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The Aryl Hydrocarbon Receptor Regulates an Essential Transcriptional Element in the Immunoglobulin Heavy Chain Gene

Michael J. Wourms
Wright State University

THE ARYL HYDROCARBON RECEPTOR REGULATES AN ESSENTIAL TRANSCRIPTIONAL ELEMENT IN THE IMMUNOGLOBULIN HEAVY CHAIN GENE

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Master of Science

By

MICHAEL JOSEPH WOURMS
B.S., Wright State University, 2010

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Michael Joseph Wourms ENTITLED The aryl hydrocarbon receptor regulates an essential transcriptional element in the immunoglobulin heavy chain gene BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Courtney Sulentic, Ph.D.

Thesis Director

Committee on

Final Examination

Norma C. Adragna, Ph.D.

Interim Chair

Department of Pharmacology and Toxicology

Katherine Excoffon, Ph.D.

Michael Leffak, Ph.D.

R. William Ayres, Ph.D.

Interim Dean, Graduate School

ABSTRACT

Wourms, Michael J. M.S., Department of Pharmacology and Toxicology, Wright State University, 2013. The aryl hydrocarbon receptor regulates an essential transcriptional element in the immunoglobulin heavy chain gene.

2,3,7,8-tetrachlorodibenzo-ρ-dioxin (TCDD) is a ubiquitous environmental contaminant that inhibits immunoglobulin (Ig) expression and Ig heavy (IgH) chain gene transcription. Transcription of the IgH gene involves several regulatory elements including the 3'*Igh* regulatory region (3'*Igh*RR) which is composed of four enhancers (hs3A, hs1,2, hs4, and hs3B). Dioxin responsive elements (DRE) in the hs4 and hs1,2 enhancers of the 3'*Igh*RR that bind the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that regulates dioxin sensitive genes suggest that the 3'*Igh*RR may be a transcriptional target of TCDD. The current study utilized an IgA secreting mouse B-cell line that stably expresses a γ2b transgene regulated by the 3'*Igh*RR (CH12.γ2b-3'*Igh*RR cells). Both shRNA knock down of AhR and an AhR antagonist (CH-223191) reduced TCDD-induced inhibition of endogenous IgA and the γ2b transgene expression. With the growing number of immune-related disorders correlated with polymorphisms of the human hs1,2 enhancer, ubiquitously found AhR ligands, and sensitivity of human Ig expression to TCDD, our findings may provide indispensible information for human health risk assessment and insight into the development of therapeutic interventions for immune-related disease.

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ACKNOWLEDGEMENTS

Although I have always had a great love for science, without the Sulentic Lab I would quite simply be without a calling or a career. The rigorous scientific training I received under Dr. Sulentic will serve me well throughout my career, wherever it may go. Dr. Sulentic will always be my mentor, colleague, and friend. I greatly appreciate the service of my committee members, Dr. Excoffon and Dr. Leffak, they have graciously provided support and feedback throughout not only my thesis research but also during my undergraduate classes. Any acknowledgements would not be complete without thanking the members of the Sulentic lab for their friendship and support. In the years to come I may not always remember at what concentration TCDD inhibits immunoglobulin expression but I will always remember the late nights, early mornings, weekends, and holidays full of laughter and camaraderie. Of course my education would not be possible without the support of my family and my unwavering wife Amy.

I. INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a ligand-activated basic-helix-loop-helix (bHLH) transcription factor most well known for mediating the toxic effects of 2,3,7,8-tetrachlorodibenzo-ρ-dioxin or TCDD (Fig. 1) as well as other dioxin congeners. In its quiescent state, the AhR resides in the cytoplasm sequestered by chaperone proteins p23, XAP2, and two heat shock 90 proteins. Once bound by ligand, AhR translocates to the nucleus heterodimerizing with the aryl hydrocarbon nuclear translocator (ARNT) protein forming a functional transcription factor that binds to *cis*-acting sequences known as dioxin responsive elements (DREs) in the regulatory regions of AhR-sensitive genes (Fig. 2) (Reviewed by Abel and Haarmann-Stemmann, 2010 and Ma et al., 2009). Although TCDD and other dioxin congeners are the prototypical compounds by which the AhR has been studied, a variety of nondioxin agonist have recently been discovered including those of pharmaceutical and dietary origins (Quattrochi and Tukey, 1993; Werlinder at al., 2000; Jeuken et al., 2003).

Canonically, AhR is known to modulate the xenobiotic enzyme battery specifically cytochrome P4501A1 (Cyp1A1) but recent evidence supports AhR regulation of genes important to adaptive immunity including the inflammatory

cytokine Interluekin 6 and immunoglobulin (Ig) (DiNatale et al., 2010, DiNatale et al., 2011, Sulentic and Kaminski, 2010). Indeed, we have recently shown variety of dioxin and non dioxin AhR agonists inhibit Ig expression in the CH12.LX murine B cell line however the BCL-1 cell line which does not express the AhR is insensitive to treatment with TCDD and other dioxin congeners (Henseler et al., 2009; Sulentic et al., 1997). Similarly, Vorderstrasse et al. (2001) demonstrated that $AhR^{-/-}$ mice generate a normal Ig response to antigens despite TCDD treatment.

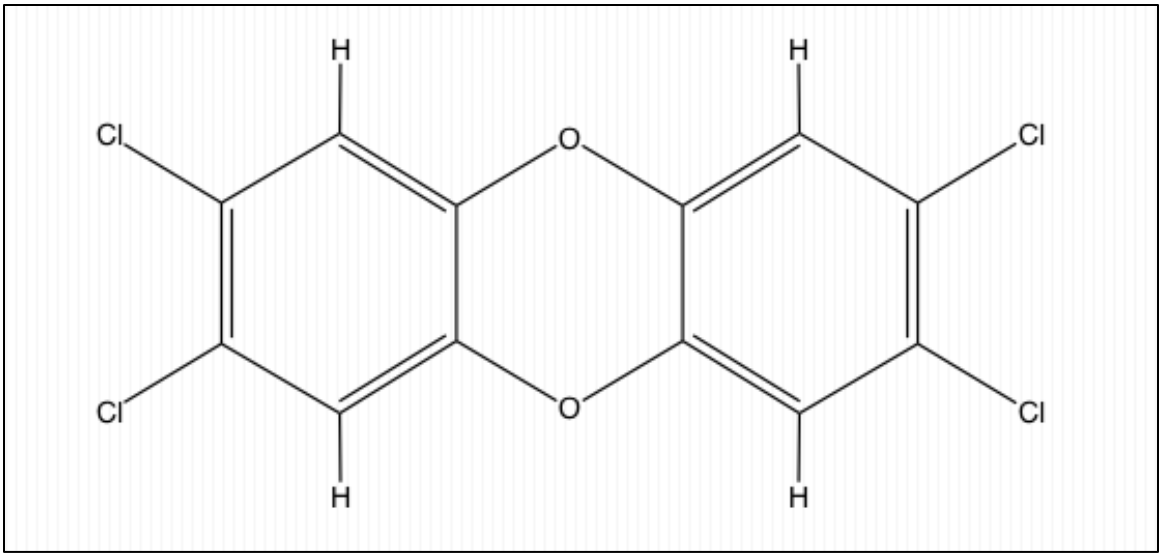

Figure 1. Chemical structure of 2,3,7,8-tetrachlorodibenzo-ρ-dioxin (TCDD).

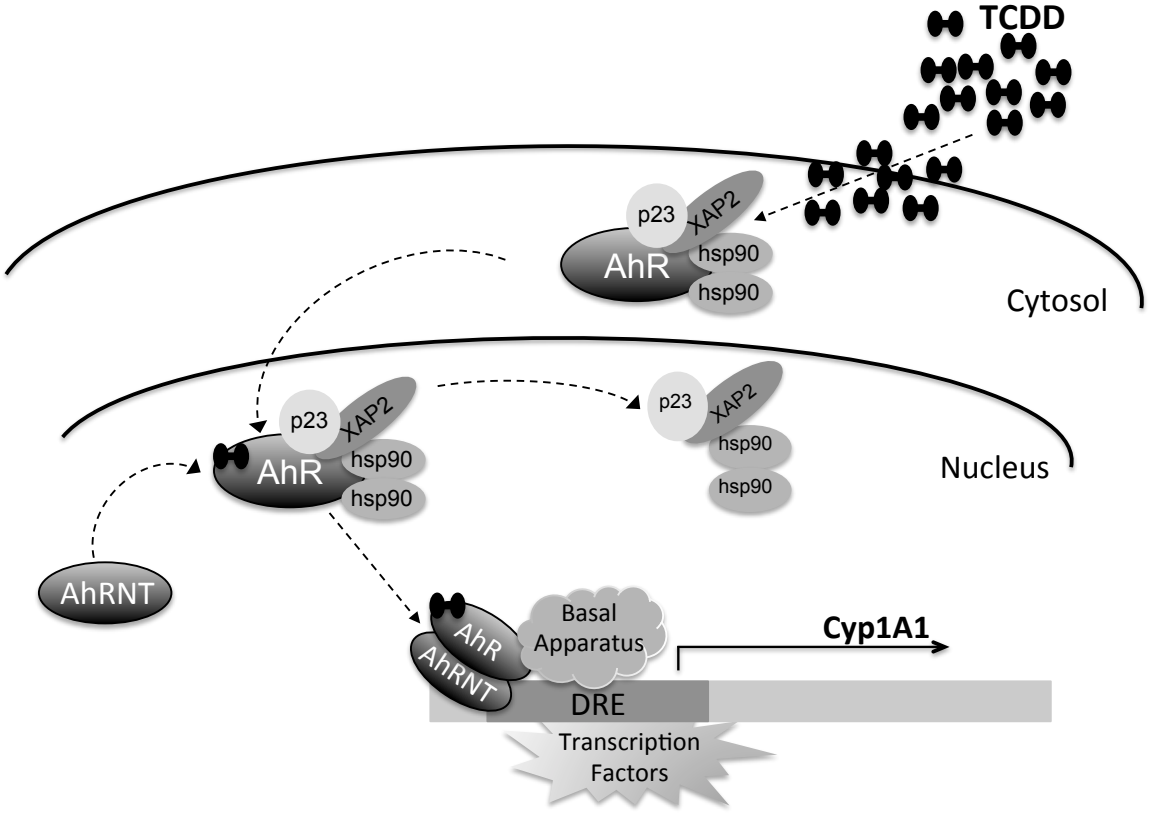

Figure 2. The aryl hydrocarbon receptor (AhR) signaling pathway. sequestered in the cytosol by chaperone proteins p23, XAP2, and hsp90, upon ligand-binding the AhR-complex translocates to the nucleus where it sheds the chaperone proteins, heterodimerizing with the aryl hydrocarbon receptor nuclear translocator protein (AhRNT). The AhR-AhRNT complex binds dioxin responsive elements (DRE) in the regulatory regions of dioxin-sensitive genes interacting with the basal transcription apparatus and other transcription factors causing changes in transcription (e.g. increasing the transcription of Cyp1A1).

Clearly, the AhR plays an obligatory role during TCDD dependent inhibition of Ig expression in B cells however a mechanism has yet to be characterized and an Ig regulatory target for the AhR not identified. Responsible for humoral immunity, the Ig is a tetrameric protein comprised of two identical heavy (IgH) and light (IgL) chain peptides (Fig. 3). The genes encoding IgH and IgL (Ig*h* and Ig*l*) require coordinated expression for effective assembly and expression. Interestingly TCDD not only inhibits Ig protein expression in CH12.LX cells but also transcription of Ig*h* and Ig*l* (Fernando et al., 2012; Henseler et al., 2009; Yoo et al., 2004; Sulentic et al., 2000).

Of the Ig genes, regulation of Ig*h* is the best understood and is achieved through a variable heavy chain promoter (V_H), an intronic enhancer (E_μ), and the 3'*Igh* regulatory region (3'*Igh*RR)(Fig. 4). Primarily activated in terminally differentiated plasma B cells, the 3'*Igh*RR provides for high *Igh* expression in plasma B cells as well as class-switch recombination (CSR), a DNA recombination event during which the type of Ig expressed is changed from IgM to one (e.g. IgG, IgE, or IgA) that interacts with a separate array of effector molecules and cells (Pinaud et al., 2001; Cogne et al., 1994, Dunnick et al., 2005). The murine 3'*Igh*RR (mo-3'*Igh*RR) contains four DNase I hypersensitivity sites (hs3A, hs1,2, hs4, and hs3B) that act as enhancers (Reviewed by Khamlichi et al., 2000). Our lab has previously characterized two DREs capable of binding ligand-activated AhR in the hs1,2 and hs4 enhancers suggesting this region is sensitive to TCDD-AhR (Sulentic et al., 2000). Indeed both transiently and stably expressed 3'*Igh*RR reporter constructs are down regulated by TCDD

in CH12.LX cells (Henseler et al., 2009). Furthermore, studies reveal that 3'*Igh*RR activity is inhibited with greater potency by ligand with higher affinity for the AhR protein (Henseler at al., 2009).

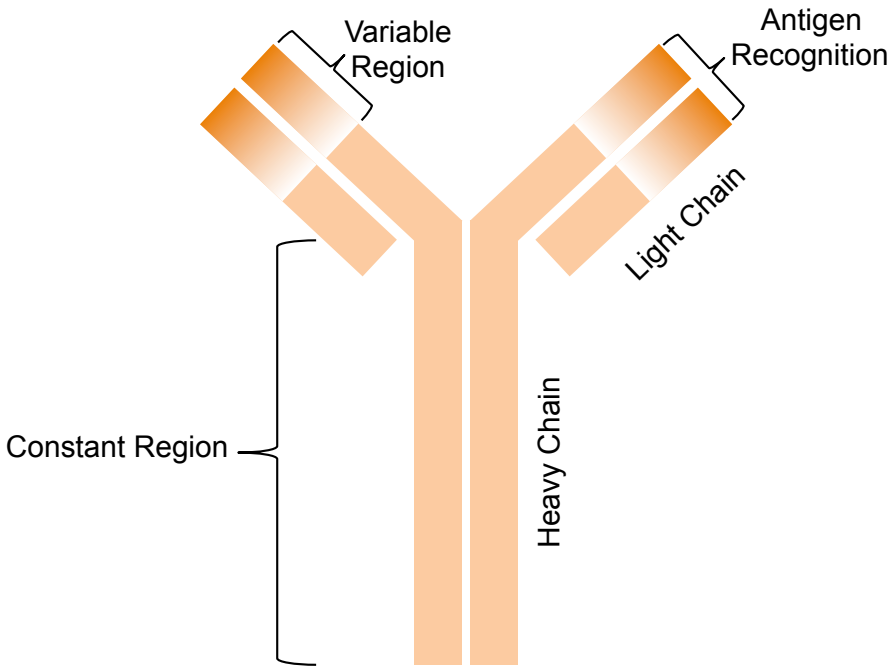

Figure 3. Structure of an immunoglobulin (Ig) protein. In its secreted form Ig is composed of two identical heavy chain and light chain proteins each with a constant region that determines the effector qualities of the Ig and a variable region that binds antigens.

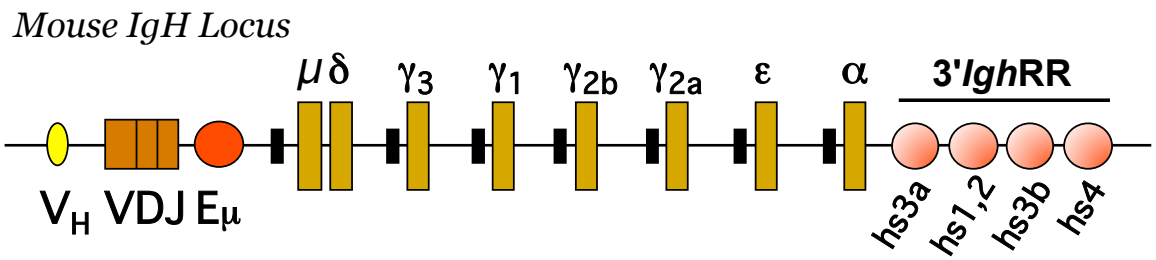

Figure 4. Schematic of the mouse immunoglobulin heavy chain gene locus. V_H, variable heavy chain promoter; Eμ, intronic enhancer (μ) enhancer; closed black rectangles, germline promoters upstream of their heavy chain constant region; 3´ *Igh*RR, 3´ immunoglobulin heavy chain regulatory region containing the hs3a, hs1,2, hs3b, and hs4 enhancers.

Although previous studies suggest AhR regulates Ig expression through modulating the 3'*Igh*RR, the data is correlative and a conclusive link has yet to be drawn. In the current study we utilized an IgA expressing CH12.LX variant that stably expresses the previously characterized 3'*Igh*RR-regulated γ2b-transgene (Henseler et al., 2009). We show that by both pharmacologically inhibiting the AhR and stably knocking down AhR expression in this cellular model, TCDD-induced inhibition of IgA expression and 3'*Igh*RR can be fully reversed. We address a critical mechanistic data gap showing that the AhR is responsible for TCDD-induced inhibition of Ig by inhibiting the capacity of the 3'*Igh*RR to increase expression of Ig. We also identify the AhR as a previously unknown regulator of the 3'*Igh*RR. The 3'*Igh*RR is a critical component of B-cell function as maintaining high levels of Ig expression in plasma cells and regulating class switch recombination are paramount to mounting an effective immune response. Any dysregulation of these processes has significant health ramifications. In addition the 3'*Igh*RR has also been associated with several diseases including Burkitt's lymphoma, celiac disease, and IgA nephropathy (Wang et al., 2005; Frezza et al., 2004; Auptetit et al., 2000). Therefore, modulation of the 3'*Igh*RR by AhR via environmentally ubiquitous dioxin and nondioxin agonists has the potential to influence the severity and/or incidence of human disease.

II. MATERIALS AND METHODS

Chemicals and Reagents

AhR antagonist (CH-223191) was purchased from EMD4Biosciences at ≥ 95% purity (Newark, NJ) and suspended in 100% dimethyl sulfoxide (DMSO). TCDD (99.1% purity) in 100% DMSO was purchased from Accustandard (New Haven, CT). DMSO and lipopolysaccharide (LPS, Escherichia coli) were purchased from Sigma-Aldrich (Atlanta, GA).

Cell Model

The CH12.γ2b-3'*Igh*RR cell line, developed by our lab (Henseler et al., 2009), is a variant of the CH12.LX murine B cell line derived from the CH12 murine B cell lymphoma arising in B10.H-2^{a}H-4^{b}p/Wts mice (Arnold at al., 1983). The CH12.γ2b-3'IghRR cell line was generated from an IgA expressing CH12.LX clone and stably expresses a 3'*Igh*RR-regulated γ2b-transgene with loxP sites flanking the hs3B and hs4 enhancer pair generously provided by Dr. Laurel Eckhardt from Hunter College, New York, NY (Figure 5; Shi and Eckhardt, 2001). PCR and ELISA analysis verified that CH12.γ2b-3'IgH cells do not contain an endogenous form of the γ2b gene. The γ2b-transgene is sensitive to activation by LPS through TLR4 signaling with maximal transgene expression 48 hours post LPS treatment (Henseler et al., 2009). All cells were grown in a 37˚C incubator with 5% CO_2 injection. Cells were maintained in RPMI 1640

(Mediatech, Herndon, VA) media supplemented with 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 13.5 mM HEPES, 100 units/mL penicillin, 50 μM 2-mercaptoethanol, and 100 μg/mL streptomycin (Hyclone Laboratories).

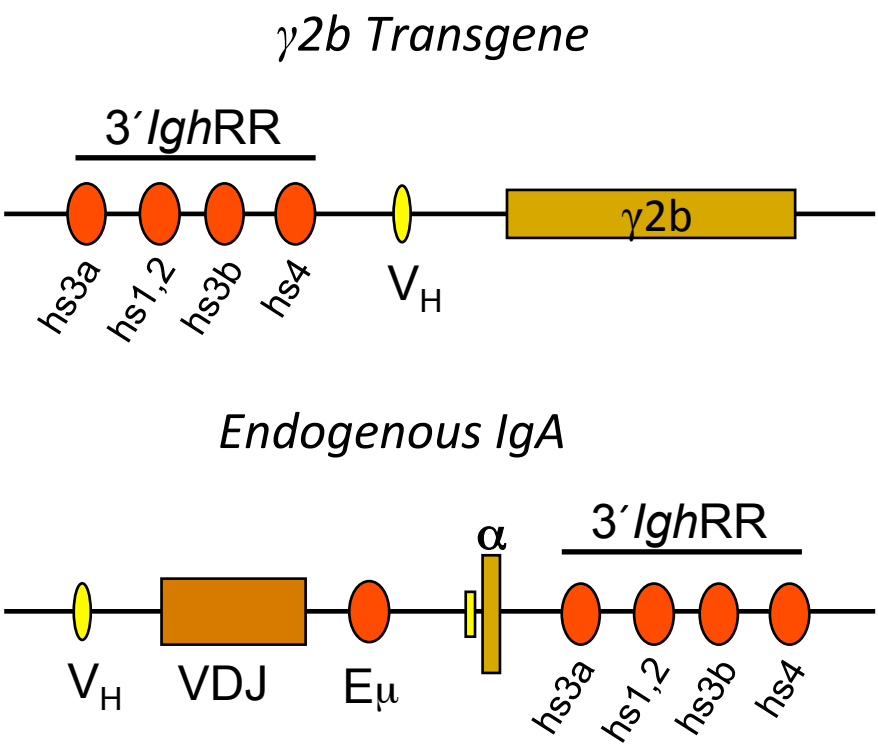

Figure 5. Schematic of the *γ2b transgene* and endogenous IgA locus in the CH12.γ2b-3'*Igh*RR cell line. Top; Schematic of the γ2b transgene, composed of the γ2b reporter protein regulated by the variable heavy chain promoter (V_H) and the 3'*Igh*RR. Bottom; Schematic of the endogenous IgA locus native to the CH12.γ2b-3'*Igh*RR cell line composed of the V_H, variable heavy chain promoter, Eμ, intronic enhancer (μ) enhancer, the germline promoter (closed small yellow rectangle) upstream of the α (IgA) heavy chain constant region followed by the 3'*Igh*RR.

shRNA Constructs

Two pLKO.1 HIV-based lentiviral vector plasmids containing shRNA sequences complimentary to AhR (shAhR) and puromycin selectable marker gene were purchased from Open Biosystems (Huntsville, AL). The shAhR sequences and target nucleotides in the AhR (Genbank Accession No. NM_013464.4) transcript are as follows; shAhR11, 5'-AATTTGCTCATGTTTCAGCGC-3', corresponding to nucleotide positions 1861-1881, shAhR12, 5'-TAATAACATCTTGCGGGAAGG-3', corresponding to nucleotide positions 527-547. Vectors were packaged into VSV-G pseudotyped fourth generation lentiviral particles by Cincinnati Children's Viral Vector Core (Cincinnati, OH) and stored at -80˚C until use.

Stable AhR Knockdown

CH12.γ2b-3'IgH cells (5 x 10^3 cells/mL) were suspended in media containing 16 µg/mL polybrene (American Bioanalytical, Natick, MA) and 0.1 mL (500 cells) immediately seeded in 96-well culture plates with either shAhR11 or shAhR12-containing lentiviral particles. Culture plates were centrifuged for 30 minutes at 1100 x g before supernatants were discarded and replaced with 200 µL media. After 24 hr the culture plates were centrifuged at 3000 rpm (5 min), the supernatants replaced with puromycin (Invivogen, San Diego, CA) selective media (1µg/mL). Puromycin selective media was replaced every 72 hr for approximately 4 weeks until cell density and culture volumes were sufficient to harvest whole cell lysate from 10 mL of cells and 1.0 x 10^6 cell stocks were frozen in liquid nitrogen for future use.

Chemical Treatment

Cells were treated with the AhR antagonist, CH-223191, or vehicle (0.1% DMSO) for one hour prior to treatment with TCDD (10 nM) or the appropriate TCDD vehicle (0.01% DMSO). For IgA and γ2b analysis, cells were stimulated with LPS (1 μg/mL), seeded in 24-well culture plates at a concentration of 3.0×10^4 cells/mL and incubated for 48 hours at 37˚C in 5% CO_2. For quantitative real-time polymerase chain reaction (qRT-PCR) of Cyp1A1 transcripts; cells were seeded at 5.0×10^5 cells/mL in 24-well culture plates and incubated for 8 h. No consistent vehicle effects were seen.

Protein Isolation and Western Blot

Cells were centrifuged at 3000 rpm then lysed with mild lysis buffer (1% NP40, 150 mM NaCl, 2 mM EDTA, 10 mM $NaPO_4$) containing protease inhibitors (Complete Mini Protease Inhibitor Cocktail; Roche, Indianapolis, IN) and frozen at -80˚C. Protein quantification was performed by thawing whole cell lysates on ice prior to centrifugation at 14,000 rpm. Supernatants were collected and protein content quantified by a Bio-Rad Assay (Hercules, CA). Protein samples were normalized to 2 μg in 100 μL of 1x PBS in preparation for ELISA analysis. Naive samples were used for SDS-PAGE and western blotting analysis of AhR protein expression. 20 μg total protein from NA samples was denatured then run on 10% polyacrylamide gel at 200 volts for ~40 minutes. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) at 100 volts for 1 h. Membranes were blocked for 1 hour in blocking buffer (3% bovine serum albumin [BSA], 0.05% tween-20, in tris buffered saline[TBS]),

incubated with either mouse anti-AhR Ab (Abcam) at a 1:1000 dilution of mouse anti-β-actin Ab (Sigma Aldrich) at a 1:10,000 dilution in 1% BSA, 0.05% tween-20 in TBS. Membrane was washed four time with TBS with 0.05% tween-20 before incubating for one hour with goat anti-mouse horse-radish-peroxidase-conjigated (Santa Cruz) at a 1:2,500 dilution in TBS with 0.05 tween-20. All incubations were performed at room temperature. Proteins were detected using Pierce Supersignal substrate (Thermoscientific Pierce, Waltham, MA) in a Fuji LAS-3000 Bioimager (Tokyo, Japan) at 30 second intervals.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IgA and γ2b in cell lysates were analyzed as described previously (Henseler, et al. 2009). Colorimetric detection was performed every minute for 1 hour using a Spectramax Plus 284 automated microplate reader with a 405-nm filter (Molecular Devices, Sunnyvale, CA). Sample concentrations of IgA and γ2b were calculated by the SOFTmax PRO analysis software (Molecular Devices) using a standard curve generated from the kinetic rate of absorption for known IgA or γ2b concentrations.

RNA Isolation

After the incubation period, cells were centrifuged at 3000 rpm, collected in 0.25 mL of TRI Reagent (Sigma-Aldrich, St. Louis, MO), and stored at -80˚C. Samples were thawed at room temperature and centrifuged at 12,000 x g for 10 minutes. The supernatant was collected into Phase Lock Gel Tubes (5 PRIME, Gaithersburg, MD), mixed with 0.1 volume of 1-bromo-3-chloropropane followed by a 10 minute room temperature incubation and the addition of 0.1 volume of

nuclease-free water. Samples were then centrifuged at 12,000 x g for 5 minutes. The aqueous phase was retained and mixed with 0.125 mL isopropanol then centrifuged at 12,000 x g for 8 minutes to precipitate RNA. The RNA pellet was washed with 0.25 mL 70% ethanol, air dried, and suspended in nuclease-free water. Samples were stored at -20˚C until analysis.

cDNA Synthesis and Real-Time PCR

RNA concentrations of samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE). One microgram of total RNA was converted to cDNA using the TaqMan Reverse Transcriptase Kit (ABI, Carlsbad, CA) and the standard manufacturer's protocol. The cycling conditions for reverse transcription were as follows, 25˚C for 10 min, 48˚C for 30 min, and 95˚C for 5 min. Cyp1A1 expression in wild type, CH223191-treated, and shAhR cell lines was determined by SYBR Green Real-Time PCR (RT-PCR) following TCDD treatment. Cyp1A1 and β-actin (endogenous control) transcripts were amplified from 5 ng cDNA utilizing the following primers: Cyp1A1 forward primer (FP), 5' AAGTGCAGATGCGGTCTTCT 3'; Cyp1A1 reverse primer (RP), 5' AAAGTAGGAGGCAGGCACAA 3'; β-actin FP, 5'-GCTACAGCTTCACCACCACA-3'; β-actin RP, 5'-TCTCCAGGGAGGAAGAGGAT-3'. cDNA was combined with 6 pmol of both FP and RP, 2 X SYBR Green, and diluted to 25 µL with nuclease-free water. Separate RT-PCR reactions were performed for Cyp1A1 and β-actin using an ABI 7500 with cycling conditions of 50˚C for 2 min, 95˚C for 10 min, and 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Relative Quantification (RQ)

values (i.e. fold-change) were determined by the ABI 7500 SDS 2.0. Because Cyp1A1 has little to no expression in the absence of an AhR ligand (i.e. naïve and vehicle-treated cells), Cyp1A1 expression is presented as a fold change (RQ) from the highest Cyp1A1-activating TCDD concentration (10 nM) which was set to one.

Statistical Analysis

The mean±SE (n=4) was calculated for all treatment groups of each ELISA experiment. IgA and γ2b expression are shown as a percent of the DMSO control. The DMSO control mean was set to 100% and the data was transformed to a percent of the mean. The IgA or γ2b figures are representative of 3-4 experiments. Significance of treatments from vehicle controls was determined by a 1-way ANOVA and Dunnett post hoc test. Significant differences in Cyp1A1 transcript expression from 10 nM TCDD (qRT-PCR) in Wild Type cells were determined by a one-tailed t test.

III. RESULTS

Stable knockdown of the AhR and the AhR antagonist CH223191 disrupt canonical AhR signaling in the CH12.LX B cell line

To determine if the AhR mediates TCDD-induced inhibition of Ig expression by disrupting 3'*Igh*RR transcriptional activity, we utilized an IgA-expressing CH12.LX cell line variant that stably expresses a γ2b transgene regulated by the 3'*Igh*RR (CH12.γ2b-3'*Igh*RR cells) (Henseler et al., 2009). CH12.γ2b-3'*Igh*RR cells were stably transduced with lentiviral particles containing shRNA targeted to the AhR. Highly variable levels of AhR-knock down were observed by western blot during the course of screening >100 cell populations from CH12.γ2b-3'*Igh*RR cells transduced with lentiviral particles containing five separate shRNA sequences targeted to AhR (data not shown). Two independent shRNA sequences (denoted shAhR11 and shAhR12) stably knocked down AhR by at least 50% (Fig.1). In order to ensure results seen in the shAhR cell populations were not the result of shRNA off target effects, we also utilized the previously characterized competitive AhR antagonist, CH-223191, to disrupt AhR signaling in the CH12.γ2b-3'*Igh*RR cells (Kim et al., 2006; Zhoa et al., 2010). Cytochrome P4501A1 (Cyp1A1) is robustly expressed as a direct result of ligand-activated AhR binding to DREs within the Cyp1A1 gene promoter (Reviewed by Beischlag et al., 2008). Concentration-response experiments were performed in order to

find the most effective concentration of CH-223191 for disrupting TCDD-AhR signaling. TCDD-induced up regulation of Cyp1A1 has been used extensively to assess functional AhR-signaling in various cell types including CH12.LX B cells and primary human B cells. Both stable AhR knockdown and CH-223191 treatment disrupted canonical AhR-signaling as evidenced by a dramatic inhibition of TCDD-induced Cyp1A1 transcript expression validating our experimental models (Fig. 6).

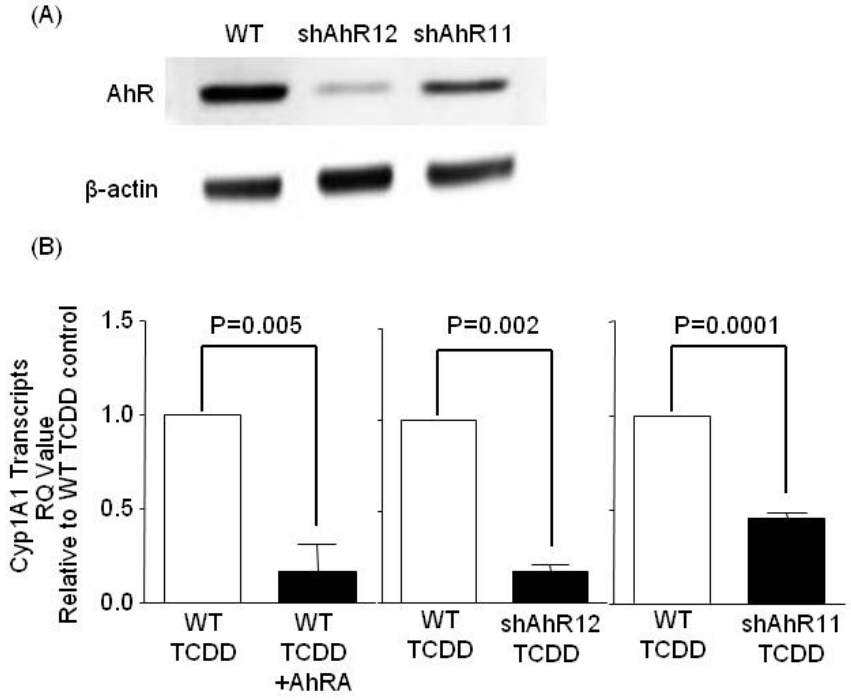

Figure 6. Effect of reduced AhR expression and a competitive AhR antagonist (CH-223191) on TCDD-induced CYP1A1 expression. "Wild Type" (WT) refers to CH12.LX murine B-cells that endogenously express IgA and stably express a 3'*Igh*RR-regulated γ2b transgene (CH12.γ2b-3'*Igh*RR cells). "shAhR" denotes CH12.γ2b-3'*Igh*RR cells that stably express shRNA to AhR (shAhR). Stable expression of shAhR was achieved using lentiviral-mediated delivery of shAhR constructs (denoted shAhR11 and shAhR12). (A); Whole cell lysates were collected from WT and shAhR cells. 20 µg of protein was subjected to western blot analysis of AhR and β-actin proteins. (B); WT, shAhR11, and shAhR12 cells (5.0×10^5 cells/mL) were culture in the presence of 10 nM TCDD, 0.11% DMSO vehicle, or left in the absence of treatment (NA) for 8 hours. WT cells were also subjected to 30 µM AhR Antagonist (CH-223191) treatment (AhRA) for 1 hour prior to TCDD. Total RNA was extracted, one microgram was reverse transcribed to cDNA, and 5 ng of cDNA was used to amplify Cyp1A1 and β-actin via SYBR Green real-time PCR. NA and DMSO treatment caused no appreciable expression of Cyp1A1 as it is only expressed when activated my ligand (i.e. TCDD), therefore results are expressed as relative quantification (RQ) values compared to TCDD-treated WT cells. The data is representative of at least two separate experiments (n=3 for each treatment group). Statistical differences compared to respective WT TCDD treatment group were determined by a one-tailed t test.

Disrupting AhR signaling reverses TCDD-induced inhibition of 3'*Igh*RR activation and Ig expression in LPS-activated B cells

AhR has long been suspected to mediate TCDD-induced inhibition of Ig expression, as supported by structure-activity relationships revealing that ligands with increasing affinity for the AhR inhibit the Ig expression with greater sensitivity (Sulentic et al., 2000). In addition, AhR null mice and an AhR-deficient murine B cell line (BCL-1) are refractory to TCDD treatment further highlighting the importance of the AhR in mediating the inhibitory effect of TCDD on Ig expression (Vorderstrasse et al., 2001; Sulentic et al., 1998). However despite extensive study, there remains a paucity of mechanistic data to explain how the AhR regulates Ig expression. Our lab has previously shown that a key regulator of *Igh* transcription and therefore Ig production, the 3'*Igh*RR, not only contains putative DRE sites but is also sensitive to a variety of AhR agonists (Fernando et al., 2012; Henseler et al., 2009). Despite a putative link, the role of the AhR in directly mediating these effects was not established.

At this time no *cis*-acting transcriptional target has been identified that links ligand-activation of the AhR to inhibition of Ig expression. Due to its sensitivity to AhR-ligands, the presence of DREs, and its paramount importance to Ig expression and CSTR, the 3'*Igh*RR is a likely transcriptional target of the AhR. As such we sought to elucidate the correlative link between the AhR and the 3'*Igh*RR using a competitive AhR antagonist (CH-223191) or shRNA targeted to AhR in CH12.γ2b-3'*Igh*RR cells (denoted shAhR11 and shAhR12) allowing for simultaneous surveillance of AhR-dependent changes in 3'*Igh*RR transcriptional activity and Ig expression. Consistent with our previous studies, TCDD inhibited

LPS-activation of both the γ2b transgene and endogenous IgA proteins (Fig. 7 and 8). Following a one hour pretreatment with CH-223191 (30 μM), TCDD-induced inhibition of both the γ2b transgene and endogenous IgA protein was completely mitigated (Fig. 7). In addition both shAhR11 and shAhR12 cell models were refractory to TCDD-treatment compared to the wild type (CH12.γ2b-3'*Igh*RR) parental cells (Fig. 8). Our data support a direct effect on the 3'*Igh*RR that is mediated either by binding of the AhR to DREs in the 3'*Igh*RR enhancers and/or interaction of the AhR with other transcription factors regulating 3'*Igh*RR activity, which results in 3'*Igh*RR inhibition and thus IgA expression.

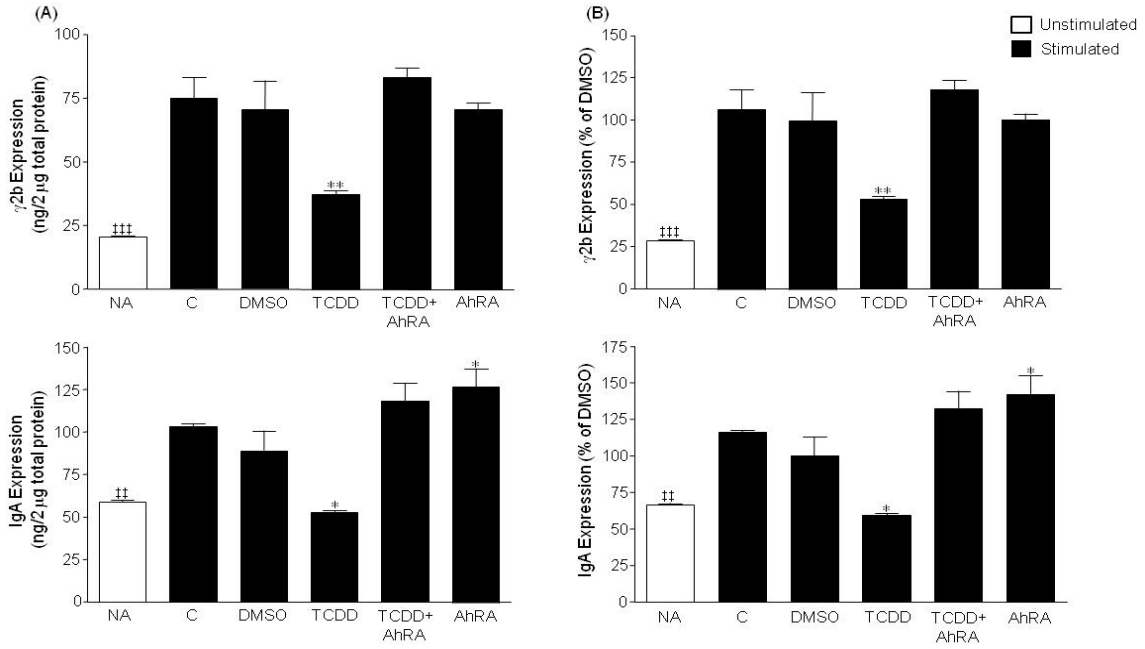

Figure 7. TCDD-induced inhibition of a 3'*Igh*RR-regulated γ2b transgene and endogenous IgA protein expression is reversed by an AhR Antagonist (CH-223191). CH12.γ2b-3'*Igh*RR cells were pretreated for 1 h with CH-223191 (30 µM) or vehicle (0.1% DMSO) followed by treatment for 48 h with TCDD (10 nM) or vehicle (0.01% DMSO) in the presence of LPS stimulation (1 µg/mL). (A); γ2b and IgA expression (mean±S.E., n=4) normalized to 2 µg total protein was determined by ELISA. (B); γ2b and IgA expression transformed to percent effect with the DMSO control set to 100%. NA denotes the naïve control. Significance was determined by a 1-way ANOVA followed by a Dunnet post hoc test. "**" and "*" denote significance from the DMSO control at $p<0.01$ and $p<0.05$ respectively. "‡‡‡" and "‡‡" denote significance of the NA control from the LPS control at $p<0.001$ and $p<0.01$ respectively.

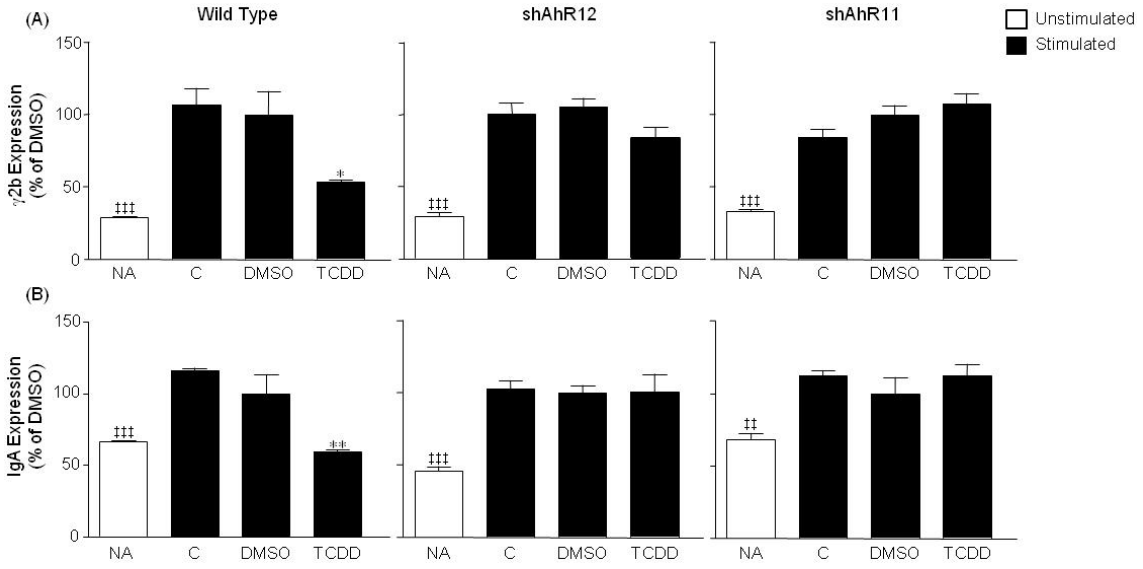

Figure 8. Down-regulation of AhR by shRNA reverses TCDD-induced inhibition of the 3'*Igh*RR-regulated γ2b transgene and endogenous IgA protein expression. CH12.γ2b-3'*Igh*RR (Wild Type) cells stably transduced with lentivirus containing one of two shRNA-containing transgenes targeted to AhR (denoted by shAhR11 and shAhR12). Wild Type and shAhR cells were treated with vehicle (0.1% DMSO) and TCDD (10 nM) in the presence of LPS (1 μg/mL) stimulation for 48 hours. Data represents γ2b (A) and IgA (B) expression as determined by ELISA in 2 μg total protein and transformed to percent effect (mean±S.E., n=4) with the DMSO control set to 100%. NA denotes the naïve control. Significance was determined by a 1-way ANOVA followed by a Dunnet post hoc test. "**" and "*" denote significance from the DMSO control at $p<0.01$ and $p<0.05$ respectively. "‡‡‡" and "‡‡" denote significance of the NA control from the LPS control at $p<0.001$ and $p<0.01$ respectively.

IV. DISCUSSION

Compromised immune function, specifically suppressed Ig expression, is one of the most sensitive toxicological consequences in TCDD-treated laboratory animals (Reviewed by Sulentic and Kaminski, 2012). Although established to be AhR-dependent, a mechanism has remained elusive and is a significant human health question given the plethora of nondioxin compounds of pharmaceutical and dietary origin shown to modulate the AhR (Reviewed by Stejskalova et al., 2011). Indeed, a recent study by Lu et al. (2010) revealed the CD40 ligand-dependent Ig response of human peripheral B cells is inhibited by TCDD highlighting the need to thoroughly identify the mechanism of AhR-dependent inhibition of Ig expression to determine risk to human health. In the current study we show that both pharmacological inhibition of the AhR and knock down of AhR protein levels fully reverses concomitant inhibition of the 3'*Igh*RR, a critical Ig gene regulator, and Ig expression in TCDD-treated CH12.LX mouse B cells. For the first time a transcriptional target for the AhR (the 3'*Igh*RR) critical for Ig expression has been identified addressing a crucial mechanistic data gap in the literature. We propose a mechanism in which the AhR inhibits the capacity of the 3'*Igh*RR to increase *Igh* transcription leading to decreased Ig expression.

The mouse 3'*Igh*RR (mo-3'*Igh*RR) is a large ~40 kb region and contains at least four enhancer domains (i.e. hs3A, hs1,2; hs3B; hs4) with binding sites for

many transcription factors including a DRE capable of binding the AhR within both the hs1,2 and hs4 enhancers (Reviewed by Pinaud et al., 2011; Sulentic et al., 2000). A single enhancer or a combination of these enhancers or specific transcription factor binding sites within the enhancers along with the AhR may direct the overall inhibition of the mo-3'*Igh*RR. Relevant to this notion is the strikingly different transcriptional behavior exhibited by the hs1,2 and hs4 enhancers when in isolation in luciferase reporter constructs. While transcriptional activity of the hs1,2 enhancer is inhibited by TCDD, the hs4 enhancer is syngeristically activated by TCDD and LPS treatment in CH12.LX B cells (Fernando et al., 2012; Sulentic et al., 2004).

Undoubtedly luciferase reporter constructs evaluating individual enhancers may not mimic enhancer behavior in the context of the entire mo-3'*Igh*RR and chromatin. We previously reported that expression of a stably-expressed γ2b reporter construct under transcriptional control of either the entire mo-3'*Igh*RR or the hs1,2/hs3A enhancer pair is inhibited by TCDD (Henseler et al., 2009; Fernando et al., 2012). In our current study we show that an AhR antagonist ameliorates TCDD- dependent inhibition of not only the mo-3'*Igh*RR but also the hs1,2/hs3A enhancer pair further suggesting that the DRE-containing, hs1,2 is the primary target of TCDD-AhR. Indeed, TCDD was recently shown to modulate the human hs1,2 (hu-hs1,2) enhancer in a concentration-dependent manner (Fernando et al., 2012). Clearly predicting the behavior (i.e. inhibition vs. activation) of AhR-sensitive genes cannot be restricted to the simple identification of a DRE (e.g. hs1,2 and hs4 enhancers)

and must be considered in the context of the entire transcriptional unit in which a DRE is located. At least in the case of mouse B cells, TCDD inhibits Ig expression in an AhR-dependent manner by modulating the mo-3′*Igh*RR, and perhaps specifically the seemingly dominant hs1,2 enhancer.

The structure of the human 3'*Igh*RR (hu-3'*Igh*RR) and the behavior of its constituent enhancers diverges slightly from that of the mo-3'*Igh*RR perhaps complicating the translation of mo-3'*Igh*RR studies to hu-3'*Igh*RR and understanding the role of the 3'*Igh*RR in possible AhR-dependent inhibition of Ig expression in human B cells. The 3'*Igh*RR is duplicated in the human *Igh* locus and consists of only the hs3A, hs1,2 and hs4 enhancers (Chen and Birshstein, 1997).

Interestingly the hu-hs1,2 is highly polymorphic, characterized by an ~38 bp invariant sequence (IS) that contains a DRE-like site and may be repeated in tandem up to three times (Denizot et al., 2001; Fernando et al., 2012). Studies using the AhR Antagonist CH-223191 revealed that hu-hs1,2 enhancers containing one, two, or three copies of the IS are activated by TCDD in an AhR-dependent manner, albeit to varying extents depending upon the number of IS (Fernando et al., 2012). However, the aforementioned study was performed using a transiently expressed reporter-plasmid system and may not be reflective of the full hu-3'*Igh*RR. Indeed, despite TCDD-dependent activation of the mo-hs4 enhancer in luciferase-reporter constructs, transcriptional activity of the stably expressing γ2b-3'*Igh*RR reporter construct inhibited. Furthermore, a recent study showed nine of twelve human donor primary B cells to have

reduced Ig expression when treated with TCDD (Lu et al., 2010). The study also reported two nonresponsive donors while one donor exhibited enhanced Ig expression with TCDD treatment. Enhanced Ig production (IgE) by TCDD has been reported in tonsillary B lymphocytes but only from atopic patients who are predisposed to type I hypersensitivity reactions, a hallmark of which is aberrant IgE production (Kimata, 2003). It is possible that an underlying genetic condition in the regulatory machinery of Ig, such as a polymorphism in the 3'*Igh*RR and/or the hs1,2 enhancer, may cause deleterious enhancement of Ig expression when activated by an AhR ligand. The combination of several single nucleotide polymorphisms (SNP) in human AhR is known to cause deficient TCDD-induced Cyp1A1 induction and may have explained the nonresponsive donors, but these SNPs were not detected in the author's study (Reviewed by Harper et al., 2001; Lu et al., 2010). It is tempting to surmise that heterogeneity of the human Ig response to TCDD (or other AhR-activators) is due to heterogeneity of the hu-hs1,2 or other elements within the 3'IghRR and/or the heterogeneity of AhR polymorphisms, however many factors, discovered and undiscovered likely play a role.

Deficient Ig expression in response to TCDD has been well established for over thirty years in mouse models however the literature contains a paucity of mechanistic data (Reviewed by Sulentic and Kaminski, 2010). To the best of our knowledge no study has identified a *cis*-acting transcriptional target for the AhR that explains AhR-dependent inhibition of Ig expression; therefore our study fills a critical mechanistic data gap. Recent evidence suggests that a proportion of the

human population is also be sensitive to the inhibitory effect of TCDD, a finding that may be due in part to AhR-dependent modulation of the hu-hs1,2 enhancer within the human 3'*Igh*RR (Lu et al., 2010; Fernando et al., 2012). Interestingly IS within the hu-hs1,2 have been correlated with the incidence of many immune-related diseases including celiac disease, IgA nephropathy, systemic sclerosis, plaque psoriasis, psoriatic arthritis, dermatitis herpetiformis, and rheumatoid arthritis (Frezza et al., 2004; Aupetit et al., 2000; Frezza et al., 2007; Cianci et al., 2008; Tolusso et al., 2009).

Given the growing number of dietary and pharmaceutical AhR-ligands and persistent presence of dioxin-related compounds in the environment, modulation of the hu-1,2 may have the capacity to alter the incidence and/or the severity of human diseases in addition to interfering with the Ig response (Reviewed by Stejskalova et al., 2011). Understanding the role of the AhR-dependent modulation of the human 3'*Igh*RR would provide valuable insight into the identification and quantification of human health risks. Furthermore, interactions of the hu-h1,2 and the AhR and/or other transcription factors that bind within the hu-hs1,2 and the 3'*Igh*RR may provide opportunities for therapeutic intervention in many immune-related disorders.

V. LITERATURE CITED

Abel, J., & Haarmann-Stemmann, T. (2010). An introduction to the molecular basics of aryl hydrocarbon receptor biology. Biol Chem, 391(11), 1235-1248. doi: 10.1515/BC.2010.128

Aupetit, C., Drouet, M., Pinaud, E., Denizot, Y., Aldigier, J. C., Bridoux, F., & CognÈ, M. (2000). Alleles of the alpha1 immunoglobulin gene 3' enhancer control evolution of IgA nephropathy toward renal failure. Kidney Int, 58(3), 966-971. doi: 10.1046/j.1523-1755.2000.00253.x

Beischlag, T. V., Luis Morales, J., Hollingshead, B. D., & Perdew, G. H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression. Crit Rev Eukaryot Gene Expr, 18(3), 207-250.

Chen, C., & Birshtein, B. K. (1997). Virtually identical enhancers containing a segment of homology to murine 3'IgH-E(hs1,2) lie downstream of human Ig C alpha 1 and C alpha 2 genes. J Immunol, 159(3), 1310-1318.

Cianci, R., Giambra, V., Mattioli, C., Esposito, M., Cammarota, G., Scibilia, G., . . . Frezza, D. (2008). Increased frequency of Ig heavy-chain HS1,2-A enhancer *2 allele in dermatitis herpetiformis, plaque psoriasis, and psoriatic arthritis. J Invest Dermatol, 128(8), 1920-1924. doi: 10.1038/jid.2008.40

CognÈ, M., Lansford, R., Bottaro, A., Zhang, J., Gorman, J., Young, F., . . . Alt, F. W. (1994). A class switch control region at the 3' end of the immunoglobulin heavy chain locus. Cell, 77(5), 737-747.

Denizot, Y., Pinaud, E., Aupetit, C., Le Morvan, C., Magnoux, E., Aldigier, J. C., & CognÈ, M. (2001). Polymorphism of the human alpha1 immunoglobulin gene 3' enhancer hs1,2 and its relation to gene expression. Immunology, 103(1), 35-40.

DiNatale, B. C., Schroeder, J. C., Francey, L. J., Kusnadi, A., & Perdew, G. H. (2010). Mechanistic insights into the events that lead to synergistic induction of interleukin 6 transcription upon activation of the aryl hydrocarbon receptor and inflammatory signaling. J Biol Chem, 285(32), 24388-24397. doi: 10.1074/jbc.M110.118570

DiNatale, B. C., Schroeder, J. C., & Perdew, G. H. (2011). Ah receptor antagonism inhibits constitutive and cytokine inducible IL6 production in head and neck tumor cell lines. Mol Carcinog, 50(3), 173-183. doi: 10.1002/mc.20702

Dunnick, W. A., Shi, J., Graves, K. A., & Collins, J. T. (2005). The 3' end of the heavy chain constant region locus enhances germline transcription and switch recombination of the four gamma genes. J Exp Med, 201(9), 1459-1466. doi: 10.1084/jem.20041988

Ema, M., Ohe, N., Suzuki, M., Mimura, J., Sogawa, K., Ikawa, S., & Fujii-Kuriyama, Y. (1994). Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors. J Biol Chem, 269(44), 27337-27343.

Fernando, T. M., Ochs, S. D., Liu, J., Chambers-Turner, R. C., & Sulentic, C. E. (2012). 2,3,7,8-tetrachlorodibenzo-p-dioxin induces transcriptional activity of the human polymorphic hs1,2 enhancer of the 3'Igh regulatory region. J Immunol, 188(7), 3294-3306. doi: 10.4049/jimmunol.1101111

Frezza, D., Giambra, V., Cianci, R., Fruscalzo, A., GiufrË, M., Cammarota, G., . . . Pandolfi, F. (2004). Increased frequency of the immunoglobulin enhancer HS1,2 allele 2 in coeliac disease. Scand J Gastroenterol, 39(11), 1083-1087. doi: 10.1080/00365520410007999

Frezza, D., Giambra, V., Tolusso, B., De Santis, M., Bosello, S., Vettori, S., . . . Ferraccioli, G. (2007). Polymorphism of immunoglobulin enhancer element HS1,2A: allele *2 associates with systemic sclerosis. Comparison with HLA-DR and DQ allele frequency. Ann Rheum Dis, 66(9), 1210-1215. doi: 10.1136/ard.2006.066597

Harper, P. A., Golas, C. L., & Okey, A. B. (1988). Characterization of the Ah receptor and aryl hydrocarbon hydroxylase induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin and benz(a)anthracene in the human A431 squamous cell carcinoma line. Cancer Res, 48(9), 2388-2395.

Harper, P. A., Prokipcak, R. D., Bush, L. E., Golas, C. L., & Okey, A. B. (1991). Detection and characterization of the Ah receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin in the human colon adenocarcinoma cell line LS180. Arch Biochem Biophys, 290(1), 27-36.

Henseler, R. A., Romer, E. J., & Sulentic, C. E. (2009). Diverse chemicals including aryl hydrocarbon receptor ligands modulate transcriptional activity of the 3'immunoglobulin heavy chain regulatory region. Toxicology, 261(1-2), 9-18. doi: 10.1016/j.tox.2009.03.015

Jeuken, A., Keser, B. J., Khan, E., Brouwer, A., Koeman, J., & Denison, M. S. (2003). Activation of the Ah receptor by extracts of dietary herbal supplements, vegetables, and fruits. J Agric Food Chem, 51(18), 5478-5487. doi: 10.1021/jf030252u

Khamlichi, A. A., Pinaud, E., Decourt, C., Chauveau, C., & CognÈ, M. (2000). The 3' IgH regulatory region: a complex structure in a search for a function. Adv Immunol, 75, 317-345.

Kim, S. H., Henry, E. C., Kim, D. K., Kim, Y. H., Shin, K. J., Han, M. S., . . . Suh, P. G. (2006). Novel compound 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide (CH-223191) prevents 2,3,7,8-TCDD-induced toxicity by antagonizing the aryl hydrocarbon receptor. Mol Pharmacol, 69(6), 1871-1878. doi: 10.1124/mol.105.021832

Kimata, H. (2003). 2,3,7,8-tetrachlorodibenzo-p-dioxin selectively enhances spontaneous IgE production in B cells from atopic patients. Int J Hyg Environ Health, 206(6), 601-604. doi: 10.1078/1438-4639-00248

Korkalainen, M., Tuomisto, J., & Pohjanvirta, R. (2001). The AH receptor of the most dioxin-sensitive species, guinea pig, is highly homologous to the human AH receptor. Biochem Biophys Res Commun, 285(5), 1121-1129. doi: 10.1006/bbrc.2001.5317

Lu, H., Crawford, R. B., Suarez-Martinez, J. E., Kaplan, B. L., & Kaminski, N. E. (2010). Induction of the aryl hydrocarbon receptor-responsive genes and modulation of the immunoglobulin M response by 2,3,7,8-tetrachlorodibenzo-p-dioxin in primary human B cells. Toxicol Sci, 118(1), 86-97. doi: 10.1093/toxsci/kfq234

Ma, C., Marlowe, J. L., & Puga, A. (2009). The aryl hydrocarbon receptor at the crossroads of multiple signaling pathways. EXS, 99, 231-257.

Pinaud, E., Khamlichi, A. A., Le Morvan, C., Drouet, M., Nalesso, V., Le Bert, M., & CognÈ, M. (2001). Localization of the 3' IgH locus elements that effect long-distance regulation of class switch recombination. Immunity, 15(2), 187-199.

Pinaud, E., Marquet, M., Fiancette, R., PÈron, S., Vincent-Fabert, C., Denizot, Y., & CognÈ, M. (2011). The IgH locus 3' regulatory region: pulling the strings from behind. Adv Immunol, 110, 27-70. doi: 10.1016/B978-0-12-387663-8.00002-8

Pohjanvirta, R., & Tuomisto, J. (1994). Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals: effects, mechanisms, and animal models. Pharmacol Rev, 46(4), 483-549.

Pohjanvirta, R., Unkila, M., & Tuomisto, J. (1993). Comparative acute lethality of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-p-dioxin and 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin in the most TCDD-susceptible and the most TCDD-resistant rat strain. Pharmacol Toxicol, 73(1), 52-56.

Pohjanvirta, R., Wong, J. M., Li, W., Harper, P. A., Tuomisto, J., & Okey, A. B. (1998). Point mutation in intron sequence causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-p-dioxin-resistant rat strain. Mol Pharmacol, 54(1), 86-93.

Poland, A., & Knutson, J. C. (1982). 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Annu Rev Pharmacol Toxicol, 22, 517-554. doi: 10.1146/annurev.pa.22.040182.002505

Quattrochi, L. C., & Tukey, R. H. (1993). Nuclear uptake of the Ah (dioxin) receptor in response to omeprazole: transcriptional activation of the human CYP1A1 gene. Mol Pharmacol, 43(4), 504-508.

Rowlands, C. J., Staskal, D. F., Gollapudi, B., & Budinsky, R. (2010). The human AHR: identification of single nucleotide polymorphisms from six ethnic populations. Pharmacogenet Genomics, 20(5), 283-290.

Stejskalova, L., Dvorak, Z., & Pavek, P. (2011). Endogenous and exogenous ligands of aryl hydrocarbon receptor: current state of art. Curr Drug Metab, 12(2), 198-212.

Sulentic, C. E., Holsapple, M. P., & Kaminski, N. E. (1998). Aryl hydrocarbon receptor-dependent suppression by 2,3,7, 8-tetrachlorodibenzo-p-dioxin of IgM secretion in activated B cells. Mol Pharmacol, 53(4), 623-629.

Sulentic, C. E., Holsapple, M. P., & Kaminski, N. E. (2000). Putative link between transcriptional regulation of IgM expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin and the aryl hydrocarbon receptor/dioxin-responsive enhancer signaling pathway. J Pharmacol Exp Ther, 295(2), 705-716.

Sulentic, C. E., & Kaminski, N. E. (2011). The long winding road toward understanding the molecular mechanisms for B-cell suppression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Sci, 120 Suppl 1, S171-191. doi: 10.1093/toxsci/kfq324

Sulentic, C. E., Kang, J. S., Na, Y. J., & Kaminski, N. E. (2004). Interactions at a dioxin responsive element (DRE) and an overlapping kappaB site within the hs4 domain of the 3'alpha immunoglobulin heavy chain enhancer. Toxicology, 200(2-3), 235-246. doi: 10.1016/j.tox.2004.03.015

Tolusso, B., Frezza, D., Mattioli, C., Fedele, A. L., Bosello, S., Faustini, F., . . . Ferraccioli, G. F. (2009). Allele *2 of the HS1,2A enhancer of the Ig regulatory region associates with rheumatoid arthritis. Ann Rheum Dis, 68(3), 416-419. doi: 10.1136/ard.2008.095414

Vorderstrasse, B. A., Steppan, L. B., Silverstone, A. E., & Kerkvliet, N. I. (2001). Aryl hydrocarbon receptor-deficient mice generate normal immune responses to model antigens and are resistant to TCDD-induced immune suppression. Toxicol Appl Pharmacol, 171(3), 157-164. doi: 10.1006/taap.2000.9122

Wang, J., & Boxer, L. M. (2005). Regulatory elements in the immunoglobulin heavy chain gene 3'-enhancers induce c-myc deregulation and lymphomagenesis in murine B cells. J Biol Chem, 280(13), 12766-12773. doi: 10.1074/jbc.M412446200

Werlinder, V., Backlund, M., Zhukov, A., & Ingelman-Sundberg, M. (2001). Transcriptional and post-translational regulation of CYP1A1 by primaquine. J Pharmacol Exp Ther, 297(1), 206-214.

Yoo, B. S., Boverhof, D. R., Shnaider, D., Crawford, R. B., Zacharewski, T. R., & Kaminski, N. E. (2004). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the regulation of Pax5 in lipopolysaccharide-activated B cells. Toxicol Sci, 77(2), 272-279. doi: 10.1093/toxsci/kfh013

Zhao, B., Degroot, D. E., Hayashi, A., He, G., & Denison, M. S. (2010). CH223191 is a ligand-selective antagonist of the Ah (Dioxin) receptor. Toxicol Sci, 117(2), 393-403. doi: 10.1093/toxsci/kfq217