TCDD REPESSES 3'*Igh*RR ACTIVATION THROUGH AN AhR-DEPENDENT SHIFT IN THE NF-κB/Rel PROTEIN COMPLEXES BINDING TO κB MOTIFS WITHIN THE HS1,2 AND HS4 ENHANCERS

A dissertation submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

By

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2014
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

16 May, 2014

I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Richard L. Salisbury Jr. ENTITLED TCDD represses 3'*Igh*RR activation through an AhR-dependent shift in the NF-κB/Rel protein complexes binding to κB motifs within the hs1,2 and hs4 enhancers BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT

Salisbury Jr., Richard L. Ph.D., Environmental Sciences Ph.D. program, Wright State University, 2014. TCDD represses 3'*Igh*RR activation through an AhR-dependent shift in the NF-κB/Rel protein complexes binding to κB motifs within the hs1,2 and hs4 enhancers

Transcriptional regulation of the murine immunoglobulin heavy chain gene involves several regulatory elements including the 3′*Igh* regulatory region (3′*Igh*RR) composed of at least four enhancers (hs3A; hs1,2; hs3B; hs4). Enhancers hs1,2 and hs4 contain binding sites for several transcription factors including NF-κB/Rel proteins and the AhR. Interestingly, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) profoundly inhibits 3′*Igh*RR and hs1,2 activation induced by the B-cell activator lipopolysaccharide (LPS), but enhances the activation of the hs4. Within the hs4, the AhR binding site overlaps an NF-κB/Rel binding site suggesting that both the AhR and the NF-κB together may modulate of the 3'*Igh*RR. The objective of the current study was to evaluate the role of NF-κB/Rel and the AhR following LPS stimulation and TCDD treatment on 3′*Igh*RR, hs1,2, and hs4. In our studies we utilized the CH12.LX B cell line; the CH12.IκBαAA cell line, which expresses an inducible IκBα super repressor (IκBαAA); the CH12.γ2b-3′*Igh*RR cell line that stably expresses a γ2b-3′*Igh*RR-regulated gamma2b transgene reporter; and splenocytes derived from B6C3F1

mice. The stimulation of the CH12.γ2b-3′*Igh*RR cell line with Toll-like receptor (TLR) agonists LPS, Resiquimod (R848), or Cytosine-phosphate-Guanine (CpG)-oligodeoxynucleotides combined with a co-treatment of TCDD significantly inhibited the TLR-induced activation of the 3'*Igh*RR. Utilizing transiently expressed luciferase reporters, we found induction of IκBαAA expression partially attenuated LPS-induced activation of the 3'*Igh*RR and hs4, partially reversed the effects of a TCDD and LPS co-treatment on the activity of the 3'*Igh*RR and hs4, and the addition of an AhR antagonist, CH223191, markedly reversed the LPS and TCDD induced inhibition of the 3'*Igh*RR and inhibited the synergistic activation of the hs4. Chromatin immunoprecipitation analysis of CH12.LX and murine splenocytes demonstrated a LPS and a LPS co-treatment with TCDD-dependent increase in RelA and AhR binding, and a significant decrease in RelB binding to the hs4 and hs1,2. These results suggest that a shift in binding of the NF-κB/Rel proteins, perhaps through an interaction with the AhR are partially responsible for 3′*Igh*RR modulation by LPS and TCDD. These results suggest that interactions between the AhR and NF-κB within the 3'*Igh*RR mediate the inhibitory effects of TCDD on immunoglobulin (Ig) expression and therefore antibody levels.

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ACKNOWLEDGEMENTS

I sincerely thank Dr. Courtney E.W. Sulentic for her mentorship, helpful career guidance, and for the opportunity she provided for me to learn and grow as a both a scientist and a person.

I am especially grateful for the enormous amount of patience and training provided to me by Dr. Eric Romer, Ph.D. He is truly dedicated to the scientific advancement of everyone that has had the fortune of working with and for him. My current level of technical ability is greatly influenced by his direct participation in my education.

Lastly, I sincerely thank my dissertation committee members, Dr. Michael Leffak, Dr. Mill Miller, Dr. David Cool, and Dr. Rodney DeKoter.

Funding Resources

Gracious financial support was provided by the Wright State University's Environmental Sciences Ph.D. program. Research funding was provided by the National Institute of Environmental Health Sciences grant R01ES014676 to Dr. Courtney E.W. Sulentic, Ph.D.

DEDICATION

This manuscript is dedicated to my family. They have provided unconditional support, childcare, and constant encouragement. My father broke free from the restrictive chains of poverty and volitionally bettered himself through the completion of a Bachelor's degree while working full time. He provided the means that allowed my mother to attend college. Through adversity, illness, and life altering events, she managed to attain her life-long goal and was awarded her Ph.D. Their efforts, trials, and sacrifices did not go unnoticed, but rather, they led by example and established a standard for their children and grandchildren.

To my wife, she has always been completely supportive of all my educational and vocational choices. She provided encouragement when I was at the lowest points during my educational endeavors, and she intellectually continues to challenge me.

To my children, life is full of choices, and you have the world before you. I hope that through your observations, you will understand that the true value of an education is not simply a degree. The true value lies in what an education can provide-the realization of your personal goals.

"Science is what we do, when we don't know what we're doing."

-Prof. Alvaro De Rujula

CHAPTER 1. BACKGROUND

Summary

The proposed studies focus on understanding how dioxin, specifically 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), impacts host resistance to infections mediated by B cells. B cells are the effector cells of the humoral immune response that produce and secrete immunoglobulins (Ig). TCDD has been shown to markedly inhibit Ig expression and secretion in vivo and in several in vitro models which appears to be dependent on activation of the aryl hydrocarbon receptor (AhR) signaling pathway (Holsapple, Dooley et al. 1986; Dooley and Holsapple 1988; North, Crawford et al. 2009). Binding of dioxin and dioxin-like compounds to the cytosolic AhR represents the first step in a series of events that results in nuclear translocation of the AhR which is a ligand-activated transcription factor. Upon translocation of the AhR, the AhR binds to dioxin responsive elements (DRE) in dioxin sensitive genes and elicits transcription (Pollenz 1996; Swanson 2002).

B-cell differentiation into immunoglobulin secreting cells is dependent on cellular activation. Cellular activation can be initiated by various extracellular stimuli leading to an intracellular signaling cascade. Of the many potential activation pathways, activation of the nuclear factor-κB (NF-κB)/Rel proteins play a significant role in cellular activation. NF-κB/Rel proteins (RelA, RelB, c-Rel,

p50, p52) are transcription factors that bind as dimers to κB sites within the DNA, and based upon the composition of the NF-κB/Rel dimers (and interactions with other transcription factors and co-factors) they can either activate or inhibit transcription of genes. Activation of the NF-κB/Rel pathway is integral to the production and release of Ig from B cells in response to bacterial insult and is required for B-cell activation and differentiation in response to a T-cell activating signal, such as through the CD40 receptor. Both Cytosine-phosphate-Guanine (CpG)-oligodeoxynucleotides and lipopolysaccharide (LPS), which are associated with bacterial DNA and bacterial cell walls respectively, activate B cells in a T-cell independent manner and lead to the production of Ig.

Results from recent research have demonstrated protein interactions between the AhR and NF-κB/Rel proteins, specifically RelA and RelB. Interaction with the AhR may alter the DNA binding profile of specific NF-κB/Rel dimers and therefore have an impact on genes that are transcriptionally regulated by NF-κB/Rel proteins (Tian, Ke et al. 1999; Vogel, Sciullo et al. 2007). Functional Ig production is dependent on the expression of the Ig heavy chain that is partially under the transcriptional regulation of the 3'*Ig heavy* chain regulatory region (3'*Igh*RR) (Klein, Sablitzky et al. 1984; Wabl and Burrows 1984; Singh and Birshtein 1993; Madisen and Groudine 1994; Saleque, Singh et al. 1997). Within the 3'*Igh*RR, both κB and DRE sites have been identified, providing a potential transcriptional target of NF-κB

and AhR proteins (Sulentic, Holsapple et al. 2000). Interestingly, treatment of B cells with TCDD has demonstrated an inhibition of *Igh* transcription and the transcriptional activity of the 3'*Igh*RR (Sulentic and Kaminski 2011), suggesting that the 3'*Igh*RR is a transcriptional target of TCDD and mediates the effect of TCDD on Ig. These transcriptional inhibitions may, in part, be mediated by an interaction between the AhR and NF-κB/Rel proteins, which could disrupt NF-κB/Rel binding, alter the NF-κB/Rel binding profiles within the 3'*Igh*RR, and/or directly modulate transcriptional control through direct AhR/DRE binding. The central hypothesis of this proposal is that TCDD-induced inhibition of 3'*Igh*RR activation is mediated by an interaction between the AhR and NF-κB/Rel proteins. Understanding how the 3'*Igh*RR becomes transcriptionally active is of great importance as the 3'*Igh*RR has been associated with human pathologies such as Burkitt's lymphoma, IgA nephropathy, Celiac disease, systemic sclerosis, dermatitis herpetiformis, psoriasis arthritis, and plaque psoriasis (Taub, Kirsch et al. 1982; Aupetit, Drouet et al. 2000; Frezza, Giambra et al. 2004; Frezza, Giambra et al. 2007; Cianci, Cammarota et al. 2008; Cianci, Giambra et al. 2008). Furthermore, understanding how TCDD modulates the expression of the 3'*Igh*RR after B cell activation is of toxicological significance to a broad range of chemicals known to modulate the AhR signaling pathway and may provide pharmaceutical approaches to managing diseases associated with the 3'*Igh*RR.

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TCDD and relevance to human health

Dioxins are persistent environmental toxins produced during the combustion of organic materials in the presence of chlorine (USEPA 2000; Hays and Aylward 2003). Dioxins have been released into the environment due to pulp bleaching in paper mills, the production of polyvinyl chloride (PVC) plastics, incineration of municipal trash and medical waste, and backyard burning of trash and foliage. Dioxin-like compounds fall under a class of chemical structures known as halogenated aromatic hydrocarbons (HAH) and include dibenzofurans, and certain polychlorinated biphenyls which exist as persistent environmental toxins in complex mixtures (Okey 1990; Safe 1995). 2,3,7,8 Tetrachlorodibenzo-*p*-dioxin (TCDD) is considered the most potent of the HAH environmental pollutants collectively known as dioxins (Franc, Pohjanvirta et al. 2001). Although there has been a gradual decrease in environmental concentrations and human body burdens of dioxin (Aylward and Hays 2002; Hays and Aylward 2003), the U.S. Environmental Protection Agency (EPA) suggested in its Dioxin Reassessment (2000) that the current average human body burdens of dioxins are 25 ppt or 25 pg TCDD Equivalent Quantity (TEQ/g lipid). However, the body burden may be up to three times higher (75 TEQ/g lipid) than the average burden and can be correlated to a diet with a high fat intake (USEPA 2000). Exposures to TCDD in non-occupational settings are almost all from the consumption of contaminated food sources (Lorber, Patterson et al. 2009). As dioxins are highly lipophilic, environmentally persistent, and bioaccumulate, the

concentration of TCDD increases in each species as it progresses through each trophic level. Concentration levels as low as 0.01ppt measured in a water column can be biomagnified to concentration levels in excess of 110 pg TEQ when measured in the species at the highest trophic level (USEPA 2000). Since humans are at the highest trophic level, the potential to bioaccumulate biologically significant concentrations of dioxin is greatly enhanced.

Dioxin exposures have generated considerable public concern in the U.S. since the late 1970's with evacuations from Love Canal in Niagara Falls, NY, Times Beach, MD, and from Seveso, Italy, as well as multiple exposures to dioxin-contaminated Agent Orange during the Vietnam War (Schecter, Quynh et al. 2006; Consonni, Pesatori et al. 2008). There have been several documented and unexpected dioxin contaminations of the food supply which are compounded by uncertainties associated with the possible sources of dioxin (Amakura, Tsutsumi et al. 2003; Hays and Aylward 2003; Mandal 2005). Human populations exposed to high levels of dioxin are subjected to alterations in liver function and tend to exhibit the hallmark response of chloracne (acne-like eruption of pustules, blackheads, and cysts) as seen in the more recent poisoning of the Ukrainian President, Viktor Yushchenko in 2004 (Neuberger, Landvoigt et al. 1991; Sweeney, Calvert et al. 1997; Mandal 2005; Linden, Lensu et al. 2010). Long term effects from exposures to TCDD in Seveso, Italy demonstrated an increase in multiple myeloma' and myeloid leukemias (Pesatori, Consonni et al. 2009). TCDD has been utilized

for the majority of dioxin research and has been studied with a greater intensity than other environmental pollutants because TCDD is highly active at low concentrations (Whitlock 1990). At low doses, TCDD has been reported to produce a diverse spectrum of adverse effects in animal models including cancer, reproductive issues, developmental issues, immunologic suppression, endocrine disruption, and death (Birnbaum and Tuomisto 2000). The biological effects following exposure to TCDD has been actively researched for more than thirty years, generating a wealth of data. These data show that toxic outcomes to TCDD exposure are species, strain/sub-strain, sex, age, dose, and duration dependent. Extensive research has identified that AhR may be responsible for most of the TCDD-induced effects (Poland and Glover 1980; Okey, Riddick et al. 1994; Fernandez-Salguero, Hilbert et al. 1996; Tuomisto, Viluksela et al. 1999; Linden, Lensu et al. 2010).

To date, there is not an established “no observable effect level” (NOEL) for TCDD burdens, and the current human body burdens may be well within the range to induce adverse health effects ranging from endocrine disruption, enzyme induction, hepatomegaly, immunomodulation, chloracne, epithelial hyperplasia, and teratogenesis (USEPA 2000; Aylward and Hays 2002; Paustenbach and Board 2002; Hays and Aylward 2003). It has been suggested that the non-cancer effects of dioxin may be a more urgent threat to human health (Stone 1994). Additionally, alterations in immune function have been observed in virtually every vertebrate species tested, and often occur at doses that fail to produce obvious signs of toxicity (Holsapple,

Snyder et al. 1991; Kerkvliet 2002; Sulentic and Kaminski 2011). Furthermore, multiple studies using animal models of human disease have demonstrated an inhibition of immune responses and increased susceptibility to bacterial, viral, and parasitic infection after host exposure to TCDD. Host resistance studies beginning in the mid-1970's through the present have demonstrated an increased susceptibility of TCDD-exposed mice and rats to gram-negative bacteria such as *Salmonella* or *Escherichia coli* (Thigpen, Faith et al. 1975; Vos, Kreeftenberg et al. 1978; Thomas and Hinsdill 1979; Hinsdill, Couch et al. 1980), gram-positive bacteria such as *Streptococcus pneumoniae* (White, Lysy et al. 1986), viral infections from *herpes simplex type II strain 33 virus* and *influenza* (Clark, Sweeney et al. 1983; White, Lysy et al. 1986; Lawrence, Warren et al. 2000; Warren, Mitchell et al. 2000), and parasitic infections from *Plasmodium yoelii 17 XNL* (Tucker, Vore et al. 1986). Exposure to TCDD inhibits T-cell dependent responses as shown by delayed hypersensitivity responses and reduced generation of cytotoxic T lymphocytes (Moos, Baecher-Steppan et al. 1994). Antigen presenting cells (APC) have been shown to have diminished capability to express common lymphocyte activation markers such as CD86 as seen in both spleen B cells and macrophage cells following exposure to TCDD (Prell and Kerkvliet 1997). Additionally, TCDD-induced inhibition of B-cell function (i.e. cellular proliferation, Ig expression and secretion) has been demonstrated using various cellular and in vivo models (Morris, Karras et al. 1993; Karras and Holsapple 1994; Masten and Shiverick 1995; Sulentic, Holsapple et al. 1998;

Sulentic and Kaminski 2011). Taken together, the immune system is a sensitive target of TCDD-induced suppression. As B-cells are directly suppressed by TCDD (Sulentic and Kaminski 2011) and exposure to TCDD directly impacts an adaptive immune response, the focus of the current research was to further investigate the mechanisms by which TCDD impacts Ig expression.

B-cell activation

B cells are lymphocytes that play a primary role in the humoral immune system. The principal functions of B cells are to make antibodies against antigens, perform as antigen presenting cells APCs, and develop into memory B cells after activation by an antigen interaction. Exposure of the antigen to a B cell elicits an activation signal that induces clonal proliferation followed by differentiation into antibody secreting cells (Figure 1). B cells can be activated through an interaction between an antigen and the B-cell receptor (BCR), activation through any one of the 10 identified Toll-Like Receptors (TLR's), co-stimulation with the BCR and an engaged TLR, co-stimulation with an antigen and CD-40 ligand (simulates a T-cell interaction), cytokines, and/or multiple combinations of these activation pathways.

B-cell activation through TLRs was recently found to play an important role in the first line of host defense by the recognition of microbial products. TLRs are capable of recognizing conserved pathogen-associated molecular

patterns (PAMPs) (Schmausser, Andrulis et al. 2004; Peng 2005), and activate B cells. Additionally, TLRs and the B Cell Receptor (BCR) have been shown to synergize to induce class switch in T-cell independent activation of B cells to enhance differentiation and antibody response (Pone, Zan et al. 2010). TLR4 is a membrane-bound protein expressed on the surface of B cells complexed with membrane-bound Radio Protective 105 (RP105) protein. The TLR4/RP105 heterodimer is a fundamental component required for the recognition of lipopolysaccharide (LPS) and T-cell independent activation of B cells (Hoshino, Takeuchi et al. 1999). TLR7, TLR8, and TLR9 are not on the cell surface but rather are associated with the endoplasmic reticulum. TLR7 and TLR8 signaling is initiated when ssRNA associates with TLR7 and TLR8. Resiquimod (R848) is an immune response modifier used in skin lesion treatment and as an adjuvant to increase the effectiveness of vaccines. R848 functions as an agonist to TLR7 and TLR8 (Lanzavecchia and Sallusto 2007). TLR9 signaling is triggered by an interaction with hypomethylated/unmethylated cytosine-phosphate-guanine (CpG**)-** oligodeoxynucleotides (ODN) which is consistent with double stranded bacterial DNA (Krieg, Yi et al. 1995; Hacker, Mischak et al. 1998; Hartmann, Weeratna et al. 2000; Hemmi, Takeuchi et al. 2000; Bauer, Kirschning et al. 2001; Takeshita, Leifer et al. 2001; Krieg 2002). Vertebrate DNA has CpG in a form that is not only highly methylated but also has a suppressed occurrence such that CpGs are found only about one quarter as often as would be predicted if base utilization was random (Krieg 2000). B-cell

activation by LPS, CpG, or R848 through TLR4, TLR9, or TLR7 and TLR8, respectively, B cells can also be activated through a membrane-bound receptor on B cells known as CD40. B-cell activation through CD40 with CD40 ligand (CD40L) which is expressed on the surface of T cells may require an additional stimulus directed against a TLR or the B cell Receptor, a membrane-bound Ig that can bind antigen. Proliferation, up-regulation of Cluster of Differentiation 86 (CD86), increased secretion of Ig and cytokines, and the cessation of spontaneous programmed cell death are all hallmarks of B cell activation (Miyake, Yamashita et al. 1994; Krieg 1995; Yi, Hornbeck et al. 1996; Chan, Mecklenbrauker et al. 1998; Krieg 2002).

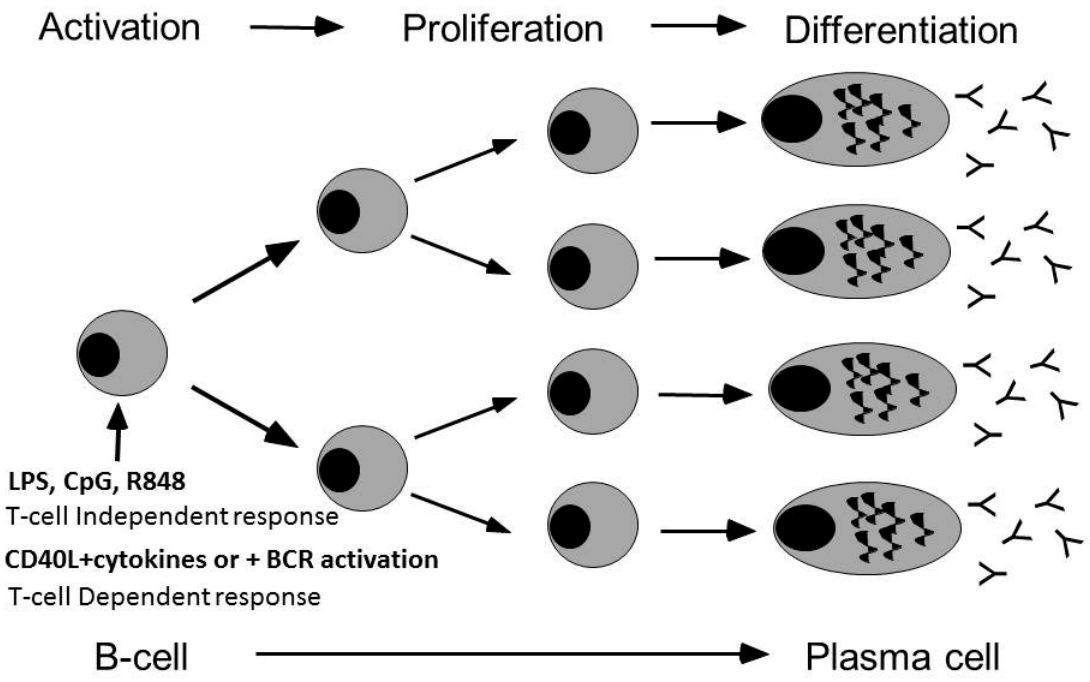

Figure 1. B-cell activation

Exposure of the antigen to a B cell elicits an activation signal that induces clonal proliferation followed by differentiation into antibody secreting cells. B cells can be activated through an interaction between an antigen and the B-cell receptor (BCR), activation through any one of the 10 identified Toll-Like Receptors (TLR's), co-stimulation with the BCR and an engaged TLR, co-stimulation with an antigen and CD-40 ligand (simulates a T-cell interaction), cytokines, and/or multiple combinations of these activation pathways.

NF-κB signaling pathway

Many similarities exist between the TLR4, TLR7, TLR8, and TLR9 signaling pathways (Figure 2). For example, they all initiate intracellular signaling through either the Toll/interleukin 1 receptor (IL-1R)-Myeloid Differentiation primary response gene 88 (MyD88) or the Toll/IL-1R domain-containing adaptor protein (TIRAP) pathway. IL-1R provides a scaffold for the recruitment of the adaptor molecule MyD88 to form a dimer and recruit the serine/threonine kinase of the interleukin-1 receptor-associated kinase (IRAK) family. Autophosphorylation of IRAK leads to TNF receptor associated factor 6 (TRAF6) adapter protein recruitment. After TRAF6 has complexed with IRAK, the TRAF6/IRAK complex disengages from the MyD88/IL-1R dimer. The TRAF6/IRAK complex then interacts with another protein complex consisting of TAK1-TAB1-TAB2 initiating phosphorylation and activation of IκB kinase (IKK). Phosphorylation of IKK leads to the phosphorylation and degradation of IκBα which ultimately induces rapid NF-κB activation (Chen and Goeddel 2002). CD40 activation combined with an antigen-induced BCR activation signaling can be transduced through the TRAF6 pathway (Zhu, Ramirez et al. 2002). Regardless of the initial activation signal, these signaling cascades result in the phosphorylation of Kinase (IKK). In the classical (canonical) pathway, NF-κB/Rel proteins are bound and inhibited by IκB proteins. IKK phosphorylates IκBα/β, ultimately leading to proteasomal degradation of IκBα/β leading to the nuclear translocation of NF-κB/Rel

proteins, RelA-p50, and to a lesser extent c-Rel-p50 (Akira and Takeda 2004). In the alternative activation pathway, IKK phosphorylation of NF-κB2 p100 leads to ubiquitination and proteasomal processing to NF-κB2/p52 creating transcriptionally active NF-κB p52/RelB. Interestingly, of the NF-κB/Rel proteins, only RelB cannot form a homodimer, and has been shown to interact with RelA, forming a transcriptionally inactive complex (Marienfeld, May et al. 2003). Upon NF-κB activation, NF-κB/Rel dimers translocate to the nucleus bind to their respective κB binding sites and exert their transcriptional effects (Figure 3) (Chen and Goeddel 2002). Interestingly, the NF-κB dimers are sensitive to the manner in which the B cell is activated. In LPS-activated B cells, RelA-p50 is the primary NF-κB/Rel protein found in the nuclear fraction; however, activation through CD40 shifts the NF-κB/Rel nuclear composition from an abundance of RelA (p65) to RelB (p68) and c-Rel (p75) (Neumann, Wohlleben et al. 1996). NF-κB/Rel activation is critical to B-cell maturation (Franzoso, Carlson et al. 1997), proliferation (Prendes, Zheng et al. 2003), and differentiation into Ig-secreting plasma cells (Liou, Sha et al. 1994).

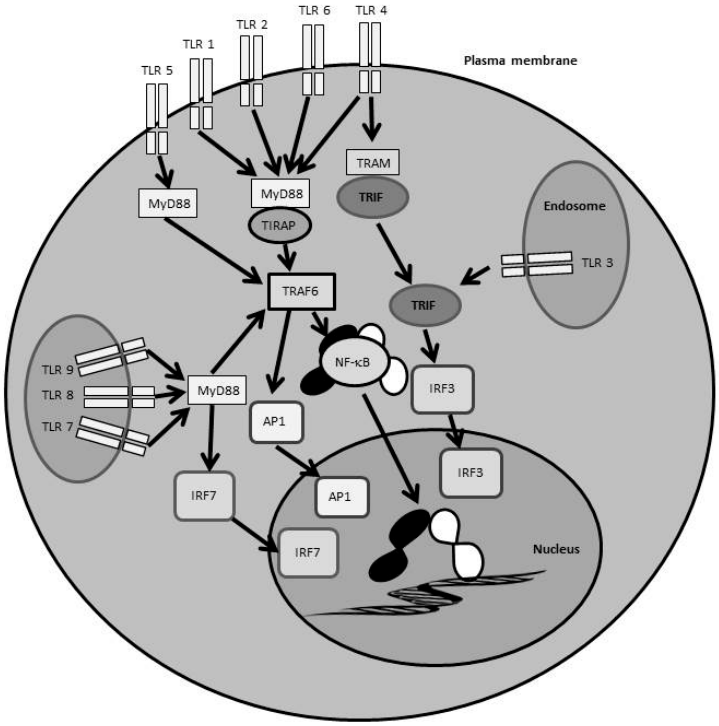

Figure 2. Toll-like receptor signaling pathway

TLR activation is initiated through either the IL-1R- MyD88 or the TIRAP pathway. IL-1R provides a scaffold for the recruitment of the adaptor molecule MyD88 to form a dimer and recruit the serine/threonine kinase of the IRAK family. Autophosphorylation of IRAK leads TRAF6 recruitment. After TRAF6 has complexed with IRAK, the TRAF6/IRAK complex disengages from the MyD88/IL-1R dimer. The TRAF6/IRAK complex then interacts with another protein complex consisting of TAK1-TAB1-TAB2 initiating phosphorylation of kinase IKKs. Phosphorylation of IKK leads to the phosphorylation and degradation of IκBα which ultimately induces rapid NF-κB activation.

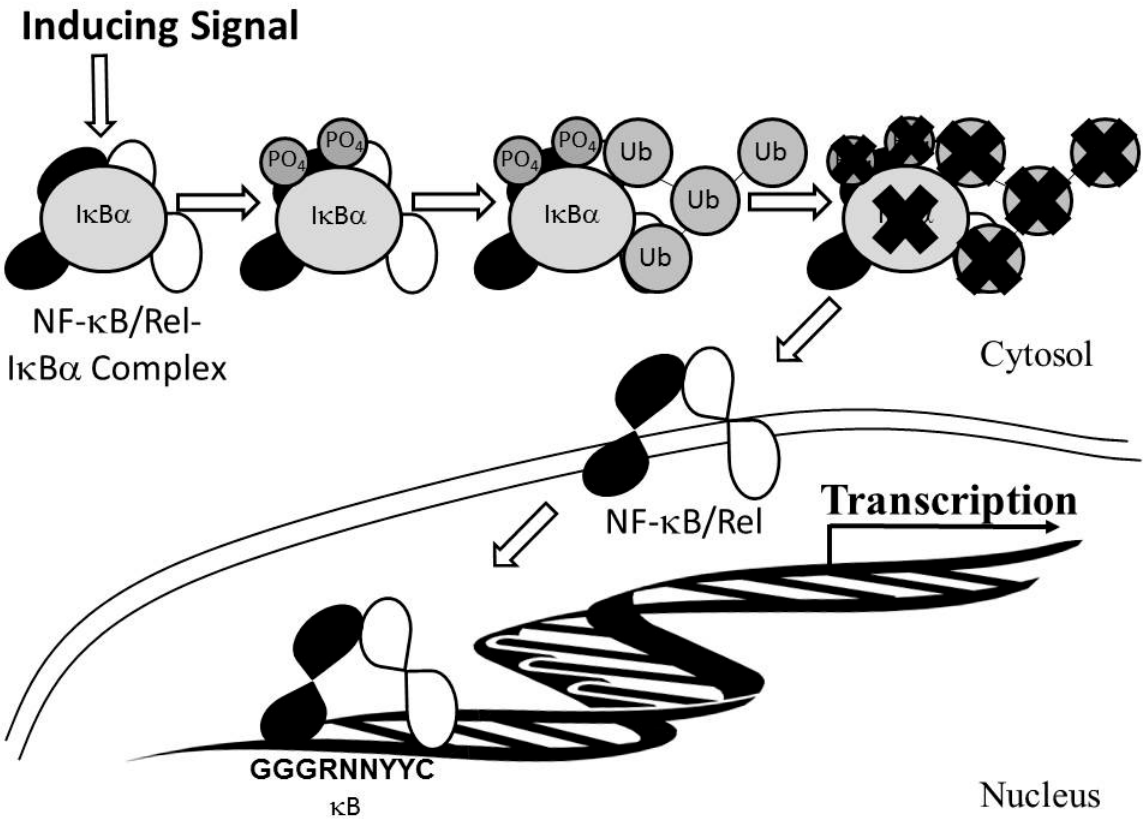

Figure 3. NF-κB Signaling Pathway

Classical activation of the NF-κB/Rel pathway is dependent upon upstream signal transduction that leads to the phosphorylation of the IκBα regulatory protein at Serines 32 and 36. Upon phosphorylation of the IκBα, IκBα is ubiquitylated, and the IκBα is subsequently targeted for proteolytic degradation. After IκBα degradation, NF-κB/Rel is liberated and is transcriptionally active.

AhR and B-cell dysfunction

Through early cell-type fractionation studies, B cells were identified as a direct target of TCDD-induced antibody suppression (Holsapple, Dooley et al. 1986; Dooley and Holsapple 1988). Several in vivo and in vitro studies have demonstrated that exposure to TCDD inhibits the process of B-cell differentiation into Ig secreting cells (North, Crawford et al. 2009). Extensive research has identified that the AhR, may be responsible for most of the TCDD-induced effects (Poland and Glover 1980; Okey, Riddick et al. 1994; Fernandez-Salguero, Hilbert et al. 1996; Tuomisto, Viluksela et al. 1999; Linden, Lensu et al. 2010). However TCDD may generate intracellular reactive oxygen species contributing to the toxicological consequences (Reichard, Dalton et al. 2005). Research using a cellular model that consisted of two surface Ig^+ (mature) B cell lines, the CH12.LX and the BCL-1, which differ in their expression of the AhR and in their sensitivity to TCDD, demonstrated that although both cell lines are significantly activated by LPS, only the CH12.LX (AhR expressing) had a marked inhibition of LPS-induced IgM secretion. In contrast, the LPS-induced IgM secretion from the AhR-deficient BCL-1 cells was unchanged by co-treatment with TCDD (Sulentic, Holsapple et al. 2000). However, these studies are somewhat limited by the comparison of two different cell lines that likely have differences distinct from AhR expression. Later studies with the AhR-null mice demonstrated a similar AhR-dependency in TCDD-induced suppression of B-cell differentiation (Vorderstrasse, Steppan et al. 2001). Additionally, studies using human

primary B cells have demonstrated an inhibition of cellular proliferation induced by co-stimulation with both CD40L (simulates a T cell interaction) and CpG (oligodeoxynucleotides containing hypomethylated or unmethylated CpG) when co-treated with a known ligand for the AhR (Allan and Sherr 2005). Sulentic et al. (Sulentic, Holsapple et al. 2000), demonstrated that in addition to IgM secretion, TCDD strongly inhibits µ heavy chain expression in the CH12.LX B cells, which also seems to be AhR-dependent, and profoundly inhibits LPS-induced activation of the 3'*Igh*RR (Sulentic, Zhang et al. 2004). The immunoglobulin heavy chain locus (*Igh*), which encodes the functional heavy chain protein of membrane-bound and secreted (antibody) immunoglobulin, is under the regulation of the 3'*Igh*RR. The 3'*Igh*RR has been shown to have AhR binding sites within the hs1,2 and the hs4 enhancers as reviewed later in the manuscript.

AhR signaling pathway

The mechanism thought to mediate the biological effects of TCDD is the AhR signaling pathway. The AhR is located in the cytoplasm (Figure 4) and exists as one component of a complex composed of two molecules of a 90-kDa heat shock protein (hsp90) (Denis, Gustafsson et al. 1988; Wilhelmsson, Cuthill et al. 1990), a 38-kDa immunophilin-related protein (XAP2), and p23 (Shetty, Bhagwat et al. 2003). Although there are multiple AhR activators including TCDD, the endogenous ligand remains elusive. When TCDD diffuses through the outer cell membrane, it binds the AhR with high affinity leading to the disassociation of XAP2, which exposes a nuclear transport signal (Kazlauskas, Sundstrom et al. 2001). Upon nuclear translocation, AhR disassociates from the hsp90 molecules and p23 and dimerizes with the AhR nuclear translocator protein (ARNT) (Pollenz 1996). The TCDD-AhR-ARNT heterodimer then binds to specific DNA sequences referred to as dioxin responsive elements (DRE) and modulates transcription of TCDD sensitive genes (Swanson 2002). The activation of AhR up-regulates a number of drug metabolizing enzymes including the well characterized induction of cytochrome P450.

Although the majority of research investigating the role of the AhR has focused primarily of the toxic effects associated with ligand activation of the AhR, there are increasing efforts to elucidate the physiological role of the AhR. That said, there is still much debate as to the endogenous ligand for the AhR. Nguyen and Bradfield (Nguyen and Bradfield 2008), have speculated

that ligand binding specificity of the AHR does not differ significantly across species, signifying that the evolutionary stressor influencing ligand specificity of the AHR is an endogenous compound. The physiological significance of the AhR was initially assed through the investigation of AhR knockout (KO) animal models. The phenotypes of newborn AhR KO mice demonstrate alterations in vascularity (Lahvis, Lindell et al. 2000), 40-50 percent neonatal lethality rate, inflammation of the bile ducts, and, 80 percent depletion of splenocytes (Fernandez-Salguero, Pineau et al. 1995), fewer lymphoid cells in the peripheral lymph nodes, increased failure to thrive, significantly reduced CyP1a1, CyP1a2, and non- inducible ethoxyresorufin-*O*-deethylase (EROD) activity (Lahvis and Bradfield 1998). Interestingly, the phenotypes of AhR KO mice and rats demonstrate uniquely and distinct difference in alterations in hepatic function, bilateral renal dilation, bilateral, ureter dilation, persistent haloid arteries in the eye, and patent ductus venosus of the liver suggesting species-dependent tissue development (Harrill, Hukkanen et al. 2013).

Indigoids (indigo and indirubin) have been suggested to be endogenous AhR ligands, and have been found in human urine and bovine serum (Adachi, Mori et al. 2001). Additional potential endogenous AhR ligands are 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) (Song, Clagett-Dame et al. 2002), arachidonic acid metabolites ((Kroetz and Zeldin 2002), heme metabolites (Phelan, Winter et al. 1998), and tryptophan metabolites (Heath-Pagliuso, Rogers et al. 1998). Based upon a review of the current literature, the endogenous ligand for the AhR still remains elusive.

Recent research has demonstrated that the AhR not only dimerizes to ARNT, but may also complex with a myriad of other transcription factors and alter functional transcription of genes. Specifically, the AhR has been shown to interact with activator protein 1 (AP-1), specificity protein 1 (Sp1), and nuclear factor-κB (NF-κB)/Rel transcription factors (Kobayashi, Sogawa et al. 1996; Tian, Ke et al. 1999; Kim, Gazourian et al. 2000; Suh, Jeon et al. 2002; Tian, Rabson et al. 2002; Vogel, Sciullo et al. 2007). Of particular interest, are the NF-κB/Rel transcription factors that regulate genes responsible for both the innate and adaptive immune response. Upon B-cell activation, NF-κB/Rel becomes activated through distinct signaling components. The activation and nuclear translocation of NF-κB/Rel proteins are critical to B-cell activation and Ig expression. Moreover, a TCDD-induced AhR/NF-κB/Rel interaction may, at least partially, mediate the well-documented inhibition of B cell activation

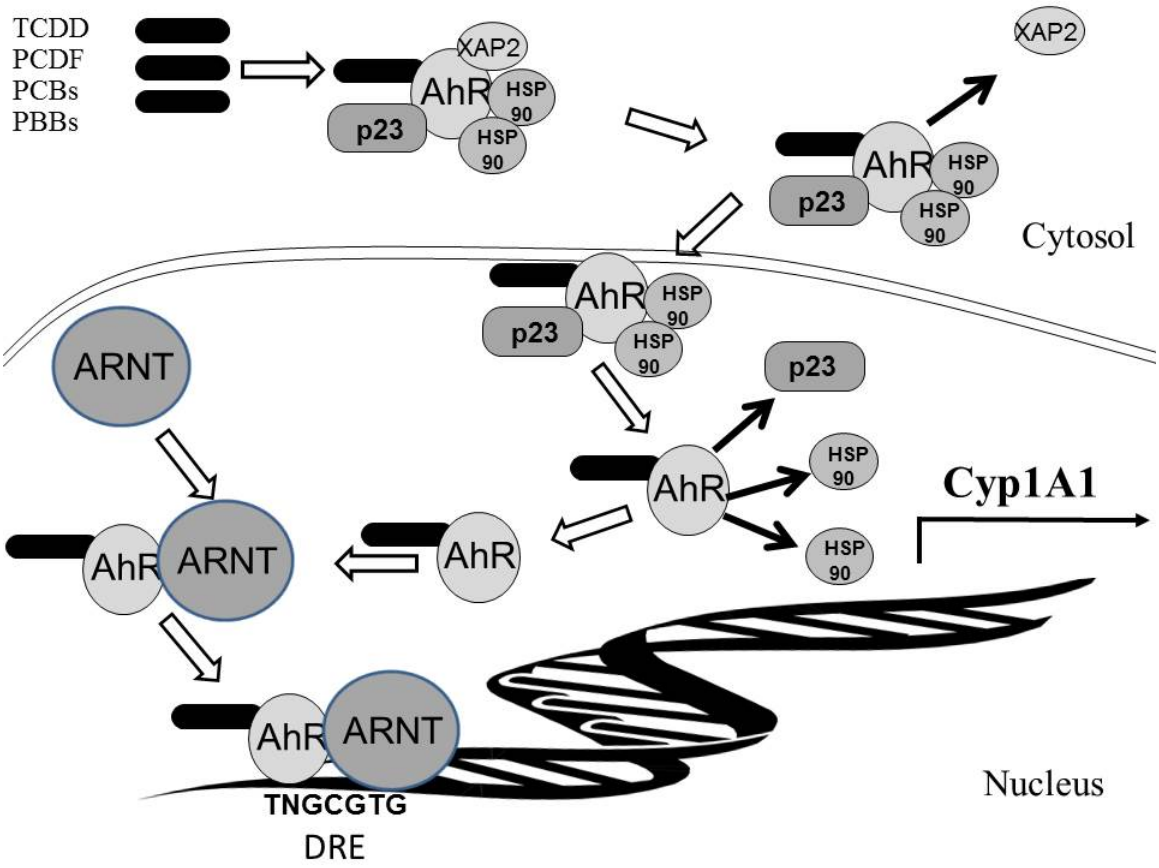

Figure 4. AhR signaling pathway

When AhR binds a ligand, it undergoes conformational changes and dissociates from the cytosolic protein X-associated protein (XAP2). The AhR then translocates into the nucleus, and dissociates from heat shock proteins, HSP90 and p23. The AhR forms a complex with the AhR nuclear translocator (ARNT). The TCDD/AhR/ARNT complex binds a dioxin responsive element (DRE). After binding the DRE, transcription of AhR sensitive genes can occur (such as transcription of Cyp1A1).

following TCDD exposure identified in both in vivo and in vitro studies. Furthermore, within the 3'*Igh*RR, the hs1,2 and hs4 enhancers have binding sites for both the AhR and NF-κB/Rel. More significantly, in the hs4 enhancer has an AhR and NF-kB/Rel binding site that overlap.

Interactions between the AhR and NF-κB/Rel proteins

When both the AhR and the NF-κB/Rel (RelA, RelB, and c-Rel) are activated, these signaling pathways have been shown to interact (Kim, Gazourian et al. 2000; Sherr 2004; Vogel, Sciullo et al. 2007). NF-κB/Rel has been shown in both the murine hepatoma Hepa1c1c7 and the monkey kidney tissue derived COS-7 cell line models to physically interact with TCDD-activated AhR leading to an AhR-mediated repression of reporter plasmids under κB regulation (Tian, Ke et al. 1999; Tian, Rabson et al. 2002). However, Kim et al. demonstrated that an AhR and RelA interaction functionally cooperate in a human breast cancer Hs578T cell line to increase c-myc gene expression (Kim, Gazourian et al. 2000). Additionally, Vogel et al. demonstrated that AhR and RelB not only associate, but over-expression of RelB can drastically increase DRE reporter activity (Vogel, Sciullo et al. 2007). These interactions may not only alter the binding of NF-κB to its respective κB binding site but may modulate the binding of the AhR to DREs (Tian, Ke et al. 1999). Additionally, Sulentic et al. demonstrated through mutational analysis in a B-cell line model that both the κB and the identified

DRE site were critical for synergistic activation of an enhancer within an immunoglobulin regulatory region known as the hs4 enhancer (Sulentic, Zhang et al. 2004). Taken together, the physical interactions between AhR and NF-κB/Rel may alter the expression of genes that are under NF-κB/Rel and/or AhR transcriptional regulation and likely lead to an alteration in cellular function.

Immunoglobulin heavy chain regulation and the 3'*Igh*RR

The *Igh* gene locus contains multiple regulatory domains including variable heavy chain promoters, a heavy chain (V_H) promoter; intronic or μ enhancer (Eμ); germline promoters upstream of each constant region (C_H); and a regulatory region 3' of the constant regions which contains at least four enhancer domains (hs3a, hs1,2, hs3b and hs4) collectively named the 3'*Igh*RR (Figure 5). Although the murine 3'*Igh*RR is approximately 16 kb downstream of Cα, (Dariavach, Williams et al. 1991) the 3'*Igh*RR has been shown to directly associate with the Eμ within the context of chromatin (Ong, Stevens et al. 1998). Activation of the 3'*Igh*RR is correlated with transcription of the rearranged Ig heavy chain gene, Ig secretion, and class switch recombination (Klein, Sablitzky et al. 1984; Wabl and Burrows 1984; Singh and Birshtein 1993; Madisen and Groudine 1994; Saleque, Singh et al. 1997).

Within the 3'*Igh*RR, there are multiple DNA binding sites for several transcriptional factors including AP-1,Oct, B-cell Specific Activator Protein

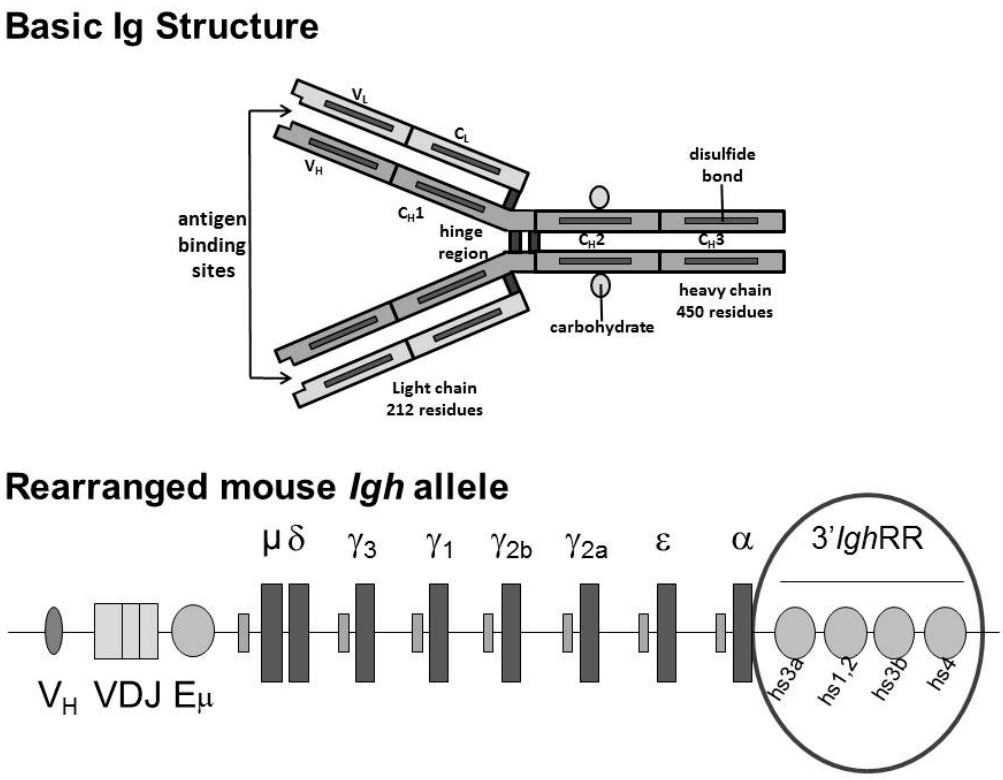

Figure 5. Basic Ig structure and murine Igh allele

Basic structure of an Ig antibody (top) produced by a B cell. The Ig heavy chains are the direct product of transcription of the *Igh* locus under the regulation of the 3'*Igh*RR. V_H, variable heavy chain promoter; Eμ, intronic or μ enhancer; open rectangles, germline promoters upstream of each C_H region.

(BSAP), and NF-κB/Rel (Arulampalam, Grant et al. 1994; Grant, Andersson et al. 1996; Michaelson, Singh et al. 1996; Saleque, Singh et al. 1999; Kanda, Hu et al. 2000; Khamlichi, Pinaud et al. 2000; Stevens, Ong et al. 2000; Zelazowski, Shen et al. 2000). Additionally, Sulentic et al. (Sulentic, Holsapple et al. 2000), identified binding of both the AhR to DREs and NF-κB/Rel proteins to κB sites in hypersensitive (hs) enhancers within the 3'*Igh*RR (Figure 6). The results from this study demonstrated that treatment of CH12.LX (AhR responsive) B cells with TCDD leads to an increase in the binding of AhR and ARNT proteins to DRE-like sites in both the hs1,2 and hs4 enhancers. These data suggest that the AhR/DRE may be involved in Ig inhibition through direct interactions within the 3'*Igh*RR.

Furthermore, additional luciferase studies demonstrate a sensitive and profound inhibition by TCDD of LPS-induced 3'*Igh*RR activity (Sulentic, Kang et al. 2004). Interestingly, neither LPS alone nor TCDD alone induce hs4 activity, yet LPS co-treated with TCDD produced a synergistic activation of hs4. In addition, a chromatin immunoprecipitation (ChIP) assay confirmed TCDD-inducible AhR binding to the hs4 enhancer domain and site-directed mutation of the hs4 DRE and/or κB decreased the effect of TCDD and LPS on hs4 activity suggesting the synergistic activation by a co-treatment of TCDD and LPS was dependent on binding to both the DRE and κB. Collectively, these data demonstrate TCDD-mediated modulation of the 3'*Igh*RR, which may be produced, at least in part, through transcriptional regulation by the

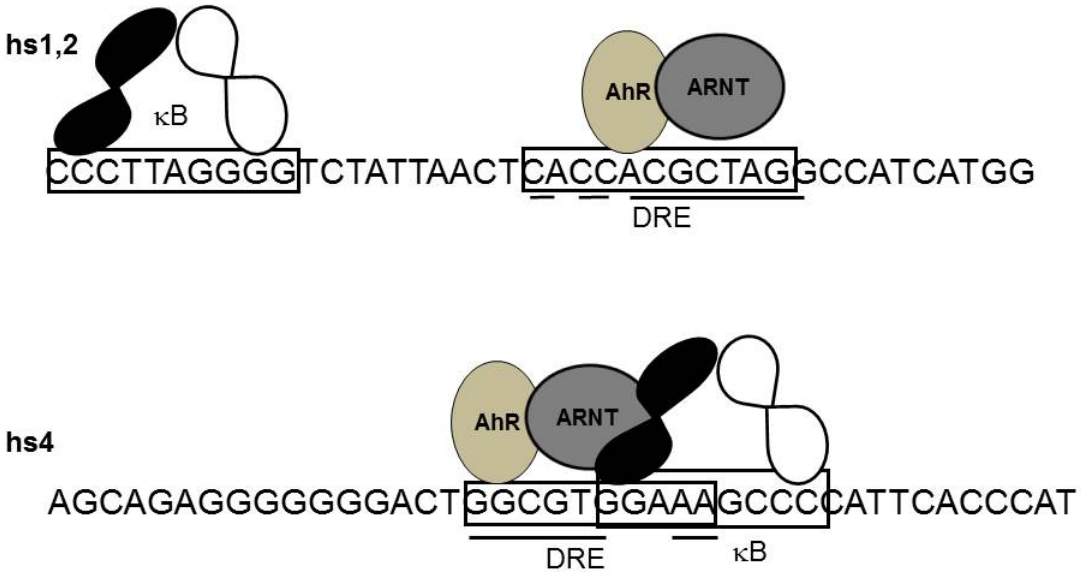

Figure 6. hs1,2 and hs4 enhancer DRE and κB binding sites

DRE and κB binding sites within the hs1,2 and hs4 for the AhR and the NF-κB/Rel transcription factors.

AhR. EMSA-Western analysis of the CH12.LX B cells demonstrated TCDD-induced binding of the NF-κB/Rel proteins RelA, c-Rel, RelB, and p50 to the κB binding motif within the hs4 enhancer. However, in the BCL-1 B cells (AhR non-responders), EMSA-Western analysis also revealed TCDD-induced binding of c-Rel, RelA, and RelB to the hs4 κB motif. These results suggested an AhR-independent mechanism for the induction of c-Rel, RelA, and RelB binding by TCDD but perhaps an AhR-dependent induction of p50 binding. These results may suggest a change in NF-κB/Rel binding profile within the hs4 enhancer when the AhR is active.

There is a cooperative interaction between the enhancers within the 3'*Igh*RR in their overall regulation of the IgH gene (Ong, Stevens et al. 1998). Interestingly, there is a dichotomy that exists between the activation states of individual hs4 and hs1,2 enhancers within the 3'*Igh*RR and the overall activation of the 3'*Igh*RR (Michaelson, Singh et al. 1996). The binding of κB, BSAP, and OCT can be both a positive regulator of hs4 activity and a negative regulator of hs1,2 activity (Michaelson, Singh et al. 1996). DRE binding also has a similar dichotomy in the effect on hs4 activity versus hs1,2 (Fernando, Ochs et al. 2012) and 3'*Igh*RR(Sulentic, Kang et al. 2004). Consistent with the previous findings, treatment with TCDD was shown to inhibit LPS induced hs1,2 activation and synergistically hs4 (Sulentic, Zhang et al. 2004). Taken together, these results suggest that the inhibition of IgM secretion by TCDD may require the induction of both DRE and κB binding

within the 3'I*gh*RR as identified in the hs4 enhancer. Because the DRE and κB binding sites overlap in the hs4, binding to either the DRE or κB motif may facilitate or stabilize binding to the other motif.

CHAPTER 2. HYPOTHESIS AND SPECIFIC AIMS

Significance of proposed research

Altered regulation of the 3'*Igh*RR by environmental toxins may pose a significant health threat to human health by limiting the ability of the immune system to respond to insult. Additionally, the 3'*Igh*RR has been associated with human pathologies such as Burkitt's lymphoma, IgA nephropathy, Celiac disease, systemic sclerosis, dermatitis herpetiformis, psoriasis arthritis, and plaque psoriasis (Aupetit, Drouet et al. 2000; Frezza, Giambra et al. 2004; Frezza, Giambra et al. 2007; Cianci, Cammarota et al. 2008; Cianci, Giambra et al. 2008). Comparisons between the mouse and human *IgH* locus and the 3'*Igh*RR, have identified structural differences and similarities including 1) unlike the mouse, the human locus has a large duplication at the 3' end of *IgH* locus which consists of several repeated constant region genes and a repeated 3'*Igh*RR; 2) the human 3'*IgH*RR (hs3, hs1,2 and hs4 homologous to mouse hs3a, hs1,2, hs4) is located 3' of both $C\alpha_1$ and $C\alpha_2$ but two hs1,2 enhancers are inverted with respect to each other; and 3) mouse and human 3'*Igh*RR has an overall core similarity in sequence (approximately 500 bp) of approximately 65% (Figure 5) (Chen and Birshtein 1997; Mills, Harindranath et al. 1997; Pinaud, Aupetit et al. 1997). Close examination of the mouse and human hs4 enhancer

sequences (Guglielmi, Truffinet et al. 2004) identified highly conserved sequences for the overlapping DRE and κB binding motifs with 10 of 11 identical nucleotides for the DRE sequences, however, as the core sequence is not conserved there may be a possibility that the AhR will not bind the human hs4. Additionally, there are 9 of 10 identical nucleotides for the hs4 κB sequences. Within the hs1,2 enhancer (Denizot, Pinaud et al. 2001), the DRE site is also fairly well conserved with 8 of 11 nucleotides identical including a consensus core DRE (GCGTG). The evolutionarily conserved close proximity of the κB and DRE binding motifs in the human hs1,2 and the overlapping κB and DRE binding motifs in the hs4 enhancers may implicate an interaction between the AhR and NF-κB/Rel proteins, supporting a role in an AhR and NF-κB/Rel mediated inhibition of the 3'*Igh*RR. As the 3'*Igh*RR is highly correlated with the secretion of Ig, and with multiple human disease processes, understanding how the 3'*Igh*RR is modulated after exposure to dioxin is of great potential relevance to human health.

The current proposal will explore the role of the AhR and NF-κB/Rel proteins in TCDD-induced modulation of the 3'*Igh*RR which may, at least partially, mediate the effects of TCDD on mouse B-cell function through an inhibition of *Igh* transcription and Ig secretion. As described earlier, current and ongoing research in our lab has indicated that the 3'*Igh*RR is a sensitive target of the suppressive effects of TCDD. Understanding how TCDD modulates transcription of the 3'*Igh*RR is of toxicological significance to a

broad range of dioxin-like chemicals and may provide pharmaceutical approaches to managing diseases associated with the 3'*Igh*RR.

Hypothesis

Immunoglobulins (Ig) are essential for maintaining immunity against a wide variety of pathogens. However, little is known regarding the impact of chemicals or disease states on Ig gene expression. Research has shown that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a well-known suppressor of B-cell differentiation, potently inhibits activation of the transcriptional regulatory region (known as the 3'*Igh*RR) found downstream of the Ig heavy chain (*Igh*) locus. In addition to its proposed role in Ig gene expression, the 3'*Igh*RR has also been associated with specific human pathologies including Burkitt's lymphoma, IgA nephropathy, Celiac disease, systemic sclerosis, dermatitis herpetiformis, psoriasis arthritis, and plaque psoriasis. Many of the toxic effects of TCDD and related chemicals have been attributed to changes in gene expression resulting from the activation of the aryl hydrocarbon receptor (AhR) which subsequently binds to dioxin-responsive elements (DRE) in affected genes. We have detected binding of AhR to DRE sites within two enhancers of the 3'*Igh*RR, hs1,2 and hs4, and find that these DRE sites are closely associated with NF-κB binding motifs. We hypothesize **that TCDD represses 3'*Igh*RR activation through an AhR-dependent shift in**

the NF-κB/Rel protein complexes binding within the hs1,2 and hs4 enhancers. The following specific aims (SA) will test this hypothesis.

Specific Aims

Specific Aim 1: **Determine if when activated by alternate B-cell stimuli, TCDD inhibits the 3'*Igh*RR.** In the CH12.γ2b-3'*Igh*RR cell line, we will evaluate the transcriptional effect of TCDD on the 3'*Igh*RR in the presence of different stimuli co-treated with TCDD. *This aim will test the hypothesis that TCDD is a general inhibitor of 3'IghRR activation and of Ig expression regardless of the activation stimuli. Additionally, we will determine if activation of different TLRs are equally affected by TCDD.*

Specific Aim 2: **Determine the TCDD and LPS-induced binding profile of the AhR and NF-κB/Rel proteins within the hs4 and hs1,2 enhancers.** In CH12.LX cells and splenocytes derived from B6C3F1 female mice, we will evaluate the binding of NF-κB/Rel proteins (RelA and RelB,) within the hs1,2 and hs4 enhancers by chromatin immunoprecipitation (ChIP) analyses. *This aim will test the hypothesis that treatment with TCDD results in both qualitative and quantitative shifts in proteins bound to the κB sites of the hs1,2 and hs4 enhancers.*

Specific Aim 3: **Determine whether the AhR and NF-κB/Rel proteins mediate TCDD's repressive effect on 3'*Igh*RR activation.** Using

repression of NF-κB/Rel proteins and chemical inhibition of the AhR, we will *test the hypothesis that both the AhR and NF-κB/Rel proteins are required for the TCDD-induced inhibition of LPS-induced 3'IghRR activation.* To this end, we will utilize the CH12.LX variants; the CH12.IκBαAA that expresses the IκBα super-repressor protein to inhibit NF-κB activation, and the CH12.γ2b-3'*Igh*RR cell line that stably expresses a γ2b transgene regulated by the 3'*Igh*RR.

CHAPTER 3. METHODS AND MATERIALS

Chemicals and Reagents

TCDD in 100% dimethyl sulfoxide (DMSO) was purchased from AccuStandard Inc. (New Haven, CT). The certificates of product analysis stated the purity of TCDD to be 99.1%. IPTG (isopropyl β-D-1-thiogalactopyranoside), LPS (*Escherichia coli*), and DMSO were purchased from Sigma Aldrich (Milwaukee, WI). IPTG and LPS were dissolved in water and 1xPBS, respectively. The AhR antagonist CH-223191 was purchased from Calbiochem (San Diego, CA) and dissolved in 100% DMSO. Resiquimod (R848) was purchased from Enzo Life Sciences (Farmingdale, NY) and dissolved in 100% DMSO. Cytosine-phosphate-guanine (CpG)-oligodeoxynucleotides (ODN) (5'-TCCATGACGTTCCTGACGTT -3') was purchased from Eurofins MWG Operon (Huntsville, Alabama) and dissolved in RNase and DNase free water.

Cell Lines

The CH12.IκBαAA B-cell line (IgM^+) was developed and generously provided by Dr. Gail Bishop (Hsing and Bishop 1999) and is a variant of the parental CH12.LX cell line (IgA^+) (Hsing and Bishop 1999), which was derived from the murine CH12 B-cell lymphoma (Arnold, LoCascio et al. 1983) in B10.H-

2^{a}H-4^{b}p/Wts mice (B10.A x B10.129). The CH12.IκBαAA cell line stably expresses an IPTG-inducible, degradation resistant IκBα super-repressor protein (IκBαAA), which sequesters NF-κB/Rel proteins in the cytoplasm (Hsing and Bishop 1999). The CH12.γ2b-3′*Igh*RR cell line (IgA^{+}) was generated from CH12.LX cells and is a subclone that stably expresses a transgene (*γ2b Igh* gene) regulated by the 3′*Igh*RR (Shi and Eckhardt 2001; Henseler, Romer et al. 2009). Cells were grown in RPMI 1640 media (MediaTech, Herndon, VA) supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 13.5 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Cells were maintained at 37˚C in an atmosphere of 5% CO_2. Cell viability was determined by assaying 1.0 mL of cell suspension for Trypan Blue exclusion staining with a ViCell instrument (Beckman Coulter, Brea, CA).

Vertebrate Animals

To validate the results from the cell line studies, splenocytes from 6 week old female B6C3F1 mice were also utilized. No in vivo treatments were performed and all animals were sacrificed by decapitation in accordance with university policy and approved under the animal use protocol number 685 (AUP). After decapitation, the spleens were aseptically removed and placed in sterile ice cold 1x PBS. The spleens were combined then homogenized with frosted microscope slides, and the debris cleared by passing the

homogenate through a screen filter. After filtration, the cells were centrifuged for 5 min at 250 x g at 4°C, the supernatant was discarded, and the cells were washed with ice cold PBS with inversion. The cells were then subjected once more to centrifugation for 5 min at 250 x g at 4°C. The supernatant was again discarded and the pellet was resuspended in 1 ml of red blood cell lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl, and 0.1 mM EDTA-pH 8.0) for 5 min, then diluted 1:10 with ice cold PBS. The cells were subjected to a final centrifugation for 5 min at 250 x g at 4°C. The supernatant was discarded and the pellet was resuspended in culture media. The cells were allowed to incubate at 37°C in an atmosphere of 5% CO_2 for 2 hr. After the incubation, the cells were slowly decanted out of the culture flask, counted with a ViCell instrument (Beckman Coulter, Brea, CA), and resuspended in culture media for use in chromatin immunoprecipitation (ChIP) assays.

Transient Transfection and Luciferase Assay

The *Igh* luciferase reporter plasmids were generously provided by Dr. Robert Roeder (Rockefeller University, New York, NY). The V_H-Luc-hs4 and V_H-Luc-3'*Igh*RR plasmids consist of an upstream variable heavy chain (V_H) promoter, a luciferase gene and the hs4 enhancer or the 3'*Igh*RR, respectively, located downstream of the luciferase gene. Plasmids were constructed using a pGL3 basic luciferase reporter construct (Promega, Madison, WI) as described previously (Ong, Stevens et al. 1998). Transient transfections were performed as previously described (Sulentic, Zhang et al. 2004; Henseler, Romer et al. 2009). Briefly, CH12.LX or CH12.IκBαAA cells

($1.0x10^7$) were resuspended in 200 μl of culture media with 10 μg of plasmid (V_H-Luc-hs4, or V_H-Luc-3'IghRR) and transferred into a 2-mm gap electroporation cuvette (Molecular BioProducts, San Diego, CA). Cells were electroporated using an electro cell manipulator (ECM 630; BTX, San Diego, CA) with the voltage at 250 V, the capacitance at 150 μF, and the resistance at 75 ohms. For each plasmid, multiple transfections were combined together in fresh media at $2.0x10^5$ cells/ml then immediately treated with the following treatment conditions and aliquoted in quadruplicate into a 12-well plate and cultured for a 24 (V_H-Luc-hs4) or 48-hr (V_H-Luc-3'*Igh*RR) incubation period in 5% CO_2 at 37°C. For the CH12.LX cells, they were pretreated for 1 hr with DMSO or 30 μM CH223191 (AhR antagonist, AhRA) then treated with DMSO or 10 nM TCDD in the presence of 1 μg/ml LPS stimulation. The final DMSO concentration was 0.11%. The CH12.IκBαAA cells were divided into two equal portions and one portion was treated with 100 μM IPTG for 2 hr to activate the IPTG-inducible IκBαAA transgene while the other portion was cultured in the absence of IPTG to provide a control that lacked IκBαAA transgene expression. An initial concentration response and time course was conducted to determine the optimum concentration and time of addition for IPTG-induced IκBαAA induction. After 2 hr of pretreatment with 100 μM IPTG, the CH12.IκBαAA cells were treated with DMSO or varying concentrations of TCDD (0.001-10 nM) with or without LPS stimulation (0.001-1 μg/mL) then immediately aliquoted in quadruplicate into a 12-well plate and cultured for 24 or 48 hr in 5% CO_2 at 37°C. After a 24 (V_H-Luc-hs4)

or 48-hr (V_H-Luc-3'*Igh*RR) incubation period, cells were lysed with a 1x reporter lysis buffer (Promega) and samples were immediately frozen at -80°C. To measure luciferase enzyme activity, samples were thawed at room temperature and 20 μl of sample lysate was mixed with 100 μl of luciferase assay reagent (Promega). Luciferase activity or luminescence was measured with a luminometer (Berthold detection systems, Oak Ridge, TN) and represented as relative light units or percent effect relative to the DMSO control.

Protein Isolation for Western Blot Analysis

Following the appropriate treatment and incubation period, CH12.IκBαAA cells were harvested using centrifugation (3000 rpm for 5 min at 4°C) and washed once with 1x PBS. The cells were resuspended in 150 μl of mild lysis buffer (150 mM NaCl, 10 mM sodium phosphate pH 7.2, 2 mM EDTA, and 1% Ipegal) and frozen at -80°C. For protein quantification, the lysate was thawed on ice and resuspended briefly then centrifuged at 14,000 rpm for 5 min at 4°C. The whole cell lysate was removed from the pelleted cell debris, quantified by the Bio-Rad Protein Assay (Bio Rad, Hercules, CA) according to manufacturer specifications, and frozen at -80°C until further analysis.

Protein Isolation for $Ig_{\gamma 2b}$ Analysis

CH12.γ2b-3'*Igh*RR cells, in the absence or presence of LPS (1 μg/ml), R848 (1 μg/ml), or CpG (1 μM) stimulation, were treated with increasing

concentrations of TCDD (0.003-30 nM) then plated into 12-well plates. Treated CH12.γ2b-3′*Igh*RR cells were plated at a concentration of $2.5x10^4$ cells/well and incubated for 48 hr. Following the incubation period, cells were centrifuged at 3000 rpm, lysed with mild lysis buffer containing freshly added protease inhibitors (Complete Mini Protease Inhibitor Cocktail; Roche Diagonostics, Indianapolis, IN). Lysed cells were centrifuged at 14,000 rpm then supernatants were collected and stored at −80 °C until analysis. To measure γ2b, cell lysates were thawed on ice and protein concentrations were determined by a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Samples were then diluted to the lowest sample concentration and 2 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 by Henseler et. al.(Henseler, Romer et al. 2009). Briefly, 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. Results are represented as percent effect relative to the stimulation with DMSO control ± S.E. (n=3).

SDS-PAGE and Western Blot Analysis

All whole cell lysates were thawed on ice and 50 μg of protein from each extract were run on a 10% polyacrylamide gel at 200 volts for 30-40 minutes. The proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) using an electric current of 100 volts for 75 min. The membrane was then immediately immersed in 3% BSA (bovine serum albumin)/TTBS (tris-buffered saline with 0.05% tween-20) and rocked overnight at 4°C. The membranes were incubated at room temperature overnight in 3% BSA/TTBS with either a mouse anti-β-actin (Sigma Aldrich) at a 1:10,000 dilution, rabbit anti-IκBα ([sc-371 (C-21), Santa Cruz, Santa Cruz, CA]) at a 1:1000 dilution, or anti-AhR (ab2770 Abcam, Cambridge, MA) at a 1:1000 dilution. The membrane was then washed four times in TTBS at 10 minute intervals, and the blot was incubated with the appropriate horse-radish-peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse at 1:8000 or goat anti-rabbit at 1:2500) for 1 hr. The blot was washed again four times in TTBS, exposed to ECL substrate (Thermoscientific, Waltham, MA) and analyzed on a Fuji LAS-3000 Bioimager (Tokyo, Japan).

Chromatin Immunoprecipitation Assay (ChIP)

1.0×10^7/treatment condition CH12.LX cells or splenocytes were treated with 1 μg/ml LPS, 0.0067% DMSO, and/or 30 nM TCDD and incubated for 90 min at 37°C with 5% CO_2. After incubation, cells were cross-linked with 1% formaldehyde for 10 min at room temperature with agitation.

Glycine at a 125 mM final concentration was then added to each treatment condition to quench the crosslinking and samples were agitated for an additional 10 min. Samples were then centrifuge at 1800 x g for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in ice cold PBS and centrifuge at 1800 x g for 5 min. at 4°C. The supernatant was again discarded and the cells were subjected to ice cold lysis buffer-1 (50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% Ipegal, 0.25% Triton X-100, and Protease Inhibitor Cocktail (PIC)) for 20 min on ice. The samples were then centrifuge at 1800 x g for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in lysis buffer-2 (10 mM Tris-HCL pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, PIC) for 10 min at room temperature. The samples were then centrifuge at 1800 x g for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in 350 μl ice cold low salt sonication buffer (40 mM Tris-HCL pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, and PIC). Samples were sonicated with a micro tip horn using a Sonicator (Heat Systems-Ultrasound, Inc. model W-225) at 40% power for eight 20 sec constant pulses with resting for 20 sec on an ice/water mix between pulses. After sonication, samples were supplemented with 5 mM $CaCl_2$, heated to 37°C, and treated with 120 units of micrococcal nuclease (MNase) for 15 min at 37°C. EDTA and EGTA at 20 mM were added to the samples after heating to quench the MNase activity. Additionally, the final concentration of NaCl was adjusted to 300 mM, and the sample placed on ice for 10 min. Samples were then centrifuged at 20,000 x

g for 10 min at 4°C, and the supernatant transferred to 1.5 mL Eppendorf tubes. The samples were then pre-cleared of proteins that non-specifically bind the IP antibodies and of free antibody by mixing with 25 μl of protein G-coated magnetic bead slurry and rotated at 4°C for 1 hr. The samples were then cleared of the magnetic beads with a magnetic Eppendorf rack. Each sample was then evaluated for total DNA by removing 10 μl of each sample, extracting the DNA with phenol-chloroform extraction, and the DNA was quantified with a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE). All cleared Pre-IP samples were diluted to same amount of DNA/sample within a treatment group and stored at -80°C until prepared for use in the ChIP assay. The immunoprecipitation (IP) antibody [(anti-AhR (ab2770 Abcam, Cambridge, MA), anti-RelA (A301-823A Bethyl Laboratories, Inc., Montgomery, TX), or anti-RelB (A302-183A Bethyl Laboratories, Inc., Montgomery, TX))] was incubated at a concentration of 3 μg of antibody to every 25 μl of protein G-coated magnetic bead slurry in 1.1 ml 1x NET buffer (150 mM NaCl, 0.5 mM EDTA, 50 mM Tris-HCL, 0.5% Ipegal) for a minimum of 4 hr at 4°C with rotation. After the first incubation, sheared salmon sperm DNA was added to the IP/bead complex and rotated an additional 1 hr. The magnetic beads were then cleared of excess/unbound antibody and salmon sperm with a magnetic Eppendorf rack. The IP/bead complex was then resuspended in 1xNET buffer and 25 μl added to each pre-cleared sample. Samples were incubated overnight with rotation at 4°C. Samples were then washed with a 150 mM NaCl wash, a 500 mM NaCl wash, a 250 mM LiCl

wash, and finally a 10 mM TE wash. The samples (IP/bead complex with the protein cross-linked to the DNA) were treated 2 times with 100 μl elution buffer (100 mM $NaHCO_3$, 1% SDS) for 15 min, and the eluent collected each time. To reverse the cross-linking, the eluent was heated to 65°C and 195 mM NaCl was added to each sample in concert with 1 μl of RNase. The samples were incubated for a minimum of 5 hr, cooled to 45°C and treated with 1 μl Proteinase K for 2 hr. The DNA from the samples was extracted with Phenol/Chloroform extraction, and the presence of DNA was validated with a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE). The PCR primers were: hs4 Forward Primer "CACACCCCACCTGTAGCAC", hs4 Reverse Primer "TGAGGAGGTTGACATGATGG", hs1,2 Forward Primer "CTGATATCTGAGCCCCCAAC", hs1,2 Reverse Primer "GTGGTCTGGGTAATGGATGG". β-actin primers were used to evaluate that the initial DNA (pre-IP samples) was equally diluted so that changes in threshold cycle (CT) could be attributed to treatment conditions within a sample set. ChIP data is reported as % input ($2^{-\Delta CT\ [normalized]}$), where $\Delta CT = (CT_{ChIP} - (CT_{input} - \log_2 (\text{Dilution Input Factor})))$ (Livak and Schmittgen 2001). The Dilution Input Factor was 1/100. The data is reported as the combined average from multiple experiments.

Statistical Analysis of Data

Mean ± S.E. was determined for each treatment group of a given experiment. A statistical difference in the fold-effect between treatment

groups and the controls was determined by 1-way ANOVA with a Dunnett's post-hoc test. Statistical differences in the fold-changes between the non-IκBαAA and IκBαAA-expressing cells were determined by either a two-tailed t-test, a one-way ANOVA with a Dunnett's post-hoc test or a two-way ANOVA with a Bonferroni post-hoc test. Statistical difference between reporters was determined by a two-way ANOVA with a Bonferroni post-hoc test. All analyzed data is represented as the average for each treatment within each experiment (each n), and combined for a minimum of n=3.

CHAPTER 4. RESULTS

TCDD is a general Inhibitor of the 3'*Igh*RR regardless of the Toll-like receptor activated

Toll-like receptors (TLRs) play an important role in the first line of host defense by recognition of microbial products. Mouse TLR4 is expressed on B cells, macrophages, T cells, epithelial cells, monocytes, myeloid DCs and is suspected to be present on many other cell populations (Iwasaki and Medzhitov 2004). Interestingly, human primary B cells have very low levels of TLR4 and express large amounts of TLR9. In agreement with this dichotomy in TLR4 and TLR9 expression in human B cells, LPS (TLR4 ligand) has a diminished ability to activate human B cells (Guijarro-Munoz, Compte et al. 2014), while hypomethylated CpG ODN (TLR9 ligand) strongly activates human B cells (Bernasconi, Onai et al. 2003). In mice, TLR9 is expressed in plasmacytoid dendritic cells (PDC), macrophage populations and B cells. In human populations, TLR9 is expressed in B cells and PDCs (Poeck, Wagner et al. 2004). As with TLR4 or TLR9-induced B cell activation, both TLR7 and TLR8 have been shown to activate both human and murine B cells and are found in dendritic cells (Iwasaki and Medzhitov 2004). Because of TLRs differences in

expression and function between species, we wanted to investigate the effect of TCDD on the activation of the 3'*Igh*RR.

To evaluate if TCDD will inhibit the activation of the 3'*Igh*RR regardless of the TLR stimuli, we elected to use the CH12.γ2b-3′*Igh*RR B cell line which is a subclone isolated from CH12.LX cells that stably express a transgene (*γ2b Igh* gene) regulated by the 3′*Igh*RR (Shi and Eckhardt 2001; Henseler, Romer et al. 2009). Briefly, upon 3′*Igh*RR activation, the transgene in the CH12.γ2b-3′*Igh*RR B cells produces Igγ2b that can be evaluated with an ELISA assay. CH12.γ2b-3′*Igh*RR B cells were treated with either; R848 (1 μg/ml), CpG (1 μM), DMSO (0.019 to 0.119%) and/or with increasing concentrations of TCDD (0.003-30 nM) (Figure 7). An LPS (1 μg/ml) control was used with either DMSO (0.119%) or a TCDD (30 μM) co-treatment for a secondary control to validate the TCDD induced inhibition of the 3'*Igh*RR. Treatment with R848 activated the 3'*Igh*RR, while the addition of increasing concentrations of TCDD demonstrated a concentration dependent inhibition of the 3'*Igh*RR reporter (Figure 7A). As with R848 treatment, CpG induced activation of the B-cells was reversed by a co-treatment with TCDD leading to significant inhibition of the CpG induced activation of the 3'*Igh*RR (Figure 7B). As all three stimuli activate the 3'*Igh*RR likely through the NF-κB/Rel signaling pathway, we elected to further investigate the role that NF-κB/Rel proteins play in the effects of TCDD inhibition on the 3'*Igh*RR by using LPS as our activation stimuli and the CH12.IκBαAA B cell line.

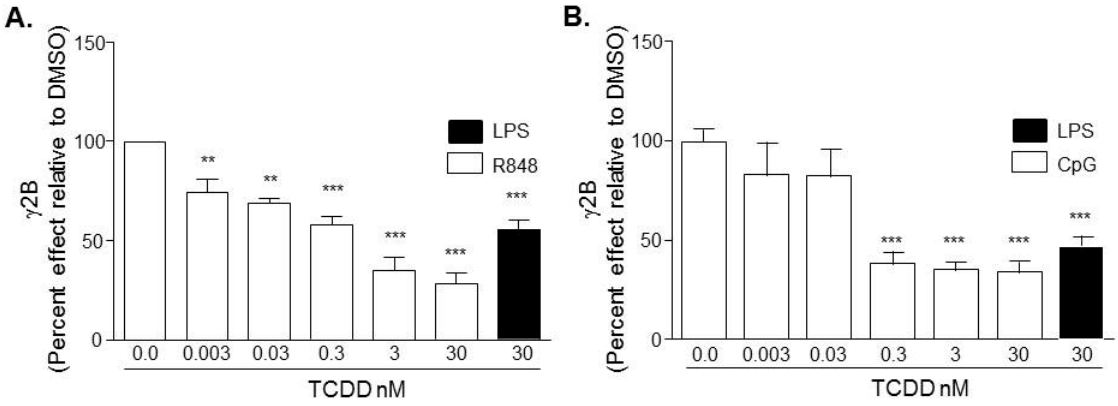

Figure 7. TCDD is a general inhibitor of TLR activation of the 3'*Igh*RR

CH12.γ2b-3'*Igh*RR B cells were treated with either; R848 (1μg/ml) (A) or CpG (1μM) (B) and co-treated with increasing concentrations of TCDD (nM). LPS (1 μg/ml) and TCDD (30 nM) co-treatment (positive control) was normalized to an LPS co-treated with DMSO that was set to 100 percent. ** p<0.01, ***p<0.001 via one-way ANOVA with a Dunnett's *post hoc* test when compared to the appropriate 0.0 (DMSO plus stimulation) control, n=3.

Characterization of the CH12.IκBαAA B cell line

To initiate studies that directly evaluate the role of NF-κB/Rel proteins in the effects of LPS and TCDD on 3'*Igh*RR activity, we chose to utilize a variant of the CH12.LX mouse B cell line, the CH12.IκBαAA B cell line. The CH12.LX B-cell line model has been extensively utilized in studying the effects of TCDD on B cell function and Ig expression (Sulentic, Holsapple et al. 1998; Sulentic, Holsapple et al. 2000; Sulentic, Kang et al. 2004; Sulentic, Zhang et al. 2004). The CH12.IκBαAA cell line stably expresses an IPTG-inducible IκBα super repressor protein (IκBαAA) which is resistant to negative feedback regulation by NF-κB/Rel proteins (Hsing and Bishop 1999; Romer and Sulentic 2011). NF-κB activation occurs when a stimulus activates an inhibitory κB kinase (IKK) which targets an inhibitory IκBα protein for proteolytic degradation. The degradation of IκBα allows the NF-κB/Rel transcription factors (e.g. RelA and to lesser extent c-Rel) to translocate to the nucleus and facilitate transcription (Figure 5) (Sen and Baltimore 1986; Baeuerle and Baltimore 1988). As the AhR and NF-κB/Rel proteins have been shown to associate ((Tian, Ke et al. 1999; Vogel, Sciullo et al. 2007) and TCDD's effects are believed to mediated through the AhR, the CH12.IκBαAA cell line was selected as an appropriate cell line to investigate the role of NF-κB/Rel on the effect of TCDD-induced inhibition of the 3'*Igh*RR.

Inducible expression of the IκBαAA

To evaluate the appropriate concentration of IPTG required to induce expression of the IκBαAA, we treated the CH12.IκBαAA B cells with 1 to 400 μM IPTG overnight followed by whole cell protein isolation and western blot analysis. An anti-IκBα antibody identified both the endogenous IκBαAA protein (approximately 37-kDa) and the slightly larger IκBαAA. Pretreatment with IPTG demonstrated a concentration-dependent increase in IκBαAA expression with concentrations of IPTG greater than 10 μM (Figure 8A). The maximal expression of the IκBαAA was induced by 100 μM IPTG, which was chosen as our working concentration.

Minimal pretreatment time induce expression of the IκBαAA

Transient transfections utilized in this investigation required 24 hr (hs4 enhancer) to 48 hr (3'*Igh*RR) incubation periods post transfection. 24 hr of IPTG pretreatment after transfection, combined with the additional treatment times resulted in a decrease in cell number and inhibited cell proliferation (data not shown). To evaluate the minimum amount of time necessary to induce IκBαAA expression, CH12.IκBαAA cells were treated with 100 μM IPTG and whole cell lysates were isolated hourly for 5 hr. Western blot analysis demonstrated a time-dependent increase in IκBαAA expression which was maximal by 2 hr of IPTG treatment (Figure 8B)**.** Therefore, subsequent studies utilized a 2 hr pretreatment with 100 μM IPTG to induce IκBαAA expression.

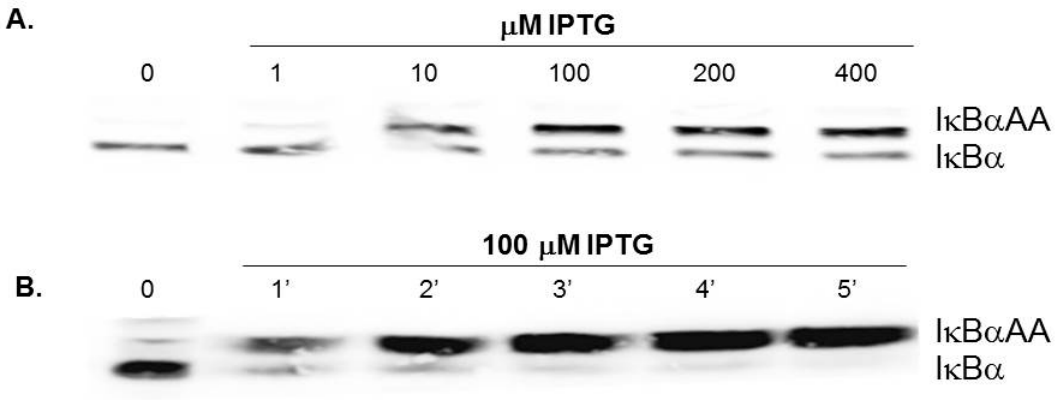

Figure 8. Inducible expression of the IκBαAA super-repressor

(A) CH12.IκBαAA B cells were treated with 1 to 400 μM IPTG overnight followed by whole cell protein isolation and western blot analysis. An anti-IκBα antibody identified both the endogenous IκBαAA protein (approximately 37-kDa) and the slightly larger IκBαAA. (B) CH12.IκBαAA cells were treated with 100 μM IPTG and whole cell lysates were isolated hourly for 5 hr. Western blot analysis demonstrated a time-dependent increase in IκBαAA expression which was maximal by 2 hr of IPTG treatment.

AhR expression and function in the CH12.IκBαAA cells

A functioning AhR signaling pathway is an important characteristic of a suitable model to study the effects of TCDD on *Igh* transcriptional regulation. Therefore AhR expression and function were evaluated in the CH12.IκBαAA cells and compared to the parental and well-characterized CH12.LX B cell line. AhR protein levels were analyzed by western blot analysis and demonstrated much lower basal levels in CH12.IκBαAA cells compared to the CH12.LX cells (Figure 9). However, LPS stimulation at 1 μg/ml for 12 hr induced AhR protein levels in both cell lines (Figure 9) as previously seen in the CH12.LX cells (Marcus, Holsapple et al. 1998) and in primary B cells (Marcus, Holsapple et al. 1998; Tanaka, Kanaji et al. 2005). Additionally, treatment with 10 nM TCDD for 12 hr decreased AhR levels in both cell lines in agreement with studies by Pollenz et. al. (Pollenz 1996). IκBαAA expression had no effect on basal AhR expression or the expression profile induced by LPS or TCDD (Figure 9B). Moreover, as a measure of AhR function, TCDD induced Cyp1a1 mRNA expression, quantified by RT-PCR analysis, in the CH12.IκBαAA cells (Figure 10). However, the level of Cyp1a1 induction by TCDD was significantly less in CH12.IκBαAA as compared to CH12.LX, which was consistent with the lower basal levels of AhR in the CH12.IκBαAA B-cell line. Taken together, these results suggest that although the basal and induced AhR levels are much lower in CH12.IκBαAA cells compared to CH12.LX cells, there are similar effects on AhR expression and

function that support the use of the CH12.IκBαAA cells as a model for evaluating the effects of TCDD and AhR activation on NF-κB-induced modulation of the *Igh*.

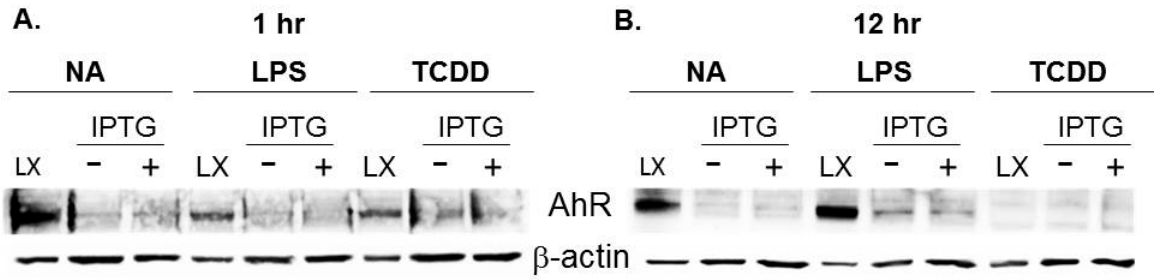

Figure 9. AhR expression and function in the CH12.IκBαAA B cells

CH12.LX (LX) and CH12.IκBαAA (-,+ IPTG) B cells were treated with 100 μM IPTG, 1μg/ml LPS, and/or 10 nM TCDD. Whole cell lysates were collected after 1 hr (A) or 12 hr (B) and 50 μg whole cell lysate was loaded for each sample. The western blot was probed with either an anti-AhR antibody or an anti-β-actin antibody (loading control).

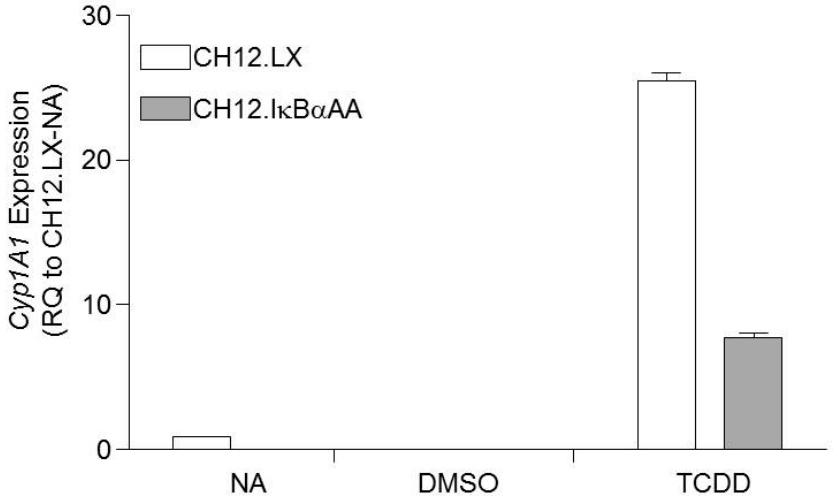

Figure 10. Cyp1A1 expression in the CH12.LX and CH12.IκBαAA cells.

Total RNA was isolated from naïve (NA) cells, or cells treated with vehicle (0.019% DMSO), or TCDD (30 nm) for 24 hr. The RNS was reversed transcribed (1μg) to cDNA, then analyzed (5ng) by SYBR Green Real-time PCR. The fold change compared to CH12.LX is represented as a relative quantification (RQ) value is relative to CH12.LX-NA (experiments conducted and analyzed by Dr. Courtney Sulentic. Ph.D.). n=2

IκBαAA expression abrogates the inhibitory effect of TCDD on 3′*Igh*RR activation and the synergistic activation of hs4

Within the 3'*Igh*RR lie four discreet enhancers; the hs1,2, hs3a, hs3b, and the hs4. As demonstrated by Fernando et. al., 2012, the murine hs1,2 activation state mirrors the activation states of the 3'*Igh*RR when activated with LPS and co-treated with TCDD (Fernando, Ochs et al. 2012). However, the dichotomy between the inhibitory effect of TCDD on 3'*Igh*RR activation and the synergistic activation of the hs4 enhancer as described by Sulentic et. al., 2004 is prevalent in our current results with the exception of a modest decrease in sensitivity to TCDD-induced inhibition of the 3'*Igh*RR (Sulentic, Zhang et al. 2004). The shift in sensitivity to TCDD is likely due to the lower levels of AhR expressed in CH12.IκBαAA as compared to CH12.LX cells. Interestingly, within the hs4 enhancer, the AhR and NF-κB/Rel proteins are shown to converge at an overlapping dioxin responsive element (DRE) and κB motif to influence synergistic transcriptional activity of the hs4 enhancer (derived through mutational analysis) (Sulentic, Kang et al. 2004). These data suggest that NF-κBs may play a significant role in concert with TCDD-induced AhR activation to synergistically activate the hs4. Equally important, are the DRE-like and κB identified motifs within the hs1,2 enhancer that may contribute to the overall inhibition of the 3'*Igh*RR. Therefore, to determine if the NF-κB/Rel proteins inhibited by IκBα (mostly RelA and to a lesser extent c-Rel) play a role in 3'*Igh*RR activation by LPS or inhibition by TCDD,

CH12.IκBαAA B cells were transiently transfected with a luciferase reporter regulated by the V_H promoter and either the 3'*Igh*RR (Figure 11) or the hs4 enhancer reporter plasmid (Figure 12). Transfected cells for each plasmid were immediately combined, split into equally into two volumes, and one volume was pre-treated with 100 μM IPTG. Following a 2 hr incubation period, transfected cells were treated with varying concentrations of LPS or TCDD in the absence or presence of IκBαAA expression.

When the CH12.IκBαAA cells were not treated with IPTG, increasing concentrations of LPS increased the activation state of the 3'*Igh*RR. Interestingly, addition of 10 nM TCDD at each increasing concentration of LPS treatment significantly reversed the LPS-induced activation of the 3'*Igh*RR greater than at the previous LPS treatment concentration (Figure 11A). Induction of the IκBαAA with IPTG lowered the overall LPS-induced 3'*Igh*RR activation, but more importantly reversed the TCDD-induced inhibition of the 3'*Igh*RR (Figure 11 A). Additionally, when the CH12.IκBαAA cells were not treated with IPTG, but treated with 1 μg/ml of LPS and increasing concentrations of TCDD (0.01-10 nM), the LPS-induced activation of the 3'*Igh*RR was inhibited by TCDD in a concentration-dependent manner (Figure 11C and D). However, with the induction of IκBαAA, the overall activation of the 3'*Igh*RR was blunted, but more importantly, the TCDD-induced inhibition of the 3'*Igh*RR was reversed (Figure 11C and D). These data suggest that the IκBα-regulated NF-κB/Rel proteins play a role in the

both the LPS-induced activation, and the TCDD-dependent inhibition of the 3'*Igh*RR.

The hs4 enhancer within the 3'*Igh*RR has overlapping binding sites for the AhR and NF-κB/Rel proteins and has been shown to synergistically activate in response to a combined treatment of LPS and TCDD (Sulentic, Kang et al. 2004) suggesting a cooperative interaction between AhR and NF-κB/Rel proteins. When the CH12.IκBαAA cells were not treated with IPTG; increasing concentrations of LPS (0.01-1 μl) marginally activated the hs4 enhancer, addition of 10 nM TCDD at each increasing concentration of LPS treatment more greatly synergistically activated the TCDD-induced activation of the hs4 enhancer greater than at the previous LPS treatment concentration (Figure 12A and B). Induction of the IκBαAA with IPTG blunted the overall LPS induced hs4 activation, but significantly inhibited and plateaued the TCDD-induced synergistic activation of the hs4 at each LPS concentration (Figure 12A and B). Additionally, when the CH12.IκBαAA cells were not treated with IPTG, but treated with 1 μg/ml of LPS and increasing concentrations of TCDD (0.01-10 nM), the TCDD-induced activation of the hs4 enhancer was synergistically activated by TCDD in a concentration-dependent manner (Figure 12C and D). However, with the induction of IκBαAA, the overall LPS induced activation of the hs4 was blunted, but more importantly, the TCDD-induced activation of the hs4 was significantly inhibited (Figure 12C and D). Consistent with previous mutational analysis studies in

the CH12.LX B-cell line (Sulentic, Kang et al. 2004) and with the data generated from the above studies with the 3'*Igh*RR, these data suggest that the IκBα-regulated NF-κB/Rel proteins play a role in the LPS and TCDD-induced synergistic activation of the hs4 enhancer.

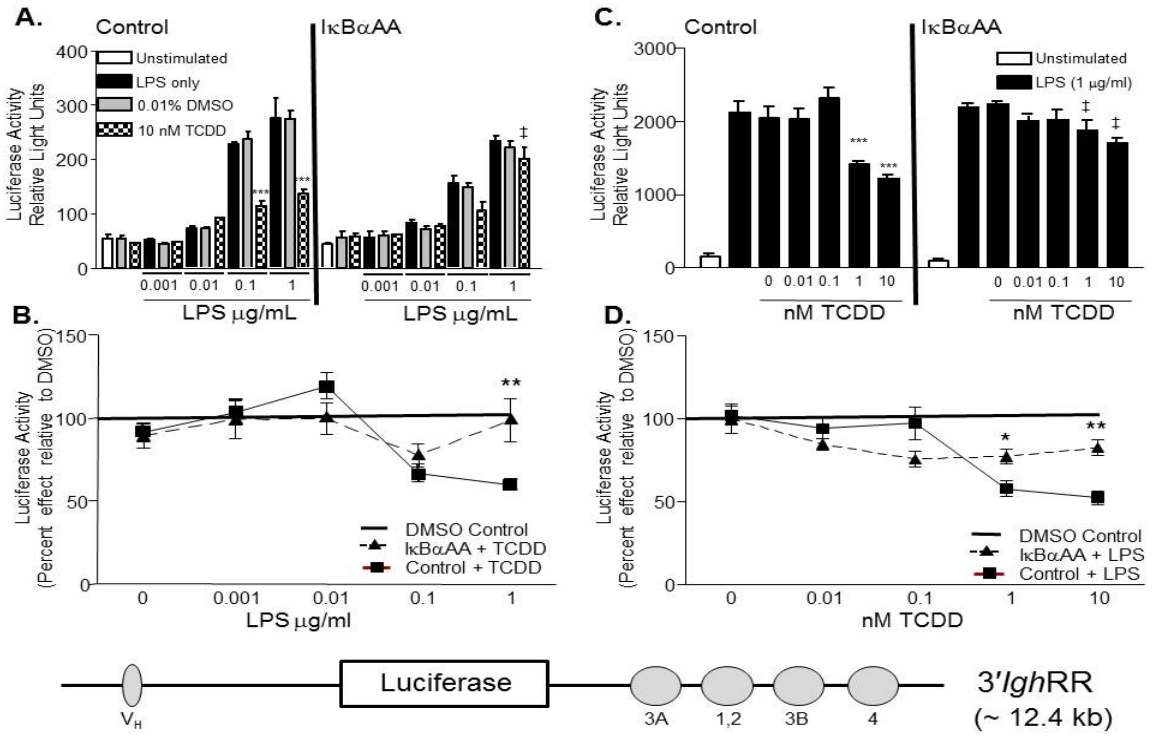

Figure 11. IκBαAA expression abrogates the inhibitory effect of TCDD on 3′*Igh*RR activation

CH12.IκBαAA cells were cultured in the presence or absence of 100 μM IPTG for 2 hr after being transiently transfected with the 3′*Igh*RR luciferase reporter. Cells were then either (A,B) stimulated with increasing concentrations of LPS (μg/mL) and co-treated with vehicle control (0.01% DMSO) or TCDD (10 nM) for 48 hr or (C,D) LPS (1 μg/mL) and co-treated with vehicle control (0.01% DMSO) or 0.01 to 10 nM TCDD for 48 hr (C, D). The percent effect relative to the appropriate DMSO control (set to 100%) for the data in A and B is represented in C and D respectively. * , **, or *** denote significance at $p<0.05$, $p<0.01$, and $p<0.001$ respectively, derived from a one-way ANOVA with a Dunnett's *post hoc* test used within a treatment group compared against the respective DMSO control. A two-way ANOVA with a Bonferroni *post hoc* test was utilized to determine significance, from the appropriate DMSO control. ‡ denotes significance between the Control group (no induced IκBαAA) and IκBαAA test group at $p<0.05$, (A, C). A two-way ANOVA Bonferroni *post hoc* test was utilized to determine significance, from the appropriate DMSO control via. *, ** denotes significance between the Control group (no induced IκBαAA) and IκBαAA test group at $p<0.05$, $p<0.01$ (B,D).

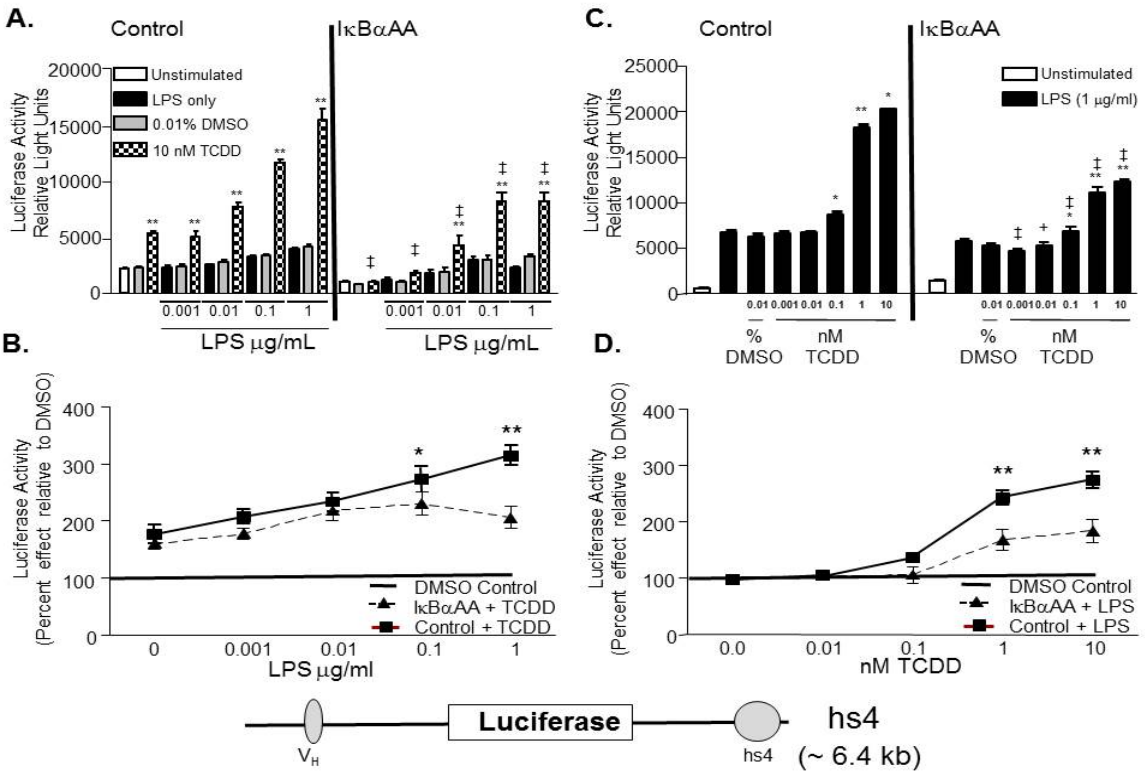

Figure 12. IκBαAA expression inhibits the synergistic activation of hs4

CH12.IκBαAA cells were cultured in the presence or absence of 100 μM IPTG for 2 hr after being transiently transfected with the hs4 luciferase reporter. Cells were then either (A,B) stimulated with increasing concentrations of LPS (μg/mL) and co-treated with vehicle control (0.01% DMSO) or TCDD (10 nM) for 48 hr or (C,D) LPS (1 μg/mL) and co-treated with vehicle control (0.01% DMSO) or 0.01 to 10 nM TCDD for 48 hr (C, D). The percent effect relative to the appropriate DMSO control (set to 100%) for the data in A and B is represented in C and D respectively. *, ** denote significance at $p<0.05$ and $p<0.01$ respectively, derived from a one-way ANOVA with a Dunnett's *post hoc* test used within a treatment group compared against the respective DMSO control. A two-way ANOVA with a Bonferroni *post hoc* test was utilized to determine significance, from the appropriate DMSO control. ‡ denotes significance between the Control group (no induced IκBαAA) and IκBαAA test group at $p<0.05$, (A and C). A two-way ANOVA Bonferroni *post hoc* test was utilized to determine significance, from the appropriate DMSO control via. *, ** denotes significance between the Control group (no induced IκBαAA) and IκBαAA test group at $p<0.05$, $p<0.01$ (B,D).

CH223191 abrogates the inhibitory effect of TCDD on 3'*Igh*RR activation and the synergistic activation of hs4

The suppressive effect of TCDD on the immune system has long been speculated to be mediated through the activation of the AhR signaling pathway. We elected to inhibit the AhR signaling pathway with the AhR antagonist, CH223191, to investigate the role the AhR on 3'*Igh*RR inhibition and hs4 activation. Addition of the (30 μM) antagonist pretreatment when combined with LPS and TCDD fully reversed the inhibitory effect of TCDD on the 3'*Igh*RR (Figure 13), suggesting that the AhR is a primary target of TCDD-induced suppression. Contrary to the complete reversal of the 3'*Igh*RR inhibition, the hs4 synergistic activation was only partially, but still significantly reduced, with the addition of the AhR antagonist (Figure 13).

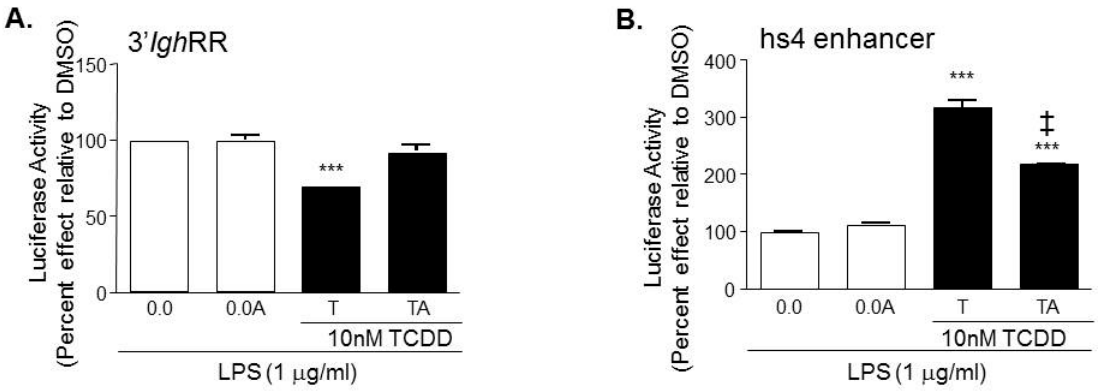

Figure 13. CH223191 reverses the effect on the 3'*Igh*RR and the hs4

The CH12.LX B cells were transiently transfected with either the 3'*Igh*RR plasmid, or the hs4 plasmid and treated with 1μg/ml LPS, 10 nM TCDD (T), 0.02% DMSO, and /or 30 μM CH223191(Antagonist [A]). Treatment of the CH12.LX B cells with CH223191 completely reversed the inhibitory effects of TCDD on the 3'*Igh*RR (A). Treatment of the CH12.LX B cells will CH223191 significantly inhibited the TCDD and LPS induced synergistic activation of the hs4 enhancer. ***=$p<0.001$ via a one-way ANOVA with a Dunnett's *post hoc* test when compared to the respective control. ‡=$p<0.01$ via a two-way ANOVA with a Bonferroni *post hoc* test when compared to the respective TCDD treatment without the CH223191 treatment, n=3.

NF-κB/Rel contributes to the TCDD induced inhibition of the 3'*Igh*RR

As the data generated from inducing the IκBαAA supported a functional role of the NF-κB's to further increase TCDD's inhibition of the 3'*Igh*RR and increase the synergistic activation of the hs4, ChIP analysis was performed to investigate alterations of NF-κB and AhR binding profiles within two enhancer regions within the 3'*Igh*RR (hs1,2 and hs4) in response treatment to TCDD. Either the CH12.LX B-cell line or the homogenate from murine spleens of female B6C3F1 mice (a strain that expresses a high affinity AhR and has a functional AhR signaling pathway) were treated with LPS, DMSO, and/or TCDD for 90-120min. The CH12.LX cell line model was chosen secondary to the high basal level of constitutively expressed AhR, thus maximizing the probability of elucidating LPS/TCDD induced alterations in protein to DNA binding profiles. After crosslinking, sonication, and MNase DNA cleavage, the lysates were subjected to immunoprecipitation (IP) with anti-RelA, anti-RelB, or anti-AhR as the IP antibody. The results demonstrate that within the hs1,2 and the hs4 (both in the CH12.LX B-cell line and in the murine spleen homogenate): NF-κB/RelA (Figure 14) binding is increased in response to TCDD treatment, and NF-κB/RelA binding is further increased when LPS is added to the TCDD treatment; NF-κB/RelB (Figure 15) binding increases in response to TCDD treatment, but interestingly, the binding of NF-κB/RelB is markedly decreased in response to treatment of both LPS and TCDD; AhR (Figure 16) binding is increased in the spleen homogenate in

response to TCDD treatment, and in all samples AhR binding was markedly increased in the presence of LPS and TCDD.

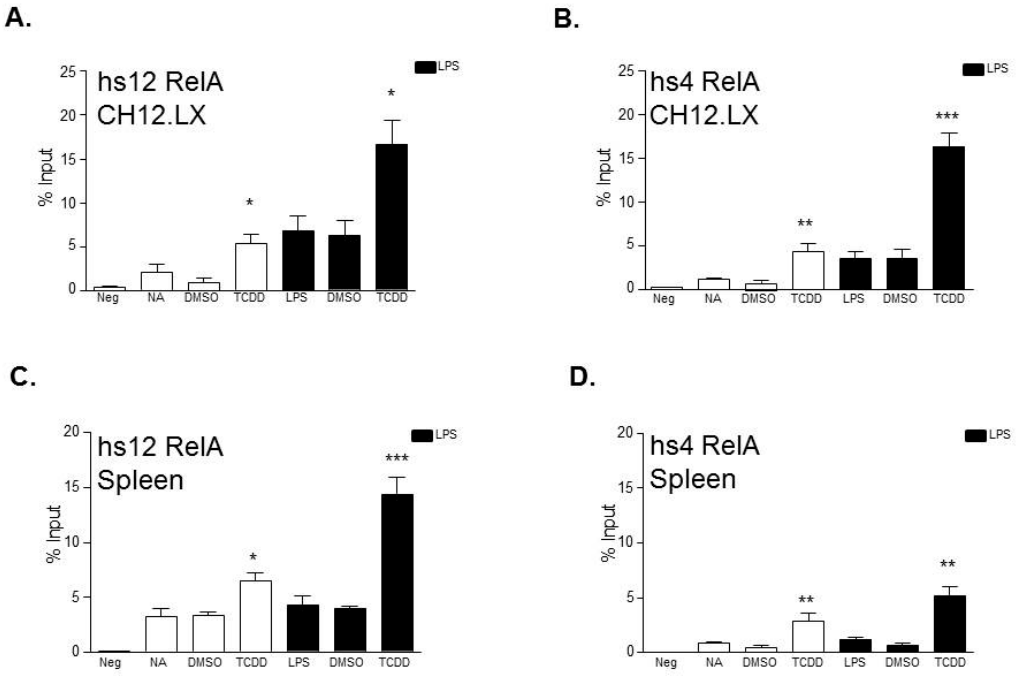

Figure 14. NF-κB/RelA binding is increased within the 3'*Igh*RR in response to TCDD treatments

Ch12.LX B cells (A, B) or the homogenate from murine spleens (C, D) were treated with LPS (1μg/ml), DMSO (0.01%), and or TCDD (30 nM). After 90-120 min of treatment, the samples were cross-linked with Formaldehyde, and prepared for ChIP analysis. The samples were subjected to an IP with an **anti-NF-κB/RelA** antibody. PCR analysis of the samples demonstrate that CH12.LX B cells and spleen homogenate when treated with either TCDD or LPS and TCDD combined significantly induced NF-κB/RelA to associate with the hs1,2 enhancer (A, C) and the hs4 enhancer (B, D). *=p<0.05, **=p<0.01, ***=p<0.001 via a one-way ANOVA with a Dunnett's *post hoc* test when compared to the respective control (TCDD compared to DMSO treatment, or LPS and TCDD compared to LPS and DMSO control).

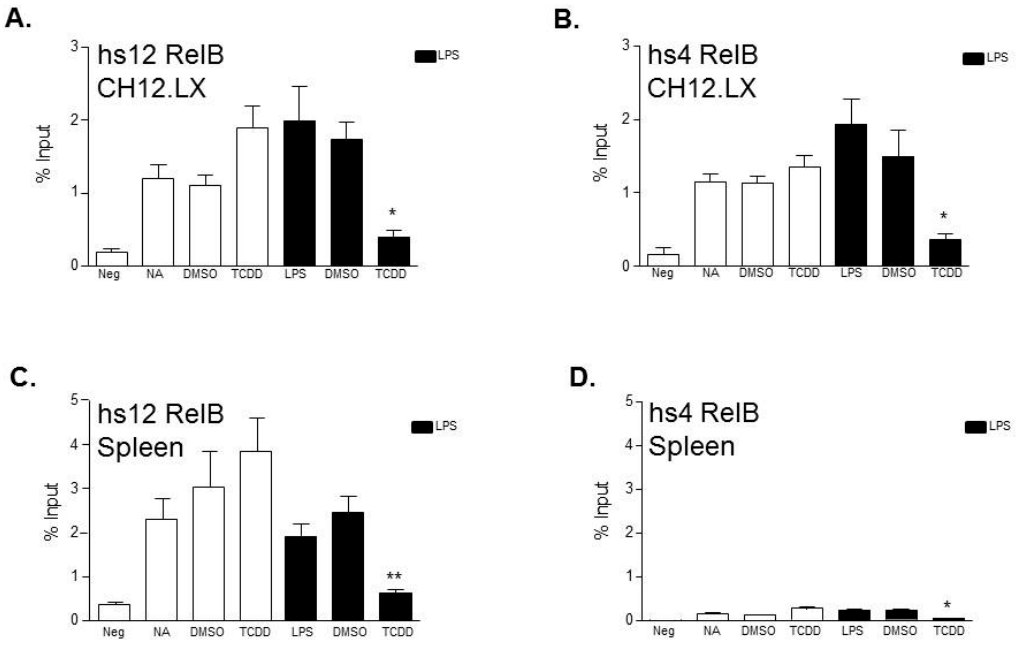

Figure 15. NF-κB/RelB binding is increased within the 3'*Igh*RR in response to TCDD treatments but inhibited by an LPS and TCDD co-treatment

Ch12.LX B cells (A, B) or the homogenate from murine spleens (C, D) were treated with LPS (1μg/ml), DMSO (0.01%), and or TCDD (30 nM). After 90-120 min of treatment, the samples were cross-linked with Formaldehyde, and prepared for ChIP analysis. The samples were subjected to an IP with an **anti-NF-κB/RelB** antibody. PCR analysis of the samples demonstrate that CH12.LX B cells when treated with TCDD significantly induced NF-κB/RelB to associate with the hs1,2 enhancer (A) and the hs4 enhancer (B). TCDD treatment of the spleen homogenate demonstrated a trend of increased association of NF-κB/RelB to both the hs1,2 (C) and the hs4 enhancer (D). When either the CH12.LX B cells or the spleen homogenate was subjected to a combined treatment of TCDD and LPS, the association of NF-κB/RelB was significantly reduced in both the hs1,2 enhancer (A, C) and the hs4 enhancer (B, D). *=$p<0.05$, **=$p<0.01$ via a one-way ANOVA with a Dunnett's *post hoc* test when compared to the respective control (TCDD compared to DMSO treatment, or LPS and TCDD compared to LPS and DMSO control).

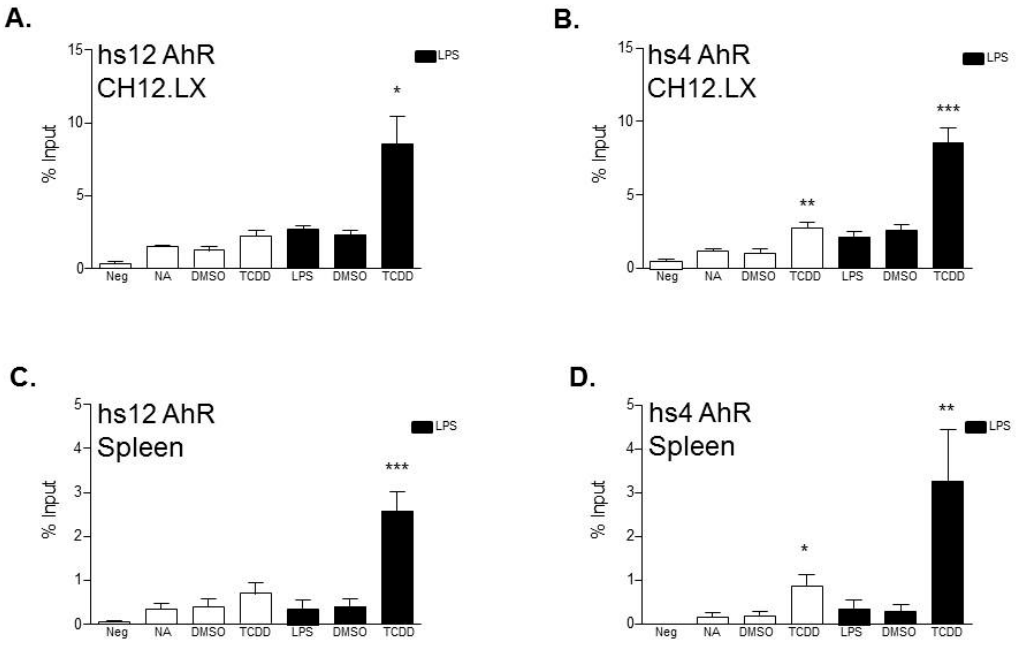

Figure 16. AhR binding is increased within the 3'*Igh*RR in response to TCDD treatments

Ch12.LX B cells (A, B) or the homogenate from murine spleens (C, D) were treated with LPS (1μg/ml), DMSO (0.01%), and or TCDD (30 nM). After 90-120 min of treatment, the samples were cross-linked with Formaldehyde, and prepared for ChIP analysis. The samples were subjected to an IP with an anti-AhR antibody. PCR analysis of the samples demonstrate that TCDD treatment of CH12.LX B cells did not alter the association of AhR within the hs1,2 enhancer (A) or the hs4 enhancer (B). However, TCDD treatment of the spleen homogenate demonstrated a significant increased association of AhR to both the hs1,2 (C) and the hs4 enhancer (D). When either the CH12.LX B cells or the spleen homogenate was subjected to a combined treatment of TCDD and LPS, the association of AhR was significantly increased in both the hs1,2 enhancer (A, C) and the hs4 enhancer (B, D). *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ via a one-way ANOVA with a Dunnett's *post hoc* test when compared to the respective control (TCDD compared to DMSO treatment, or LPS and TCDD compared to LPS and DMSO control).

CHAPTER 5. DISSCUSSION

The combustion of organic material in the presence of chlorine produces halogenated aromatic hydrocarbons (HAH). Chlorine is found as a primary constituent in plastics, textiles, solvents, agrochemicals, insecticides, and in a myriad of common use substances. TCDD is the most potent of the environmentally persistent halogenated aromatic hydrocarbons, and has been the greatest HAH investigated secondary to high profile contaminations involving both humans and wildlife (White and Birnbaum 2009). Although there are significant differences in both intra-species and inter-species sensitivity to TCDD, exposure to TCDD in sensitive models is correlated to a plethora of disease states. The focus of our current study was to investigate the mechanism by which TCDD alters the activations states of the 3'*Igh*RR.

Consistent with studies using T-cell independent activation of B cells through the use of LPS, CpG, or R848, our results demonstrated a TCDD co-treatment dependent inhibition of 3'*Igh*RR activation. The inhibition of the 3'*Igh*RR activation mirrored a TCDD co-treatment dependent reduction of the production of IgM in response to activation through the Toll-like receptors by LPS, CpG, and R848 (North, Crawford et al. 2010). The authors of the study

demonstrated alterations in the phosphorylated states of signal transduction proteins upstream of the transcription factors, NF-κB/Rel and AP-1, in response to TCDD co-treatments with the TLR agonists. Multiple transcription binding sites have been identified within the 3'*Igh*RR including sites for NF-κB/Rel, Pax5, Oct, and AP-1 (as reviewed by (Sulentic and Kaminski 2011)).

To initially investigate the role that NF-κB/Rel proteins played in the modulation of TCDD dependent inhibition of the 3'*Igh*RR, we elected to use the well characterized CH12.IκBαAA cell line that stably expresses an IPTG-inducible IκBα super repressor protein (IκBαAA). The induced IκBαAA is resistant to negative feedback regulation by NF-κB/Rel proteins (Hsing and Bishop 1999). The use of CH12.IκBαAA cell line provided a tool to selectively liberate or retarded the transcriptional active form of NF-κB/Rel proteins in response to a treatment with LPS. Although the CH12.IκBαAA cell line demonstrated a markedly lower level of constitutively expressed AhR, the cell line was sensitive to treatments with TCDD, and mirrored previous results by Sulentic et al., using the parental CH12.LX cell line (Sulentic, Zhang et al. 2004). However, the degree of inhibition of the 3'*Igh*RR in the CH12.IκBαAA cell line in response to a LPS and TCDD co-treatment was modestly less when compared to the parental CH12.LX cell line (data not shown). These results support the theory that TCDD toxicity is not only dependent on the AhR, but equally important, low levels of constitutively expressed AhR can have a significant impact on toxicity in dioxin sensitive models. Interestingly, the results from the

expression of the IκBαAA in the CH12.IκBαAA cell line suggested that both the AhR as well as NF-κB/Rel play a functional role in overall inhibition of the 3"*IgH*RR in response to a co-treatment with TCDD. In the absence of TCDD, LPS significantly enhances the activation state of the 3'*Igh*RR, and has little effect on the activation of the hs4. However, the addition of TCDD in concert with LPS markedly inhibits the 3'*Igh*RR activation and synergistically activates the hs4. Interestingly, when a co-treatment involved increasing the concentration of LPS or TCDD, the percent of 3'*Igh*RR inhibition or the synergistic activation of the hs4 was significantly increased. These results suggest that both the AhR and intra cellular proteins released in response to TLR activation are functioning together to enhance the suppressive effect of TCDD. Moreover, the expression of IκBαAA significantly altered the effect that TCDD combined with LPS had on both the 3'*Igh*RR and the hs4. Increasing the concentration of either LPS or TCDD had significantly less of an effect on the suppression of 3'*Igh*RR or the synergistic activation of the hs4. Taken together, these results further suggest that the proteins that are regulated by the IκBα (NF-κB/RelA and to a lesser extent NF-κB/c-Rel) have a contributing role in the suppression of the TCDD induced inhibition of the 3'*Igh*RR and the synergistic activation of the hs4. The activation of NF-κB/RelA by LPS significantly enhances the activation state of the 3'*Igh*RR. Conversely LPS-induced NF-κB/RelA activation significantly enhances the inhibition of the 3'*Igh*RR when B-cells are activated by LPS in the presence of TCDD. Furthermore, we were able to demonstrate a full reversal of the TCDD dependent inhibition of the LPS induced activation of the 3'*Igh*RR with the AhR

antagonist CH223191, and a partial, yet significant reversal of the synergistic activation of hs4. These data further demonstrates that the effect of B cell immune suppression by dioxin is dependent on a functional and sensitive AhR. These results suggest that NF-κB/RelA and AhR pathways converge within the 3'*Igh*RR and cooperatively mediate TCDD suppressive effect on TLR-induced B cell activation.

The entire 3'*Igh*RR is necessary for LPS induced *Ig* transcription and CSR in all B cells (Vincent-Fabert, Fiancette et al.). Additionally, the 3'*Igh*RR is associated with multiple disease states such as: dermatitis herpetiformis, plaque psoriasis, psoriatic arthritis, IgA renal nephropathy, coeliac disease, systemic sclerosis, and Burkitt's lymphoma (Madisen and Groudine 1994; Aupetit, Drouet et al. 2000; Frezza, Giambra et al. 2004; Frezza, Giambra et al. 2007; Cianci, Cammarota et al. 2008). Furthermore, the 3'*Igh*RR has been implicated in dysregulation of the oncogene, c-*myc,* via chromosomal translocation. The dysregulation of c-*myc* is highly correlated with mature B-cell neoplasms (Vincent-Fabert, Fiancette et al. 2010). Interestingly, and consistent with 3'*Igh*RR dysregulation, NF-κB dysregulation and activation has been implicated in: plaque psoriasis, psoriatic arthritis, IgA renal nephropathy, coeliac disease, and Burkitt's lymphoma (Knecht, Berger et al. 2001; Ashizawa, Miyazaki et al. 2003; Bell, Degitz et al. 2003; Maiuri, De Stefano et al. 2003; Abdou and Hanout 2008; Lories, Derese et al. 2008; Trynka, Zhernakova et al. 2009). Review of the current literature demonstrated conflicting emphasis for the role of the hs4 enhancer. The hs4 enhancer has been shown to be an essential and necessary

enhancer in class switch recombination (CSR) and Ig transcription in plasma cells, provided it is paired with hs3b (Manis, van der Stoep et al. 1998; Pinaud, Khamlichi et al. 2001; Vincent-Fabert, Truffinet et al. 2009). However, Collins et al. demonstrated that hs4 plays a significant role in CSR for the Ig isotype of Igγ3 and Igγ2b in transgenic C57BL/6 mice splenic B cells (Dunnick, Shi et al. 2011). Alterations of the physiological "normal" state of the 3'*Igh*RR (including alteration of activation states of the 3'*Igh*RR's hs enhancers) may lead to the exacerbation of disease pathology and a lowered ability to effectively respond to an immunologic insult.

Many studies have demonstrated an association between the AhR and NF-κB/Rel proteins ((Tian, Ke et al. 1999; Kim, Gazourian et al. 2000; Jensen, Leeman et al. 2003; Vorderstrasse, Dearstyne et al. 2003; Vogel, Sciullo et al. 2007). However, the AhR and NF-κB/Rel associations were cell-type specific, dependent upon the measured outcome, and either enhanced transcription or suppressed transcription of the selected gene targets. Consistent with the results demonstrated by those studies, our lab was also able to identify an association between the AhR and the NF-κB proteins, RelA (p65) and RelB (p68), in the CH12.LX murine B-cell line (data not shown), however, we were unable to consistently establish a treatment dependent association. Interestingly, we were able to demonstrate an NF-κB/RelA and NF-κB/RelB association with AhR if the AhR was immonoprecipitated (Ip'd), but we were unable to visualize the AhR association with NF-κB/Rel if we Ip'd NF-κB/Rel. Both Vogel, and Tian, have

published excellent reviews that explore the potential functional roles that involve the AhR/NF-κB interaction and how those unique interactions may play a vital role in intracellular processes (Vogel, Sciullo et al. 2007; Tian 2009). Interestingly, Sulentic et. al., demonstrated that within the hs4 enhancer, and in the presence of a functional AhR pathway, treatment with TCDD alone preferentially induced binding of NF-κB/RelA over the binding of RelB. However, in the absence of a functional AhR, TCDD induced preferential and significant binding of NF-κB RelB over RelA to the hs4 enhancer (Sulentic, Holsapple et al. 2000). Taken together, these results would support that not only does an AhR dependent shift in the binding profile of transcription factors modulate the activity of the unique enhancers within the 3'*Igh*RR, but an LPS-induced NF-κB dependent shift in binding profiles is altered by the activation of the AhR with TCDD. The results from the ChIP data support that upon LPS activation, the presence of TCDD leads to a further increase in NF-κB/RelA binding to both the hs4 and hs1,2 enhancer. In addition to the increase in NF-κB/RelA binding, NF-κB/RelB binding is markedly decreased in response to the addition of LPS combined with a treatment of TCDD suggesting that the alterations in NF-κB/RelA binding are a prime candidate that contributes to the overall inhibition in the murine 3'*Igh*RR induced transcription of the *Igh*. That said, we cannot rule out that the epitope on the NF-κB/RelB may have been mask by an interaction of NF-κB/RelB and the AhR. The masking of the epitope could have rendered our anti-RelB antibody ineffective in the ChIP assay. Furthermore, The ChIP

experiments were conducted at early treatment times (cells were subjected to crosslinking after approximately 90 min of treatment). Bode et. al., investigated the kinetics of RelA activation in response to LPS and CpG treatment in dendritic cells (Bode, Schmitz et al. 2009). The data demonstrated that IκBα (RelA activation) was rapidly degraded (within 5-10 min) in response to LPS activation, whereas IκBβ (RelB activation) failed to show degradation within the first 2 hr of LPS treatment. It is highly possible that the fast LPS-induced degradation of the IκBα allowed for NF-κB/RelA to "out-compete" NF-κB/RelB for κB binding within the enhancers. Additionally, as AhR has been shown to inhibit the activation state of the 3'*Igh*RR, activation of the AhR by TCDD is a powerful tool to elucidate the underlying mechanisms by which the 3'*Igh*RR is inhibited. The overall inhibition of the 3'*Igh*RR may prove to have a therapeutic benefit in the aforementioned disease states. Furthermore, the convergence of these two transcriptional pathways may prove to be a therapeutic target to enhance suppression of the 3'*Igh*RR.

In conflict to the present study, Fernando et al., demonstrated that there are species dependent variations within the 3'*Igh*RR that may contribute to altered and conflicting sensitivities to TCDD. Fernando et al., demonstrated that the LPS induced activation of the: mouse hs1,2 (ms-hs1,2), the 3'*Igh*RR, and the endogenous transcription of the *Ig* Heavy chain were all significantly inhibited by a co-treatment with TCDD when examined in a mouse derived cell line. However, when the human (hu-hs1,2) enhancer was transfected into either CH12.LX cell line or a human IM9 B cell line, the hu-hs1,2 was significantly *activated* in

response to LPS and TCDD (Fernando, Ochs et al. 2012). The authors of the study speculated that of the many possibilities as to why the hu-hs1,2 enhancer functioned in a manner inconsistent with the ms-hs1,2, was secondary to the lack of a high-affinity Pax5 binding site within the ms-hs1,2. Interestingly, Lu et al. demonstrated that 75% of human donor primary B cells samples that were tested demonstrated a significant reduction in Ig expression in response to a treatment with TCDD. In the same study, 16.6% of the samples were insensitive to TCDD, and 8% of the samples showed an increase in Ig expression in response to TCDD treatment (Lu, Crawford et al. 2010). Taken together, these data suggest that; the well-known alterations in the structure of the AhR (altering the sensitivity of the AhR to an agonist), alterations in the non-conserved sequences within the 3'*Igh*RR (both between intra and interspecies), a symptomatic disease state (i.e. autoimmune) in a tested subject, the concentration of an AhR agonist, and the degree to which the immune system is activated by an antigen can impact the outcome of an immune response to an AhR agonist. It would be of great scientific interest to transiently transfect a hu-*3'IgHRR* into both the CH12.LX and IM9 B cell line to further elucidate alterations in sensitivity to TCDD, as there is much work yet to be done.

CHAPTER 6. CONCLUSION

There is still much work that needs be accomplished to elucidate the mechanisms by which TCDD dysregulates the immune system. Although, we are gaining a stronger understanding of the roles that NF-κB/Rel and AhR contribute to alterations in transcription of sensitive genes, transgenic mice may prove to be a beneficial model. Collins, et. al. has developed a C57BL/6 transgenic mice that contains either the complete 3'*Igh*RR, or multiple variations of the 3'*Igh*RR hs enhancer regions (Dunnick, Shi et al. 2011). The C57BL/6 mouse model has historically been sensitive to dioxin. Splenocytes harvested from the transgenic model would allow for the investigation of the impact of dioxin on each unique hs enhancer, the effects of dioxin on *Igh* transcription, and the impact that dioxin has on Ig production. Furthermore, an extensive review of the literature failed to provide any information on any studies that investigated the "quality" of antibodies produced by dioxin challenged mammals. The majority of the research correlated the reduction of antibody production to reduced immunologic capability. Provided that the hs3b and hs4 activity can induce V_H gene mutations and given the importance of these regions for somatic hypermutation (Laurencikiene, Tamosiunas et al. 2007), could activation of the hs4 with dioxin impact the overall affinity and avidity of the resultant Ig. Although technically

challenging, it would be of great interest to evaluate the quality of the resultant antibodies to effectively bind an antigen after dioxin exposure.

CHAPTER 7. REFERENCES

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CHAPTER 8. APPENDIX

LIST OF ABBREVIATIONS

3'*Igh*RR: 3'*Ig* heavy chain regulatory region

AhR: Aryl hydrocarbon receptor

APC: Antigen presenting cells

AP-1: Activator protein 1

ARNT: AhR nuclear translocator protein

BCR: B-cell receptor

CD40L: CD40 ligand

C_H: Constant region

ChIP: Chromatin Immunoprecipitation Assay

CpG: Cytosine-phosphate-Guanine (CpG)-oligodeoxynucleotides

DRE: Dioxin responsive elements

ELISA: Enzyme-Linked Immunosorbent Assay

Eμ: μ enhancer

HAH: Halogenated aromatic hydrocarbons

HSP: Heat shock protien

Ig: Immunoglobulin

Ip'd: Immunoprecipitated

I**κK:** I kappa Kinase

IL-1R-MyD88: Toll/interleukin 1 receptor-Myeloid Differentiation primary response gene 88

KO: Knockout

LPS: Lipopolysaccharide

NF-κB: Nuclear factor-kappaB

OCT: Octamer-binding transcription factor

P23: Heat shock protein 23

PVC: Polyvinyl chloride

R848: Resiquimod

SP1: Specificity protein 1

TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

TLR: Toll-Like Receptors

TIRAP: Toll/IL-1R domain-containing adaptor protein

V_H: Variable heavy chain promoter

XAP2: X-associated protein

Contributions to dissertation

All the experimental procedures, interpretation, and design contained within this dissertation were solely performed by Richard L. Salisbury Jr., Assistance and guidance was kindly provided by Dr. Courtney E.W. Sulentic, Ph.D. Initial experimental models and design outlines were derived from the Environmental Health Sciences grant R01ES014676 authored by Dr. Courtney E.W. Sulentic, Ph.D.

Additional contributions by Richard L. Salisbury Jr., were the equally shared experimental procedures, interpretation, design, and co-first authorship with Dr. Monita Sharma, Ph.D., in the accepted manuscript titled “Gold nanoparticles induce transcriptional activity of NF-kappaB in a B-lymphocyte cell line” (Sharma, Salisbury et al. 2013).