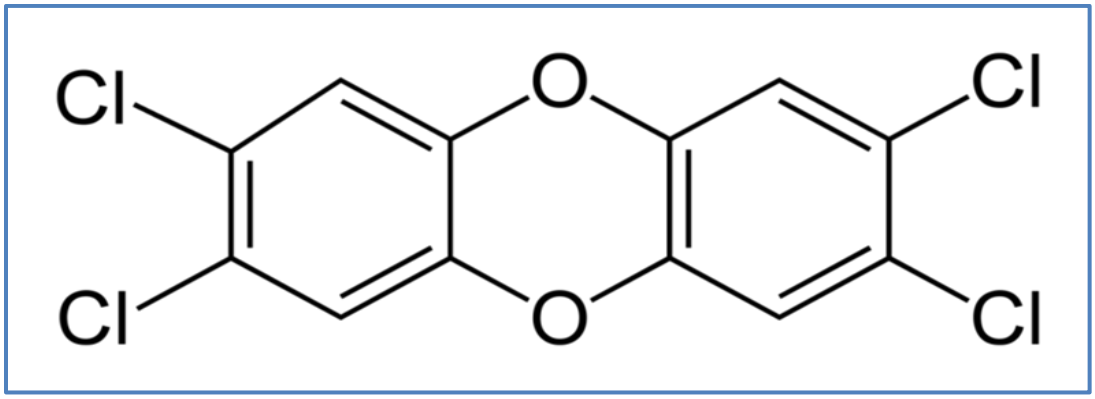

Figure 1: Chemical structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

Aryl Hydrocarbon Receptor Signaling Pathway

The aryl hydrocarbon receptor (AhR) is a constitutively expressed transcription factor that is encoded by the AhR gene and consists of 848 amino acids. It regulates xenobiotic metabolizing enzymes such as cytochrome P450. AhR is an inactive cytosolic protein belonging to the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family of transcription factors (Abel & Haarmann-Stemmann, 2010; Nebert & Karp, 2008). The AhR binds to ligands that include; (1) chemicals including aromatic environmental pollutants and several pharmaceuticals, (2) endogenous ligands or, (3) synthetic and compounds (Denison & Nagy, 2003; Nebert & Karp, 2008; Strucinski et al., 2011; Whitlock, 1999).

In the absence of a ligand such as TCDD, AhR stays in the cytoplasm in an inactive form bound to a multi-protein complex which consists of a dimer of HSP90, co-chaperone p23, and XAP2. In order to stabilize the whole complex, XAP2 binds with AhR and HSP90, while p23 directly binds to HSP90 and plays an essential role in the process of AhR activation (Whitlock, 1999) (Fig 2). Upon ligand binding, the AhR protein undergoes conformational changes and dissociates from the multi-protein complex. The AhR then translocates into the nucleus and binds to the AhR nuclear translocator (ARNT) forming the heterodimer complex, TCDD-AhR/ARNT (Abel & Haarmann-Stemmann, 2010). The TCDD-AhR/ARNT heterodimer complex is transcriptionally active and binds to dioxin responsive elements (DRE) in the promoter and enhancer regions of target genes resulting in alterated gene expression. The AhR protein can be

downregulated by either negative feedback inhibition or ubiquitination. Negative feedback inhibition is mediated by the AhR repressor or nuclear export of the receptor by proteosomal degradation (Abel & Haarmann-Stemmann, 2010). The induction of cytochrome P4501A1 (Cyp1a1) has been used as a biomarker for AhR activation (Hansen, Esakky, Drury, Lamb, & Moley, 2014; Hu, Sorrentino, Denison, Kolaja, & Fielden, 2007; Whitlock, 1999). Furthermore, up-regulation of metabolic enzymes by TCDD led to the discovery of the AhR and its signaling pathway and the succeeding efforts to determine if the AhR was responsible for the toxic effects of TCDD (Delescluse, Lemaire, de Sousa, & Rahmani, 2000). The AhR also appears to play an important role in mediating most of the toxic and carcinogenic effects of TCDD and other dioxins (Dertinger, Nazarenko, Silverstone, & Gasiewicz, 2001). The AhR also has roles in steroid hormone metabolism, cellular proliferation and differentiation, and cell death. The immune system has also been identified as a sensitive target of TCDD and various components of the immune system are affected, including the differentiation of B cells into antibody forming cells (Zhang et al., 2013).

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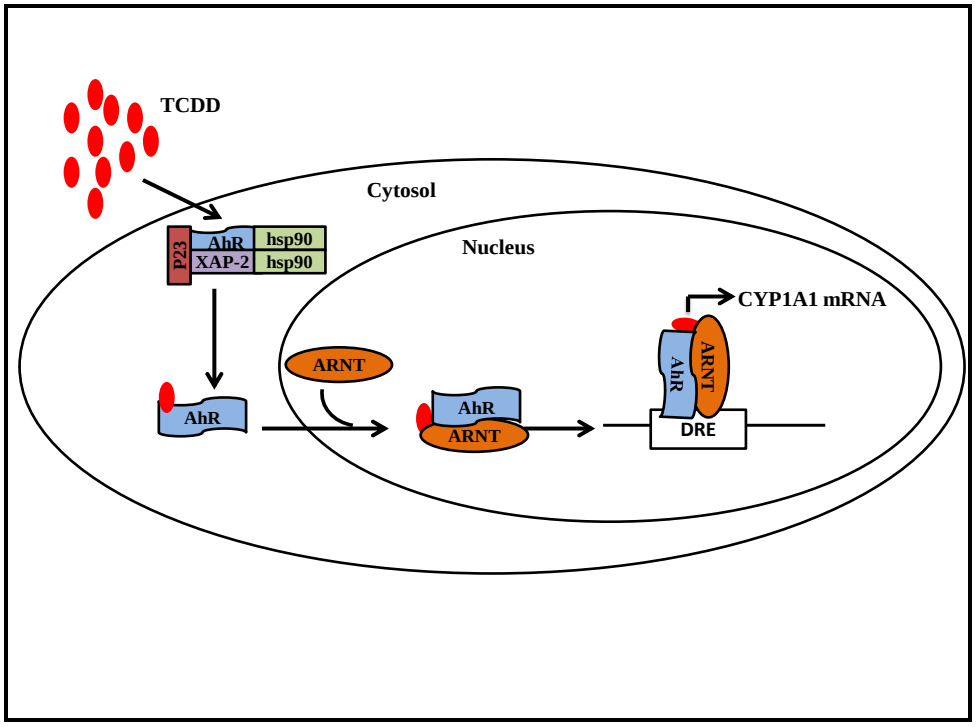


Figure 2: Aryl hydrocarbon receptor signaling pathway. Dioxin binds to the AhR to initiate a series of events leading to modulation of gene expression. CYP1A1 is a sensitive indicator of AhR activation.

The Immune system

The immune system is a network of cells, tissue, and organs that works together to defend the body against foreign invaders. The human body provides a perfect environment for many microbe, such as bacteria, viruses, fungi, and parasites. The immune system, which is crucial to human survival, prevents and limits the microbial entry and growth. The immune system is classified into innate and adaptive immune system.

Innate immunity is always present and ready to fight microbes at the site of infection. It is the first line of defense and employs a non–specific response against pathogens. The main components of innate immunity include physical barriers, phagocytes (such as macrophages, dendritic cells, and neutrophils), and natural killer cells. On the other hand, adaptive immunity is an antigen-specific defense. Both innate and adaptive immunity often work together and there is a lot of crossover between the two mechanisms. Adaptive immunity eliminates specific antigens by either antibody-mediated immunity or cell-mediated immunity. Antibody-mediated immunity involves the production of antibody molecules in response to an antigen and is mediated by B cells, while cell-mediated immunity involves the activation of cytotoxic T-lymphocytes in response to an antigen and is mediated by T cells.

T cells either express CD4 or CD8 co-receptors. The CD4+ T cells are essential for both antibody-mediated immunity and cell-mediated immunity, and bind to epitopes presented by class II major histocompatibility complex (MHC II). The MHC II molecules

normally found only on antigen-presenting cells (macrophage, dendritic cells, and B cells) and are specialized to fight extracellular sources of the infection such as bacteria. Alternatively, CD8+ T cells bind to epitopes presented by a class I MHC and secrete molecules that destroy the cell to which they have bound. The MHC I molecules are expressed by nearly every nucleated cell of the body and are specialized to fight intracellular infections such as viruses.

B cells

B cells, or B lymphocytes, are essential in the humoral immune response of the adaptive immunity. B cells are produced from the bone marrow and then migrate as mature cells to the spleen and other secondary lymphoid tissue. B cell receptors (BCR) are transmembrane receptor proteins located on the outer surface of B-cells that interact with antigen. In order to activate B cells, the cells need a secondary activation signal provided by either the antigen itself or helper T cells. After stimulation, B cells eventually undergo proliferation and differentiation to plasma cells that secrete antibodies. The antibody, or secreted immunoglobulin (Ig), is used by the immune system to identify and neutralize foreign antigens such as bacteria and viruses. Ig is composed of two identical heavy chains (H) and two identical light chains (L) connected together by disulfide bonds (Fig 3). Antibodies can be divided into five different effector classes (IgM, IgG, IgA, IgD, IgE). The heavy chains differ in structural and functional properties, and determine the class and subclass of antibodies. The two isotypes of light chains are either kappa or lambda; however there are no significant differences between them.

V(D)J recombination, also known as somatic recombination, is a mechanism of genetic recombination specific to B cells that takes place in bone marrow and randomly combines three gene segments (V, D, J) of the IgH gene and two segments (V, J) of the IgL gene to encode for the variable region of the heavy and light chain, respectively. Maturation of immature B cells to mature B cells produces membrane-bound IgM and IgD, whereas the B cells that fail to pass any of the maturation steps will die by apoptosis. Production of Ig can also involve the transition to expressing different classes of antibodies.

Following maturation B cells stay in the peripheral tissues until they encounter an antigen and are activated. Upon activation, B cells differentiate into plasma cells or memory B cells. Plasma cells can secrete huge amounts of Ig every minute for several days. A large amount of antibody is released into the circulation to respond to the infection. The initial burst of antibody production progressively decreases as the stimulus is removed (e.g., by recovery from infection). However, some antibody continues to be present for several months afterward. Alternatively, memory B cells are long-lived lymphocytes that have previously encountered a given antigen. When B is re-exposed to the same antigen, it rapidly initiates an immune response, and then B cells will proliferate and produce large amounts of specific antibodies, memory B, against the agent and therefore providing lasting immunity.

Class switch recombination (CSR) is a biological mechanism that changes a B cell's expression of Ig from one class to another. CSR occurs after activation of a mature B cell. As a result of helper T cell recognition of antigen on the B cell surface through MHC II, the T cell becomes activated and provides a secondary stimulus to B cell to

differentiate into IgM producing cells. Furthermore, the B cell can undergo antibody class switching to produce IgG, IgA or IgE antibodies. During this process, the constant region of the heavy chain is changed, whereas the variable region stays the same, meaning the class switch is limited to changing the heavy chain only, so CSR will not affect antigen specificity. The variable region of heavy and light chain determines antigen specificity where the population of antibody molecules are able to react with only one antigen.

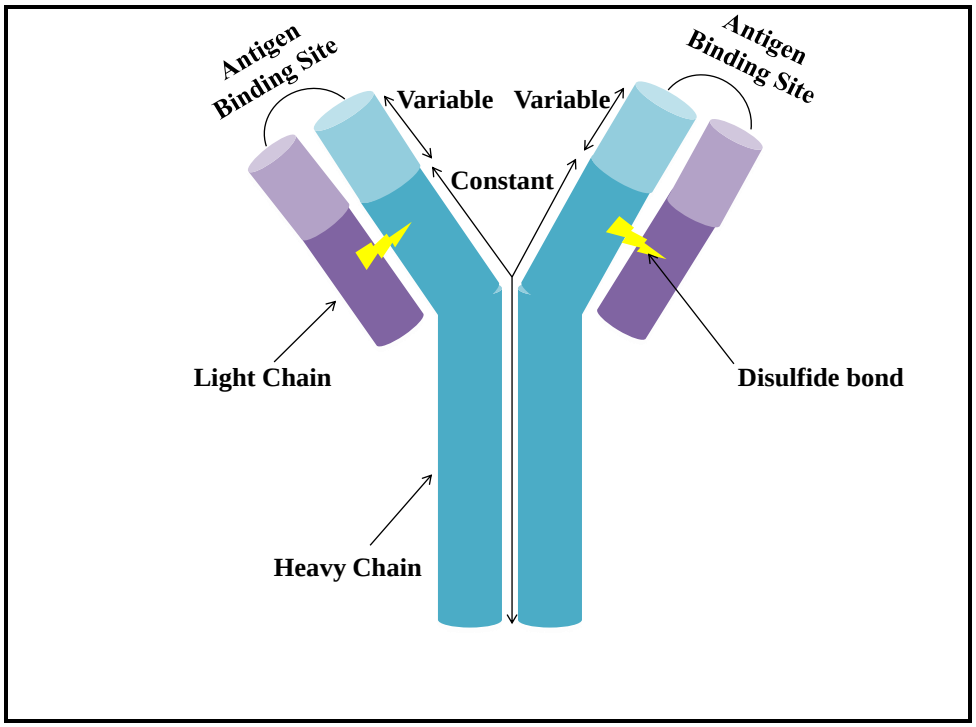


Figure 3: Immunoglobulin (Ig) structure. Secreted Ig (antibody) is composed of a heavy chain and light chain that binds a specific antigen. Each chain contain constant region that determines the effector qualities of the Ig and a variable region that binds antigens.

TCDD-Induced Immunological Effects

The immune system is a sensitive target of TCDD. The effects of TCDD in experimental animals include suppression of both innate and adaptive immune responses and decreased host resistance to infectious agents (Morris & Holsapple, 1991; Safe, 1998). The majority of effects have been reported based on either accidental exposures or in vitro analysis. However, humans seem to be less sensitive to immunotoxic effects by TCDD than many laboratory animals (Okey, Riddick, & Harper, 1994). TCDD-induced immunological effects on human beings include severe skin rash called chloracne, effects on thymus cells such as human thymic epithelial cells (TEC) which affects immune response, porphyria, transient hepatotoxicity, peripheral and central neurotoxicity, and significant decrease in IgG levels (Dertinger et al., 2001; Silbergeld & Gasiewicz, 1989). In addition, there are some effects of TCDD on human serum such as elevations in GGT and triglyceride levels, and alterations in FSH and LH (Sweeney & Mocarelli, 2000). TCDD-induced immunotoxicity such as suppression B development and decrease antibody secretion is mediated by the aryl hydrocarbon receptor but the mechanism is still unclear (Hansen et al., 2014). Moreover, previous studies showed that the affinity of TCDD binding to the AhR is much lower in human than in animals and that might explain why humans seem to be less sensitive to immunotoxic effects by TCDD than many laboratory animals (Okey et al., 1994).

TCDD-Induced B cell Dysregulation

The B cell is a sensitive and direct cellular target of TCDD (Sulentic, Holsapple, & Kaminski, 1998). B cell differentiation into antibody forming cells is significantly inhibited by TCDD in various animal models and this effect appears to be AhR-dependent (Mimura & Fujii-Kuriyama, 2003; Sulentic et al., 1998). TCDD-induced suppression of mouse B cell maturation, activation, and differentiation is mediated by the immunoglobulin heavy chain (IgH) locus (Fernando, Ochs, Liu, Chambers-Turner, & Sulentic, 2012; Sulentic et al., 1998). Inhibition of antibody secretion can occur either with or without T-helper function. B cells can be activated via lipopolysaccharide, LPS, (T-cell independent) or sheep red blood cells (T-cell dependent) (Holsapple, Dooley, McNerney, & McCay, 1986). Furthermore, mouse B cells (CH12.LX) activated by LPS increase AhR expression, which may increase the ability of AhR to bind to TCDD and increase the sensitivity of B cells to TCDD (Sulentic et al., 1998). One study showed that TCDD decreases IgM secretion under LPS stimulation in AhR expressing mouse B cells (CH12.LX), while it did not inhibit IgM secretion when activated by LPS in AhR-deficient mouse B cells (BCL-1), implicating an important role for AhR in inhibition of B cells by TCDD (Sulentic et al., 1998). Another research study confirmed that knocking down AhR or using an AhR antagonist in an IgA secreting mouse B cell line reduced TCDD-induced inhibition IgA secretion, which adds more evidence that AhR plays an essential role in inhibition of Ig expression by TCDD (Sulentic , data not published).

Immunoglobulin Heavy Chain *(IgH)* Locus

The human *Ig* heavy chain *(IgH)* locus is located on chromosome 14, encodes the heavy chains of human antibodies, and undergoes VDJ recombination, class switch recombination (CSR), and somatic hypermutation (SHM) (Pinaud et al., 2011). A rearranged IgH gene is comprised of the variable heavy chain promoter (V_H), VDJ region, intronic enhancer (Eμ), heavy chain constant region (C_H) with germline promoters, and the 3' immunoglobulin heavy chain regulatory region (3'*IgH*RR) (Mills, Harindranath, Mitchell, & Max, 1997) (Fig 4). The V_H promoter initiates transcriptional regulation of the *IgH*, while the VDJ encodes the antigen binding site. The intronic enhancer (Eμ) is necessary for efficient V to DJ joining and Cμ expression. The mouse 3'*Igh*RR, located downstream of the *Igh* locus, is a key regulator of the *Igh* locus and plays an important role in modulating the transcription of the *Igh* gene, and CSR but does not affect VDJ rearrangement (Cogne et al., 1994; Ju, Chatterjee, & Birshtein, 2011). There are some differences between the 3'*IgH*RR in the mouse and human. While the mouse has one 3'*Igh*RR that contains four enhancers (hs3a; hs1,2; hs3b; hs4) that display DNase I hypersensitivity, the human 3'*IgH*RR has two regulatory regions, α_1 3'*IgH*RR and α_2 3'*IgH*RR, and each of these regions has three enhancers (hs3; hs1,2; hs4) (Chauveau & Cogne, 1996; Mills et al., 1997; Sepulveda, Emelyanov, & Birshtein, 2004; Sepulveda, Garrett, Price-Whelan, & Birshtein, 2005).

In the mouse, the 3'*Igh*RR was identified as a sensitive transcriptional target of TCDD, which may mediate the inhibitory effect of TCDD on Ig expression (Sulentic, Holsapple, & Kaminski, 2000). The 3'*IgH*RR has stronger activity in surface Ig B cells and plasma cells than in pre-B cells (Ong, Stevens, Roeder, & Eckhardt, 1998). Utilizing the CH12LX mature B-cell line, TCDD suppresses the mouse 3′*Igh*RR activation and Ig secretion, and induces AhR binding to dioxin response elements (DRE) within the 3′*IgH*RR hs1,2 and hs4 enhancers (Sulentic, Kang, Na, & Kaminski, 2004)**.** In addition, antagonist studies supported an aryl hydrocarbon receptor-dependent inhibition of 3′*IgH*RR activation (Sulentic, data not published). The mouse enhancer hs1,2 is the most active enhancer within 3'*IgH*RR's enhancers in mature B cells and plasma cells whereas the hs3 enhancer has slight and hs4 has modest activity in activated B cells (Chauveau, Pinaud, & Cogne, 1998; Saleque, Singh, & Birshtein, 1999). Moreover, the hs1,2 and hs4 enhancers are regulated by many different transcription factors such as NFkB, Oct, and Pax5. Overall, the enhancers of 3'IgH RR display stronger synergistic activity when they are together than they do individually (Ong et al., 1998; Stevens, Ong, Kim, Eckhardt, & Roeder, 2000). Previous studies have demonstrated a species difference in the effects of TCDD on hs1,2 enhancer activity. TCDD inhibits mouse hs1,2 enhancer activation, which correlates with the inhibitory effects of TCDD on mouse 3'*Igh*RR and Ig, while TCDD activates the human hs1,2 enhancer, which is contradictory to the inhibitory effects of TCDD on mouse Ig expression (Fernando et al., 2012).

In humans, a polymorphism of the hs1,2 enhancer in the 3'*IgH*RR has been associated with Burkitt's lymphoma and many different human immune-related disorders such as IgA nephropathy, celiac disease, systemic sclerosis, plaque psoriasis, psoriatic arthritis, dermatitis herpetiformis, and rheumatoid arthritis (Aupetit et al., 2000; Cianci et al., 2008; Pinaud et al., 2011; Tolusso et al., 2009). The B cell malignancies, such as human Burkitt's lymphoma, contain a chromosomal translocation between proto-oncogenes, c-myc and bcl-2, and the 3'*IgH*RR which induces c-myc and bcl-2 deregulated gene expression and increases resistance to cell death (Heckman et al., 2003).

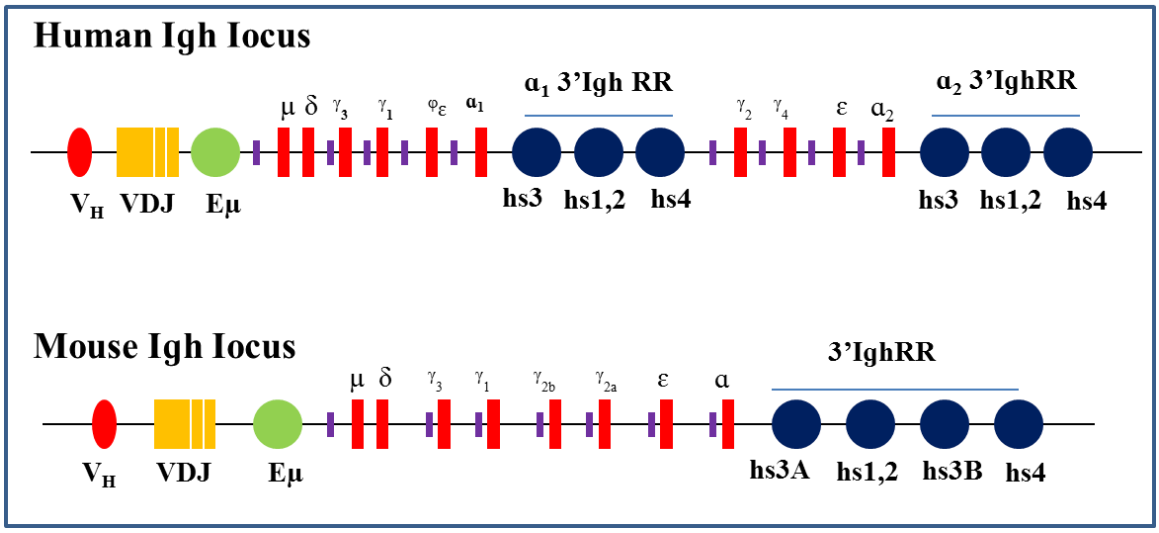


Figure 4: Human and mouse immunoglobulin heavy chain (IgH) gene locus. VH, variable heavy chain promoter; Eμ, intronic or μ enhancer; open rectangles, germline promoters upstream of each heavy chain constant region.

The hs1,2 Human Polymorphic Enhancer.

The human 3' *IgH*RR contains three transcriptional enhancers called hs3, hs1,2 and hs4 that are located downstream of the 3'*IgH* locus (Fig. 4). Among them, the human hs1,2 enhancer plays a critical role in the control of transcription and is considered the strongest the three enhancers (Cogne et al., 1994; Guglielmi, Truffinet, Magnoux, Cogne, & Denizot, 2004). Although the mouse and human hs1,2 enhancers share about 90% similarly in DNA sequences, there are some notable differences between them (Mills et al., 1997). These differences may explain the diverse behaviors of both human and mouse hs1,2 enhancers under the effect of TCDD. TCDD inhibits the mouse hs1,2 enhancer while it activates the human hs1,2 enhancer in both a mouse and human B-cell line (Fernando et al., 2012). Interestingly, Paired box protein (Pax5), an important regulator of B cell development and differentiation, highlights the most notable difference between the human and mouse hs1,2 enhancer (Fig 5). While the human hs1,2 enhancer does not have Pax5, the mouse hs1,2 enhancer contains two copies of Pax5 binding sites (Schneider, Manzan, Yoo, Crawford, & Kaminski, 2009). Pax5 is a negative regulator of B-cell differentiation into antibody secreting cells and this may explain the divergent effects of TCDD between the human and mouse hs1,2 enhancer.

The human α_1 hs1,2 enhancer is polymorphic and contains one to four copies of an invariant sequence (IS) of 53 bp. The invariant sequence (IS) could be repeated one (α_{1A}), two (α_{1B}), three (α_{1C}), or four (α_{1D}) times and each repeat

has several binding sites for transcription factors such as AP-1, SP1, NF-κB, and DRE (Denizot et al., 2001; Mills et al., 1997). Previous research has shown that an increase in the number of 53 bp repeats may result in an increase in sensitivity to TCDD (Fernando et al., 2012). Polymorphisms in the human hs1,2 enhancer correlate with a number of immune disorders, such as plaque psoriasis, psoriatic arthritis, systemic sclerosis lupus, rheumatoid arthritis, coeliac disease, herpetiform dermatitis, and IgA nephropathy. Of the different hs1,2 alleles, α_{1B} significantly correlates with increased severity of these diseases (Aupetit et al., 2000; Cianci et al., 2008; Pinaud et al., 2011; Tolusso et al., 2009). Furthermore, past studies have shown that the allelic frequency of all four alleles of the hs1,2 enhancer is distributed differently among ethnic populations suggesting the hs1,2 polymorphism may be considered a reliable anthropogenetic marker. For example, α_{1B} has the highest frequency among Asian and Europeans, α_{1C} and α_{1D} alleles are at their highest frequencies among Africans (Giambra et al., 2006). Elucidating the role of the polymorphic hs1,2 enhancer in 3'*IgH*RR activity and the effect of TCDD on the enhancers of the 3'*IgH*RR may provide insights into the etiology of autoimmune diseases associated with the hs1,2 polymorphism.

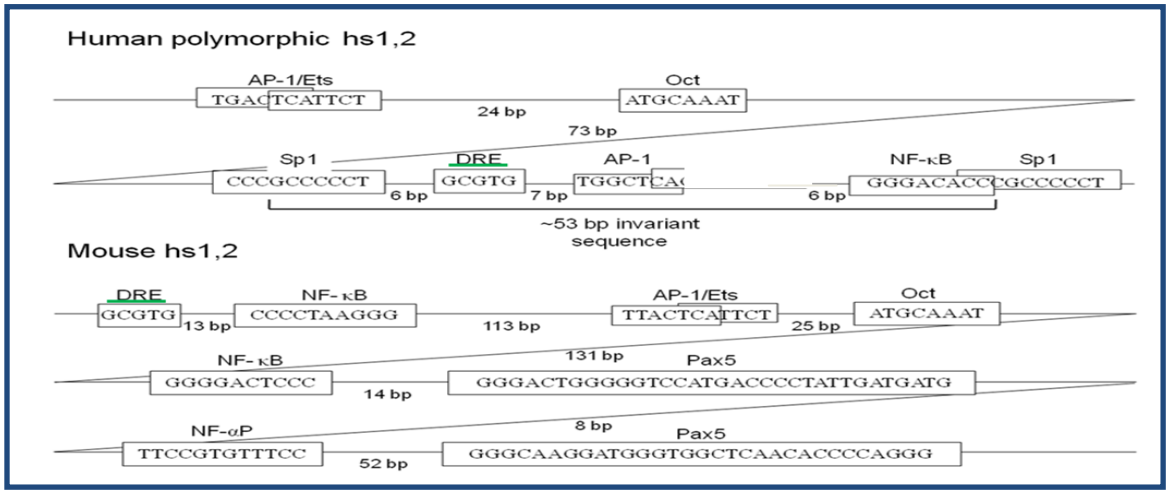


Figure 5: Comparison of DNA sequences for the human and mouse hs1,2 enhancers with predicted transcription factor binding sites.

Significance, Objectives, and Hypothesis

A previous study showed the activation of the human hs1,2 enhancer by TCDD using a mouse B-cell line (CH12.LX) and a human B-cell line (IM-9) (Fernando et al., 2012). In the IM-9 human cell line, cells could not be activated with either R848, a ligand for TLR 7/8, or CpG, a ligand for TLR 9 whereas LPS, a ligand for TLR 4, stimulated the basal activity of the human hs1,2 enhancer and antibody secretion in the mouse cell line, CH.12LX. Activating B cells is very important for differentiation into plasma cells and production and secretion of antibodies IgD and IgM with lipophilic tail are constitutively expressed in mature, unactivated B cells. For this project, I am using a different cell line Cl-01 that was isolated from a Burkitt's lymphoma patient and has antigen specificity to human monoclonal B cell line that expresses surface IgM and IgD. CL-01 cells can undergo somatic hypermutation and Ig class switching in vitro, which makes the CL-01 cell line a better model for in vitro studies of human B cell differentiation and CSR (Cerutti et al., 1998). The CL-01 cell line should offer a suitable model to study the effects of TCDD on the hs1,2 alleles and correlate these effects with Ig expression and CSR. Based on preliminary data, my hypothesis is that the AhR is a significant positive regulator of the human hs1,2 enhancer and that the hs1,2 enhancer is a negative regulator of 3'*IgH*RR activity and Ig expression. In order to test this hypothesis, I had three objectives. Objective one was to determine the effect of TCDD on enhancer activity of the human hs1,2 alleles. Objective two was to determine the effect of B cell stimulation by R848 and CpG on enhancer activity of the human hs1,2 alleles. Objective three was to determine what role the AhR plays in TCDD's effects on the activity of the human hs1,2 alleles.

The current study has significant implications for several reasons. First of all, the human hs1,2 enhancer is polymorphic and has been associated with a number of autoimmune diseases such as dermatitis herpatiforms, plaque psoriasis, psoriatic arthritis, systemic sclerosis, coeliac disease, rheumatoid arthritis, lupus, sclerosis, schizophrenia, and IgA nephropathy. Elucidating the role of the polymorphic hs1,2 enhancer in 3'*IgH*RR activity and the effect of TCDD on the enhancers of the 3'*IgH*RR may provide insights into the etiology of autoimmune diseases associated with the hs1,2 polymorphism as well as greater understanding of Ig regulation in general. Secondly, studying the human polymorphic hs1,2 enhancer as a part the 3'*IgH*RR is very important since 3'*IgH*RR is related to chromosomal translocations that lead to result in an oncogene (i.e. c-myc or bcl2) under the transcriptional regulation of the 3'IghRR leading to specific cancers, such as Burkitt's and diffuse large cell lymphoma. Finally, studying the AhR's role in human hs1,2 enhancer activity is important in assessing risk of human exposure to AhR ligands and potentially offering new therapeutic avenues for diseases associated with the 3'IghRR.

II. MATERIALS AND METHODS

Chemicals and Reagents

TCDD, dissolved in 100% dimethyl sulfoxide (DMSO), was purchased from Accustandard Inc. (New Haven, CT). This product has a certificate of analysis that reports 99.1% purity. DMSO was purchased from Sigma-Aldrich (St. Louis, MO). The AhR antagonist (CH-223191), dissolved in 100% DMSO, was purchased from Calbiochem (Carlsbad, CA). R848, a TLR 7/8 ligand, was purchased from Enzo Life Sciences and was dissolved in water. CpG, a TLR 9 ligand, was purchased from Eurofins MEG Operon (Huntsville, AL) and was dissolved in water.

Cell Line Model

The CL-01 human B-cell line that was utilized in all experiments and isolated from a Burkitt's lymphoma patient. The CL-01 cell line is a non-antigen specific human monoclonal B cell line that expresses surface IgM and IgD. Moreover, CL-01 cells can undergo somatic hypermutation, and CSR in vitro, which makes CL-01 a better model for in vitro studies of human B-cell differentiation and CSR (Cerutti et al., 1998).

Cell Culture Conditions

The CL-01 cell line was grown in RPMI 1640 medium (Mediatech, Inc., Manassas, VA) supplemented with 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 β-mercaptoethanol. Cl-01 cell line was maintained at 37°C in an atmosphere of 5% CO_2 for 24 hours.

Reporter Plasmid Constructs

Human polymorphic hs1,2 reporter plasmids (α_{1A}, α_{1B}, α_{1C}, and α_{1D}) were generously provided by Dr. Michel Cogne (Laboratoire d'Immunologic, Limoges, France). As described previously, the human polymorphic hs1,2 reporter plasmids contain the variable heavy chain promoter and either one (α_{1A}), two (α_{1B}), three (α_{1C}) or four (α_{1D}) repeats of the 53 bp invariant sequence (IS) (Fig 6) (Denizot et al., 2001). The human polymorphic hs1,2 reporter plasmids utilize the pGL3 basic luciferase reporter construct (Promega, Madison, WI) containing ampicillin resistance and the luciferase gene.

Site-directed Mutagenesis

Site-directed mutagenesis was performed on the α_{1D} human hs1,2 luciferase reporter construct according to the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to avoid a confounding contribution of a DRE within the multiple cloning site (MCS) of pGL3 the backbone as described previously (Fernando et al., 2012) Oligonucleotide primers (Table 1) were designed to delete the DRE binding motif in the multiple cloning site. The deletion reaction used 5 µl of 10X reaction buffer, 100 ng of genomic DNA extracting from CL-01 cell line, 1 µl of dNTP mix, 125 ng each of forward and reverse mutation primers, 1.5 µl of QuikSolution reagent, and ddH_2O to a final volume of 50 µl in PCR tubes. This was followed by adding 1 µl of QuikChange Lightning Enyzme before placing the tubes on thermal cycling machine. PCR conditions were 95º C for 2 min followed by 18 cycles at 95º C for 20s, 60º C for 10s, 68º C for 2 min and 30 sec (30 sec/kb of plasmid length), then 68º C for 5 min. Following the PCR reaction, 2 µL of the Dpn I restriction enzyme was added to each 50 µL reaction and

incubated at 37ºC for 5 minutes. 2 µl of Dpn I-treated DNA was transformed into 45 µl of XL10-Gold® ultracompetent cells (Retrogen, Inc., San Diego, CA).

Forward Primer	*GGTACCGAGCTCTTAC----------CTAGCCCGGGC*
Template sequence	CCGAGCTCTTAC**GCGTG**CTAGCCCGGG
Reverse Primer	CCCGGGCTAGG----------TAAGAGCTCGGTACC

Table 1: Oligonucleotide primers for site-directed mutagenesis to remove MCS-DRE from α_{1D} hs1,2 reporter. Primers are italicized, and the MCS-DRE in bold is deleted.

Transient Transfection

CL-01 cells (1.0×10^7) were pelleted by centrifugation at 500 x g for 5 minutes at 4° C. The pellet was re-suspended with 10 µg of plasmid and enough complete medium to make the final volume 200 µl. The 200 µl mixture was transferred to a 2mm electroporation cuvette and electroporated at 150 V, 1500 µF, and 75 ohms. For each plasmid, multiple transfection cuvettes were pooled and diluted to a seeding concentration of 1×10^5 cells/ml, then treated as follows: naïve (NA), 0.01% DMSO vehicle (0 nM TCDD), or TCDD (0.03 nM, 0.3 nM, 3 nM, 30 nM) in the absence or presence of R848 (1 µg/mL) or CpG (5 nM) stimulation. Cells were aliquoted into 12-well plates, each well contained 2 ml of pooled transfected cells (n=3) and was incubated at 37°C in 5% CO_2 for 24 hours. In the AhR antagonist experiments (AHRA), we treated as follows: naïve (NA), 0.05% DMSO vehicle (0 nM AhR), or AhR (30 µM) in the absence or presence of R848 (1 µg/mL). Cells were pre-treated with 30 µM AhRA and incubated at 37°C in 5% CO_2 for 1 hr before adding other treatments. Following a 24 hr of incubation, the cells were pelleted and lysed with 1x reporter lysis buffer (Promega),

then immediately frozen at -80°C for a minimum of 2 hours. To measure luciferase enzyme activity, samples were thawed on ice and centrifuged at 14000 x g for 5 minutes at 4°C then100 µl of luciferase substrate (Promega) was mixed with 20 µl of sample lysate in a glass tube and luminescence (luciferase activity) was measured by a Sirius luminometer (Berthold Detection Systems, Oak Ridge, TN) and presented as relative light units (RLU)

Transfection Efficiency

To determine the transfection efficiency, as previously described Sulentic et al., 2004, we measured the number of plasmids per cell using quantitative real-time PCR. DNA was isolated using a genomic DNA extraction kit (Sigma Aldrich) from naïve cells. TCDD and cellular activation treatments do not affect transfection efficiency (Sulentic, Zhang, Na, & Kaminski, 2004). Two hours following transfection, the DNA was diluted 10-fold and 2 µl was mixed with 12.5 µl 2x SYBR Green (Applied Biosystems, Warrington, UK), 1 µl 10 pmol/µl forward primer-pGL3 luciferase (5'-ACTGGGACGAAGACGAACACTT-3'), 1 µl 10 pmol/µl reverse primer-pGL3 luciferase (5'-TCAGAGACTTCAGGCGGTCAA-3'), and 8.5 µl purified water. Samples were compared to a standard luciferase reporter plasmid (α_{1A}) with concentrations ranging from 0.1 ng/µl to $1x10^6$ ng/µl. The plasmid number per cell was calculated using the following equation: (ng of plasmid) x (molecules of plasmid/ng of plasmid) /cell number,. Luciferase activity was normalized to the control plasmid based on plasmids per cell.

Statistical Analysis of Data

A one way ANOVA followed by a Dunnett's Multiple Comparison post test was used to analyze the treatments groups (n=3) for significant differences to the appropriate vehicle control "*", "**", "***" represent significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. A two way ANOVA followed by a Dunnett's Multiple Comparison post test was used to analyze significant differences between different plasmids. Results are represented as either relative light units (RLU) normalized to transfection efficiency or fold-change relative to the appropriate vehicle control. The mean fold-change ± S.E. was determined by averaging the means from four to five separate experiments.

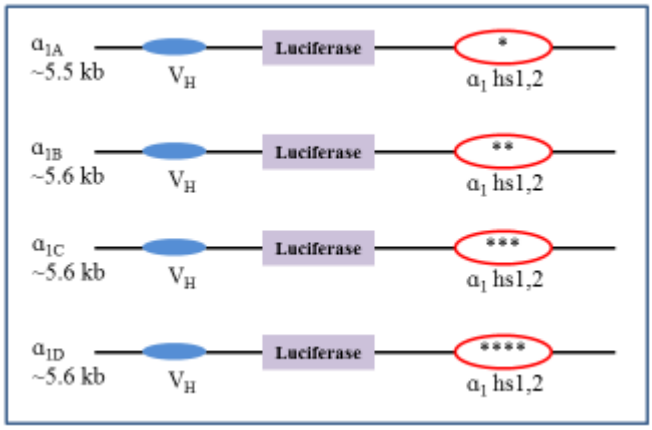


Figure 6: Human polymorphic hs1,2 reporter plasmid constructs. The asterick (*) represents the IS that may be present one (α_{1A}), two (α_{1B}), three (α_{1C}) or four (α_{1D}) times. V_H, variable heavy chain promoter

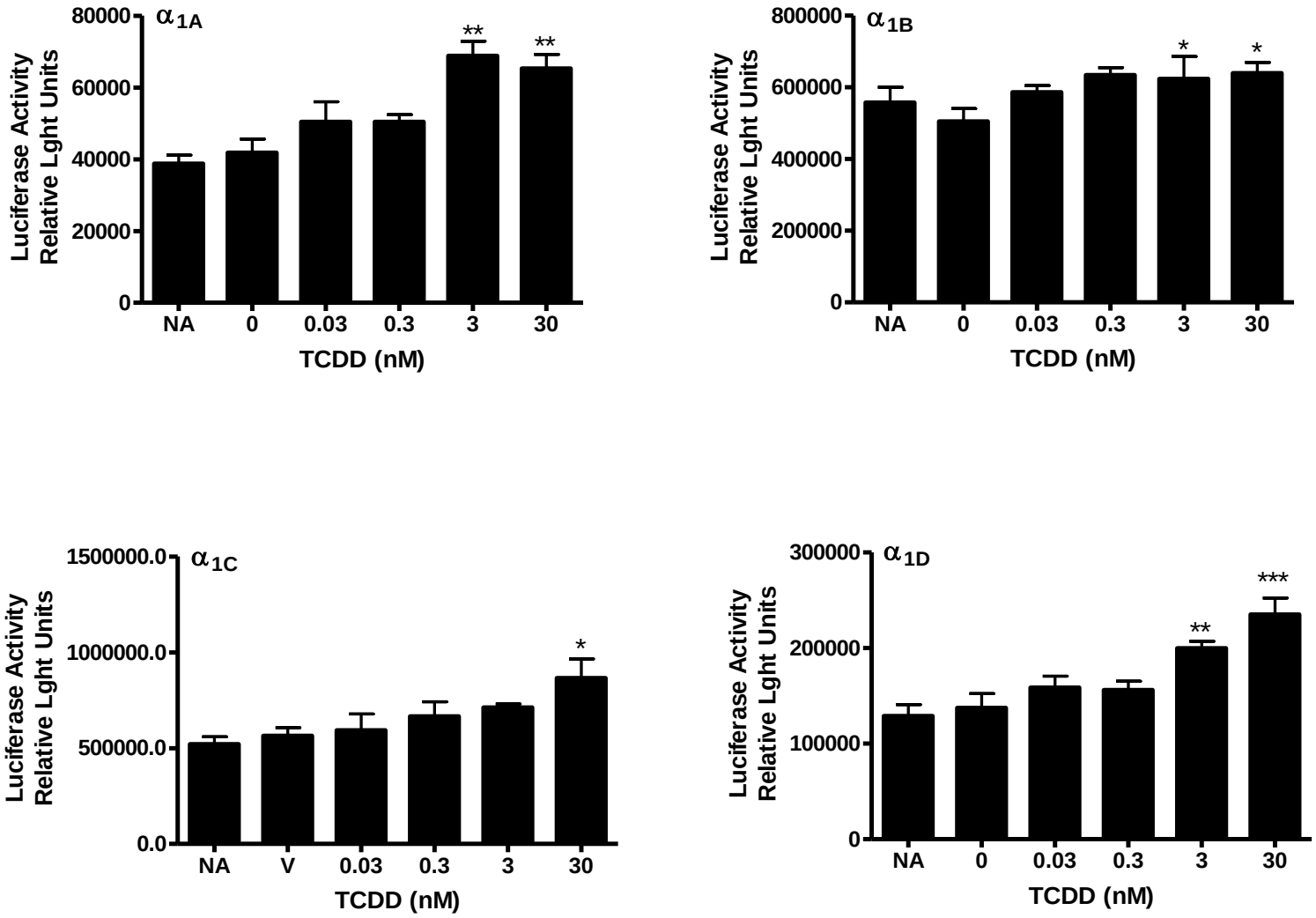
III. RESULTS

TCDD activates the human polymorphic hs1,2 enhancer in the CL-01 human B cell line

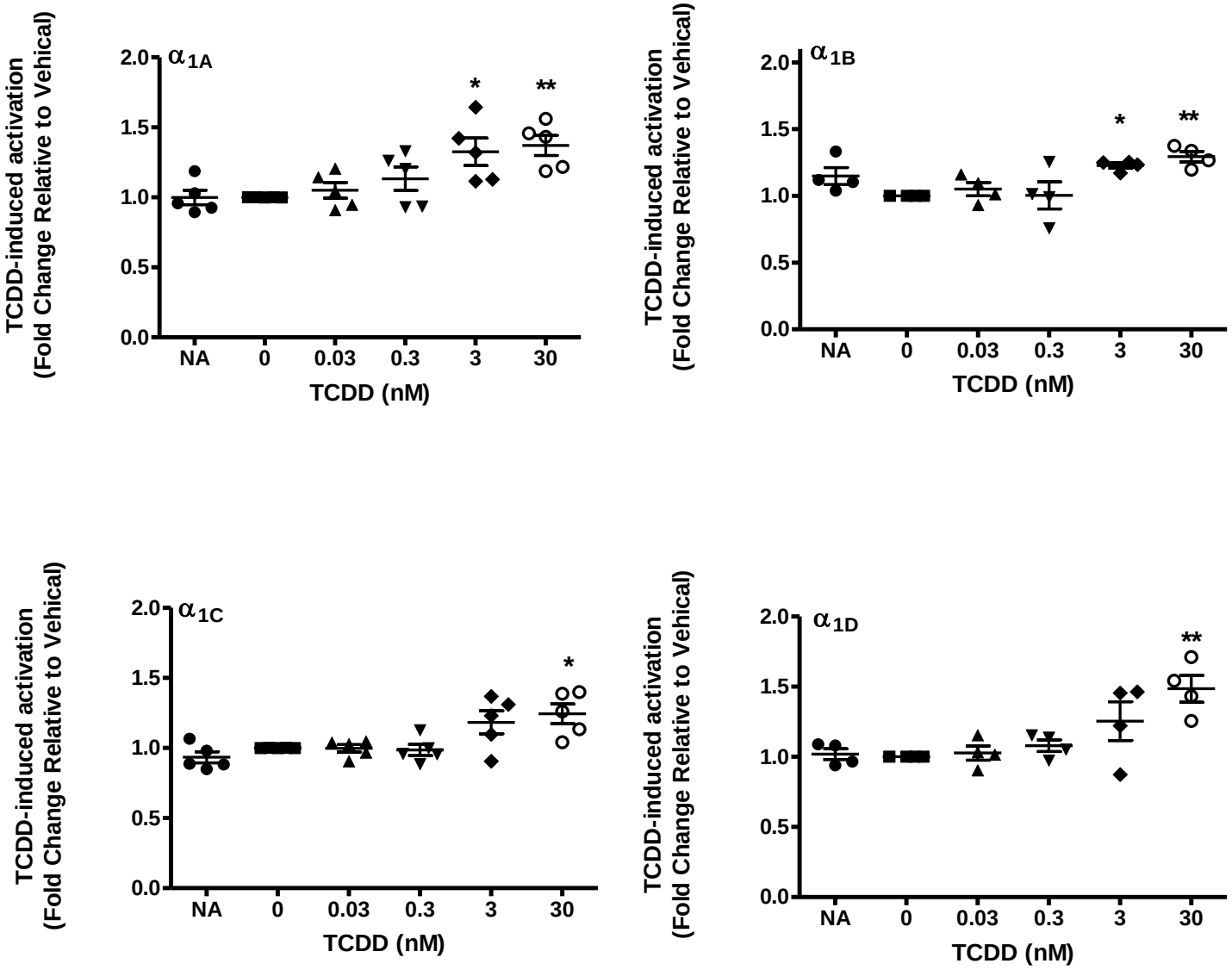
CL-01 cells were transiently transfected with luciferase reporter plasmids containing the human polymorphic hs1,2 enhancer (α_{1A}, α_{1B}, α_{1C}, and α_{1D}), then treated with different concentrations of TCDD (0.03-30 nM). Luciferase reporter results were normalized to transfection efficiency as determined by quantitative real-time PCR targeting the luciferase gene. Expectedly, TCDD significantly activated the human polymorphic hs1,2 enhancer (α_{1A}, α_{1B}, α_{1C}, and α_{1D}) in a concentration-dependent manner (Fig.7 A). On the other hand, TCDD inhibited IgM and IgG secretion (Brooke Johnson, data not published). The fold change of TCDD-induced activation for each luciferase reporter plasmid (α_{1A}, α_{1B}, α_{1C}, and α_{1D}) was relatively similar (Fig.7 B) Whereas increase in the number of invariant sequences doesn't greatly impact TCDD-induced activation (Fig.7 C). Each repeat of IS has several binding sites for transcription factors such as AP-1, SP1, NF-κB, and DRE (Denizot et al., 2001; Mills et al., 1997). Increasing the number IS will raise the chance for AhR-TCDD to bind to more transcription binding sites within the IS's and makes stronger effect of TCDD on the basal activity of human hs1,2 enhancer. Therefore, the effect of both R848 and TCDD was evaluated on the human polymorphic hs1,2 enhancer by utilizing reporter plasmids (α_{1A}, α_{1B}, α_{1C}, and α_{1D}) (Fig. 8). Interestingly, the basal transcriptional activity in human CL-01 cells increased with increasing number of IS repeats up to three (α_{1A}, α_{1B}, and α_{1C}), while the basal activity of four IS repeats (α_{1D}) was similar to the basal activity of two IS repeats (α_{1B}). Unexpectedly, R848 stimulation inhibited the basal activity of each hs1,2 allelic reporter (Fig 8). Previous studies have shown increases in basal activity of the human

polymorphic hs1,2 enhancer that correlated with an increased number of these 53 bp sequences for (α_{1A}, α_{1B}, and α_{1C}) (Mimura & Fujii-Kuriyama, 2003).

A)



B)


TCDD-induced activation
(Fold Change Relative to Vehical)
α1A
α1B
α1C
α1D
2.0
1.5
1.0
0.5
0.0
NA
0
0.03
0.3
3
30
TCDD (nM)
*
**

C)

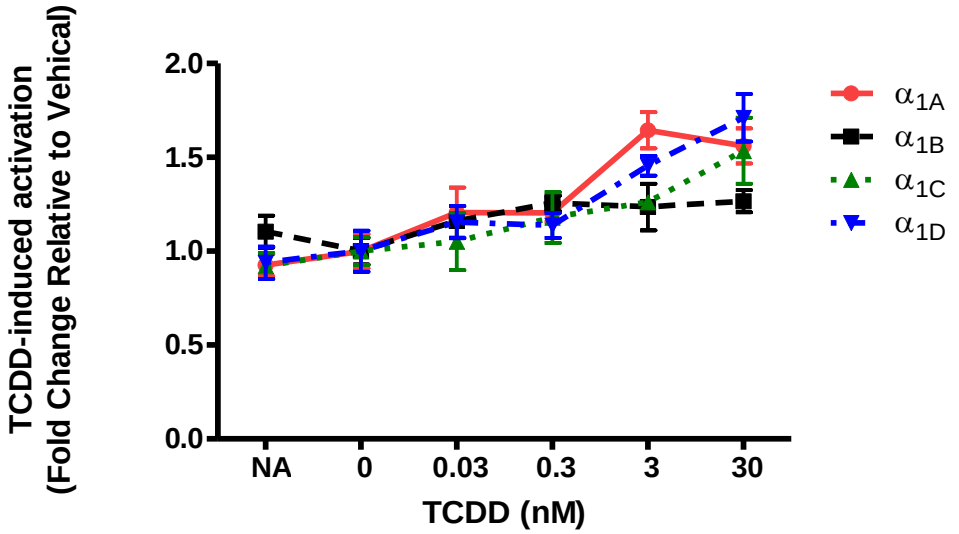


Figure 7: TCDD activates the human polymorphic hs1,2 enhancer in the CL-01 human B cell line and an increase in the number of invariant sequences doesn't greatly impact TCDD-induced activation. CL-01 cells were transiently transfected with α_{1A}, α_{1B}, α_{1C}, or α_{1D} luciferase reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.03-30.0 nM). **(A)** Luciferase enzyme activity (mean SEM, n=1) is represented on the y-axis as relative light units (RLU) normalized to transfection efficiency. **(B)** TCDD-induced activation (mean SEM, n≥3) is represented on the y-axis as fold change relative to the vehicle control (0 nM TCDD). **(C)** TCDD-induced activation is represented on the y-axis as fold change relative to the appropriate vehicle control. Comparisons between treatment groups were analyzed using a 1-way ANOVA for A and B followed by a Dunnett's Multiple Comparison post test . Asterisks, "*", "**", or "***" denote significance compared to the corresponding vehicle set to 1 at $p<0.05$, $p<0.01$ or $p<0.001$, respectively. For C, followed by a Dunnett's Multiple Comparison post test .

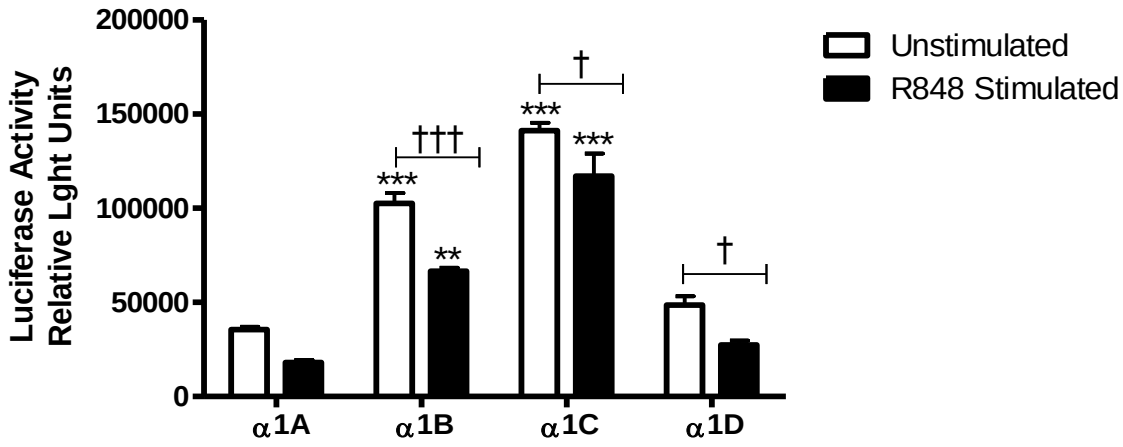
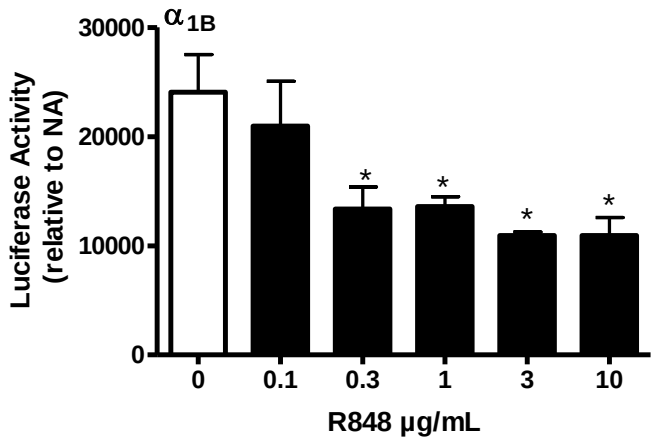


Figure 8: The basal activity of human hs1,2 enhancer increased with increasing number of IS repeats up to three (α1A, α1B, and α1C), while the basal activity of four IS repeats (α1D) was similar to the basal activity of two IS repeats (α1B). The CL-01 cells were transiently transfected with the α_{1A}, α_{1B}, α_{1C}, and α_{1D} reporter plasmids. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 hr with 1.0 μg/mL R848. Luciferase activity is represented on the y-axis as relative light units normalized to transfection efficiency ($n \geq 3$). Comparisons between the treatment groups were analyzed using a one-way ANOVA followed by a Dunnett's two-tailed *t*-test. Asterisks, "*", "**", or "***" denote significance compared to α_{1A} at $p<0.05$, $p<0.01$ or $p<0.001$, respectively. Comparisons between unstimulated and R848 stimulated of each plasmid was analyzed using a two-way ANOVA followed by a Bonferroni's two-tailed *t* test. "†", "††", and "†††" denote significance compared with the unstimulated at $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

Cellular stimulation inhibits basal hs1,2 activity but TCDD co-treatment reverses this inhibition

To better characterize the effects of cellular stimulation on hs1,2 activity, we evaluated the concentration-dependent effects of R848 on the α1B hs1,2 reporter in the CL-01 cells with varying concentrations of R848 (0.1 - 10 μg/mL). R848 inhibited the basal activity of the α1B hs1,2 enhancer reporter in a concentration-dependent manner (Fig 9). However, 1ug/mL R848 induces IgM and IgG stimulation (Brooke Johnson, unpublished data). Therefore R848 is inducing cellular activation of the CL-01 cells, perhaps suggesting that the hs1,2 enhancer is a negative regulator of the 3'IgHRR. Another possibility is that the cells have not reached a sufficient cellular activation phase when the luciferase is read. Recall early work of Roeder showing a dichotomy in the regulation of hs1,2 that was stage dependent.

A)



B)

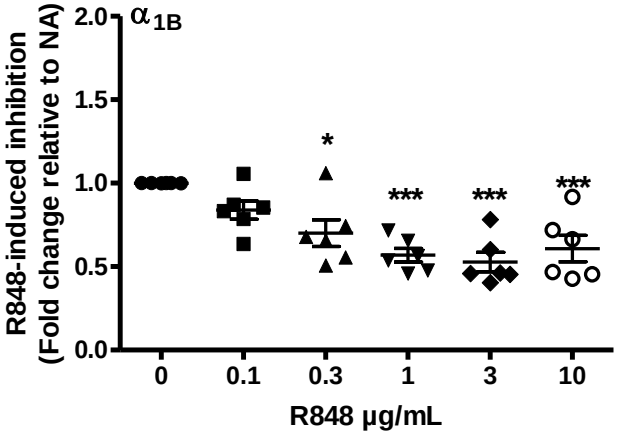


Figure 9: R848 inhibit basal activity of α_{1B} hs1,2 enhancer. CL-01 cells were transiently transfected with the α_{1B} luciferase reporter plasmid. Transfected cells were either cultured in the absence of any additional treatment (naïve, 0 µg/ml) or stimulated for 24 h with R848 (0.1 - 10 µg/ml). Luciferase enzyme activity (mean SEM, n=3) is represented on the y-axis as relative light units normalized to transfection efficiency. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, *, **, or *** denote significance compared corresponding vehicle at $p<0.05$, $p<0.01$ or $p<0.001$, respectively

R848 stimulates B cells by targeting TLR 7/8 to increase immunoglobulin levels (Tomai, Imbertson, Stanczak, Tygrett, & Waldschmidt, 2000). However, having such an unexpected inhibitory effect of R848 on the activity of the human hs1,2 enhancers led us to check whether R848 can activate NFkB, a universal endpoint of TLR activation. The CL-01 cells were transiently transfected with a luciferase reporter regulated by three NF-kB binding motifs. R848 increased 3X NF-κB activation indicating that R848 is able to activate TLRs individually even though it inhibits overall activity of the hs1,2 enhancer (Fig 10).

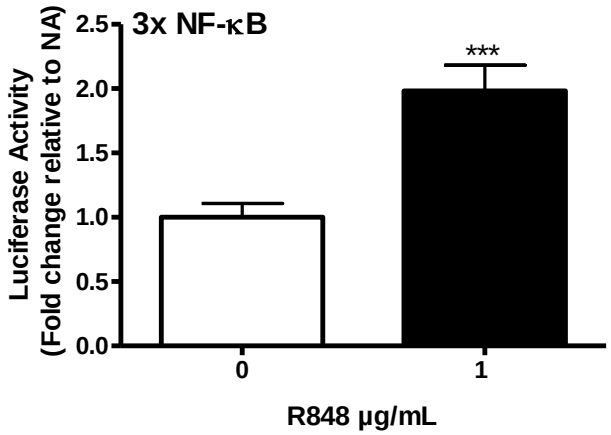
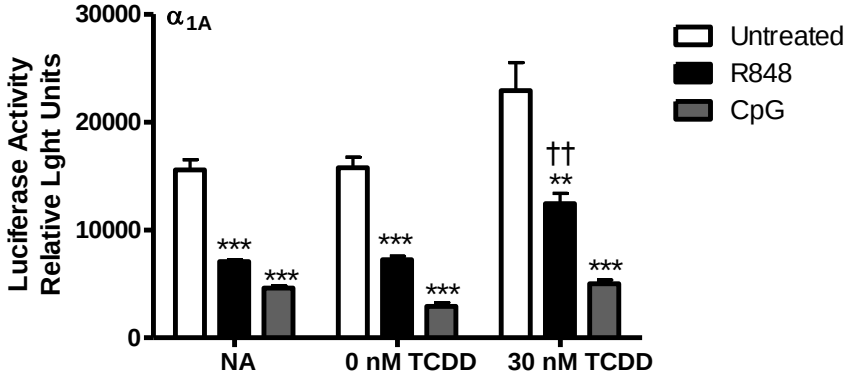
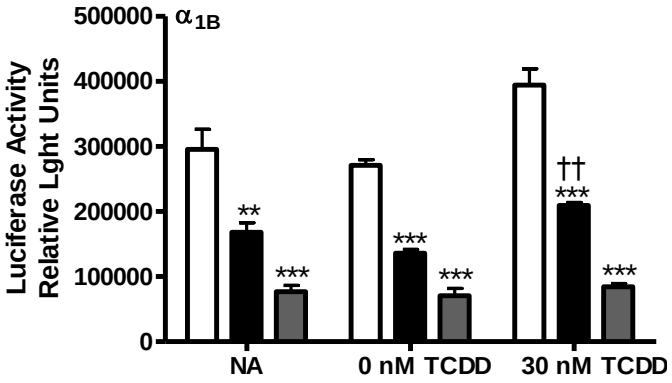
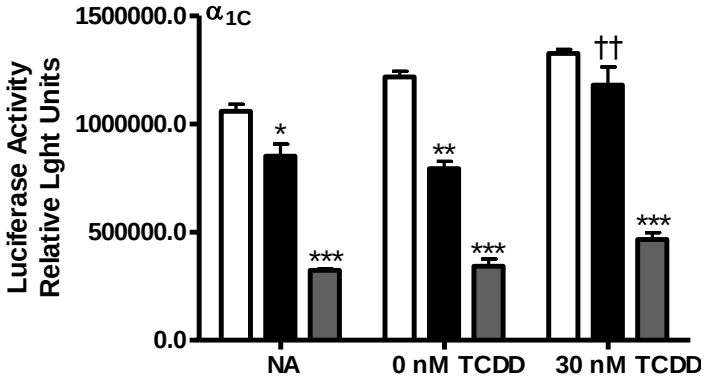


Fig. 10: R848 activates a 3x NF-κB luciferase reporter in the CL-01 human B-cell line. CL-01 cells were transiently transfected with a 3x NF-κB luciferase reporter plasmid. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with R848 (1 µg/ml). Luciferase enzyme activity (mean SEM, n=3) is represented on the y-axis as fold change relative to vehicle. Comparisons between treatment groups were analyzed using a 2-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "***" denote significance compared to the corresponding vehicle set to 1 at $p<0.001$

We also designed an experiment to check if the unexpected inhibitory effect of R848 on hs1,2 is just limited to R848 or includes other aspects of B-cell stimulation such as CpG, a ligand for TLR 9. CpG inhibits the basal activity of the human hs1,2 enhancer in the same way as R848. This result indicates that B-cell activation, in general, inhibits the basal activity of the human hs1,2 enhancer suggesting that the hs1,2 enhancer is a negative regulator of the 3'IgHRR (Fig 11). According to these results, human hs1,2 enhancer clearly inhibited by both R-848 and CpG, with CpG being superior for hs1,2 enhancer inhibition compared to R-848.


α1A
Luciferase Activity
Relative Lght Units
30000
20000
10000
0
Untreated
R848
CpG
***
***
***
***
***
††
**
***
NA
0 nM TCDD
30 nM TCDD


α1B
Luciferase Activity
Relative Lght Units
500000
400000
300000
200000
100000
0
**
***
***
***
***
††
***
NA
0 nM TCDD
30 nM TCDD


α1C
Luciferase Activity
Relative Lght Units
1500000.0
1000000.0
500000.0
0.0
*
***
**
***
††
***
NA
0 nM TCDD
30 nM TCDD

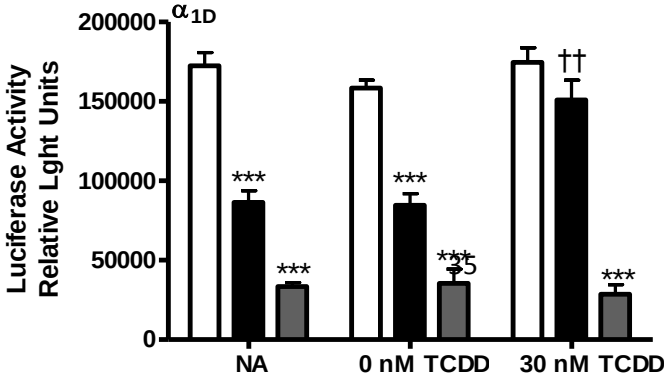

α1D
Luciferase Activity
Relative Lght Units
200000
150000
100000
50000
0
***
***
***
***
††
***
NA
0 nM TCDD
30 nM TCDD

Figure 11: R848 and CpG inhibit basal activity of human hs1,2 and TCDD reverses this inhibition. CL-01 cells were transiently transfected with α1A, α1B, α1C, or α1D luciferase reporter plasmids luciferase reporter plasmid. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (30 nM) in the absence or presence of R848 (1 μg/ml) or CpG (5 nM) stimulation. Luciferase enzyme activity (mean ± SEM, n=3) is represented on the y-axis as relative light units normalized to transfection efficiency. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "*", "**", or "***" denote significance compared between untreated to the corresponding R848 and CpG stimulated with vehicle (0 nM TCDD) or TCDD (30 nM) at $p<0.05$, $p<0.01$ or $p<0.001$, respectively. Also, "††" denotes significance compared between R848-stimulated (0 nM TCDD) and R848- stimulated (30 nM TCDD) at $p<0.01$.

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AhR Antagonist (AhRA) inhibits basal and TCDD-induced human hs1,2 enhancer activity

The AhR antagonist (AhRA) was used to test if AhR is necessary for TCDD-induced activation of the human polymorphic hs1,2 enhancer in the human CL-01 cell line. The AhRA functions by sequestering AhR in the cytoplasm upon binding to the receptor (Kim et al., 2006). CL-01 cells were transiently transfected with the hs1,2 reporters (α_{1A}, α_{1B}, α_{1C}, and α_{1D}) then treated with the AhRA. Unexpectedly, AhRA inhibited basal activity of the hs1,2 enhancer and TCDD reversed this inhibition (Figure 12**)**. Interestingly, R848 increased IgG secretion but did not affect IgM secretion (Brooke Johnson data not shown). Overall, the results indicate that the AhR is very important in mediating TCDD's effect on the human hs1,2 enhancer and AhR may also be an important biological regulator of hs1,2.

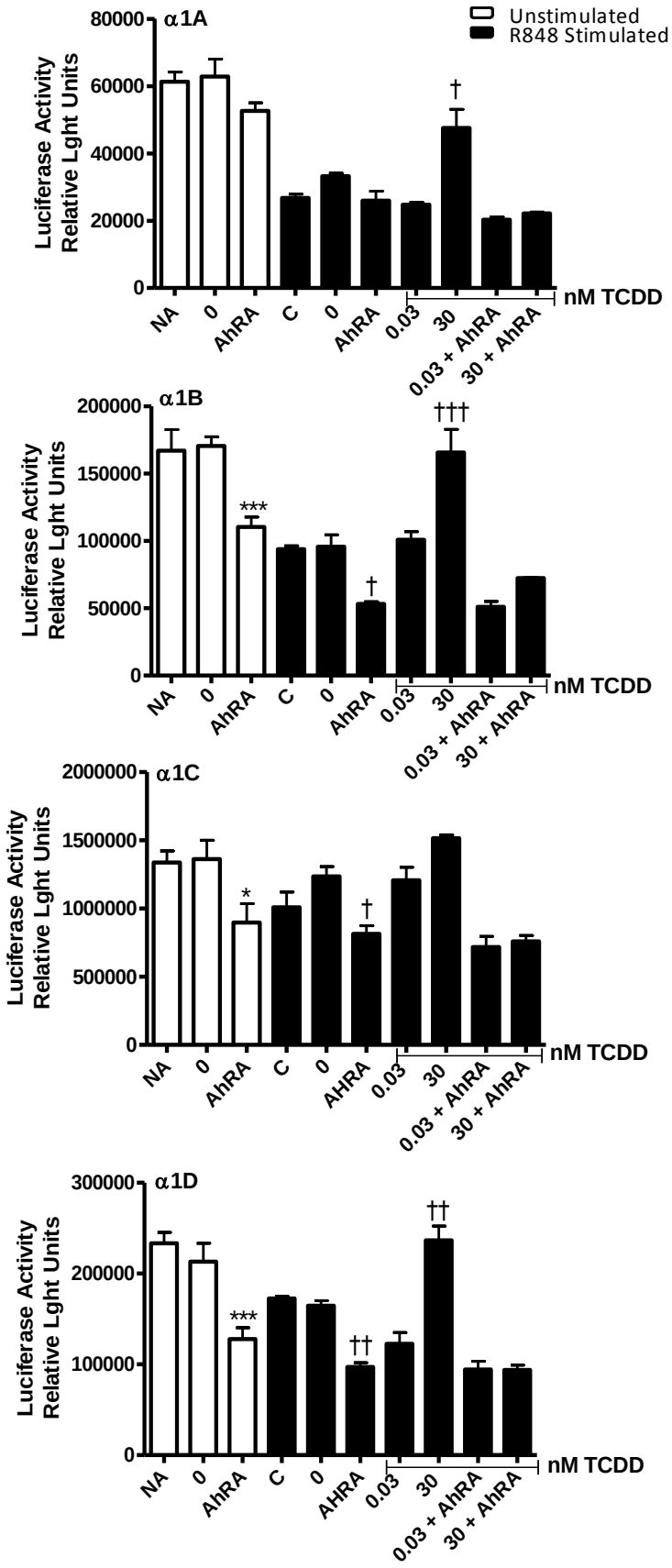
α1A
Unstimulated
R848 Stimulated
Luciferase Activity
Relative Lght Units
80000
60000
40000
20000
0
†
NA
0
AhRA
C
0
AhRA
0.03
30
0.03 + AhRA
30 + AhRA
nM TCDD
α1B
200000
150000
100000
50000
0
***
†
†††
α1C
2000000
1500000
1000000
500000
0
*
†
AHRA
α1D
300000
200000
100000
0
***
††

Figure 12: AhR Antagonist (AhRA) inhibits basal and TCDD-induced human hs1,2 enhancer activity. CL-01 cells were transiently transfected with the α1B luciferase reporter plasmid. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or stimulated with R848 (1 μg/ml), then treated for 24 h with 0.05% DMSO vehicle or TCDD (0.03, 30 nM) in the absence or presence of AhRA (30 μM). Luciferase enzyme activity (mean SEM, n=3) is represented on the y-axis as relative light units normalized to transfection efficiency. C represents the R848 alone control. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Asterisks, "*", "**", or "***" denote significance compared to the corresponding unstimulated vehicle control at $p<0.05$, $p<0.01$ or $p<0.001$, respectively. "†" or "††"denote significance compared to the corresponding stimulated vehicle control at $p<0.05$, or $p<0.01$, respectively

DISCUSSION

TCDD targets Ig expression and decreases Ig heavy and light chain transcription in mouse B-cells. Although, the AhR signaling pathway seems to mediate TCDD's effect on gene expression, the mechanism of TCDD's inhibitory effect on Ig expression is still unclear (Sulentic & Kaminski, 2011) .The mouse *Igh* locus, which codes for the heavy chain of antibodies, contains several regulatory elements including the 3'*Igh*RR region, which contains four enhancers (hs3A; hs1,2; hs3B; and hs4) (Pinaud et al., 2011). Our lab identified the mouse 3'*Igh*RR as a sensitive target of TCDD-induced inhibition and TCDD-induced AhR binding to dioxin responsive elements (DRE) in the mouse hs1,2 and hs4 enhancers (Sulentic et al., 2000; Sulentic, Zhang, et al., 2004). In addition, by using a competitive AhR antagonist (CH-223191) and knock down of AhR protein levels, our lab confirmed that TCDD-induced inhibition of mouse *Igh* expression is mediated by an AhR-dependent mechanism, in that preventing AhR activation by TCDD fully reversed TCDD-induced inhibition of 3'*Igh*RR and antibody secretion (Wourms, Sulentic, in revision).

There is some uncertainty as to whether the experimental results from mouse 3`*Igh*RR studies can be translated to the human *IgH* gene. The human *IgH* locus contains a duplication of the 3'*IgH*RR (α_1 3'*IgH* RR and α_2 3'*IgH* RR) and each region has three enhancers (hs3; hs1,2; and hs4) (Pinaud et al., 2011). Additionally the α_1 hs1,2 enhancer is polymorphic and has one to four 53 bp invariant sequences. The invariant sequence contains several transcription factor binding sites including DRE, NF-κB, SP-1, NF-1 and AP-1 (Frezza, Rubino IA et al. 2009). Interestingly, the hs1,2 enhancer polymorphism has been associated with several autoimmune diseases such as IgA nephropathy, celiac

disease, systemic sclerosis, plaque psoriasis, psoriatic arthritis, dermatitis herpetiformis, and rheumatoid arthritis (Aupetit et al., 2000; Cianci et al., 2008; Pinaud et al., 2011; Tolusso et al., 2009). Furthermore, previous work in our lab demonstrated a species difference between the mouse and human hs1,2 enhancer under the effects of TCDD using the well-characterized CH12.LX mouse B-cell line model. Notably, TCDD inhibited mouse hs1,2 enhancer activation and activated basal human hs1,2 enhancer activity (Fernando, Ochs et al. 2012). Recently, our lab discovered that the NF-1 binding site could be involved in the TCDD-induced activation of the human hs1,2 enhancer. Mutation of the NF-1 binding site in the human hs1,2 enhancer decreased TCDD-induced activation. Also, others have shown a direct interaction between Sp1, NF-κB, or AP-1 and the AhR/ARNT complex (Kobayashi, Sogawa, & Fujii-Kuriyama, 1996).We speculate that potential protein-protein interactions between the transcription factors that bind within the hs1,2 enhancer are sensitive to environmental influences (i.e. exposure to dioxin and other AhR ligands, etc.) leading to altered hs1,2 activity and Ig expression. Our current results suggest a dominant role of the AhR and NF1 in TCDD-induced activation of the human hs1,2 enhancer, but does not rule out a contribution of the other transcription factors binding within the hs1,2 enhancer (data not published). Activation of the human hs1,2 enhancer by TCDD may provide a link between the human hs1,2 enhancer and the above mentioned autoimmune diseases. In other words, AhR ligands could alter human 3'*IgH*RR activity through the hs1,2 enhancer and potentially modulate disease states associated with the hs1,2 polymorphism, particularly due to the fact that most of the transcription factors binding site within the invariant sequence have been shown to be modulated by TCDD (Frericks, Burgoon, Zacharewski, & Esser, 2008;

Sulentic et al., 2000). Furthermore, a functional humoral immune response requires B-cell activation and previous studies with mouse or human B cells have demonstrated an increase in AhR protein levels due to cellular activation (Marcus, Holsapple, & Kaminski, 1998; Sherr & Monti, 2013). For that reason, increased AhR expression in activated B cells and differences in the number of AhR binding sites in the hs1,2 alleles may influence individual sensitivity to AhR ligands and to autoimmune diseases associated with the polymorphic hs1,2 enhancer. However, studies to date have been limited to examining the human hs1,2 enhancer in the well-characterized CH12.LX mouse B-cell model. Therefore, the objective of this study was to elucidate the effects of TCDD and cellular stimulation on the polymorphic human hs1,2 enhancer in a human B-cell line (CL-01) and to determine if these effects are AhR dependent and similar to previous results using the CH12.LX mouse model.

As seen in the CH12.LX cells, the current results demonstrate TCDD-induced activation of the human polymorphic hs1,2 luciferase reporter constructs in the CL-01 human B-cell line. However, activation of the human hs1,2 enhancer contrasts markedly with the inhibitory effect of TCDD on human IgM and IgG secretion (Fig.7 and Brooke Johnson, unpublished data). On the other hand, cellular activation through TLR7/8 or 9 via R848 or CpG, respectively, inhibits human hs1,2 enhancer activity, which also contrasts with the induction of IgM and IgG secretion by R848 (Fig.12 and Brooke Johnson, unpublished data, summarized in Fig. 13 and 14). R848 has been reported to activate immune cells, including neutrophils, T cells, dendritic cells, and B cells (Tomai MA, Waldschmidt TJ et al. 2000 North, Crawford et al. 2010), which corresponds to the activation of IgM and IgG secretion in the CL-01 cells. However, the inhibition of the

human hs1,2 enhancer by R848 not only contrasts with the effects on Ig secretion but also contrasts with the effects in the mouse B cell line where LPS stimulation activated the human and mouse hs1,2 enhancers. Perhaps there are differences in TLR signaling pathways between human and mouse or different TLR pathways influence the human hs1,2 enhancer differently. R848 activates human B cells through TLR7/8 while LPS activates mouse B cells through TLR4, which is not present in human B cells. TLR7/8 activation may have a different mechanism than TLR4 has on the hs1,2 enhancers. Interestingly, R848 and CpG have no effect on the human hs1,2 enhancer activity when evaluated in the mouse B cell line CH12.LX (data not published) which lead us to believe that the differences in hs1,2 activation are related to species difference in TLR signaling pathways. Furthermore, R848 (TLR7/8) and CpG (TLR9) increased NF-κB activation in the CL-01 cells, which was consistent with the effects of these stimuli on Ig secretion. However, the effect of these stimuli on other transcriptions factors that may bind within the human hs1,2 enhancer have not been evaluated. Therefore, future studies should focus on evaluating the different TLR signaling pathways in human and mouse cells to identify any differences in the activation of transcriptions factors such as AP-1, AhR, Sp1, and NF-1 or any differences in the levels of TLR expressed following TLR activation and/or TCDD treatment.

The basal activity of the enhancer increases along with increasing numbers of 53 bp invariant sequences in the human hs1,2 enhancer with the exception of the α_{1D} allele (four IS). The basal activity of α_{1D} is the same as α_{1B} ($\alpha_{1A} < \alpha_{1B} \sim \alpha_{1D} < \alpha_{1C}$). Each 53 bp IS has many transcription factor binding sites such as NFκB, NF-1, AP-1, and DRE. TCDD-activated AhR could bind the DRE binding site to directly increase the basal

activity of the hs1,2 enhancer or the AhR could alter the signaling pathway of other transcription factors associated with the hs1,2 enhancer. The lower basal activity of α_{1D} could be because not all binding sites, especially the DRE located in the fourth repeat, are conserved. To better evaluate the impact of the fourth repeat and of potential binding sites within the human polymorphic hs1,2 enhancer, mutational analysis of the α_{1D} human hs1,2 reporter construct should be performed in which the non-conserved binding sites (i.e. DRE) are mutated to be identical to the conserved sites within the other allele. This would determine if loss of conserved sites has a functional impact. Secondly, we could delete the fourth IS repeat in the α_{1D} allele and see if the reporter after deletion behaves like the α_{1C} under the effects of TCDD and R848. If it is the same then the fourth repeat is not as active as the first three repeats. Also the fourth 53 bp IS may even be inhibitory since α_{1C} has more activity than the $\alpha_{1D.}$

As previously mentioned, R848 and CpG both inhibit the hs1,2 enhancer. Interestingly, CpG is a stronger inhibitor than R848 and TCDD reverses the R848 inhibitory effect but not that of CpG. These data might suggest that CpG and therefore TLR9 activation inhibits the human hs1,2 enhancer through NFκB and AP-1(Kawai & Akira, 2006), and additional differences in TLR signaling pathways. Alternatively, CpG could induce a greater NFκB response and a stronger inhibitory signal for the hs1,2 enhancer that is too powerful for a 30 nM TCDD treatment to reverse. To test this theory, a CpG concentration response with a fixed 30 nM TCDD concentration would determine if the increased inhibitory effect of CpG is due to pharmacokinetic rather than pharmacodynamic reasons.

AhR is a protein located in the cytosol in an inactive form bound to a dimer of the HSP90 molecules, p23, and XAP2. Upon TCDD binding, the AhR becomes active and translocates to the nucleus to bind with ARNT and either directly or indirectly influences the hs1,2 enhancer and the 3'IgHRR. It is still unclear whether binding of the TCDD/AhR/ARNT complex to DREs or to other transcription factors affects the basal activity of the human hs1,2 enhancer. Therefore, it was very important to characterize the role of the AhR on the hs1,2 enhancer to determine if TCDD-induced inhibition of *IgH* is mediated by an AhR-dependent activation of the human hs1,2 enhancer. Using a competitive AhR antagonist (AhRA), we demonstrated inhibition of basal and TCDD-induced hs1,2 activity. However, other studies in our lab demonstrated a surprising result where the AhRA increased the levels of R848-induced IgG secretion with no effect on IgM secretion (Brooke Johnson, unpublished data). On the other hand, AhRA unexpectedly inhibited basal activity of the hs1,2 enhancer and TCDD reversed this inhibition (Fig. 14). By using TCDD, AhR becomes activated and translocates to the nucleus and activates the hs1,2 enhancer but inhibits IgM and IgG secretion. The AhRA, on the other hand, competes with TCDD to keep the AhR in the cytosol in its inactive form resulting in no activation of the hs1,2 enhancer and no inhibition of IgG secretion. Surprisingly, the AhRA in the absence of TCDD inhibited basal hs1,2 enhancer activity and increased IgG secretion suggesting endogenous activation of the AhR perhaps by an as of yet unidentified endogenous ligand. This data indicates that the effects of TCDD on the human polymorphic hs1,2 enhancer may be mediated by the AhR and that some level of endogenous AhR activity is present that may inhibit CSR from IgM to IgG. Taken together, these results support our hypothesis that the AhR is a negative regulator of class

switch recombination (CSR) and Ig expression. Additional studies are necessary to confirm a physiological role of the AhR in CSR and Ig expression including knocking down the AhR using shRNA and thoroughly evaluating the effect of less AhR on the complicated CSR process in the CL-01 cells.

In conclusion, the present study has evaluated the human hs1,2 enhancer in human cells under TCDD. Interestingly, the polymorphisms in the human hs1,2 enhancer, which results in a varying number of tandem repeats of a 53 bp sequence, correlate with a number of immune disorders, such as plaque psoriasis, psoriatic arthritis, systemic sclerosis lupus, rheumatoid arthritis, celiac disease, herpetiform dermatitis, and IgA nephropathy (Aupetit, Drouet et al. 2000, Cianci, Giambra et al. 2008, Tolusso, Frezza et al. 2009, Pinaud, Marquet et al. 2011). Elucidating the role of the polymorphic hs1,2 enhancer in 3'*IgH*RR activity and the effect of TCDD on the enhancers of the 3'*IgH*RR may provide insights into the etiology of autoimmune diseases associated with the hs1,2 polymorphism. Previous work from the lab identified the mouse 3'*Igh*RR as a sensitive target of AhR ligands including TCDD and non-dioxin ligands such as ICZ, primaquine, carbaryl, and omeprazole. These non-dioxin chemicals have the same inhibitory effect as TCDD on both 3'*Igh*RR activation and *Igh* protein expression (Sulentic 2008). Also, our studies with an AhR antagonist strongly support an important role for AhR ialsoalson the effects of TCDD on human hs1,2 activity. Interestingly α_{1B} increased severity of several autoimmune diseases such as IgA nephropathy, celiac disease, systemic sclerosis, plaque psoriasis, psoriatic arthritis, dermatitis herpetiformis, and rheumatoid arthritis (Aupetit, Drouet et al. 2000, Cianci, Giambra et al. 2008, Tolusso, Frezza et al. 2009, Pinaud, Marquet et al. 2011), which could be altered by AhR

ligands. This work demonstrates Ig expression and CSR can be modulated by AhR ligands through the 3'IgHRR.Therefore non-persistent AhR ligands that do not produce the plethora of toxicities produced by TCDD could provide insight in designing an AhR ligand drug for autoimmune diseases (Hu et al., 2007).

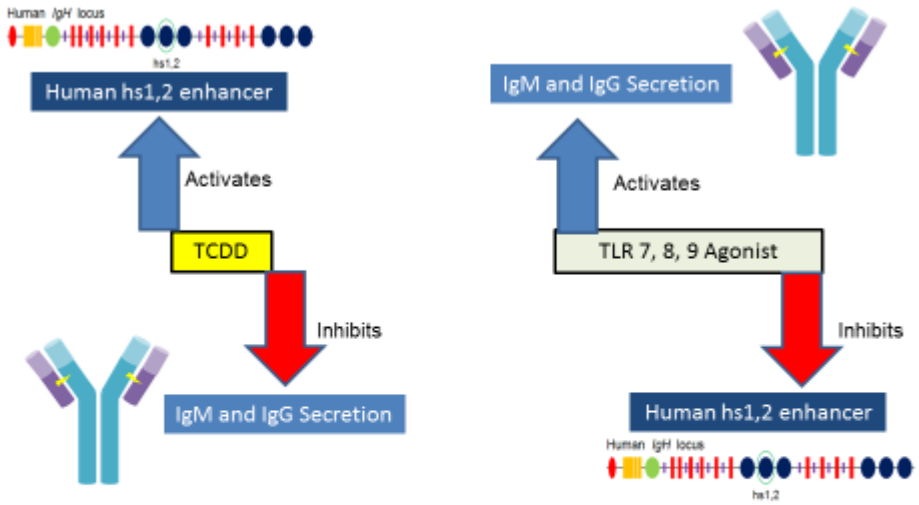

Figure 13. Contradictory effects of TCDD versus cellular stimulation through TLR on the the human polymorphic hs1,2 enhancer and Ig expression in the CL-01 human B cell line

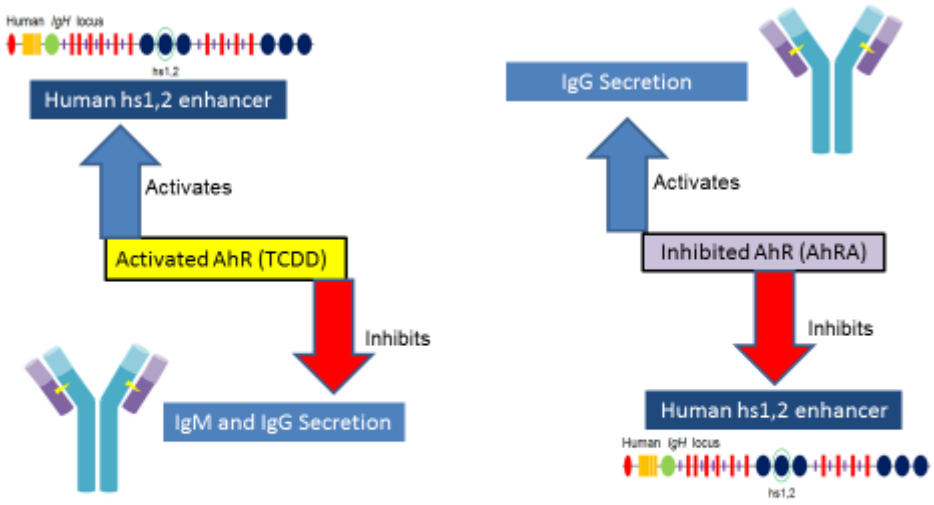

Figure 14. Contradictory effects of TCDD versus AhR agonists on the human polymorphic hs1,2 enhancer and Ig expression in the CL-01 human B cell line.

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