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Environmental Toxicants and Human B Cells: Insights from CRISPR Editing and Genomic Sequencing

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ENVIRONMENTAL TOXICANTS AND HUMAN B CELLS: INSIGHTS FROM CRISPR EDITING AND GENOMIC SEQUENCING

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Clayton Allex-Buckner ENTITLED Environmental toxicants and human B cells: insights from CRISPR editing and genomic sequencing. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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The human immunoglobulin heavy chain gene locus (*IGH*) has two 3 prime regulatory regions (3'*IGH*RR), each containing three enhancers (hs3, hs1.2, hs4). In animal models, the 3'*Igh*RR regulates *IgH* expression and class switch recombination (CSR) to different Ig isotypes. The 3'*IGH*RR hs1.2 enhancer in humans is polymorphic in that an invariant sequence (IS) can be repeated one to four times in tandem. The hs1.2 polymorphism is of interest due to its association with several human autoimmune disorders and its potential sensitivity to exogenous substances such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin). In mouse models, TCDD inhibits the hs1.2 enhancer and 3'*Igh*RR activation through the aryl hydrocarbon receptor (AHR), which correlates with Ig inhibition. However, in humans, TCDD activates the hs1.2 enhancer, decreases IgG secretion and increases IgE secretion, suggesting species differences in hs1.2 activity and overall *IGH* regulation. Furthermore, in the human CL01 B-cell line, that can be induced to secrete antibodies and undergo CSR, loss of the AHR via siRNA and CRISP/Cas9 gene editing results in a significant decrease in IgG secretion. We utilized CRISPR/Cas9 gene editing to target the hs1.2 IS repeats in the CL01 cell line. Several clones exhibited a functional effect on *IGH* expression with a reduction in the number of hs1.2 IS repeats within one or both of the 3'*IGH*RRs. Treatment with TCDD resulted in modulated IgG secretion profiles dependent on the hs1.2 IS genotype changes. We also developed a bioinformatics pipeline to

characterize the genomes of our parent cell line, CL-01, and an AHR CRISPR/Cas9-edited clone that only expresses a functional transactivation domain (TAD) due to the editing. Finally, to further assess the sensitivity of the 3'*IGH*RR to environmental chemicals we exposed our CL01 cell line model to two additional environmental toxicants, perfluorooctanoic sulfonic acid (PFOS) and naphthalene. Decreased IgA was observed with these exposures. This work further implies that the *IGH* is a target of a variety of chemicals which may be mediated through the number of signaling pathways that converge at the 3'*IGH*RR.

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ACKNOWLEDGEMENTS

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I. Literature Review

An Introduction to the Immune System

The first line of defense against microbial infections are physiological and anatomical barriers. These barriers include intact skin, bacterial lysozymes in secretions, and mucus production. If a breakdown occurs in this first line of defense, the immune system is activated to clear the infection. The immune system is an organization of specialized cell types and effector molecules that can be categorized into two distinct responses: the innate response and the adaptive response. Innate immunity is rapid and uses phagocytic cells such as macrophages and neutrophils, to recognize conserved pathogen-associated molecular patterns (PAMPs), or antigens, via pattern recognition receptors (PRRs). This response occurs within hours of infection, and once activated, produces inflammatory cytokines to initiate a protective inflammatory response. Although rapid, the innate immune system is a broad protection that does not provide antigen-specific protection. Because of this, innate immunity is unchanged no matter the frequency of exposure to a particular pathogen. Another key function of innate immunity is in aiding the activation of the adaptive immune response. Through the production of inflammatory cytokines and chemokines, the innate cells begin to signal to the adaptive immune system (Mackay & Rosen, 2000, Akira et al., 2006).

As opposed to the rapid, non-specific innate immune response, the adaptive immune system provides a slower, antigen specific response to microbial infection. The adaptive immune response is broadly mediated by two cell types, T-lymphocytes and B-lymphocytes, that have different but interconnected cellular responses. T-lymphocytes provide cell-mediated immunity through recognition of presented antigens via major histocompatibility complexes (MHCs). Originating in the bone marrow (BM) from hematopoietic stem cells (HSCs), T-lymphocytes migrate to the thymus to mature into either naïve $CD4^+$ or $CD8^+$ T cells. Antigen presenting cells (APCs), such as dendritic cells, macrophages, and B cells, provide the stimulation to activate these cells to proliferate, differentiate into effector cells, and produce memory cells. Cytotoxic T cells ($CD8^+$) can recognize infected or cancerous cells and destroy them to mitigate further injury. In contrast, activated $CD4^+$ T cells differentiate into T helper (Th) cells. These Th cells go on to augment the innate and adaptive immune response through the release of different cytokines dependent on the Th subtype. In the innate response, Th1 cells can activate phagocytic macrophages, increasing local clearance of infection through production of IL-12 and IFNγ. Th2 cells can also up-regulate the production of surface markers and cytokines to initiate the activation of B-lymphocytes. For example, during an immune challenge, Th2 cells that recognize the antigen presented by B lymphocytes will up-regulate CD40 ligand (CD40L) on their surface, which will interact with the CD40 receptors on the surface of B-lymphocytes. Through increased production of cytokines such as IL-4 and IL-2 in conjunction with surface cell-cell interaction via CD40L-CD40 receptor, Th2 cells can activate the B-lymphocytes to initiate a humoral immune response (Luckheeram et al., 2012).

B-lymphocyte Maturation and V(D)J Recombination

As an essential portion of host immunity, B cells facilitate the protective qualities of humoral immunity through production of immunoglobulins (Igs) and establishing immunological memory. Antibodies are the secreted form of Ig. The process in which B cells produce Igs is highly regulated and marked by three genomic alteration events. These three genomic alteration events occur during different B cell maturation stages. Through these genomic alterations, Igs can establish antigen specificity, a specialized immunological function, and antigen affinity maturation (LeBien et al., 2008, Pieper et al., 2013).

Like T-lymphocytes, B-lymphocytes begin their maturation in the BM from HSCs (De Grandis, Lhoumeau, Mancini, & Aurrand-Lions, 2016). The first step in B-cell maturation is the rearrangement of the variable (V), diverse (D), and joining (J) segments within the Ig heavy chain gene and the V and J segments in the light chain gene. This process, called V(D)J recombination ultimately results in the antigen specificity of Igs produced by B-lymphocytes. There are 3.5 million V(D)J recombination possibilities that exist to allow B-lymphocytes to potentially recognize all antigens through the Ig variable regions in humans and mice (Schatz, D.G et al., 2011).

The first maturation step is the rearrangement of the D-J segments in the heavy chain in Pro-B-cells. This maturation step is followed by the rearrangement of the V segment to the newly formed DJ segment in pre-B cells (Soulas-Sprauel et al., 2007). V(D)J recombination is mediated by the RAG1/RAG2 endonuclease and nonhomologous end-joining (NHEJ) repair (Scahts et al., 2011, Soulas-Sprauel et al., 2007, Brecht et al., 2020). Successful V(D)J recombination results in the formation of Ig,

which is composed to two heavy chains and two light chains. The first Ig expressed in naïve mature B cells is IgM which is expressed on the cell surface as the B-cell receptor (BCR) (Brack et al., 1978). The BCR is protein complex expressed on the surface of B cells and is comprised of two heavy chain proteins and two light chain proteins. It is crucial for specific antigen recognition, B cell stimulation, and overall function in the humoral immune system (Krangle, 2003, Piper et al., 2013).

Production of a BCR that is reactive against self-antigen is a possibility in the random recombination of the V(D)J segments. Several checkpoints during B-cell maturation exist to eliminate autoreactive B-cells (Keenan et al., 2008). B lymphocytes with a functional BCR can enter the periphery and those B-cells that have specificity for antigen will be activated and differentiate into antibody secreting cells (Cinamon, et al., 2008, Masaaki et al., 2000). To increase antigen specificity, antigen-activated B lymphocytes undergo somatic hypermutation (SHM), the second DNA mutational event that introduces point mutations to the V(D)J of both the heavy chain and light chain (Weigert et al., 1970, Gearhart, et al., 1983). Activated B cells either terminally differentiate into antibody secreting cells or memory cells. Memory B cells are long lived, quiescent cells that can be found in the secondary lymphoid organs and circulating within the blood. Upon reinfection, memory B-cells quickly recognize previously encountered antigen and help facilitate rapid activation of the innate and adaptive responses while aiding in clearance of antigen.

Antibodies All Have Similar Structures but Different Functions

During an immune challenge, antibodies provide protection through specific antigen recognition that can aid in clearance of the challenge. Antibodies are formed by linking two identical heavy chain proteins (HC) proteins and two identical light chain proteins (LC) into a 'Y' shaped molecule through disulfide bonds (figure 1). Antibodies are comprised of three functional components, two antigen binding domain fragments (Fab), and the crystallizable fragment (Fc) (figure 1). The amino terminal ends of the HC and LC contain a variable amino acid sequence that forms the antigen binding pocket of the Fab. The amino acid sequence is encoded by the recombined V(D)J sections of the HC and LC genes as previously discussed. Due to the diverse nature of amino acids in the Fab binding pocket, B cells can produce antibodies that recognize a broad spectrum of antigens.

Continuing to the tail of the 'Y', the Fc fragment of the antibody is comprised of only the heavy chain. The Fc fragment interacts with Fc receptors on other immune cells and proteins involved in the complement cascade to aid in clearance of the immune challenge. Comprised of three to four relatively conserved amino acid domains, this region is also referred to as the constant (C_H) region. Expression of the C_H is controlled by the Ig heavy chain gene locus (*IGH*) on chromosome 14 in humans and chromosome 12 in mice (D'Eustachio, 1984; Stavnezer et al., 2008, Blutt et al., 2013, Horton et al., 2013). These C_H regions dictate the structure and function of antibodies, which can be broadly categorized into five antibody isotypes: IgM, IgD, IgG, IgA, and IgE (figure 2).

Each of the antibody isotypes have a unique structure and function that allow them to interact with compartments of the innate and adaptive immunity while opsonizing and neutralizing infections. IgM can take have two forms, a monomer (general structure shown in figure 1) that forms part of the previously mentioned BCR and a pentamer that is the secreted form. The pentamer structure of IgM, increases avidity and enhances complement binding (Czajkowsky, et al., 2009; Liu et al., 2019). Like IgM, the IgD isotype acts as a membrane receptor on naïve mature B cells and can also be secreted. Secreted IgD plays a role in mucosal homeostasis through interaction with mast cells and basophils to produce inflammatory cytokines and antimicrobial peptides (Chen et al., 2009; Choi et al., 2017; Gutzeit et al., 2018). IgG and its sub-isotypes (IgG_{1-4}) account for up to 20% of total protein found in serum. In general, they aid in the further stimulation of the adaptive immune system through enhancing binding to antigen and Fc receptors (Ramsland et al., 2011; Redpath et al., 1998). IgA is found in the serum in a monomeric form but is primarily found lining the mucosal surfaces as a dimeric shape. These dimers provide active protection from incoming pathogens within the airways while also helping maintain microbiota homeostasis within the gut (Bunker et al., 2017; Nakajima et al., 2018). Lastly, IgE, has the shortest half-life and lowest circulating serum concentrations out of the other isotypes. IgE is mostly associated with hypersensitivity and allergen response, as it potently binds specific Fc receptors that mediate the allergen response (Chan et al., 2014; Galli and Tsai, 2012).

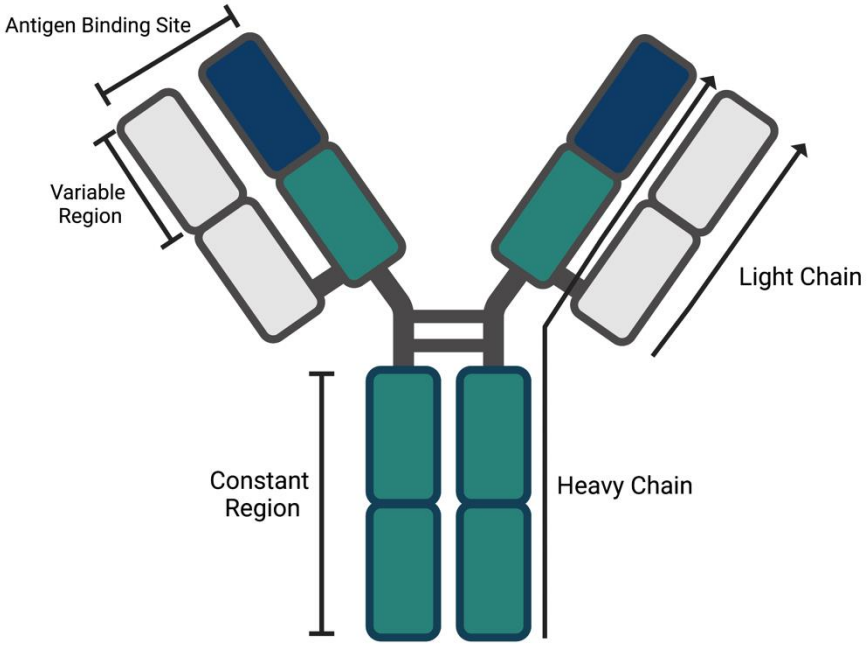


Figure 1. **General Immunoglobulin Structure.** The function of the Ig is determined by the constant (C_H) region of the *IGH* gene. Antigen specificity is determined by the V(D)J of the *IGH* and the VJ of the light chain gene. Figure made with biorender.com.

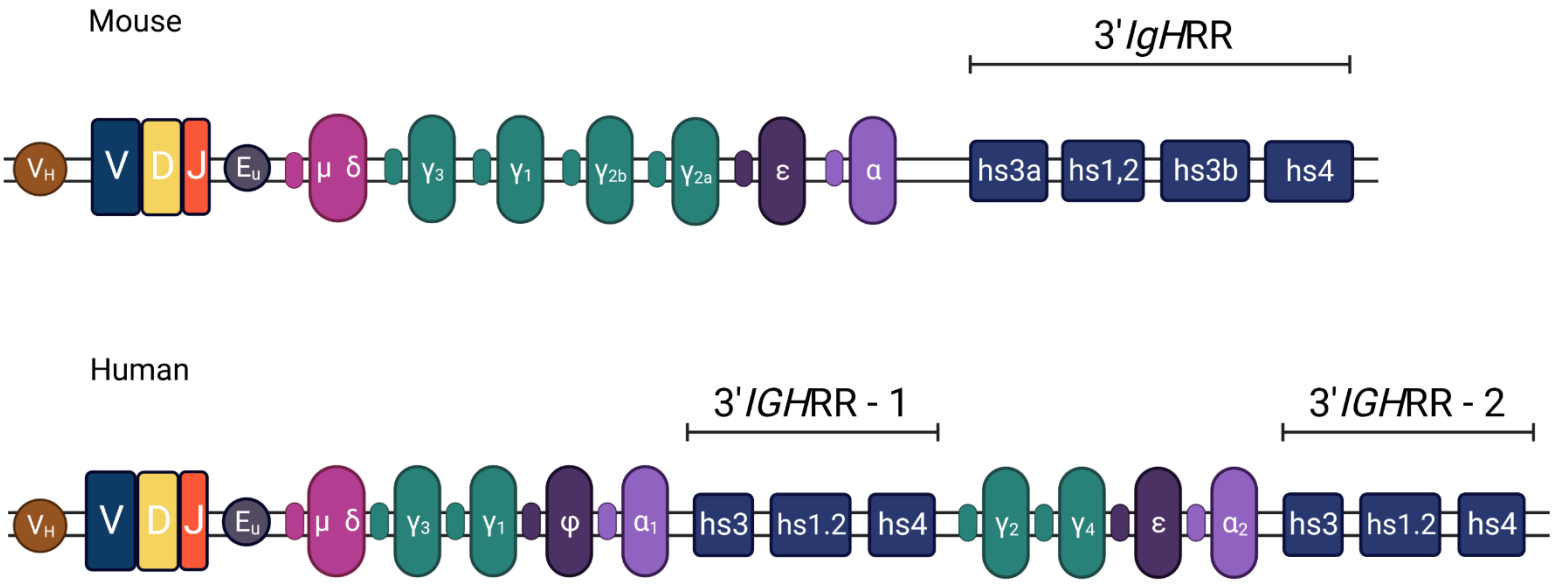


Figure 2. Mouse vs. Human *IGH* Gene Loci. Comparison of the mouse and human Ig heavy chain locus. The human *IGH* gene locus contains a duplicated regulatory region, 3'*IGH*RR-1 and 3'*IGH*RR-2. Function of the duplicated 3'I*GH*RR in upstream regulation has not been adequately studied. Figure made with biorender.com

Class Switch Recombination Changes Antibody Isotype

The final genomic alteration event, class switch recombination (CSR), results in mature activated B lymphocytes switching their expressed Ig isotype to a secondary isotype. The constant region of the heavy chain determines the Ig type and its immunological function (Blutt, S.E., et al. 2013; Gadermier, E, et al., 2014; Vidarsson, G. et al., 2014; Shan, M., et al., 2018). The *IGH* locus contains all of the constant region genes (C_H) that encode for the different Ig isotypes (μ, δ, $\gamma_{1\text{-}4}$, $\alpha_{1\text{-}2}$, ε; figure 2). Initially, the Ig isotypes produced by mature B lymphocytes are IgM and IgD as the BCR. IgM positive B lymphocytes can switch their expressed Ig to a secondary Ig isotype (i.e. IgG or IgA) when stimulated. CSR results in the intervening sequences between the C_μ and the downstream C_H to be excised and recombined to the V(D)J.

Initiation of CSR through B lymphocyte stimulation results in the initial transcription of the intervening region (I_H) promoter, the switch (S) region, and the C_H exon cluster or C_H region. This process forms a germline I_H-S-C_H transcript (GLTs), that undergoes normal splicing and polyadenylation, but does not encode for a functional Ig (Yang, S. & Schatz, D.G., 2007; Kothapalli, N. R. & Fugmann, S.D., 2011). B lymphocyte stimulation also induces expression of activation-induced cytidine deaminase (AID), another key component in CSR (Schrader, C. E., et al., 2007; Nagaoka, H., et al., 2010; Pone, E.J., et al., 2012). AID functions by deaminating deoxycytosines within 5'-AGCT-3' and 3'-TCGA-5' tandem repeats found within the core of the S regions to deoxyuricil (Muramatsu, M., et al., 2000; Thientosapol, E. S., 2017). The deoxyuracils are removed by uracil DNA glycosylase (UNG), an integral protein in the base excision repair pathway (BER), inducing DSBs in the S regions of the *IGH* (Yousif, A. S., et al., 2014).

The intervening sequences between the C_{μ} and the downstream C_H are excised, resulting in the donor C_H recombined to the V(D)J to result in a functional class switch (Schrader, C. E., et al., 2007; Pan-Hammarstrom et al., 2005; Wuerffel et al., 2007).

Regulation of the mouse *IgH* locus and CSR is partially controlled by the ~40kb 3'Ig Regulatory Region (3'IgHRR), found downstream of the constant regions (Ju, Z., et al., 2007). The 3'*IgH*RR comprises of four DNase 1 hypersensitive sites in mice and three in human that exhibit enhancer activity in both species. The human 3'*IGH*RR is also duplicated with the first duplication sitting downstream of the $C_{\alpha 1}$ and the second duplication sitting downstream of the $C_{\alpha 2}$ (Figure 3). Long-range regulation by the 3'*IgH*RR via interaction with the V_H promoter and the I_H intronic promoter regions has been observed in mice (Wuerffel, R., et al., 2007; Ju, Z., et al., 2007; Degner, S.C., et al., 2011). Deletion of the mouse 3'*IgH*RR results in an overall reduction of Ig secretion and CSR (Vincent-Fabert, C., et al., 2010; Pinaud, E., et al., 2001; Dunnick, W. A., et al., 2005). However, the role of the 3'*IGH*RR in humans is still under investigation.

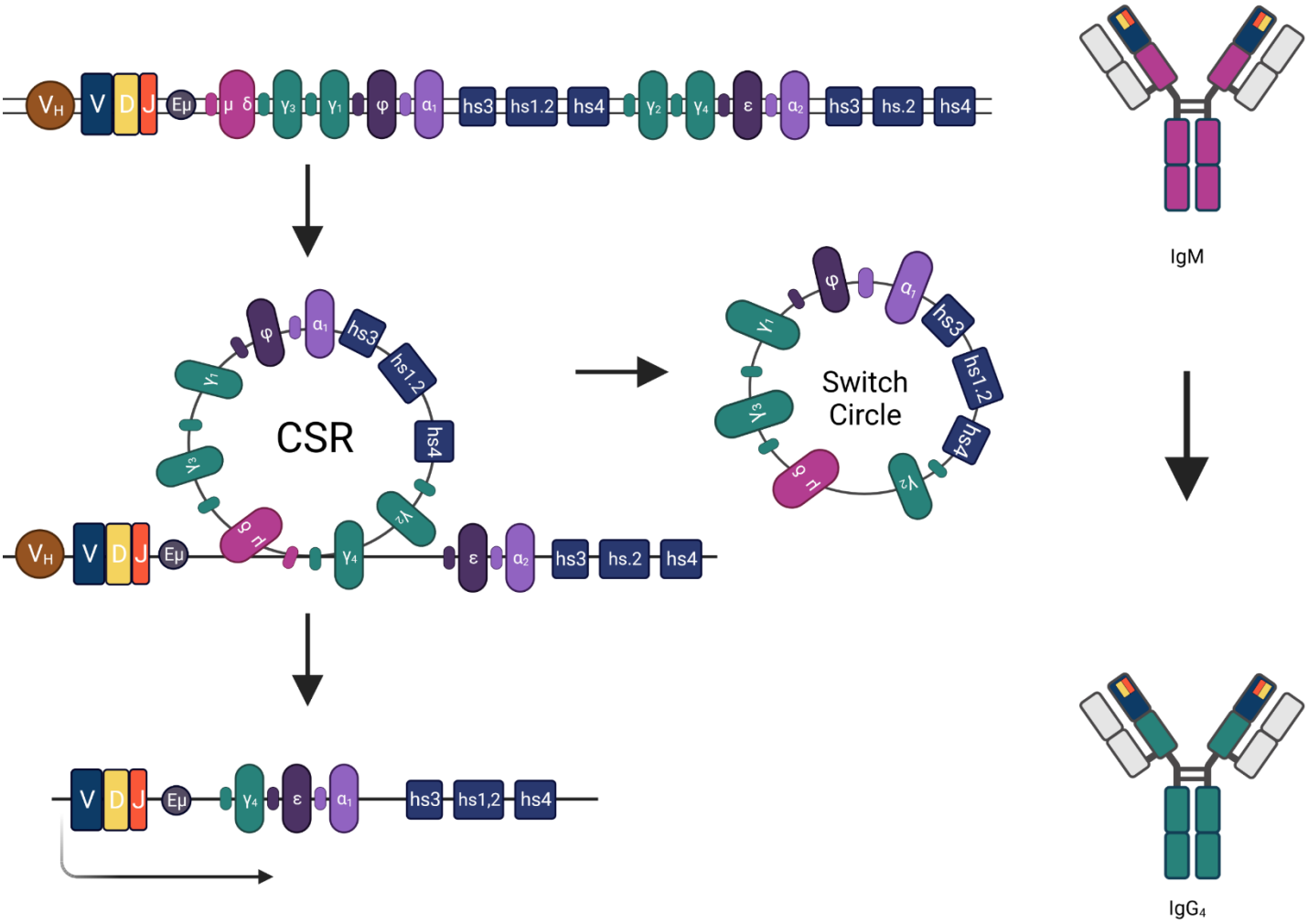


Figure 3. Class Switch Recombination of the *IGH* Gene. CSR from IgM antibody production to IgG_4 antibody production. Germline transcription originating from the I_H immediately 5' of their respective constant regions is required for successful AID targeting to the switch regions. Excision of the intervening sequence results in the switch C_H region to be recombined to the V(D)J, resulting in functional transcription and production of the secondary Ig isotype (i.e. IgM to IgG_4). Figure made with biorender.com.

The hs1.2 is Polymorphic and Associated with Human Immune Disorders

As stated previously, the mouse and human *IGH* loci differ through a duplication of the human *IGH* locus (figure 2). Within the single mouse *IgH*RR are four enhancers: hs3a, hs1.2, hs3b and hs4. The duplicated human 3'*IGH*RRs each only contain three of these enhancers, hs3, hs1.2, and hs4. Each of the human enhancers alone have been shown to exhibit weak regulatory control over the *IGH* in the context of CSR and VDJ recombination (Chauveau, Pinaud, & Cogne, 1998; Sepulveda, M., et al., 2005). The intervening sequences between the hs3, hs1.2, and hs4 are comprised of large palindromic regions that are conserved in other amniote species (D'Addabbo et al., 2011; Frezza et al. 2020).

The human 3'*IGH*RR enhancers contain several transcription factor (TF) binding motifs, such as OCT, AP-1, and NF-kB (Pinaud et al., 2011; Salisbury and Sulentic, 2015). Most of these TF binding sites are located within the central enhancer, the hs1.2. This is due to the hs1.2 being polymorphic for a ~53bp invariant sequence (figure 4). This means that dependent on the population, the invariant sequence can be repeated in tandem one to four times (Frezza, et al., 2020). In brief, the first duplication of the 3'*IGH*RR may have one to four repeats with one repeat being denoted as allele A, two repeats allele B, three allele C and four with allele D. The second 3'*IGH*RR region in most populations contains only three or four repeats, maintaining the same nomenclature of allele C or allele D (figure 4; Denizot et al., 2001; Giambra et al., 2005). Association between the hs1.2 alleles and multiple diseases has been observed. These include autoimmune disorders, such as lupus and its association with allele A and allele B, and rheumatoid arthritis with allele B (Tolusso et al., 2009; Frezza et al., 2012).

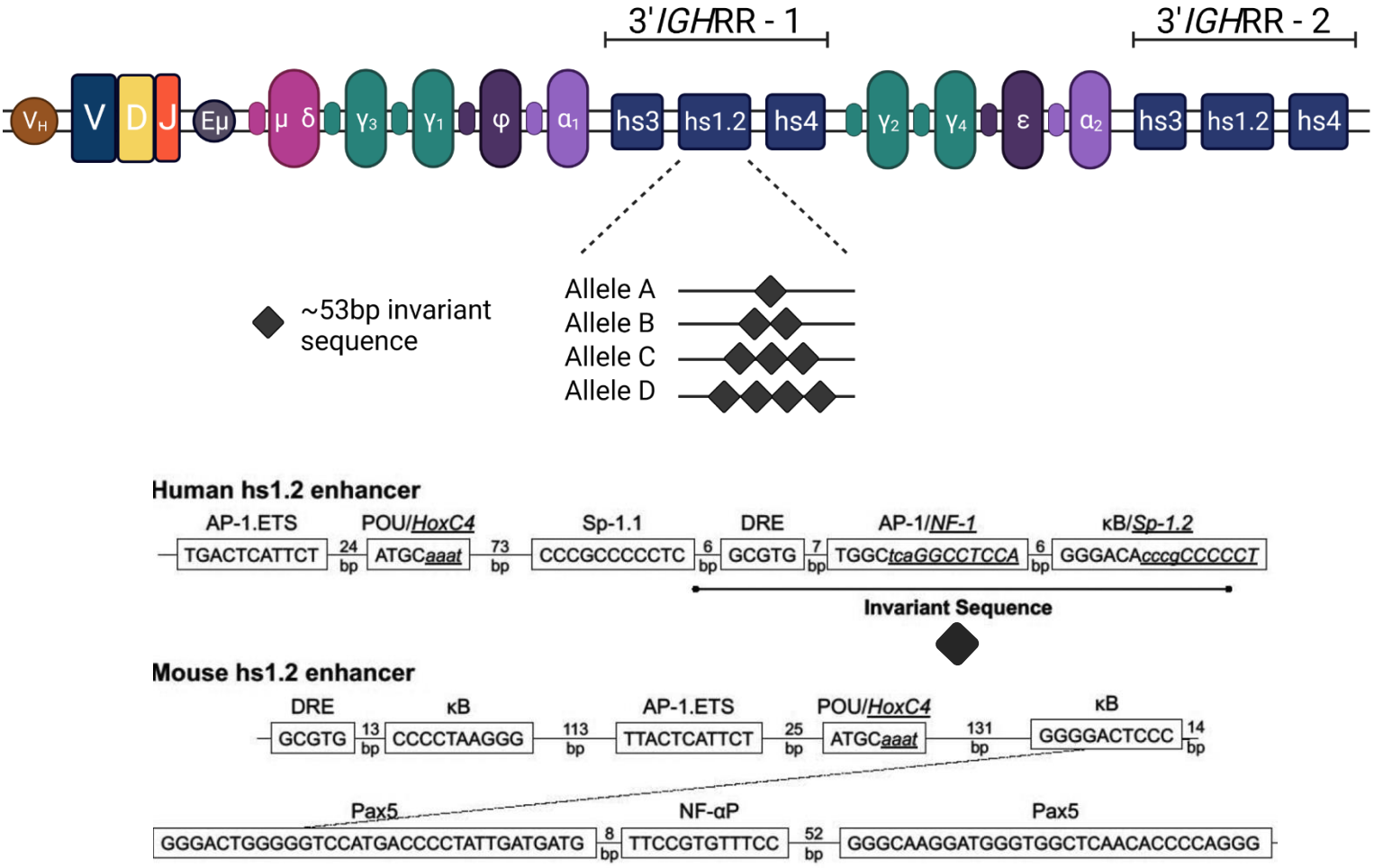


Figure 4: The Human hs1.2 Enhancer is Polymorphic. The human hs1.2 enhancer contains an invariant sequence that may be repeated one to four times depending on the population (top). Comparison of the human and mouse hs1.2 enhancers, with the invariant sequence underlined (bottom). Transcription factor motifs are boxed. Adapted from Snyder et al., 2020. Figure made in biorender.com.

Ligand Activation of the Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AHR) is a cytosolic, ligand-activated transcription factor. It was first discovered in the 1970s with the ability to bind 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and would later be described as a mediator of other environmental toxicants. Shortly thereafter, the AHR was found to induce the expression of aryl hydrocarbon hydroxlyase enzyme, CYP1A1 (Poland, A.P et al., 1974; Poland, A.P et al., 1976). Structurally, the AHR is a member of the basic-helix/loop/helix per-Arnt-sim (bHLH/PAS) family of transcription factors (Schulte, K et al., 2017).

Once a ligand is bound to the AHR, the chaperone repressor proteins dissociate from the nuclear localization signal (NLS) and nuclear export signal (NES) (Kudo et al., 2017). This leads to conformational changes of the AHR-ligand complex that allow it to be shuttled from the cytosol into the nucleus (figure 4) The AHR then forms a heterodimer with the AhR nuclear translocator (ARNT), forming the AHR-ARNT complex. At this stage, domains on the AHR and ARNT complex can bind to dioxin response elements (DREs), a unique DNA motif (5'- TNGCGTG - 3'), and regulate expression of target genes (DeGroot et al., 2014; Schulte, K. et al., 2017).

Several key domains comprise the AHR and provide it functional context in the cell. On the C-terminal end of the protein is the basic helix-loop-helix (bHLH) that is responsible for DNA binding (Schulte, K., et al., 2017). Immediately following the bHLH are the two PAS domains (PAS-A and PAS-B) that are partially responsible for protein-protein interactions and ligand binding respectively. Following the PAS domains is the transactivation domain (TAD). The TAD is responsible for recruiting co-transcriptional machinery in conjunction with the AHR repressor (AHRR), and is the main driving force

in regulating transcription after activation (Kumar et al., 2001; Rothhammer, V., et al., 2019; Schulte, K., et al., 2017).

As stated previously, the AhR was first discovered as a receptor for exogenous/environmental toxincants. Classically, these toxincants include the halogenated aromatic hydrocarbons (HAHs) and the polycyclic aromatic hydrocarbons (PAHs), such as TCDD (Bohonowych, J et al., 2007; Stejskalova et al., 2011) . However, recent work has revealed that a large range of natural-derived ligands, including those from diet and commensal microflora, can activate the AhR (Lamas, B., et al., 2020; Han, H., et al., 2021;). Because of this broad range of ligands, different physiological roles have been observed for the AhR depending on the tissue type.

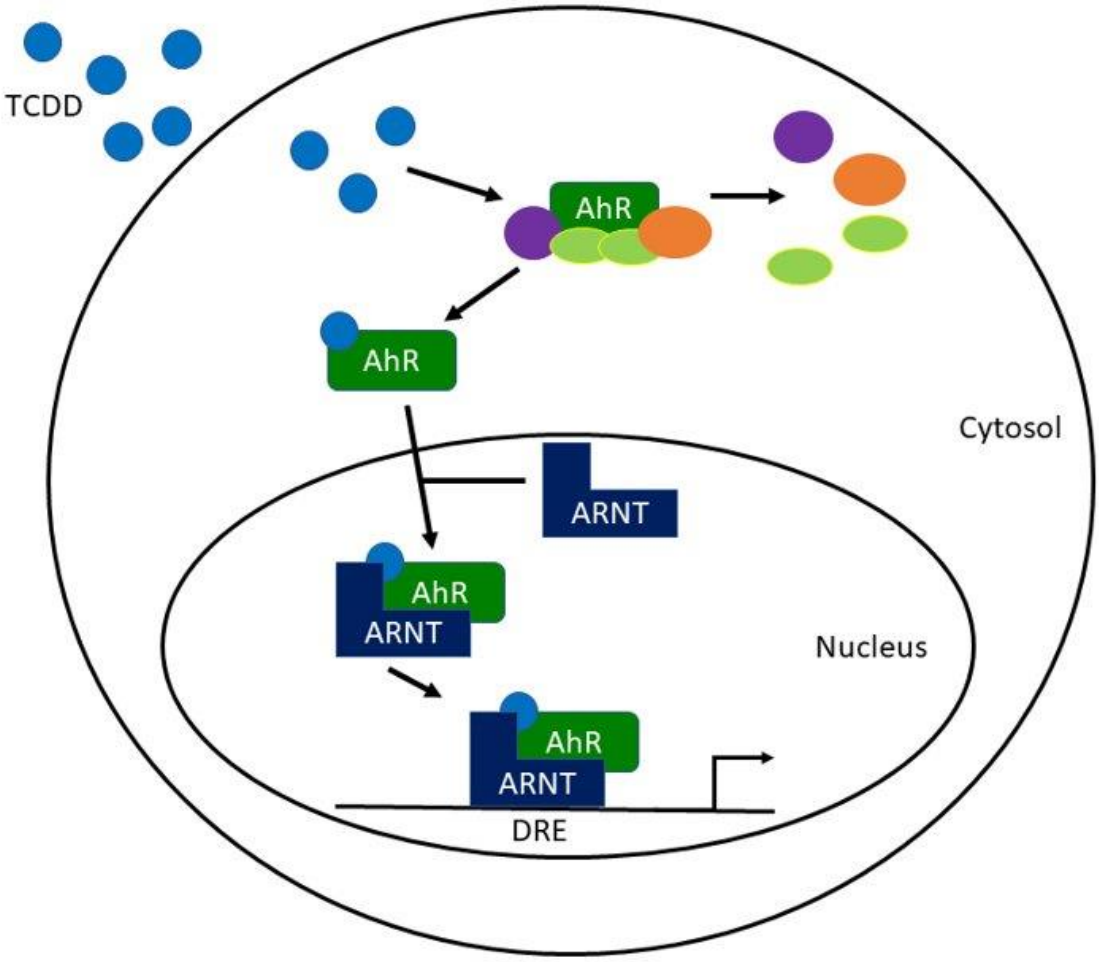


Figure 5. The Classical AHR Signaling Pathway. Binding of ligand to the AHR induces translocation from the cytoplasm to the nucleus and dimerization with the AHR nuclear translocator (ARNT). Once in the nucleus, the AHR-ARNT complex can bind to DRE motifs within promoter and enhancer regions of AHR sensitive genes and modulate gene expression.

B-lymphocyte Function May be Modulated by AHR Activation

As stated previously, the AHR was first discovered in the context of mitigating the toxic effects of HAHs, a class of organic compounds found in petroleum and the by-product of burning fuels sources. More recently, the AHR has been shown to regulate other physiological processes, such as cell proliferation, to gut microbiota homeostasis, and has been a recent target in cancer biology research (Bekki et al., 2014; Kou and Dai, 2021; Yin et al., 2016). Special attention has recently been paid to the AHR and its role in the regulation of the immune system.

Murine models have been used extensively to study the effects of AHR activation and environmental toxicant exposure on B-lymphocytes. During T-cell dependent activation, the AHR is upregulated in B-lymphocytes, potentially increasing the susceptibility towards endogenous and exogenous ligands of the AHR (Allan & Sherr, 2005). Fate determination of terminal B-lymphocytes has also been shown to be regulated by the AHR in the precesence of TCDD (Vaidyanathan et al., 2017; Zhang et al., 2013). AHR activtion via TCDD was also shown to inhibit IgM production in murine B cells, while mouine B cells lacking AHR expression tolerated this immunosuppression (Sulentic, Holsapple, & Kaminski, 1998). Further mouse work also explored the difference between exogenous and endogenous ligands of the AHR effects on murine B-lymphocytes. The tryptophan metabolite, 2-(1'H-indole-3'-carbonyl) thiazole-4-carboxylic acid methyl ester (ITE), was shown to suppress IgM, IgG1, and IgE production in purified mouse B-lymphocytes (Yoshida et al., 2012).

The effects of AHR and it’s ligands on humn B cells is relatively unknown. Physiological effects via AHR activation is different between mouse and human models.

Primary human studies have found that TCDD may decrease IgM secretion, but was not ubiquitous between donors (Kovalova et al., 2017). However, in murine B cells, AHR activation by TCDD inhibits IgM secretion (Worms & Sulentic, 2015). When the AHR was knocked down, TCDD exposure was tolerated and IgM secretion was unchanged. As mentioned previously, the human hs1.2 enhancer polymorphism contains multiple TF binding sites. Of these TF binding sites is a putative DRE site. Recent work from Snyder et al. used a murine cell line with human reporters and found that the flanking AP1 and POU binding sites played a major role in the basal and stimulated effects in B cells (Snyder et al., 2020). Although driect binding of the AHR to the DRE was not observed, this suggests that the AHR still plays a antibody secretion through an non-canonical pathway interaction.

Another feature of the human AHR is that it is polymorphic within exon 10 of the gene, which encodes for the TAD domain. Three possible SNPs can be present within exon 10: P517S, R554K, and V570I. Different combinations of these SNPs can partially determine the immunosuppresive effects of the AHR. In example, people with all three SNPs have been shown to slightly mitigate TCDD induced effects of the AHR on IgM secretion in human B cells (Kovalova et al., 2016). The AHR activation and its effects on the other antibody isotypes (IgG_{1-4}, IgA_{1-2}, IgE) in human B cells has not been adequately studied. Although direct binding within the 3'*IGH*RRs has not been observed, previous work suggests a interaction between the AHR and other pathways that can influence antibody production.

Enviornmental Pollutants and Humoral Imminuity Impairment

Recently, exposure to environmental toxicants has become more of a concern with recent chemical spills, mega fires, and drinking water contamination. One such chemical that has been shown in high levels of drinking water is perflurooctane sulfonic acid (PFOS), a “forever chemical” that is related to the perfluorinated alkylated substances (PFAS) (Cordner et al., 2019). First used in the 1940s, PFOS and its related compounds were utilized in military applications due to its oil repellent properties. This quickly shifted to mass production and PFOS has been used in products such as fire-extinguishers and as a coating agent in non-stick cookware (Sajid & Ilyas, 2017). The main route of exposure for PFOS is primarily ingestion through drinking water sources, as reported by the environmental protection agency (EPA) (Cordner et al., 2019).

Another environmental chemical of concern is the polycyclic aromatic hydrocarbon naphthalene. Regarded as a “high-production volume chemical”, naphthalene is used as an intermediary chemical for plastics production, is found in fossil fuels, jet fuels, burn pits, and biomass combustions (Yost et al., 2021). Due to its presence in both biomass combustion and fossil fuels, inhalation toxicity is a risk to human health.

Because of the ubiquitous nature of exposure to PFOS and naphthalene, human health effects are a major concern. For PFOS there exists a breadth of work done in animal models trying to understand the potential method of action (MOA) and systemic physiological effects of PFOS exposure. Human exposure studies have also revealed potential physiological effects of Naphthalene including association with type-2

diabetes, endocrine disruption, and impaired immune function (Han et al., 2021; Looker et al., 2014). In the context of human humoral immunity and PFOS exposure, epidemiological studies have shown decreased overall serum antibody levels in children and decreased efficacy of influenza vaccination via lowered antibody titers in adult sera (Grandjean et al., 2012; Looker et al., 2014)

Understanding naphthalene's effects on humoral immunity has not been adequately studied. Epidemiological studies have found associations between naphthalene exposure and allergen response by increased IgE serum concentrations and diminished IgA and IgG serum levels (Lin et al., 2018). However, dose-response data from epidemiological studies of naphthalene have not been successful in understanding dose-dependent immunotoxic effects of naphthalene (Yost et al., 2021). An explanation for these low-confidence studies of direct naphthalene impairment of humoral immunity is the potential need for naphthalene metabolism before toxic effects are observed (Kawabata & White Jr., 1990).

Epidemiological studies have shown immunotoxic effects of exposure to both PFOS and naphthalene. However, these studies have not directly assessed dose-dependent impairment of B-cell function and immunoglobulin production. This is a crucial gap in knowledge as exposure to these chemicals is widespread and diminished immune function can lead to detrimental human health effects.

Significance and Hypothesis

Significant differences exist between the murine *IgH* and human *IGH* gene loci. Work within murine models has shown a direct regulation of the *IgH* gene but this has

not been extensively studied in humans. The hs1.2 polymorphism present in humans but not mice, is associated with several immune such as celiac disease, rheumatoid arthritis, and systemic sclerosis (Cianci et al., 2008; Frezza et al., 2009; Frezza et al., 2007; Tolusso et al., 2009). It also contains multiple TF binding sites, including a DRE that can be repeated one to four times. The hs1.2 polymorphism makes it a potent target for multiple toxicants. Previous work in our lab demonstrated an inhibition of IgG expression via TCDD in the human CL01 cell line (Burra & Sulentic, 2015).

Here I hypothesize those **genetic variations within the hs1.2 enhancer are susceptible to exogenous toxicants and will modulate antibody production.** To test this hypothesis, I first generated sequencing data for a AHR edited clone that expresses only a functional TAD and compared it to the parental cell line. I confirmed that their variant profiles were almost identical and that this cell line does not contain deleterious mutations through variant effect prediction. I also confirmed that our cell line and subsequent clone is heterozygous for three SNPs within the TAD domain, that result in one allele coding for a non-functioning TAD and the other allele encoding for a functional TAD. Next, within clones edited in the hs1.2 IS, I surveyed the effects of AHR activation via TCDD on Ig production. Lastly,

A modest reduction in Ig production could have an impact on the hosts ability to clear infections and toxicants. Several human immune disorders possess modulated levels of Ig production resulting in impaired immunocompetence, which is correlated with a diminished ability of B lymphocytes to undergo CSR (Doffinger et al., 2001, Imai et al., 2003, Durandy et al., 2007). Therefore, it is essential for B-cells to tightly regulate both the production of Igs and the ability to switch between the different Ig isotypes.

Understanding how exposures to different toxicants may dysregulate B lymphocytes to class switch and produce Igs is imperative for creating protective therapies for potential immune disorders caused by these disease states.

II. Materials and Methods

Chemicals and Reagents

Naphthalene, PFOS, and 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) was purchased from AccuStandard (New Haven, CT). Terbutaline and Methanol were purchased from Fisher Scientific (Waltham, MA). Human recombinant CD40 ligand (MEGACD40L) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY). Human interleukin 4 (hIL-4) was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Cell Culture

The human EBV transformed B-lymphocyte cell line, CL-01, was purchased from Novus Biologicals (Littleton, CO). CL-01 cells and all CRISPR/cas9-edited clones were grown in RPMI-1640 (Corning) supplemented with 2mM L-glutamine, 10% bovine calf serum (ThermoScientific Laboratories, Logan, UT), 13.5mM HEPES, 1.0mM sodium pyruvate, and 1.0mM non-essential amino acids. Culturing conditions were maintained at 37^{o}C with 5% CO_2 atmospheric conditions. All cells were maintained between 1x10^5 cells/mL to 3x10^5 cells/mL and media was replaced every two to three days.

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

All cell lines (1x10^5 cells/mL) were treated with the following ranges of PFOS, Naphthalene, Terbutaline, and TCDD. Each treatment condition was aliquoted in triplicate to 12-well cell culture plates at 1mL/well and incubated at the previously mentioned cell culture conditions. All treatments were carried out for 96 hours, which

has previously been shown to be effective for demonstrating *IgH* expression and previously published for evaluating changes in antibody secretion (Cerruti et al., 1998). High-bind 96-well ELISA plates were first coated with Goat Anti-Human Ig-UNLB antibody (SouthernBiotech; Birmingham, AL) at a 1:1500 dilution factor in 0.1M sodium carbonate bicarbonate buffer. Plates were coated overnight followed by washing with 1X PBS + 0.05% Tween20 and ddH_2O. A blocking solution was prepared using 1X PBS with 3% (w/v) of BSA and added to the plate for 1.5 hours at room temperature. After blocking, the plates were again washed as above. The following standards were diluted in media and applied to the washed and blocked plates: purified human IgG (Bethyl Laboratories; Montgomery, TX), purified human IgA (Bethyl Laboratories; Montgomery, TX), and purified human IgM (Southern Biotech; Athens, GA). Supernatants from each well were added to plates at 100uL/well following addition of standards and incubated for 1.5 hours at 37^{O}C. Above wash steps were repeated again, and detection antibodies were diluted in 1X PBS with 0.05% Tween20 and 3% BSA (w/v), and then added to the plate. Goat anti-human IgG HRP-conjugated antibody was diluted 1:10,000 (Bethyl Laboratories), Goat anti-human IgM HRP-conjugated antibody was diluted 1:4000 (Southern Biotech, Birmingham, AL), and goat anti-human IgA HRP-conjugated was diluted at 1:20,000 (Southern Biotech). A final wash step was conducted as above and TMB substrate (Milipore Sigma; St. Louis, MO) was added to the plates. Each plate was incubated for 30 minutes in no-light conditions at room temperature. To stop the reaction, 100uL/well of 4N H_2SO_4 was added. Quantification of antibody concentration was performed using the Spectramax Plus 384 UV/VIS (Molecular devices; Sunnyvale, CA) with the read conditions set to OD values of the wells at 450nm. SOFTmaxPRO

software (Molecular Devices) was used to generate standard curves and calculate concentrations of IgG, IgM and IgA.

Genomic DNA Isolations

Isolation of high molecular weight (HMW) genomic DNA (gDNA) was performed for Illumina sequencing at HudsonAlpha Genomic (Huntsville, AL). HMW gDNA isolations were carried out using the Qiagen Genomic tips in conjunction with the QIAmp DNA mini kit (Qiagen, Germantown MD). Cells were first lysed using the provided lysis buffer and proteinase K and incubated at 56^{o}C for 10 minutes. Secondary detergents provided by the kit were then added and the mixture was added to the genomic tips for gravity filtration. Following initial filtration, the binding column within the tips was washed and then DNA eluted using 1X TE buffer at a pH of 8.0. Isolated DNA was immediately frozen and sent to HudsonAlpha for whole genome sequencing.

PCR Genotyping of hs1.2 Enhancer

Genotype of the hs1.2 enhancers was accomplished using PCR amplification. In short, 100ng of gDNA was added to a PCR master mix containing 1X standard taq buffer (NEB), 10nM dNTPs (ThermoFisher), 10uM of forward and reverse primers (IDT; Coralville, IA) nuclease-free water, and standard taq polymerase (NEB; Ipswich, MA). PCR cycle conditions are as follows: 95^{o}C for 30 seconds, 67 ^{o}C for 30 seconds, and 68 ^{o}C for 30 seconds repeated 30 times followed by a final extension at 68 ^{o}C for 5 minutes. The expected product sizes are 209 bp for allele A, 264 bp for allele B, 319 bp

for allele C, and 374 bp for allele D (figure 4), PCR products were viewed using a 2% agarose gel with ethidium bromide.

Whole Genome Sequencing and Analysis

Sample quality was determined via nanodrop and concentration was quantified via qubit fluorometer broad range DNA assay (Thermo Fisher Scientific; Waltham, MA). Samples were sent to HudsonAlpha Institute (Huntsville, AL) for HiSeq and NextSeq sequencing respectively. Data was returned as preprocessed fastq files. Briefly, analysis was automated through an in lab developed Snakemake pipeline (see results). Reads were preprocessed with Trimmomatic (Bolger, A. et al., 2014) to remove sequencing library specific adaptors. Trimmed reads were then mapped using the Burrows-Wheeler aligner (BWA) algorithm with the most recent human reference genome, version hg38 (Li, Heng, and Durbin, 2009). Mapped reads where quality checked using FastQC (Andrew, S., 2010), followed by duplicate read marking using GATK4.0. BCFtools was used to produce a single nucleotide polymorphism (SNP) and insertion deletion (INDEL) call file and calls were filtered using standard parameters (Danecek et al., 2021). Annotation of the predicted SNPs and INDELs was accomplished using the ENSEMBL database and their variant effect predictor (VEP) (Oscanoa et al., 2020).

Statistical Analysis of Data

Statistical analysis was carried out using GraphPad prism software (San Diego, CA) and the open-source statistical language R. Comparisons between treatment groups and corresponding vehicle were done using a one-way ANOVA with a

Bonferroni multiple comparisons test. Significance between naïve and stimulated cells is denoted by a “dagger” representing a p-value <0.05. Significant differences between the chemical treatments and corresponding vehicle controls is denoted by “*”, “**”, and “***” representing p-values < 0.05, < 0.01, and < 0.001 respectively.

III. Results

Building a Reproducible Whole Genome Sequencing Analysis Pipeline

As omics data becomes cheaper and more accessible to generate, development of analysis pipelines is critical for reproducibility of work. Several languages exist to aid in the production of reproducible workflows, such as nextflow and snakemake. Here I developed a snakemake pipeline, as shown in a graphical representation in figure 6**.** This pipeline takes raw FASTQ files from Illumina whole genome sequencing and processes them to analysis ready variant call format (VCF) files. In brief, reads were first trimmed using trimmomatic software to remove sequencing specific adapters (Bolger et al., 2014). Mapping of trimmed reads was accomplished using the Burrors-Wheeler algorithm with default parameters against the latest hg38 human genome reference from ensemble (Li and Durbin, 2009). Reads were then quality checked using FASTQC and MULTIQC after alignment and HTML reports were generated (Andrews, 2010). Duplicate reads were marked and removed using GATK MarkDuplicates, as these can confound downstream variant calling (Poplin et al., 2017). Coverage statistics at the per-nucleotide resolution was then calculated using the samtools mpileup function (Li et al., 2009). Finally, single SNPs and INDELs were called using VarScan2.0 (Koboldt et al., 2012). To ensure confidence in SNP and INDEL calls, several thresholds were used: p-value < 0.005, average coverage of > 20 reads, and a minimum number of supporting reads of at least 15. Corresponding SNP and INDEL VCF files were generated and downstream analysis and visualization were performed. Access to code

and modules developed for this pipeline can be found in the following github repository: https://github.com/CAllex-Buckner/wgs_vdj_pipe .

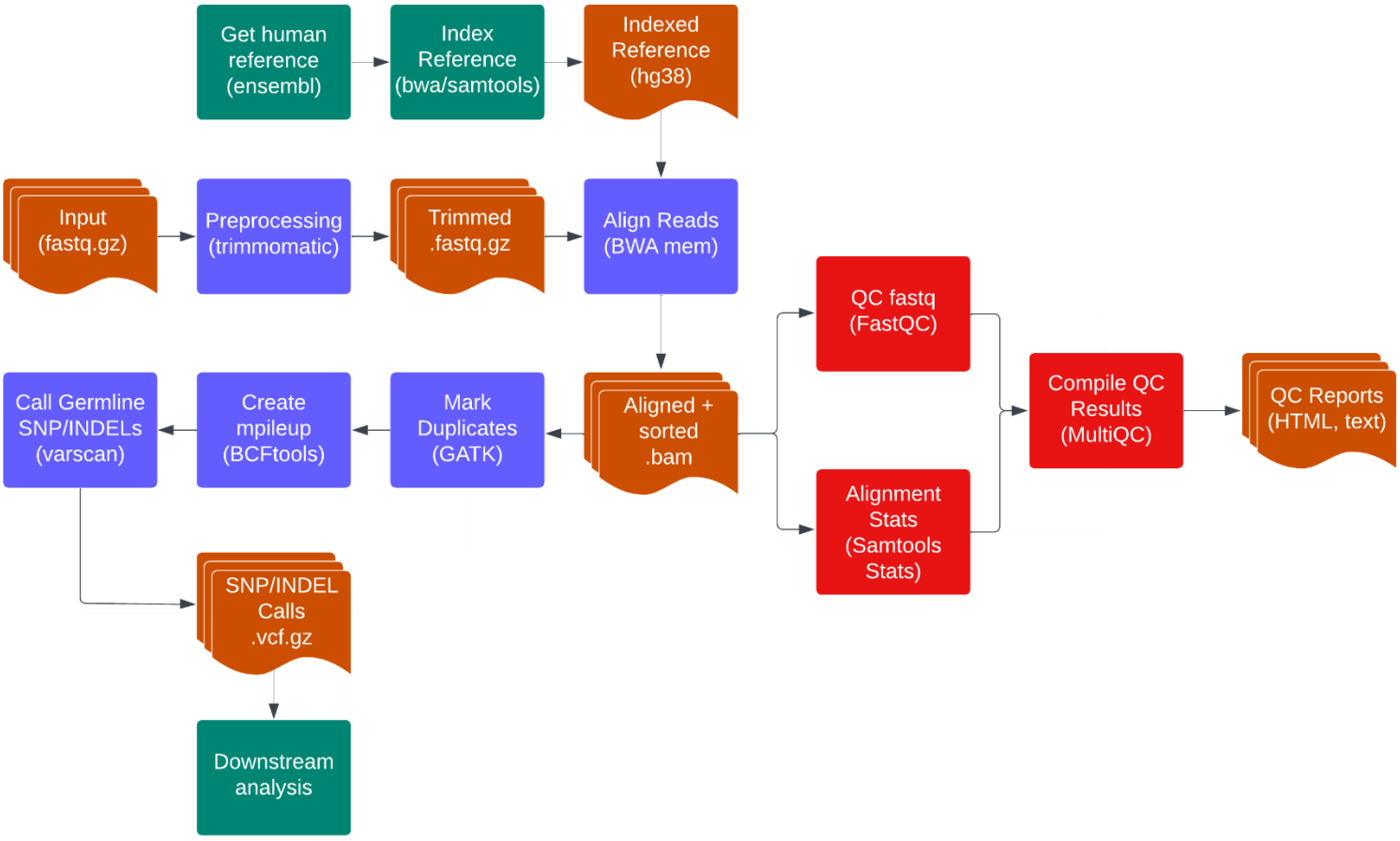


Figure 6. A Graphical Representation of the Whole Genome Sequencing Pipeline. Steps in green represent functions that call to outside databases and scripts. Orange files represent input and outputs from connected processes. Blue functions represent corresponding tools used to generate input and output files. Red processes are quality control and evaluation steps.

Genome Wide Characterization of CL01 and 10F10 Cells

The CL-01 cell line is an EBV transformed Burkett's lymphoma cancer line that was first described in Cerruti et al., 1998. It has been used in several studies for B-cell maturation and differentiation (Bernstein et al., 2004; Cerruti et al., 1998). In the presence of CD40L and IL-4, this cell line can be induced to secrete higher levels of Ig and potentially undergo CSR. Because of this, it is an excellent human cell line model to study CSR and Ig production in the context of the AHR and environmental chemical exposures.

To better understand the regulatory role the AHR plays in B cells, our lab attempted to generate an AHR knockout clone from the CL01 cells using CRISPR/Cas9 gene editing (Nasser & Sulentic, 2017). A clone positive for successful editing as determined by western blot was selected. However, over time the clone's AHR expression returned. Luciferase reporter assays using a 6X DRE binding site found that the AHR was still functional and had higher Luciferase reporter activity in the presence of TCDD compared to the parent CL01 cells. This suggested a mono-allelic edit, resulting in the clone expressing a functional TAD domain, which will be referred to as TAD^{+}.

To better characterize both the CL01 parent cell line and the TAD^{+} clonal population, we isolated HMW gDNA and sent it for Illumina HiSeq and Nova short read sequencing. Analysis was performed in house with the pipeline described in the previous section (figure 6). Both sequencing experiments resulted in > 30X coverage of both genomes and was ideal for downstream SNP and INDEL profiling and annotation. Finally, all called SNPs and INDELs were used for variant effect prediction using the

ensemble database. Percentage of combined and intersecting SNPs and INDELs from both CL01 and TAD^{+} lines were annotated, and variant predictions made.

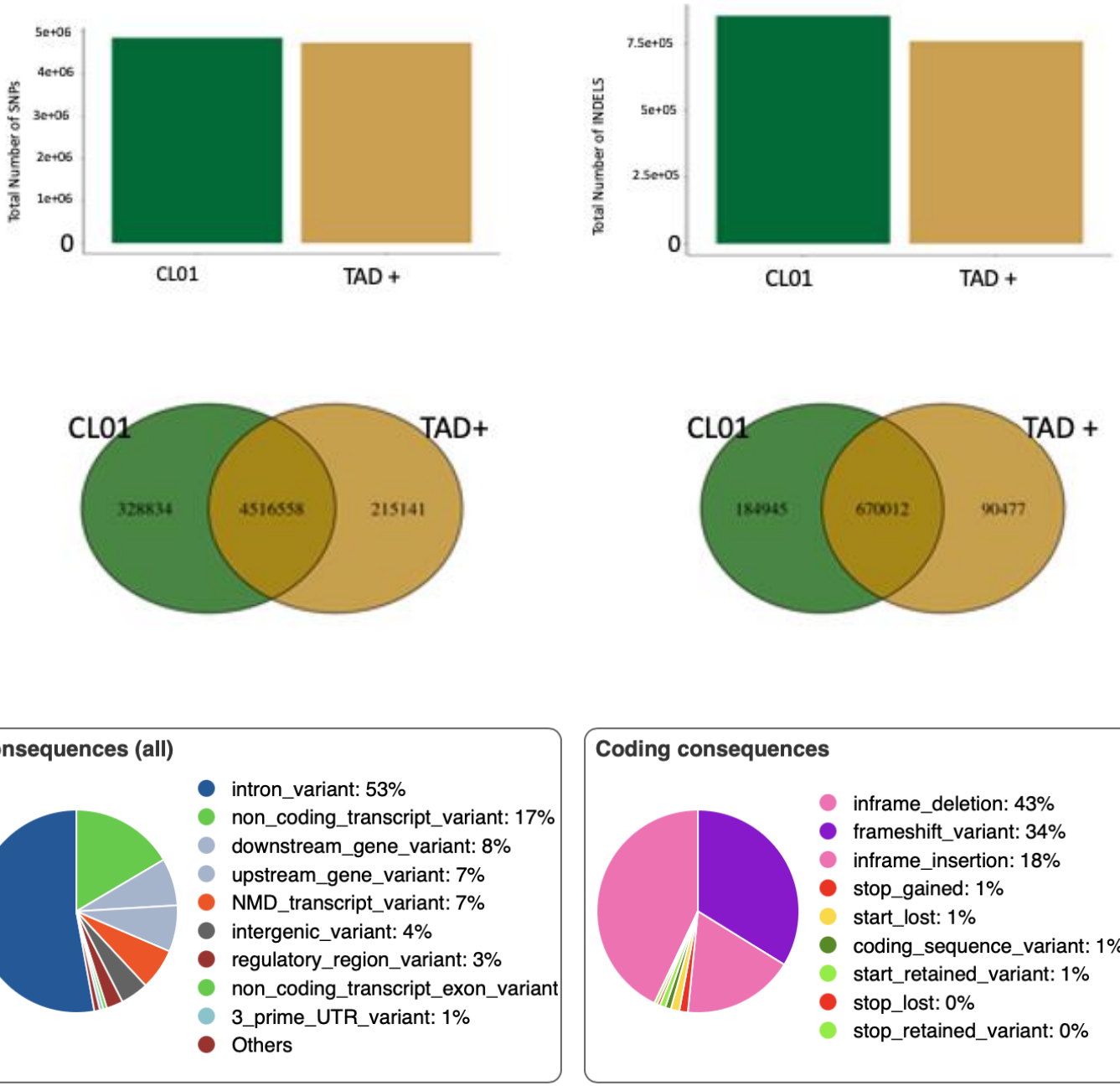
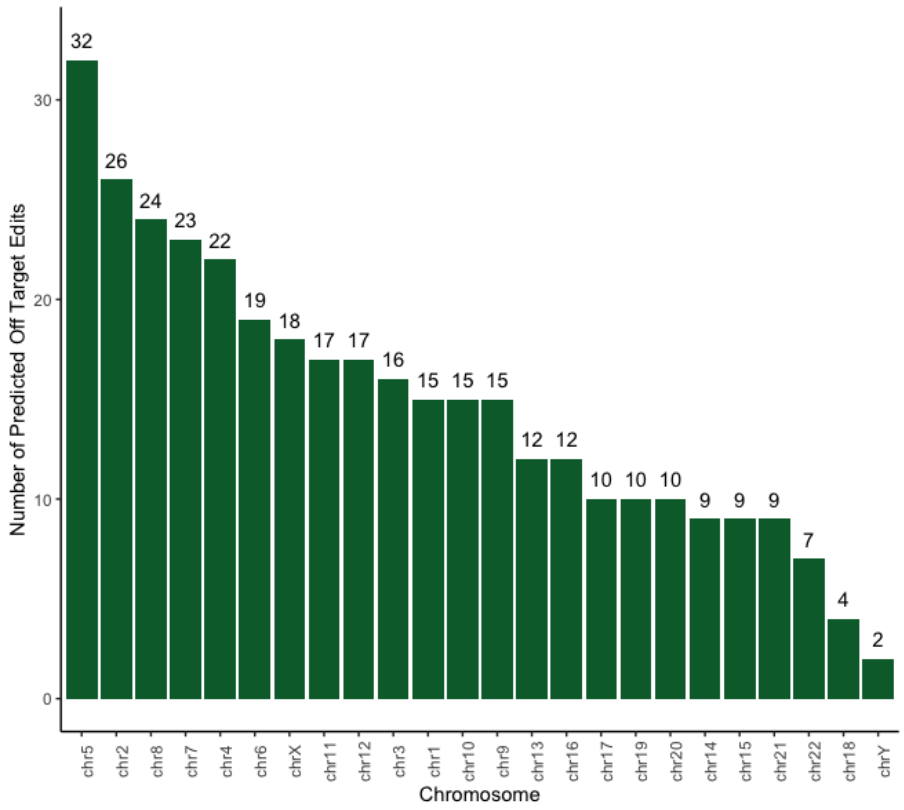


Fig 7. Comparison of Wildtype and TAD⁺ Clone SNP/INDEL Profiles. Total number of called SNPs (Top Left) by cell type and the number of overlapping SNPS (Top Right) determined by genomic coordinate. Similarly, the total number of called INDELS (Middle Left) by cell type and the number of overlapping INDELS (Middle Right) determined by genomic coordinate. VEP predictions by consequence and coding consequence (Bottom).

CRISPR/Cas9 Editing of CL01s Resulted in No Off-Target Edits

Genome editing with CRISPR has become an invaluable tool for research and therapeutic applications. In principle, the guidance RNA (gRNA) should be specific enough to facilitate site specific cuts at the desired genomic site. However, multiple factors could cause off-target editing, including wobbling at the 5' or 3' ends of gRNA during binding. The potential for deleterious off-target editing is low but should still be surveyed for when generating edited cell populations (Bock et al., 2022). One methodology for this is using a predictive program to take your gRNA sequence and predict across an assembled genome potential off targeting sights dependent on wobbling at the ends of the gRNA.

Here I surveyed our CRISPR/Cas9 edited TAD^{+} clone for potential off target editing using the Cas-OFFinder software (Bae et al., 2014). In brief, the gRNA for the study was provided and predictions made against the hg38 human genome were made. Allowed mismatches were set to three and DNA and RNA bulge sizes were set to one each. Cas-OFFinder produces potential targets and their coordinates based on the genome reference provided. Number of predicted off target edits is shown by chromosome. Variant data previously generated by my analysis pipeline (figure 6) was then used to determine if the predicted edits were present in both the CL01 and TAD cell lines. No corresponding off-target edits were found within the called SNP/INDEL in both variant datasets (figure 8).

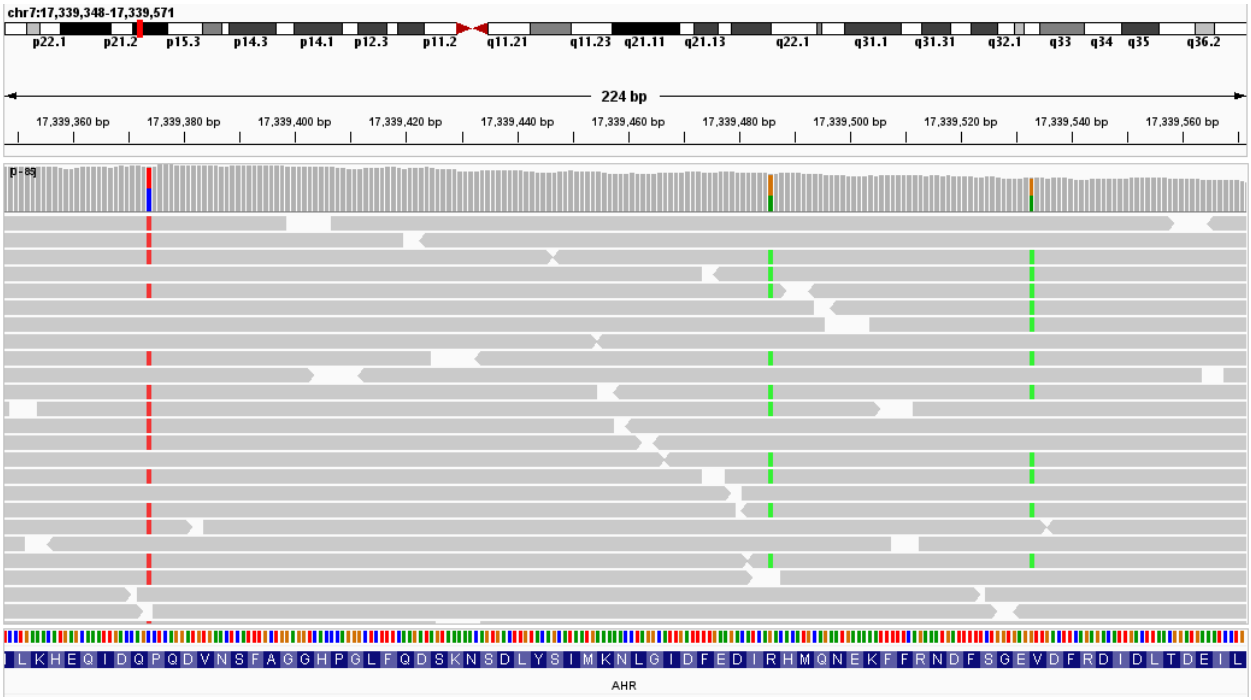


	Cell Type	
	Wild Type	AHR KO
Predicted Number of Off-Target Editing Events	375	
Number of Matching Sites w/ SNPs or INDELS	0	0

Figure 8. Validation of CRISPR/Cas9 Gene Editing Specificity in Edited Cells. (Top) Count of Cas-Offinder predicted off-target CRISPR/Cas9 edits by chromosome. Cas-Offinder software parameters used: Mismatch number of 5, DNA/RNA bulge of 0. (Bottom) Table of total number of matched SNP and INDEL genomic coordinates from both Wildtype and AHR KO called SNPs and INDELS.

CL01 and TAD^{+} are Heterozygous for Three SNPs in AHR TAD Domain

The AHR is polymorphic in humans in that different mutations have differential effects on the AHRs physiological function depending on the cell type (Aftabi et al., 2021). Within our cell line, as found from the whole genome sequencing experiments three potential SNPs within the AHR TAD are located within 160 base-pairs of one another in exon 10. These three SNPs (R554K, V570I, and P517S) have been previously reported to disrupt TAD function in the context of TCDD activation and IgM secretion. Generally, determination of allele specific SNPs is done through transcriptomic studies on expressed RNA of interest. Fortunately, from our WGS experiments, the average length of each read was 151 base pairs. This means significant overlap of reads with the called SNPs could be isolated out and assembled. Following assembly, contigs were assessed for the presence of the SNPs. All contigs were unanimous in calling the SNPs together, and no contigs were produced that contained a SNP separate from the others. This indicates that our CL01 cell line is truly heterozygous for the three TAD SNPs, and that one allele contains all three SNPs while the other allele does not (figure 9).

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TAD+ 1 CCAGCCTCAGGATGTGAACTCATTTGCTGGAGGTCACCCAGGGCTCTTTC     50
       ||||.||||||||||||||||||||||||||||||||||||||||||||
TAD- 1 CCAGTCTCAGGATGTGAACTCATTTGCTGGAGGTCACCCAGGGCTCTTTC     50

TAD+ 51 AAGATAGTAAAAACAGTGACTTGTACAGCATAATGAAAAACCTAGGCATT    100
        ||||||||||||||||||||||||||||||||||||||||||||||||||||
TAD- 51 AAGATAGTAAAAACAGTGACTTGTACAGCATAATGAAAAACCTAGGCATT    100

TAD+ 101 GATTTTGAAGACATCAGACACATGCAGAATGAAAAATTTTTCAGAAATGA   150
         ||||||||||||||||.||||||||||||||||||||||||||||||||
TAD- 101 GATTTTGAAGACATCAAACACATGCAGAATGAAAAATTTTTCAGAAATGA   150

TAD+ 151 TTTTTCTGGTGAGGTTGACTT    171
         |||||||||||||.|||||||
TAD- 151 TTTTTCTGGTGAGATTGACTT    171
```

Figure 9. CL01 Cells are Heterozygous for Three TAD SNPs. IGV viewer snapshot of CL01 WGS sequence alignments within the AHR (Top). SNPs R554K, V570I, and P517S are shown as red and green respectively. Multiple sequence alignment with regards to the two TAD alleles (Bottom). Both CL01 cells and the TAD$^+$ clone were heterozygous for the three SNPs within exon 10 of the AHR. Dots determine the position of the SNP and what allele it is located on.

Naphthalene Significantly Reduces IgM Secretion

As discussed previously, exposure to environmental toxicants has been of greater concern recently. This is due to detection of high levels of forever chemicals, such as PFOS in drinking water, naphthalene being present in fuel combustion, and production of high amount of dioxins from mega fires (Salamanca et al., 2016). Previous work in human primary cells and mouse models have shown reduction of IgM after exposure to TCDD. Epidemiological studies of PFOS and naphthalene exposure have shown reductions in serum levels of antibodies and began to elucidate the immunotoxic effects of these compounds. However, these studies are considered “low-confidence” as they are not suitable for dose-response analysis due to their bio-marker metrics (Yost et al., 2021).

Here we use dose-responses to determine if exposure to PFOS and naphthalene significantly reduce antibody production via ELISA assays in CL01 cells after stimulation with CD40L and IL4. The first antibody isotype observed was IgM (figure 10). PFOS and TCDD did not significantly reduce IgM secretion in CL01 cells. This effect with TCDD cells has been observed in our lab before (Kaulini & Sulentic, 2016). Naphthalene, however significantly reduced IgM secretion as compared to vehicle. The most significant reduction was observed in the smallest dosage. Although a vehicle effect was observed, this was not found to be significantly different as compared to stimulated only control (figure 10).

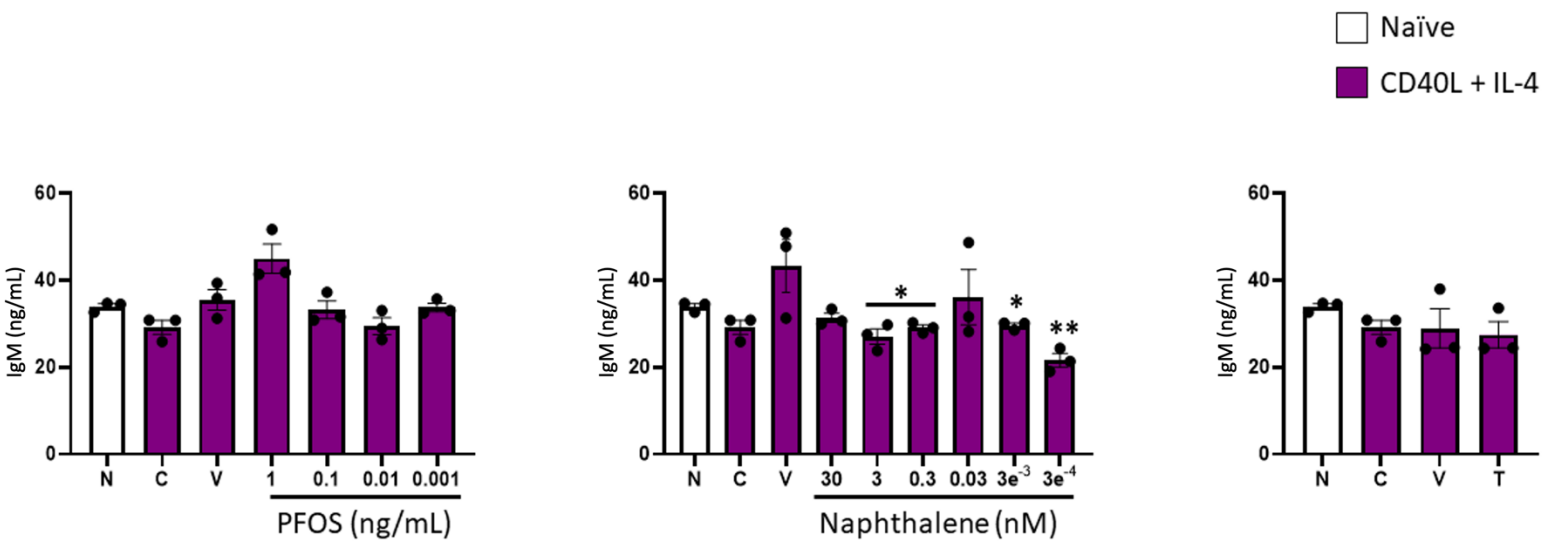

Figure 10. IgM Secretion is Significantly Reduced by Naphthalene. CL01 cells were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and either treated with vehicle (1% MeOH, 0.001% MeOH, 0.01% DMSO), various concentrations of PFOS and Naphthalene as indicated on graphs, or TCDD (30nM). IgM was measured by ELISA. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Bonferroni post-test. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Graphs are a representative or two repeats. Graphs show individual points (n=3) and SEM of individual points.

PFOS and Naphthalene Significantly Reduce IgA Secretion

Reduction of IgM secretion was only observed in naphthalene treatment, especially at the lowest molar concentrations of naphthalene. To determine if these same concentrations had an impact on other Ig isotypes, we measured for IgA antibody secretion in stimulated and environmental toxicant treated CL01 cells.

Here we observed that both PFOS and naphthalene significantly reduce IgA secretion in stimulated CL01 cells (figure 11). TCDD however, does not significantly alter IgA secretion as compared to vehicle control or stimulation alone. Again, the lowest molar concentration of naphthalene (0.3pM) does most significantly reduces IgA secretion. TCDD did significantly reduce IgA secretion compared to vehicle control, but it does seem to trend towards lowering it to basal secretion levels as compared to naïve control.

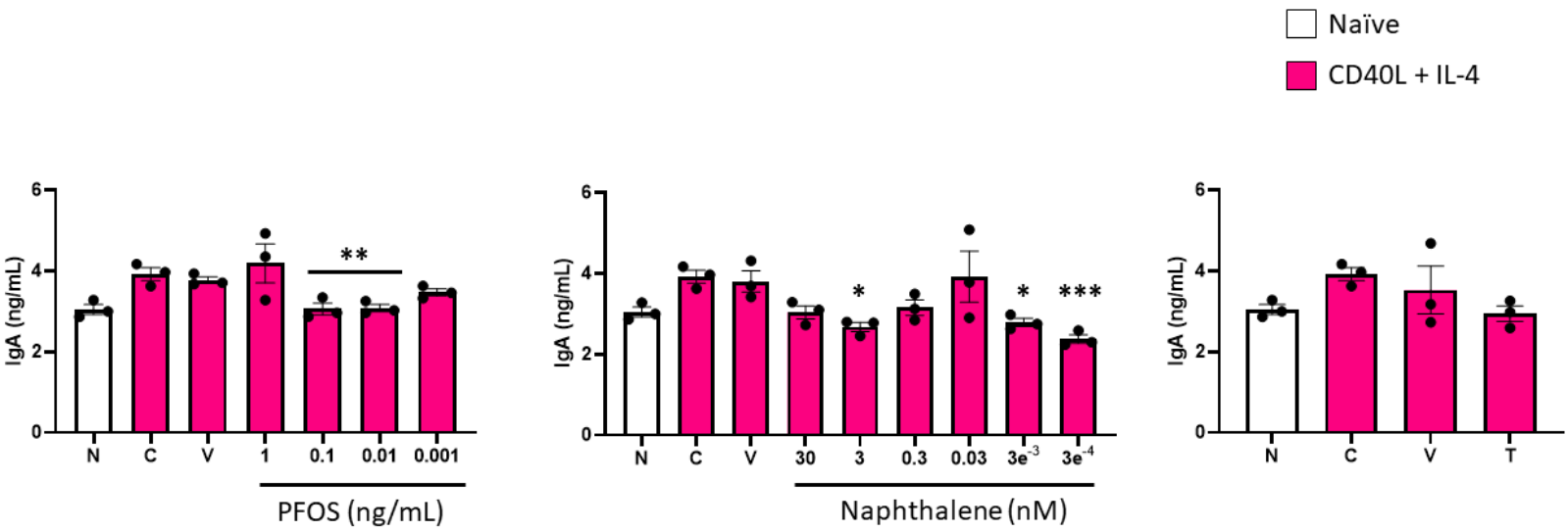

Figure 11. PFOS and Naphthalene Significantly Reduce IgG Secretion. CL01 cells were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and either treated with vehicle (1% MeOH, 0.001% MeOH, 0.01% DMSO), various concentrations of PFOS and Naphthalene as indicated on graphs, or TCDD (30nM). IgA was measured by ELISA. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Bonferroni. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Graphs are a representative or two repeats. Graphs show individual points (n=3) and SEM of individual points.

PFOS and TCDD Significantly Reduce IgG Secretion

In the previous two experiments, naphthalene was show to have a significant reduction of IgM and IgA. PFOS was only shown to significantly reduce IgA secretion, while TCDD treatment does not significantly reduce IgM and IgA secretion. Because of these results, we wanted to observe exposure to these environmental toxicants resulted in dose-dependent reduction of IgG secretion.

Here we observed that PFOS and TCDD were able to reduce IgG secretion (figure 12). TCDD reduction of IgG in CL01 cells has been previously observed in our lab (Kaulini & Sulentic, 2016). Surprisingly, naphthalene did not result in significant change of IgG secretion, even though it had significantly reduced secretion of both IgM and IgA. Conversely, PFOS treatment resulted in significant reduction IgG secretion (figure 12). These results in combination point to a dose-dependent and Ig isotype specific reduction of secretion for each of the environmental toxicants in our CL01 cell model.

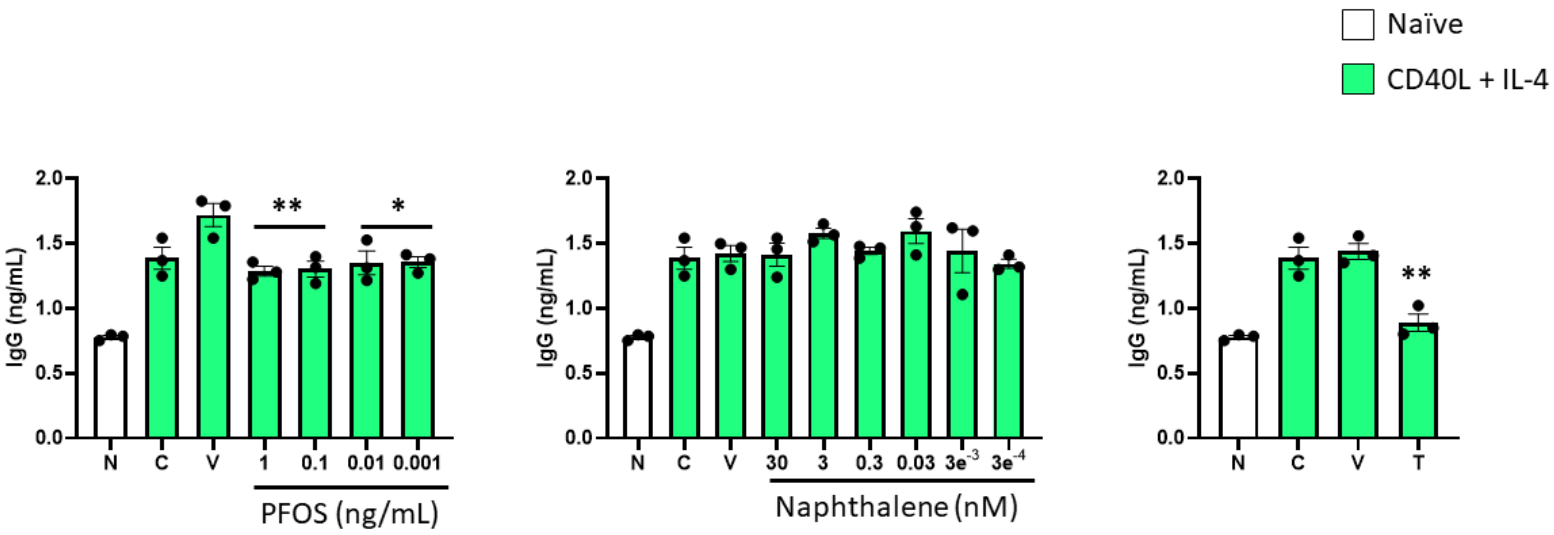

Figure 12. PFOS and TCDD Reduces IgG Production. CL01 cells were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and either treated with vehicle (1% MeOH, 0.001% MeOH, 0.01% DMSO), various concentrations of PFOS and Naphthalene as indicated on graphs, or TCDD (30nM). IgG was measured by ELISA. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Bonferroni. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Graphs are a representative or two repeats. Graphs show individual points (n=3) and SEM of these individual points.

CRISPR/Cas9 Editing the hs1.2 Enhancer Yields Differential TCDD Sensitivity

As previously discussed, there exists several structural differences between the human and mouse *IGH* gene loci. The most prominent of these differences is the duplication of the 3'*IGH*RR in humans as compared to mice. Furthermore, the hs1.2 enhancer is polymorphic for the ~53bp IS in humans but not mice. There also exists slight differences between the two human hs1.2 enhancers. The 3'*IGH*RR-1 (3'RR-1) IS can be repeated one to four times where the 3'*IGH*RR-2 (3'RR-2) hs1.2 enhancer always contains three or four repeats (Mills et al., 1997; Pinaud et al., 1997).

Previous work in our lab sought to elucidate the regulatory role of the hs1.2 enhancers on Ig production and CSR via CRISPR/Cas9 (Snyder & Sulentic, 2016). They found that specific combinations of hs1.2 IS reduction resulted in differential Ig isotype profiles (Snyder & Sulentic, 2016). However, this work only assed the role of the hs1.2 in B-cell stimulation. As previously mentioned, the hs1.2 IS contains several AHR DRE binding motifs. Editing the hs1.2 IS may results in reduced number of DREs, which in turn could modulate the response of Ig production in the presence of TCDD. Here we survey a few of these previously generated clones and asses IgG secretion profiles in the presence of TCDD.

From the genotyping experiments, PCR of the hs1.2 in the 3'RR-1 does not yield bands in clone 2-F3 while the 3'RR-2 does not seem to have been edited (figure 12). This would indicate that at least a partial genomic deletion occurred by 5' resection near the induced double stranded break induced in the 3'RR-1 hs1.2. When challenged with TCDD there was no significant difference between this condition and its corresponding

vehicle control. Addition of an AHR antagonist (AHRA) however resulted in significantly elevated IgG production and even more so when AHRA was combined with TCDD and Comparatively, in the CL01 cells, AHRA also significantly increased IgG production, but this effect was blunted when TCD was cotreated with AHRA.

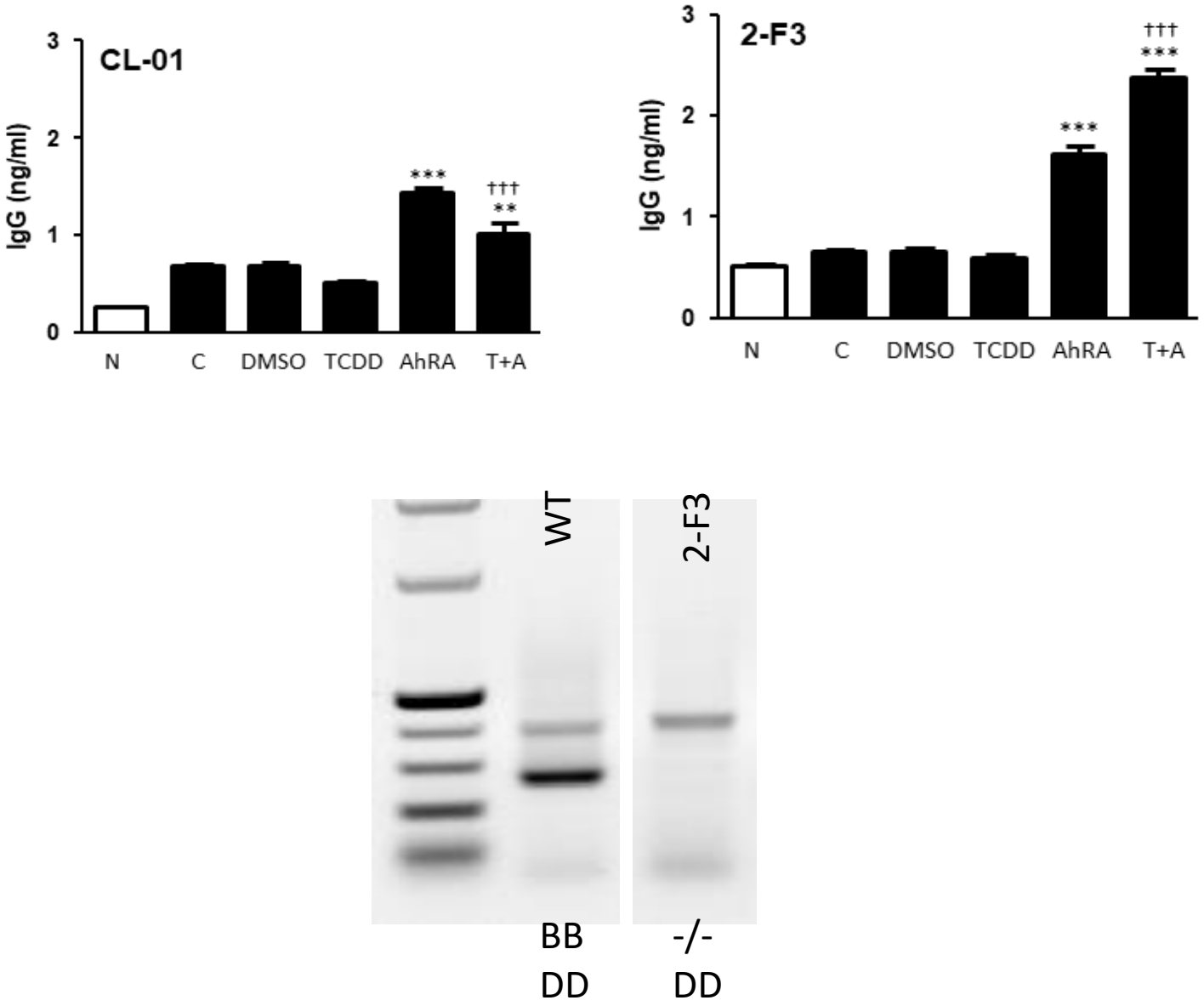

Figure 13. CRISPR hs1.2 Clone 2-F3 has a Deletion in the RR-1. CL01 wildtype cells and hs1.2 clone 2-F3 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and either treated with vehicle (0.01% DMSO), TCDD (30nM), AhR antagonist (AhRA, 10uM), or a co-treatment of TCDD and AhRA (T+A). IgG was measured by ELISA. The genotype of the hs1.2 enhancers in the modified clones versus wild type CL-01 cells. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three, 264 bp for two, and 209 bp for one. IgG graphs show a representative of three repeats. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Bonferroni post-test for IgG secretion. One, two, or three asterisks (*) denote significance compared to the corresponding DMSO control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Three (†) denote significance compared to the corresponding AHRA alone at $p<0.001$**.**

The hs1.2 edited clone 1-A6 appears to have the same genotype (BB/DD) as the CL01 parent cell line (figure 13). However, the IgG profile in naïve cells versus CD40L and IL-4 stimulated cells is dramatically different as compared to CL01 cells. Basal IgG secretion is lost in 1-A6 and stimulated IgG secretion is slightly diminished as compared to CL01 cells. Like clone 2-F3, IgG secretion is increased when treated with AHRA. In contrast to clone 2-F3, cotreatment of AHRA and TCDD does not increase or decrease IgG secretion. Although clone 1-A6 seems to share a genotype via PCR analysis, these results indicate that a small INDEL must be present within the clones hs1.2 enhancer. If so, this indicates that such a small edit could have a profound effect on tolerance to exogenous chemicals and overall regulation of Ig production. Determination of hs1.2 edits is still underway. As will be discussed later in this work, conventional 3rd generation short read sequencing is unable to provide consensus sequences for this polymorphic and variable region. Studies utilizing long-read single-molecule sequencing are currently ongoing.

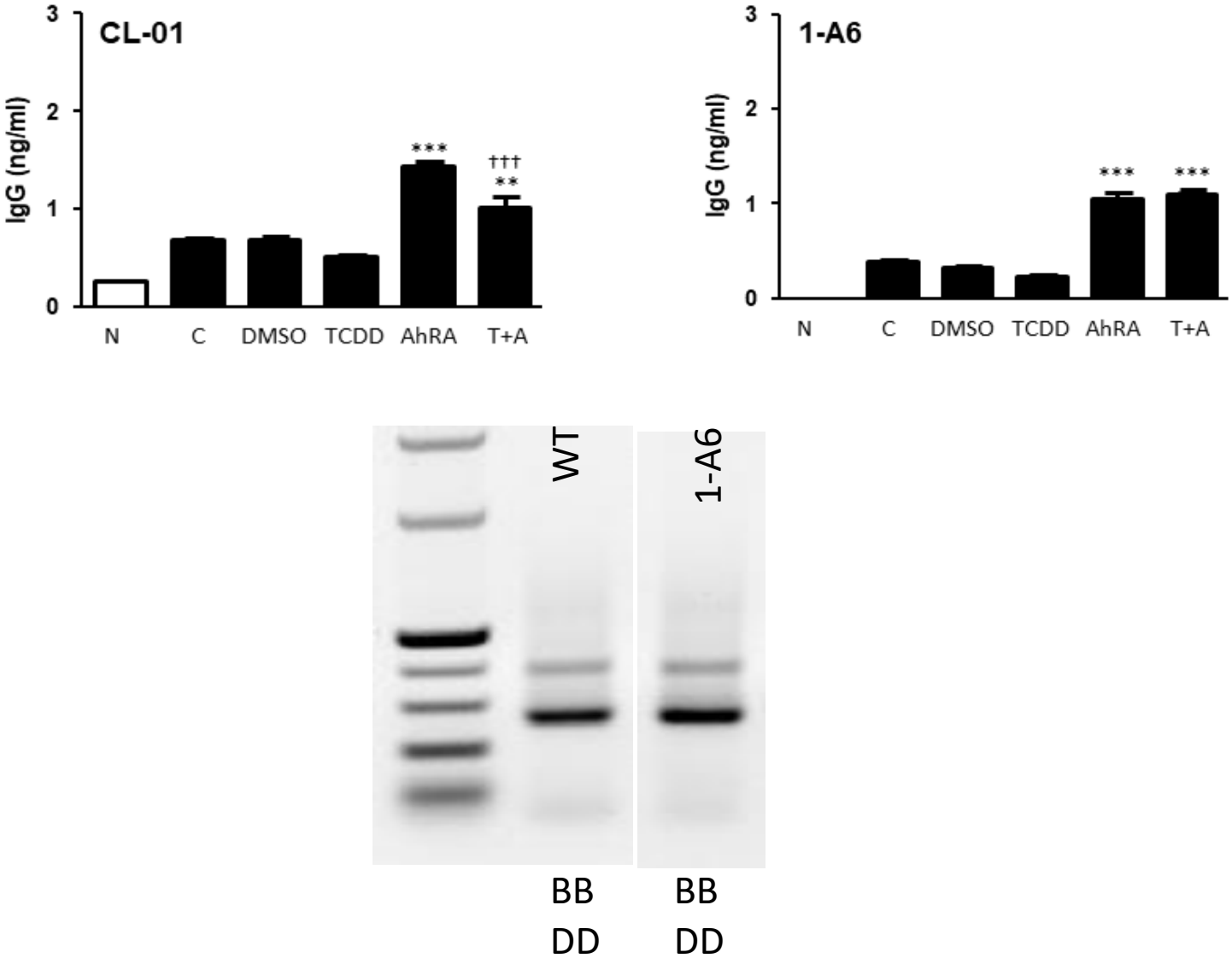

Figure 14. CRISPR hs1.2 Clone 1-A6 has a similar Genotype to WT Cells. CL01 wild type cells and hs1.2 clone 1-A6 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and either treated with vehicle (0.01% DMSO), TCDD (30nM), AHR antagonist (AhRA, 10uM), or a co-treatment of TCDD and AhRA (T+A). IgG was measured by ELISA. The genotype of the hs1.2 enhancers in the modified clones versus wild type CL-01 cells. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three, 264 bp for two, and 209 bp for one. IgG graphs show a representative of three repeats. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Bonferroni post-test for IgG secretion. One, two, or three asterisks (*) denote significance compared to the corresponding DMSO control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Three (†) denote significance compared to the corresponding AHRA alone at $p<0.001$**.**

Clone 2-F11 is unique in that it has been edited in both 3'RRs. In the 2-F11 clone the 3'RR-1 band (lower band) runs at roughly 209bp, which is indicative for one hs1.2 IS repeat (AB). However, the upper band for the 3'RR-2 is unique in that it is lies between the predicted genotyping for two and three IS repeats as compared to the CL01 parent cells. Basal IgG secretion was lower in 2-F11 cells as compared to CL01 cells. Interestingly, AHRA treatment resulted in slightly higher IgG secretion. It appears though that 2-F11s do not produce as much IgG when stimulated when compared to the parent CL01 cells. This clone does maintain the trend of increased IgG secretion in the presence of AHRA and this response is abrogated in cotreatment with TCDD. These findings are interesting, as they point to the effect that small changes in the hs1.2 IS repeats can play in IgG production as compared to the other two clones surveyed here.

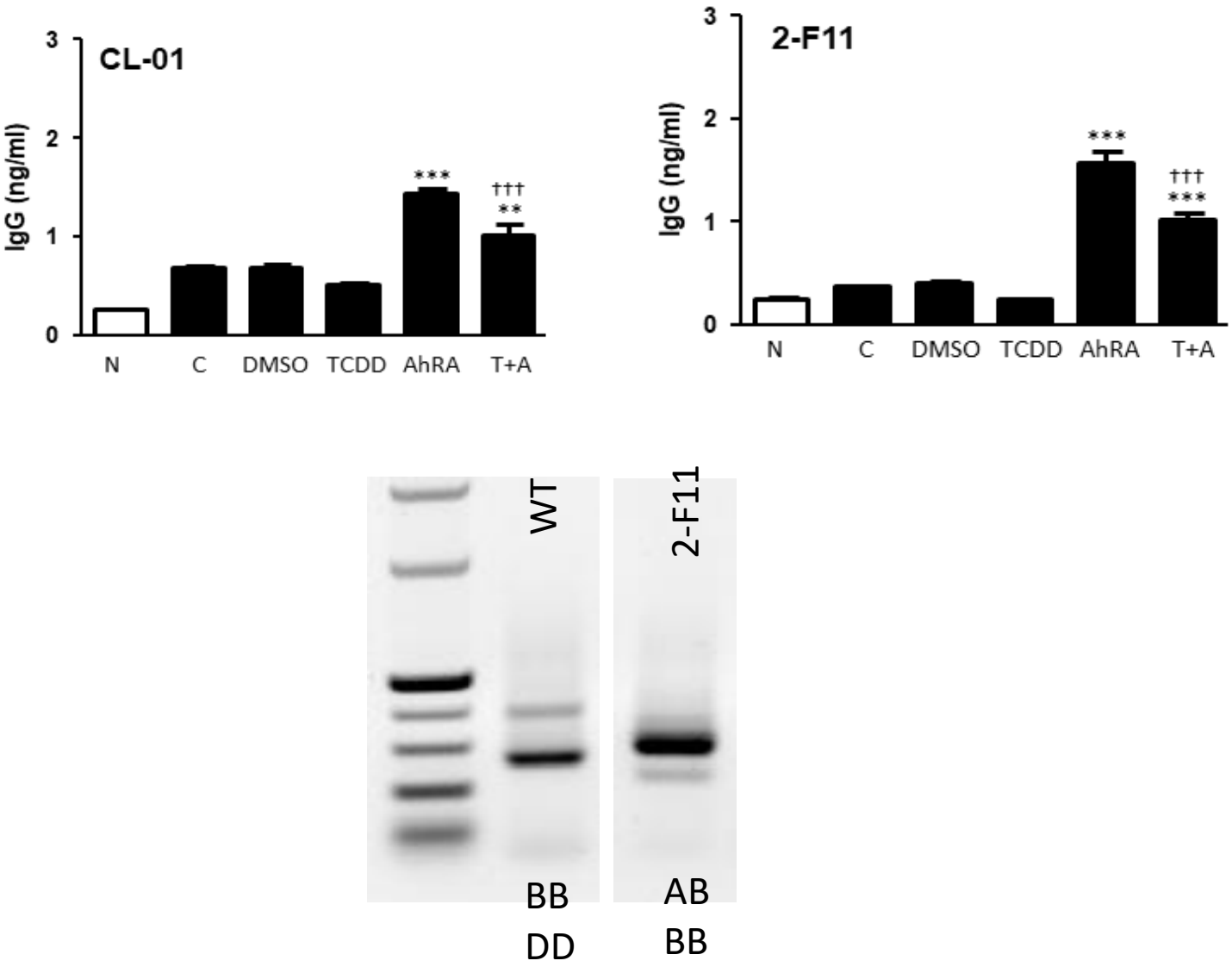

Figure 15. CRISPR hs1.2 Clone 2-F11 has an Unexpected Genotype. CL01 wild type cells and clone 2-F11 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and either treated with vehicle (0.01% DMSO), TCDD (30nM), AHR antagonist (AHRA, 10uM), or a co-treatment of TCDD and AhRA (T+A). IgG was measured by ELISA. The genotype of the hs1.2 enhancers in the modified clones versus wild type CL-01 cells. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three, 264 bp for two, and 209 bp for one. IgG Graphs show a representative of three repeats. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Bonferroni post-test for IgG secretion. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Three (†) denote significance compared to the corresponding AhRA alone at $p<0.001$.

Short Read Sequencing is Insufficient for Determining hs1.2 Genotype

Although simple genotyping was possible with PCR amplification, we wished to genetically characterize the hs1.2 enhancers using our WGS data. The 3'*IGH*RRs are both roughly 20kb in length. The intervening sequences between the three enhancers (hs3, hs1.2, hs4) are comprised of semi-palindromes and simple nucleotide repeats. Using short read sequencing resulted in less than desirable results. Assembly and alignment with these reads were inconclusive due to poor mapping quality and fragmented contig generation. As an example of these, we compared the AHR gene to the hs1.2 site in figure 15. In simple protein coding genes like the AHR sequence coverage was above all mapping thresholds and provided consensus consistent with the human hg38 reference. However, the hs1.2 however did not map within threshold parameters and did not provide a consensus sequence within confidence parameters. This points to the need for single molecule/long read sequencing methods to fully understand the edits made within the hs1.2 edited clones as short read sequencing technology cannot properly sequence this highly repetitive and palindromic region.

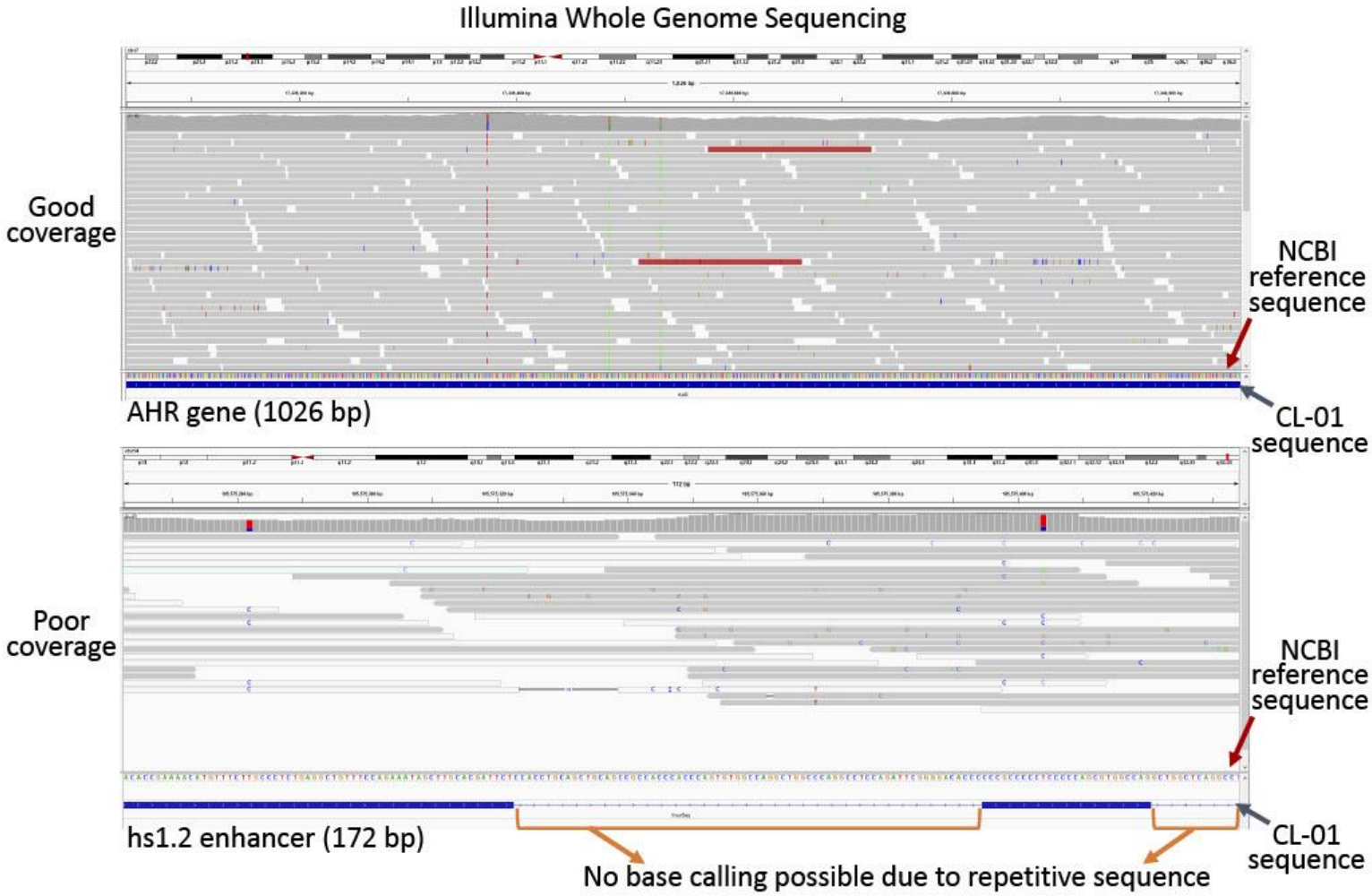

Figure 16. Comparison of hs1.2 Gene Mapping with the AHR. 3rd generation short read sequencing does not provide accurate coverage of the hs1.2 enhancer. For comparison, the hs1.2 and AHR regions were mapped to the hg38 human reference. Aligned reads are shown as gray horizontal bars.

IV. Discussion

In general, the regulation of the human *IGH* gene is poorly misunderstood. A wide breadth of work has been done in murine B cells on regulation of the *IgH*, which has been shown to have a direct regulation of the upstream elements of the *IgH* gene (Pinaud et al., 2001, Vincent-Fabert et al., 2010). The duplication of the human 3'IGHRR in combination with the polymorphic hs1.2 enhancer has made mechanistically understanding human *IGH* regulation hard to study. Furthermore, elucidating the complex relationships between the human gene and environmental exposure were studied. The work presented here was focused on model development to allow for future studies that will elucidate aimed the interplay between the hs1.2 polymorphism, the AHR, and exposure to environmental toxicants.

Our lab has previously tried to understand the relationship between the AHR and the hs1.2 via TCDD in human antibody production. In their study, IgM production was not changed, but IgG production was significantly reduced (Burra & Sulentic, 2015). To further test the effects of the AHR Ig production, knockdown of the AHR gene in CL01 cells by both shRNA and CRISP/Cas9 resulted in a significant decrease in IgG production (Alhmadan & Sulentic, 2017; Kashgari & Sulentic, 2015). Although these results are profound, these studies were done in one cell line, human Burkitt's lymphoma B cell line, CL01. To validate these results, another B cell line (SKW6.4) that does not express AHR was used to validate these original studies (Bhakta & Sulentic, 2020). The SKW6.4 cell line did not produce IgG under CD40L and IL-4 stimulation, suggesting that the AHR plays a physiological role in Ig production.

The AHR is polymorphic in the human population, with different SNPs being reported to have a wide arrange of effects depending on the SNPs location and the tissue type (Aftabi et al., 2021). As stated previously, our lab generated several AHR knockout (KO) clones to study the physiological role of the AHR on Ig production (Alhamdan & Sulentic, 2017). Although these clones initially had little to no AHR expression via western blot, one clone began expressing the AHR again over time. This clone resulted in higher AHR activity compared to CL01 parent cells via DRE luciferase reporter studies. These results suggested a monoallelic edit within the AHR that resulted in the increased AHR activity. To better understand both the parent CL01 cell line and the potential off-target edits we made within the TAD^{+} cell line, we performed whole genome sequencing on both cell populations. Each sequencing experiment resulted in a greater than 30X theoretical coverage of the genome, which is more than suitable for high fidelity variant calling.

Reproducibility and replication of analysis is critical for the rigor of research. To ensure this standard was met, we first produced an analysis pipeline in Snakemake (figure 6) (Mölder et al., 2021). One of the first questions we asked from this data was what the variant profiles looked like in both cell populations. Furthermore, we expected that the TAD^{+} clone variant profile should be almost identical to CL01 profile, as the TAD^{+} clone originated from the CL01 cells. After running the analysis pipeline (figure 6), we obtained variant call files with parameters to gate for only high confidence variants (see methods). Pairwise comparison based on variants genomic coordinates revealed that most variants overlapped with one another (figure 7). However, there was a small portion of variants that did not overlap based on genetic coordinates. Possible reasons

for this were two-fold. First, the parent cell line, CL01 cells are a cancerous cell line that was originally isolated from a Burkitt's lymphoma patient (Cerruti et al., 1998). Cancerous cell lines have altered regulation within multiple families of genes that can add to the load of structural variants that are present. Additionally, repeated subculturing over long periods of time may allow edits to form, though the nature and impact of these edits on cellular function can be silent. The second reason for the observed difference in variant profiles is the TAD^{+} clone was isolated from a monoculture during the initial editing experiments while CL01 cells are a homogenous population that have been sub-cultured over a long period of time. This could have resulted in specific variants being selected for during isolation of this clone.

From these called variants, we wished to understand the potential consequences of each variant. To accomplish this, use of the ensemble variant effect predictor was utilized on the overlapping SNPs within both cell populations (figure 7). Predicted consequence of these edits are found mostly within intronic portions of genes, upstream and downstream of genes, and are non-coding transcript variants (figure 7). The predicted consequences of these variants are largely in-frame deletions and frameshift variants. Although these have the possibility for causing deleterious effects, most of these edits resided in non-coding regions of the genome and in pseudo genes.

Whenever performing genomic editing, a concern is the presence of off target editing that could cause deleterious effects. Our whole genome sequencing data of both parent cell line and edited clone provided an excellent chance to study the potential for off-target editing within our generated clone. Use of a predictive software (Bae et al., 2014) produced coordinates for all potential off target editing events. By matching up

the coordinates from the predicted edits to our called variants, we first were able to determine that no variants were present within the non-edited CL01 cells. Fortunately, the TAD$^+$ line that was edited also had no corresponding variants within predicted off target sites. This provides higher confidence in the data produced by this CRISPR edited clonal population.

As previously stated, the AHR is polymorphic in humans, and we identified three SNPs within exon 10 which encodes for the TAD: R554K, V570I, and P517S. However, the allelic distribution of these three SNPs within the CL01 cells was unknown. Fortunately, these three SNPs lay within ~160bp of each other, which means that the chance of NGS reads containing two of the SNPs is high. Using this, we subset the reads within this region and built a network of which reads contained one or two of the SNPs. Unfortunately, no reads contained all three SNPs. However, all subset reads always contained two of the SNPs on a single read if the SNPs were within range of each other. Because of this, we are confident that all SNPs are located on one allele, (figure 9). Work previously done in our lab confirms the functional difference due to heterozygous nature of the TAD in CL01 cells as evidenced by increased DRE luciferase reporter activity in the edited TAD$^+$ clones as compared CL01 parental cells (Nasser & Sulentic, 2017).

Experiments in primary human B cells has shown a TCDD induced reduction of IgM via the AHR (Lu et al., 2010). This reduction however is variable in primary cells. One reasoning for this variability is due to the polymorphism of the AHR in different human populations, to confirm these results, Kovalova et al. tested the effects of the previously mentioned SNPs in another human B lymphocyte line (Kovalova et al.,2016).

Their work demonstrated that the three SNPs were needed in tandem to abrogate TCDD induced inhibition of IgM, but only the presence of R554K was enough to alter sensitivity to TCDD in the human B cell line (Kovalova et al., 2016).

The human hs1.2 IS polymorphism contains several transcription factor binding sites, including a putative DRE binding site (figure 4). Repeats of the IS occur one to four times in the human population, with the CL01 cell lines containing a two repeat in the 3'RR-1 and a four repeat in the 3'RR-2. Variable numbers of repeats could result in increased or decreased sensitivity to TCDD due to the DRE motifs. Our lab utilized CRISPR/Cas9 sequencing again to edit our CL01 cells, but targeted the hs1.2 IS (Snyder & Sulentic, 2016). The resulting clones showed modulated IgG secretion and *IGH* constant region transcription (Snyder & Sulentic, 2016). To test if the repeats had increased or decreased susceptibility to TCDD and AHR activation, we treated these clones for 96 hours with CD40L and IL-4 stimulation. We also applied an AHR antagonist in conjunction with TCDD treatment.

All three clones tested: 1-A6, 2-F3, and 2-F11 have modulated IgG responses in the presence of TCDD and stimulation. Clone 2-F3, which appears to have a deletion of the entire 3'RR-1 hs1.2, did not exhibit decreased sensitivity to TCDD via IgG secretion (figure 12). Interestingly, both AHRA treatment and co-treatment with TCDD and AHRA resulted in significantly higher IgG production in both the CL01 cells and the hs1.2 edited clones. The increase in IgG production in the presence of AHRA is a trend that is kept between the other two clones, 1-A6 (figure 13) and 2-F11 (figure 14). Clone 1-A6 does not appear to have been edited by our genotyping experiments. However, here we see a loss of basal IgG as compared to CL01 control. Finally, clone 2-F11s genotype

showed editing within both 3’RRs (figure 14) decreasing the 3’RR-1 to have allele A and the 3’RR-2 to have allele B. The IgG production profiles though mimic CL01s, and again display an increase in production in the AHRA treated condition.

Addition of the AHRA increasing IgG production has been previously observed in our lab (Kaulini & Sulentic, 2015). Antagonism of the AHR sequesters it in the cytoplasm, which should prevent the canonical AHR signaling pathway. Studies have suggested that the AHR can work in conjunction to other signaling pathways (Wright et al., 2020). A murine macrophage study found that induction of IL-22 was increased by AHR activation via TCDD (Ishihara et al., 2022). The murine IL-22 gene contains three DRE binding sites, a NF-kB binding site, and a RelB/AHR responsive element. Deletion of the NF-kB site decreased IL-22 promoter activity but did not change TCDD-induced AHR activity (Ishihara et al., 2022). Furthermore, mutations of the first two DREs had no change in IL-22 promoter activity, but mutation of the third DRE and RelB/AHR responsive element did significantly decrease IL-22 promoter activity. Another study observed a synergistic regulation of the CYP1A1 gene through recruitment of SP1 in human cells (Ye et al., 2019). The DRE within the hs1.2 enhancer IS is proximal to an SP-1.1 binding motif. These studies suggest that the AHR may play a regulatory role that is either synergistic with other signaling pathways or involves direct recruitment of other TFs with close by TF binding sites.

Understanding how other classes of environmental toxicants impact human B lymphocytes and immunoglobulin production is an area of ongoing study.
Our experiments in the hs1.2 edited clones demonstrated a modulated IgG response in the presence of TCDD. To further understand how a diverse range of environmental

toxicants effect immunoglobulin production, two other environmental toxicants we examined the effect of PFOS and Naphthalene on Ig production. As previously discussed, these two chemicals are prevalent in the environment and have both been associated with impairment of immune function. Both exhibited modulated Ig responses dependent on the concentration and Ig isotype. We first measured total IgM secretion, which did not result in significant change (figure 10). This is not abnormal for this cell line, as it is a naïve, mature B cell, meaning that when stimulated, it should switch from IgM to a downstream constant region. In contrast to IgM, IgA production was significantly impacted when challenged by all three toxicants (figure 11). Overall, a decrease was observed in IgA production when challenged with PFOS and Naphthalene. Reduction of IgA can potentially result in reduced protection of the mucosal surfaces and lead to increase susceptibility to infection. Finally, PFOS and TCDD produced a significant reduction to IgG (figure 12). We have previously seen reduction of human Ig via TCDD in our lab and the literature (Kavolova et al., 2016; Nasser & Sulentic, 2017). This reduction in Ig through TCDD is mediated by the AHR. However, PFOS and Naphthalene have not been reported to activate the AHR pathway. In the literature, PFOS has been associated with diminished serum Ig levels in exposed children (Grandjen et al., 2012).

The method of action in for these chemicals is also sparsely understood. PFOS has been previously reported to activate PPAR, a ligand activated transcription factor that function in insulin sensitization and glucose metabolism (Tyagi et al., 2011). Overall reduction in antibody concentrations is concerning for human health. These

reductions potentially increased risk of infection and maintenance of long term immunity.

The work presented here accomplishes three things. Firstly, we show that the CL01 cell line as a cancerous cell type does not harbor any variants that could potentially confound results produced by this cell line. This provides confidence in working with this cell line as a model for studying human Ig production. We also show that CRISPR/Cas9 editing in this results in precise editing with appropriate guidance RNAs. Secondly, we demonstrate that small changes in the hs1.2 enhancer can impact the effect of AHR ligands and antagonism of Ig production. Significantly, small edits within the hs1.2 results in modulated Ig response both in the presence of the AHR and in stimulation alone. Finally, we show that Ig production of specific isotypes in a human mature B cell can be decrease in the presence of other classes of environmental toxicants. Future work should be done to see how the different genotypes of the hs1.2 enhancer change Ig production in the presence of these toxicants. Additionally, understanding the precise edits through long range sequencing within the hs1.2 enhancer using single molecule sequencing, which is currently underway, is integral in elucidating the relationship between the hs1.2 polymorphisms and their role in human *IGH* regulation.

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