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Potential Role of AhR in Antibody Production

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POTENTIAL ROLE OF AhR IN ANTIBODY PRODUCTION

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ABSTRACT

Bhakta, Mili. M.S., Microbiology and Immunology Graduate Program, Wright State University, 2020. Potential role of AhR in antibody production.

Aryl hydrocarbon receptor (AhR) mediates the immunosuppressive effects of 2,3,7,8 -tetrachlorodibenzodioxin (TCDD) in murine B cells. The effects of AhR activation on the regulation of expression of human immunoglobulin isotypes (μ, γ1-4, α1-2 and ε) and Ig secretion is unclear. Our previous results using CL-01 cell-line originating from a Burkitt's lymphoma patient, demonstrated an inhibitory effect of TCDD on IgG expression but a surprising and marked loss of IgG secretion when the AhR was knocked out by siRNA or CRISPR/Cas9 gene editing. To determine if the AhR is a critical mediator of IgG expression, current study is focused on characterizing IgG expression in another human B-cell line (SKW 6.4 or SKW WT) originating from a different, non-related Burkitt's lymphoma. We confirmed that SKW WT cells do not have endogenous expression of AhR using PCR analysis and Western blotting. We also demonstrated that SKW 6.4 cells can be stimulated in-vitro using CD40L and IL-4 to produce more IgM antibodies as detected by ELISA assays. Further, we demonstrate that total IgG secretion induced by CD40L and IL-4 stimulation is severely impaired in SKW WT cells. Conversely, the Qt-PCR studies show that the expression of ε, γ2-4 transcripts that code for IgE and IgG2-4 respectively is significantly increased with stimulation as compared to

un-stimulated SKW cell lines. The expression of γ1 was low in naïve as well as stimulated SKW WT cells. The α1-2 transcripts coding for IgA1-2 respectively are not expressed at all in SKW cells regardless of stimulation. To further investigate, we used CL-01 AhR^{TA} cells that express AhR with functional TAD, to compare the expression of different isotypes. It was found that the expression of γ1-4 and ε transcripts was significantly higher in AhR expressing CL-01 AhR^{TA} cells as compared to SKW WT cells. Our observations imply that AhR plays a critical role in expression of the *IgH* gene.

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INTRODUCTION

Immune System is body's defense mechanism

The immune system is a defense mechanism of mammalian bodies that recognizes and provides protection against bacteria, fungi, viruses and other infectious agents. The immune response is multilayered and improves gradually as the infection proceeds. The two main phases of the immune response are the innate immune response and adaptive immune response. Although both innate and adaptive immune responses are distinct in function, there is a lot of coordination and interaction between the two responses. The innate immune system is a well-organized first line of defense that prevents the entry of foreign particles/pathogens by providing physical barriers (such as skin and epithelium linings in gut and nasal tract) and chemical barriers (anti-microbial proteins continuously secreted in the gut). Pathogens that enter the body by evading the physical and chemical barriers of the innate immune system, are recognized by the innate immune cells as non-self or foreign substances. Certain molecules present on the surface of the pathogens are recognized as characteristic pathogen-associated molecular patterns (PAMPs) by the pattern recognition receptors (PRRs) present in innate immune cells. Once the pathogen is recognized as "foreign", phagocytes and natural killer (NK) cells elicit a quick immune response at the site of infection. The innate immune response is not antigen specific and the response remains the same against all kinds of pathogens. If the innate immune

response fails to clear the infection, adaptive immunity is stimulated by effector cells and cytokines from the innate immune response (Turvey & Broide, 2010).

The adaptive immune response is antigen-specific and involves cell-mediated immunity and humoral immunity. Cellular immunity is mediated by T lymphocytes or T cells. T cells are activated by the recognition of antigen presented by the major histocompatibility complex (MHC) molecules on other cells of the body. T cells proliferate and differentiate in the thymus to generate different effector T cells with unique functions. The CD8+ (cytotoxic) T cell recognizes intracellular pathogens for instance, viruses that replicate inside of a host cell. The infected host cells present a portion of the virus via the MHC type I complex expressed on the surface. The T cells that are activated by the same antigen recognize the antigen and MHC type I complex via the T cell receptor (TCR). This interaction triggers proliferation and differentiation to effector cytotoxic T cells, which destroy the host cells bearing the intracellular pathogen and thus successfully eliminate the source of infection. The CD4+ (helper) T cells recognize the MHC type II complex expressed on antigen-presenting cells such as dendritic cells, macrophages and B cells. Activated T helper cells not only influence the type of adaptive immune response to be stimulated for a distinct pathogen but also help other lymphocytes to carry out their effector functions, one example is amplifying the phagocytic activity of macrophages (Kumar, Connors, & Farber, 2018). Adaptive immunity provides long term immunity by generating long-lived memory cells against each antigen encountered. If the host gets infected by the same pathogen again, these memory cells rapidly become activated to generate a secondary immune response which is very vigorous and rapid at clearing the infection.

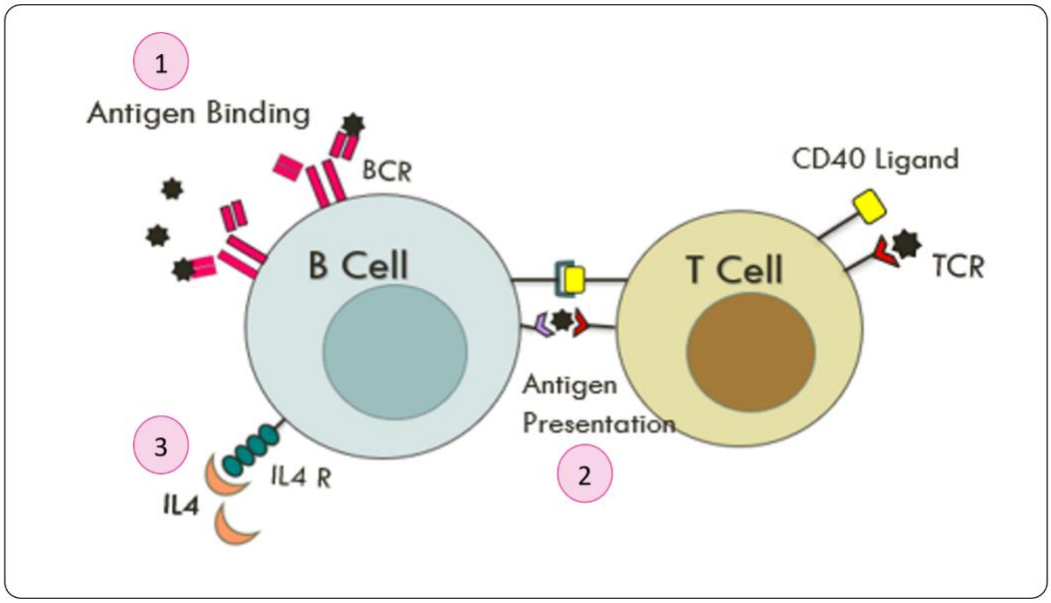

Figure 1: B cell Stimulation via BCR, IL-4 and CD40 Ligand.

The B cells (blue) are stimulated in various ways: 1) binding of antigen to the B-cell receptor (BCR), 2) antigen presentation by B cells and interaction with T cells (peptide loaded MHC on B cell interacts with TCR on T cell), 3) IL-4 secreted by activated T cells and CD40L on T cells binding to CD40 on B cells.

B cells undergo several stages before differentiating to antibody secreting plasma cells

Humoral immunity is mediated through antibodies secreted by B cells. B cells are derived from hematopoietic stem cells (HSC) in the bone marrow. The milieu in the bone marrow directs the sequential development of B cells from HSC (De Grandis, Lhoumeau, Mancini, & Aurrand-Lions, 2016). Naïve mature B cells can be stimulated to proliferate and differentiate into plasma cells committed to producing antibodies against a specific antigen. Naïve B cells circulating in blood migrate to secondary lymphoid tissues, like the spleen, where B cell receptor (BCR) on B cells recognize an antigen by one of the two ways. First, by binding of the antigen itself to the BCR or second, by recognition of antigen presented by dendritic cells. The BCR usually binds to a specific portion of the antigen called an epitope. The thymus independent (TI) antigens can stimulate a B cell response without the help of T cells by extensively cross-linking the BCRs due to displaying repeating epitopes, for instance, polysaccharides on the bacteria. Sometimes, B cells require help from T-helper cells to stimulate the response against certain antigens, such antigens are termed as thymus dependent (TD) antigens.

B cells internalize the TD antigen and degrade the antigen by proteolytic enzymes. The peptide portion of the antigen is presented to the T helper cells via MHC type II, triggering the expression of surface and secreted effector molecules by T cells that 3'stimulate B cells to proliferate and differentiate. For instance, firstly, the upregulation of CD40 ligand (CD40L) on the surface of T cells that interact with CD40 receptor on B cells. Secondly, the upregulation of IL-4 secretion. IL-4 acts in synergy with CD40L-CD40 interactions and induce B cell to undergo cell proliferation and produce antibody (fig. 1). The clonal expansion of B cells occurs at the border of the T-cell zone in lymphoid tissue

and generate ‘germinal centers’ with a few T helper cells and a cluster of B cells. After several rounds of the cell cycle, B cells undergo class switch recombination to change the antibody isotype; and somatic hypermutation further modifies the antigen binding pocket of the antibody (fig.3).

The affinity of antibody for the antigen is continuously tested in the germinal centers and only the B cells producing high affinity antibodies can survive, this process is called ‘affinity maturation’. B cells expressing high-affinity antibody are preferentially selected for proliferation and differentiation. The B cells leaving the germinal centers are completely differentiated into plasma cells or memory cells. Plasma cells are short lived and return to the bone marrow and continue to secrete antibodies. Memory cells circulate in the blood waiting for a subsequent exposure to the antigen upon which they will generate a rapid and highly efficient secondary immune response.

Antibodies have generic structure

Immunoglobulins or antibodies secreted by B cells are effector protein molecules that mediate the humoral immune response. Antibodies bind to epitopes on the surface of antigens or pathogens to either neutralize or opsonize the pathogen to clear it from the body. Antibodies are Y shaped proteins consisting of two identical heavy chains linked to each other by a covalent disulfide bond and two identical light chains linked to each heavy chain via disulfide bonds (fig. 2) The amino terminal of each heavy chain and light chain has a protein domain with a variable amino acid sequence, which forms the antigen binding pocket at the ends of the arms of the Y-shaped antibody. The variability of this ‘variable’

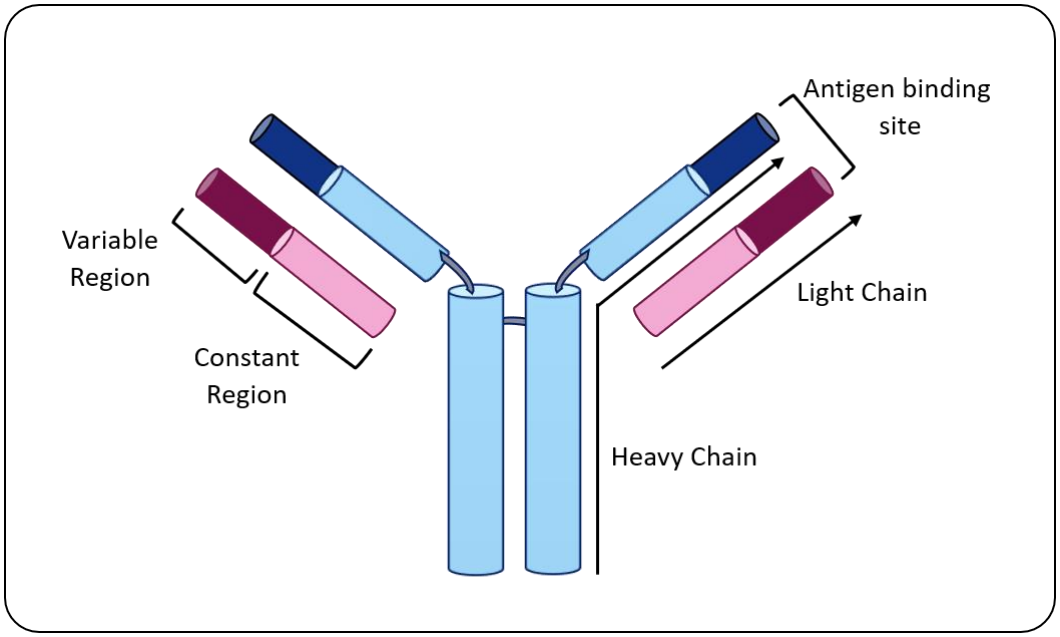

Figure 2. The structure of an Immunoglobulin

Immunoglobulin or antibody consists of two light and two heavy chains. Each chain has a constant region, which mediates the antibody effector functions, and a variable region, which forms the antigen binding site.

sequence is a result of a recombination phenomenon discussed later. Because of the high variability of the antigen binding domain, B cells can generate diverse antibodies to target a broad spectrum of epitopes on infectious agents and toxic compounds. The rest of the heavy chain is comprised of three to four protein or Ig domains with relatively conserved amino acid sequence that form the stem of the Y-shape of the antibody, which is also known as constant (C_H) region or the F_C fragment.

The F_C fragment interacts with other immune cells, contributing to the effector functions of an antibody. The C_H region expressed from the Ig heavy chain (*IGH*) gene locus determines the isotype of the antibody. There are five isotypes/classes of antibody: IgM, IgD, IgG, IgA and IgE. Each isotype is structurally and functionally distinct. The Ig light chain comprises of only one C_L region which is coded either by the κ (*IGK*) or λ (*IGL*) gene locus. Each of the Ig light chain gene loci and the Ig heavy chain gene locus cannot be expressed in their germline configuration. The germline configuration of these genes is in a fragmented form, meaning each gene is segregated into families of 'gene segments', which require one or two recombination events to put together these fragments or gene segments to create a continuous coding joint for successful expression (fig. 3).

VDJ recombination confers diversity to antibody repertoire

The *IGH*, *IGK* and *IGL* gene loci have several variable (V), joining (J) and constant (C) gene segments. The *IGH* locus has additional diversity (D) gene segments. The antigen binding portion of the heavy chain is expressed by linkage of one from each V, D and J

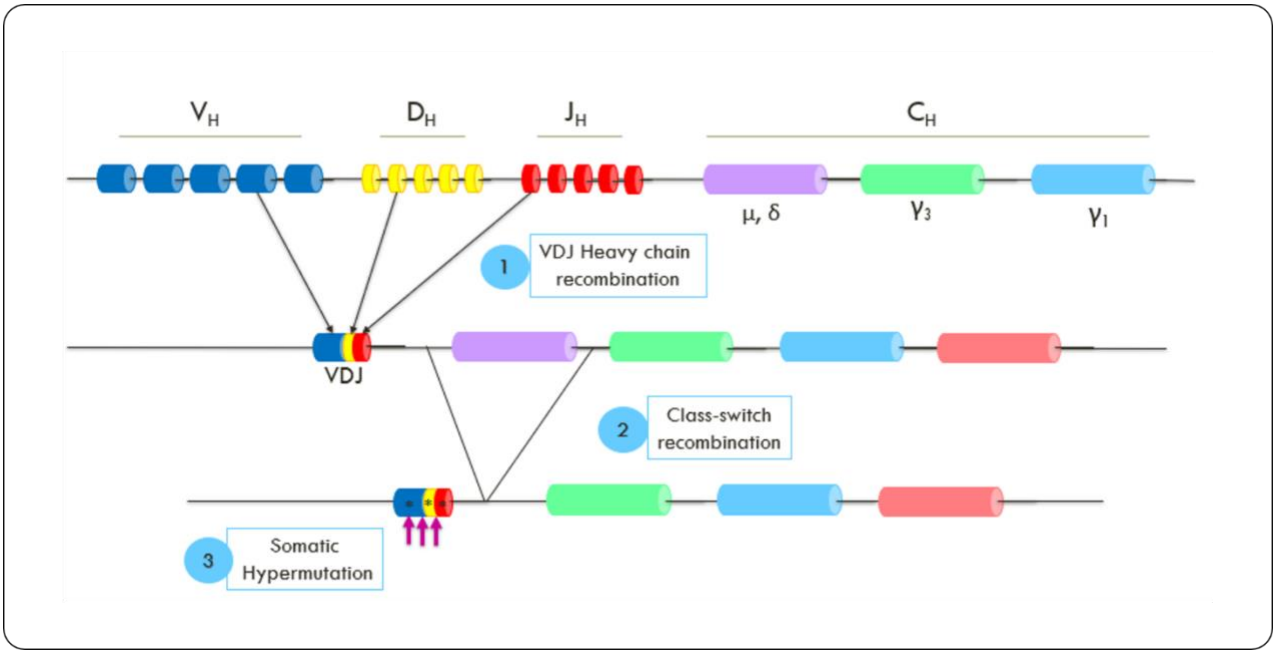

Figure 3: Overview of recombination events at IGH locus.

(1) VDJ recombination at IGH locus to form the coding joint for the variable region of the heavy chain. (2) Class-switch recombination from μ to the γ_3 constant region changes the antibody isotype from IgM to IgG3. (3) Somatic hypermutation involves point mutations within the VDJ region.

gene segments to form a coding joint (VDJ) by a somatic recombination event known as VDJ recombination.

VDJ recombination is a crucial event for survival during the early B-cell developmental stages in the bone marrow. During the very initial stages, pro-B cells express recombination activating genes (RAG) generating the RAG-1 and RAG-2 recombinase proteins, which initiate the somatic recombination process (Mombaerts et al., 1992; Shinkai et al., 1992). These recombinase proteins recognize the recombination signal sequences (RSSs) that are present near each and every V, D and J gene segment. Each RSS is comprised of a heptamer sequence and a nonamer sequence with either a 12bp or 23bp spacer sequence. The recognition of both 12 bp and 23 bp spacers by RAG-1 and RAG-2 is required for signaling the cleavage of the DNA for recombination. According to 'the 12/23 rule', the recombination occurs only between the gene segments with different spacer sequence lengths ensuring the linking of D gene segments to J gene segments and V to DJ linkage (Hiom & Gellert, 1998; van Gent, Ramsden, & Gellert, 1996)

VDJ recombination initiates when the RAG proteins associate with the RSSs of one D and one J segment which are randomly selected and then recruit a group of proteins known as high mobility group (HMG) group proteins that promotes localization of RAG complex at RSS (Mo, Bailin, Noggle, & Sadofsky, 2000; Swanson, 2002). The intervening DNA between the two RSSs is bent to form a loop which eventually is cleaved off by introducing a double strand break within the heptamer sequences of both the RSSs. This double stranded break creates blunt ends of the DNA which are eventually closed by the hairpin loop. The ends of DNA are held together by the RAG complex (McBlane et al., 1995; van Gent, Hiom, Paull, & Gellert, 1997). The two hairpin ends of the DNA are joined

together by non-homologous end joining (NHEJ) using the ubiquitous DNA repair machinery of the cell. Briefly, the artemis protein complexes with DNA-dependent protein kinase (DNA-PK) opens the hairpin structure such that a palindrome sequence is created at the ends. Terminal deoxynuclease transferase (TdT) continues to add new nucleotides to fill the gap and complement the flanking palindrome sequence while the unmatched nucleotides are removed by nuclease. The DNA ligase and several other proteins participate to join the two open ends of the DNA eventually linking the D and J gene segments (Bassing, Swat, & Alt, 2002; Ma, Pannicke, Schwarz, & Lieber, 2002).

During the entire VDJ recombination event, the germline nucleotide sequence at the junction of the gene segments is irreversibly altered, which confers diversity to the antigen binding region and increases the antibody repertoire. First, the selection of V, D and J gene segments at random introduces combinatorial diversity. Meaning, different combinations of V, D and J segments generate different variable region sequences. Second, junctional diversity occurs during the joining of each segment (i.e. D to J and V to DJ). Junctional diversity is added in two ways. Firstly, the nick to open the hair-pin loop can occur at any nucleotide on a strand to create a palindromic sequence, thus altering the original germline sequence by adding nucleotides (P nucleotides) or deleting nucleotides. Secondly, nucleotides are randomly added to the junction by the TdT enzyme (N nucleotides), which adds to the diversity of the sequence. Both P and N nucleotides contribute to the junctional diversity and increases the diversity of the antibody repertoire.

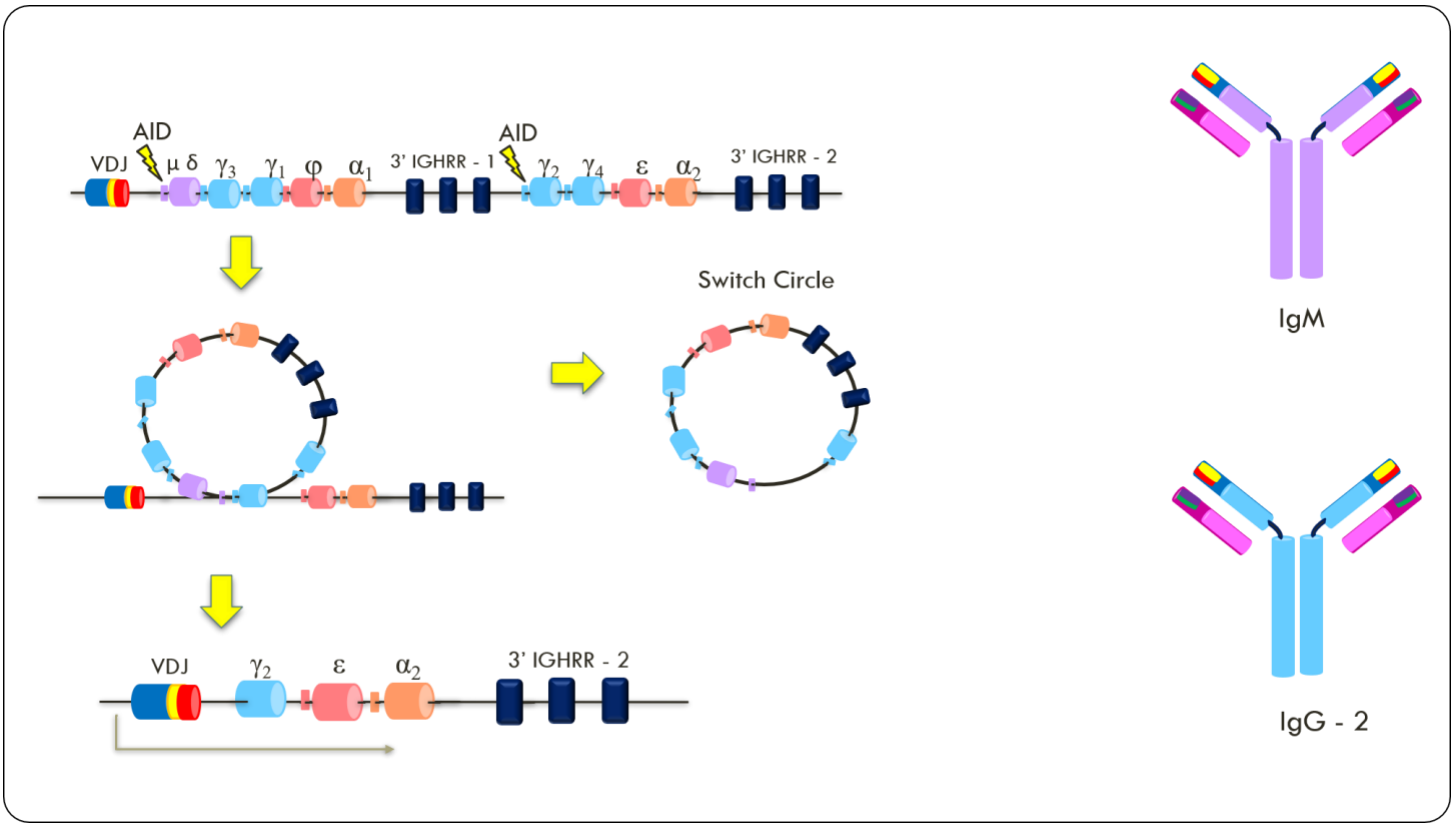

Figure 4: Class Switch Recombination event from IgM to IgG2.

CSR begins with AID mutating the DNA in switch (S) regions followed by the interaction of the switch regions. The Switch Circle is cleaved off bringing the VDJ near $C\gamma_2$ region which codes for IgG2.

Class switch recombination alters the functional properties of antibody

The effector functions of antibodies depend on their constant region, which is encoded by the C_H segments on the *IGH* gene. Naive B cells produce IgM and IgD until stimulated to undergo Class Switch Recombination (CSR) to a different antibody isotype. Class switch recombination alters the constant region and hence functional properties of the antibody, while the VDJ sequence and antigen specificity remains the same. CSR is a DNA recombination event that replaces the Cμ segment adjoining the rearranged VDJ region with a different C_H (i.e. Cγ, Cε, or Cα) segment to code for a different type of antibody (IgG, IgE or IgA respectively).

Each C_H segment is preceded by a switch (S) region and an intronic promoter (I_H). Germline transcription of the C_H segment is induced by stimulation through T-cell derived cytokines, interleukin-4 or by direct interaction between B cells and T cells via CD40 and CD40L (Tangye, Ferguson, Avery, Ma, & Hodgkin, 2002). Germline transcription of each C_H segment results in non-coding sterile transcripts comprised of an intronic promoter, S region, first exon through 3' end of C_H segment (Fear, McCloskey, O'Connor, Felsenfeld, & Gould, 2004; Kim, Lim, Kang, Hillsamer, & Kim, 2005; Lane et al., 1992). Certain ex vivo studies imply that germline transcription plays a critical role in CSR. Transcription through the G-rich portions of the S region generates transiently stable secondary structures of DNA leaving long stretches of DNA single stranded (Chaudhuri et al., 2003; J. Stavnezer et al., 1988; L. Wang, Wuerffel, Feldman, Khamlichi, & Kenter, 2009). Recent studies reveal that post-transcriptional events including the formation of branched DNA structures like R-loop and G-quadruplex are critical in recruiting activation-induced cytidine deaminase (AID) for CSR (Qiao et al., 2017; Zheng et al., 2015). Recruitment of AID

which deaminates the cytidine moieties converting them to uracil is critical for CSR (Muramatsu et al., 2000). Modification of the cytidine nucleotide is recognized by uracil DNA glycosylase (UNG) and mis-match repair mechanism of the cell (Rada, Di Noia, & Neuberger, 2004). The ubiquitous DNA repair machinery of the cell eventually converts the nick to a double stranded break in the switch region sequences preceding the two constant regions, one 5' of Cμ and the other 5' of the target constant region (e.g. $C\gamma_2$) (fig. 4). The intervening DNA is subsequently excised as a switch circle, which results in the joining of the VDJ to the targeted C_H segment (e.g. $C\gamma_2$) forming a new coding sequence (fig. 3). The joining can occur within or near the switch regions by non-homologous end joining (Dunnick, Hertz, Scappino, & Gritzmacher, 1993; Pan-Hammarstrom et al., 2005; Soulas-Sprauel et al., 2007; Janet Stavnezer, Guikema, & Schrader, 2008).

Somatic hypermutation enhances the affinity of the antibody for the antigen

Somatic hypermutation is a process by which the antigen binding region of the immunoglobulin is further diversified by introduction of point mutations in V regions of each heavy and light chain. During cell division, single-stranded DNA within the V region is targeted by AID, which converts any cytidine to uracil by deamination. UNG removes the uracil moiety and DNA polymerases insert any one of the four nucleotides at the site altering the original sequence by one nucleotide change (Maul & Gearhart, 2010). The frequency of SMH is higher in the sequence that codes for the protein domain that directly interacts with the antigen (reviewed in (Papavasiliou & Schatz, 2002)). After undergoing SMH, some B cells express Ig receptors with higher affinity for the antigen. In germinal

centers, the B cells are constantly exposed to antigens and B cells expressing high-affinity antibody for a particular antigen are preferentially selected for proliferation and differentiation into antibody-secreting plasma cells or memory cells. This process of improving affinity of the antibody as the immune response advances is known as affinity maturation.

Germline transcription of *IGH* gene and CSR may be regulated by 3' *IGH* regulatory region

In murine B cells, the assembly of four enhancer regions (hs3a, hs1.2, hs3b and hs4) known as the 3' *Igh*RR is thought to regulate the transcription of *Igh* gene and Ig isotype switching process (Vincent-Fabert et al., 2010). The transcription of seven constant regions is partly regulated by their respective intronic promoters and partly by the 3' *Igh*RR.

There are major differences in mice *IgH* locus and human IGH locus. Primarily, human *IGH* gene have a set of two regulatory regions, each located on the 3' of the $C\alpha_1$ and $C\alpha_2$. Both regulatory regions are almost indistinguishable from one another in terms of the sequence (Mills, Harindranath, Mitchell, & Max, 1997) and are named as 3' *IGH*RR -1 and 3' *IGH*RR -2. Secondly, each 3' *IGH*RR is comprised of three enhancer regions namely: hs3, hs1.2 and hs4. Collectively, these enhancer regions differentially regulate the activity of intronic promoters of each C_H segment and hence their germline transcription (Hu et al., 2000; Pan, Petit-Frére, Stavnezer, & Hammarström, 2000). Lastly, human *IGH* gene comprises of more C_H segments compared to mouse *IgH* gene (fig. 5).

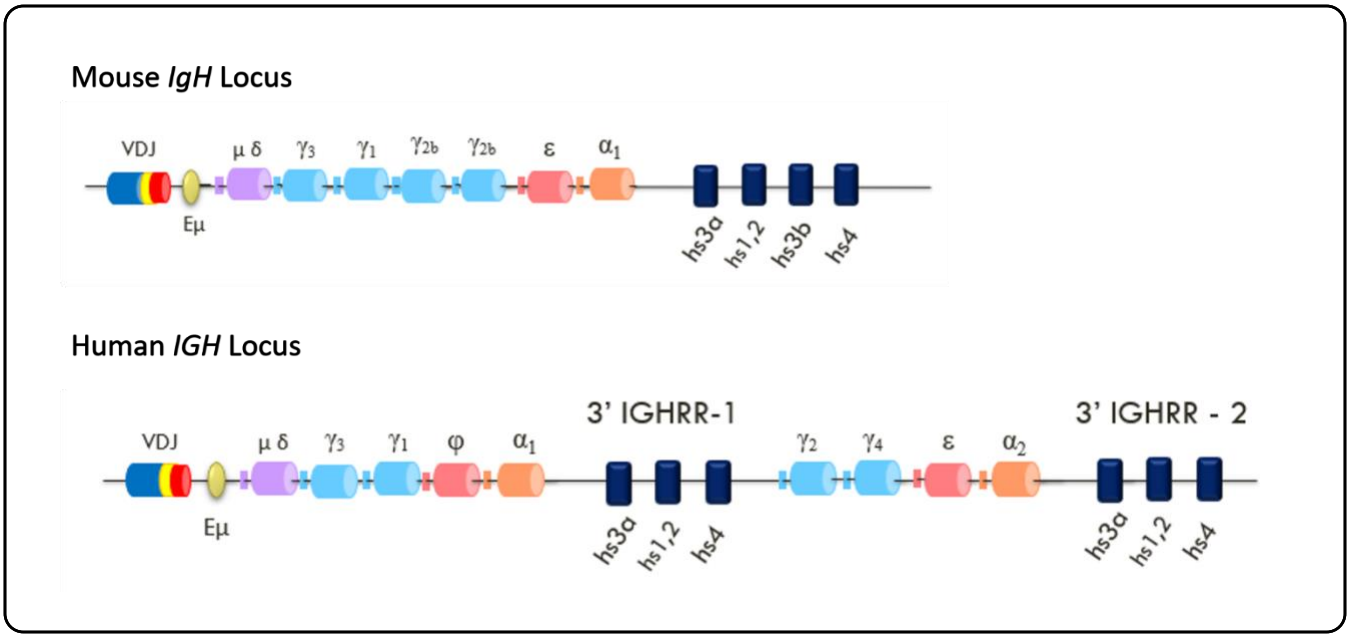

Figure 5. Mouse *IgH* and Human *IGH* Loci.

Expression of the murine heavy chain (*IgH*) gene is regulated by 3' regulatory region, 3'*IgH*RR which contains four enhancer regions (hs3a, hs1,2, hs3b and hs4). The human heavy chain (*IGH)* gene is regulated by two 3' regulatory regions, each 3' *IGH*RR is comprised of three enhancer regions (hs3a, hs1.2 and hs4).

Antibodies function in multiple ways to clear the infection

The main component of humoral immunity is antibody secreted by plasma cells. Antibodies circulate in blood and facilitate the clearance of infection from the host. Different infections require different means for clearance. Therefore, antibodies act in more than one way depending upon the characteristics of the cause of infection. The main functions of antibodies are neutralization and opsonization.

Antibodies neutralize the pathogen by directly binding to the pathogen. Neutralizing antibodies play an important role in clearing viral infections like HIV, influenza and more (Corti et al., 2017; Jardine et al., 2016). Antibodies neutralize viral particles by binding epitopes required for entering a host cell. Binding of the antibody prevents the virus from entering the host cell and the virus is eventually cleared by phagocytic cells. Neutralizing antibodies also act by literally neutralizing toxins secreted by certain microbes. For example, skin infections caused by a toxin from *Staphylococcus aureus* can be neutralized by antibodies (Le et al., 2016). Antibody binds to the toxin preventing it from binding to the cellular components and thus impairing its toxic effects. For example, a recent study showed that binding of intact antibodies neutralizes tetanus toxin by sequestering it and obstructing it from binding to its receptors (Ghotloo et al., 2020),

The other effector function of antibodies is opsonization. Opsonizing basically means tagging the pathogen as a ‘foreign particle’ so that the phagocytic cells of the immune system can clear the infection by ingesting the pathogen. Another way to eliminate the pathogen is to induce respiratory burst, in which the lymphocytes introduce reactive

oxygen species or superoxide into the pathogen. Phagocytic cells like macrophages and neutrophils express F_C receptors on their surface that recognizes the F_C fragment of the antibody bound to the pathogen. This method is beneficial especially when microbes like *Streptococcus pneumoniae* evades complement-mediated immunity and escapes from phagocytosis (Andre et al., 2017). Therefore, opsonizing antibodies against the capsule of *S. pneumoniae* is crucial for its recognition and elimination by the phagocytic cells (Viðarsson, Jónsdóttir, Jónsson, & Valdimarsson, 1994). Another example is the malarial parasite, *Plasmodium falciparum*, which is targeted by neutrophils after opsonization by antibodies (Jäschke, Coulibaly, Remarque, Bujard, & Epp, 2017). Recent studies have shown that the opsonizing function of the antibody is also very effective in diminishing different types of tumors by recruiting macrophages and neutrophils for the tumoricidal activity (Matlung et al., 2018; Velmurugan, Challa, Ram, Ober, & Ward, 2016).

Aryl hydrocarbon receptor is a ligand-activated transcription factor

The aryl hydrocarbon receptor or AhR is a ligand-activated transcription factor that is known to mediate the biological effects of several environmental toxins. AhR is classified under basic region/helix-loop-helix-PER-ARNT-SIM (bHLH–PAS) nuclear receptors based on its structure (Gu, Hogenesch, & Bradfield, 2000). The AhR protein is comprised of many functionally distinct domains, like the N-terminal bHLH domain that binds DNA, the PAS ligand binding domain, nuclear localization signal (NLS), nuclear export signal (NES), and C-terminal transactivation domain (TAD). Repressor proteins like

HSP90 and XAP2 form a complex with the AhR such that the NLS and NES domains remains hidden or unexposed and thus renders the AhR inactive and sequestered in the

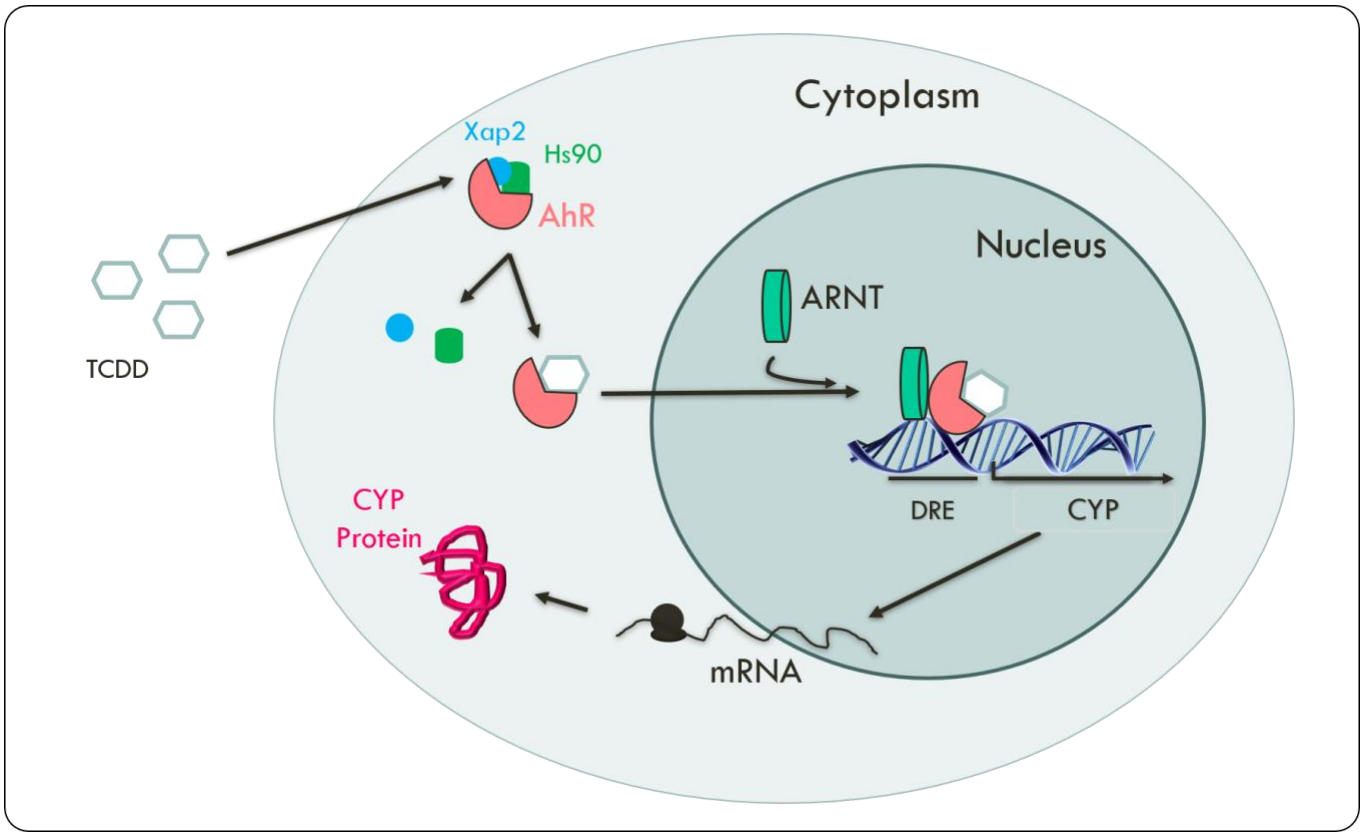

Figure 6. AhR Signaling Pathway.

Ligand binding to the AhR induces nuclear translocation and dimerization with the AhR nuclear translocator (ARNT). The AhR/ARNT nuclear complex binds to DRE motifs in the promoter and enhancer regions of genes sensitive to modulation by the AhR. CYP1A1 induction is a hallmark response of AhR activation.

cytosol (Denis, Cuthill, Wikstrom, Poellinger, & Gustafsson, 1988; Reyes, Reisz-Porszasz, & Hankinson, 1992). Once a ligand binds to the AhR, the repressor proteins dissociate from the AhR and conformational changes occur such that the nuclear localization signal (NLS) on the AhR is exposed which leads to translocation of the AhR-ligand complex (Kudo et al., 2017). Once inside the nucleus, the AhR complex forms a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT) creating a DNA-binding AhR-ARNT complex. The AhR-ARNT complex regulates expression of target genes by binding to unique DNA motifs (5'- TNGCGTG-3') named dioxin responsive elements or DREs. Protein domains within both ARNT and AhR recognize and bind the DRE (Reyes et al., 1992; Schulte, Green, Wilz, Platten, & Daumke, 2017; Whitelaw, Pongratz, Wilhelmsson, Gustafsson, & Poellinger, 1993).

Exogenous ligands of the AhR include polycyclic aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons (HAH) (Stejskalova, Dvorak, & Pavek, 2011). AhR can also be activated by various endogenous ligands such as bilirubin (Sinal & Bend, 1997), tryptophan

catabolites (Opitz et al., 2011) and other byproducts from the microbiota. Apart from these molecules, AhR also recognizes pathogen-associated molecular patterns (PAMPs) of certain bacteria, such as naphthoquinone phthiocol from Mycobacterium (Moura-Alves et al., 2014) indicating a possible role of AhR in immunity. The AhR is still considered an orphan nuclear receptor due to a lack of a distinct physiological ligand and role. However, a plethora of genes are regulated by the AhR, the most well-characterized being the detoxifying enzymes. Usually the induction of detoxifying enzymes like cytochrome 4501A1 (CYP1A1) is considered a hallmark response of AhR activation. The AhR is now becoming increasingly popular in the field of immunology.

Humoral immunity and B cell functioning can be modulated by activation of AhR

AhR was originally discovered as a transcription factor that mediates the biological effects of HAHs and other xenobiotics (Nebert, Dalton, Okey, & Gonzalez, 2004). It was later discovered that the AhR also regulates the genes that influence cell proliferation, differentiation, and apoptosis (Marlowe & Puga, 2005; Sartor et al., 2009). Recently, the AhR has become more prevalent in the field of immunology as a key player in regulating immune cell development and differentiation and mediating the immunosuppressive effects of dioxins.

Dioxins like TCDD target multiple developmental stages of B cells ultimately reducing the count of antibody secreting plasma cells. When human primary B cells are treated with PAH compounds or dioxins, AhR mediates the metabolism of these toxic compounds. Also, activated AhR upregulates other target genes that modulate cell

proliferation, one such example is upregulation of suppressor of cytokine signaling (Socs2) (Boverhof et al., 2004), which can diminish cytokine and growth factor production. In mice models, the knockout of AhR results in an increased number of pre-and pro-B cells but the number of mature B cells remains the same. This suggest that AhR plays some role in regulating the population of B cells in their early stages in the bone marrow (Thurmond, Staples, Silverstone, & Gasiewicz, 2000).

B cells undergo many changes when activated by T helper cells or by pathogen itself. One of the changes is upregulation of AhR expression making the cells more susceptible to the effects of TCDD or other AhR ligands (Allan & Sherr, 2005). AhR mediates the effects of TCDD by suppressing the expression of the human B cell activation markers like CD80, CD86 and CD69 (Lu, Crawford, Kaplan, & Kaminski, 2011). One of the possible mechanisms for the suppression of B cell activation might be increase in BCL-6 and SHP-1 expression by activated AhR (Phadnis-Moghe, Li, Crawford, & Kaminski, 2016). Both these proteins are repressed upon B cell activation implying that the upregulation might hinder the activation of B cells. Moreover, recent studies confirm that AhR activation in human B cells reduces the expression of the essential transcription factors for human B cell commitment and development like early B cell factor I (EBF1) and paired box 5 (PAX5). EBF-1 and PAX5 are essential for the differentiation of B cells to antibody producing plasma cells (Li et al., 2017).

TCDD-mediated AhR activation inhibits IgM production by murine B cells and the cells without AhR expression are resistant to immunosuppression by TCDD (Sulentic, Holsapple, & Kaminski, 1998). AhR activation by 2-(1'H-indole-3'-carbonyl) thiazole-4-carboxylic acid methyl ester (ITE), an endogenous ligand suppresses the production of

IgM, IgG1 and IgE by purified mouse B cells (Yoshida et al., 2012). Immunosuppression by TCDD is thought to be mediated by the inhibition of the 3' regulatory region of the Ig heavy chain gene. The activated AhR binds to the enhancer region of the 3'*IgH*RR to inhibit it's activity in mice models (Salisbury & Sulentic, 2015).

Little is known about the effects of TCDD and AhR activation in human B cells. A study on four patients showed that TCDD suppresses IgM production by human B cells (Wood & Holsapple, 1993). However, recently it was found out that AhR activation have different outcomes in human vs. mouse B cells. In humans, AhR target the genes involved in cell to cell communication like integrins (Kovalova, Nault, Crawford, Zacharewski, & Kaminski, 2017). A study on primary B cells from 12 patients shows that TCDD decreases IgM secretion in most of the population, but not all. IgM secretion by B cells of two donors was unaffected; and increased in one donor by TCDD treatment (Lu, Crawford, Suarez-Martinez, Kaplan, & Kaminski, 2010). The AhR is polymorphic in the human population and AhR with different SNPs combinations, effects the extent of immunosuppression by inhibition of IgM secretion. For instance, the AhR with all three SNPs slightly mitigates the inhibition of IgM secretion by TCDD (Kovalova, Manzan, Crawford, & Kaminski, 2016). Lastly, AhR activation by PAH compounds heightens IgE production in human B cells already class switched to IgE (Takenaka, Zhang, Diaz-Sanchez, Tsien, & Saxon, 1995). The effect of AhR activation on expression of other isotypes like IgG1-4, IgA1-2 and IgE is still unclear. The possible role of AhR in modulation of the 3' regulatory region of *IGH* gene in humans is not yet explored.

HYPOTHESIS AND SIGNIFICANCE

AhR mediated inhibition of antibody production by TCDD treatment in mice models has been researched extensively. However, the effect of TCDD or the role of the AhR on human antibody expression is not yet delineated. Previous studies in our lab, using a human B-cell line originating from a Burkitt lymphoma, CL-01 cells, demonstrated an inhibitory effect of TCDD on IgG expression in a concentration-dependent manner (Burra & Sulentic, 2015). Further, the AhR knocked out by siRNA or CRISPR/Cas9 gene editing resulted in significant inhibition of IgG secretion (Alhamdan & Sulentic, 2017; Burra & Sulentic, 2015; Kashgari & Sulentic, 2015).

These studies led to the hypothesis of the current study: **The AhR plays a physiological role in antibody production by human B cells**. To test this hypothesis, current studies utilized another human B-cell line (SKW6.4) originating from a different, non-related Burkitt lymphoma patient to further explore the role of the AhR in IgG expression and secretion. The SKW6.4 cells lack the endogenous expression of AhR protein, which makes them a good model to conduct these studies.

Significance of the current study is threefold. Firstly, AhR ligands are readily available in the environment as pollutants and are also found in several pharmaceutical and dietary items (Stejskalova et al., 2011). Some metabolites of the gut microbiota can also activate AhR, such as tryptophan and its catabolites (Opitz et al., 2011). Secondly, the alteration in the antibody levels by AhR activation can have harmful consequences on individual's health. Decreased antibody levels makes an individual susceptible to infectious disease. The alteration of IgE levels can present serious hypersensitivity

conditions (Maurer et al., 2018). Lastly, the comparison of IgG secretion as well as Ig isotype profiles of B cells in the absence of the AhR protein vs. B cells that express the AhR suggests a significant contribution of the AhR in the antibody production process, especially class switching to IgG and other isotypes. A physiological role of the AhR in IgG expression would be novel and have profound implications regarding the understanding of normal human antibody regulation.

MATERIALS AND METHODS

Chemicals and Reagents

2,3,7,8-Tetrachlorodibenzodioxin (TCDD) was purchased from AccuStandard (New Haven, CT) and dimethyl sulfoxide (DMSO) was purchased from SIGMA-Aldrich (St. Louis, MO). For stimulation studies, human recombinant CD40 ligand (MEGACD40L®) was purchased from Enzo Life Sciences, Inc., (Farmingdale, NY); and human interleukin (IL) 4 was purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Cell Culture

Novus CL-01 cells are EBV transformed human B cells derived from a Burkitt lymphoma patient. These monoclonal CL-01 cells express surface IgM and IgD and can be stimulated in vitro to undergo CSR (Cerutti et al., 1998). The ability of the cell line to undergo CSR in vitro makes them an ideal model to study the mechanism of antibody

production. The CL-01 cell-line was purchased from Novus Biologicals (Centennial, CO). The concentration of cells was maintained between $1x10^5$ cells/ml to $3x10^5$ cells/ml.

CL-01 cells are heterozygous for the AhR gene. CRISPR/Cas9 targeting of the AhR in CL-01 cells generated multiple clones, out of which one clone was discovered that appeared to have a monoallelic knockout of the AhR allele with the dysfunctional TAD resulting in expression of a TAD functional AhR (CL-01 AhR^{TA}), meaning that the Cl-01 AhR^{TA} cells express AhR with a functional TAD domain (Panstingel, data not published).

The SKW 6.4 (SKW WT) cells are another EBV-transformed B cell line derived from a different Burkitt's lymphoma patient. The SKW 6.4 (WT) cell line (ATCC® TIB-215™) was purchased from ATCC® (Manassas, VA). SKW WT cells do not express AhR endogenously. A variant of the SKW WT cells was created by (Kovalova et al., 2016) that stably expresses a functional AhR gene fused with GFP for detection. The SKW AhR^+ cells were cultured under puromycin (0.5 μg/ml) selection. The concentrations of both SKW WT and SKW AhR^+ cells were maintained between $2x10^5$ cells/ml to $1x10^6$ cells/ml.

All the cell lines were cultured in RPMI complete media supplemented with 13.5 mM HEPES, 1.0 mM sodium pyruvate, 1.0 mM non-essential amino acids, 50 μM 2-mercaptoethanol and 10% (v/v) bovine calf serum (Thermo scientific Laboratories, Logan, UT). The cells were incubated at 37 ^oC with 5% CO_2 and media was replaced every two to three days.

Sandwich enzyme linked immuno-sorbent assay

The sandwich ELISA technique was used to determine the amount of secreted IgG and IgM by measuring the immunoglobulins levels in the cell supernatants after 96 hours of stimulation and treatment. A 96-well ELISA plate was coated with Goat Anti-Human Ig-UNLB (SouthernBiotech; Birmingham, AL) at 1:1500 dilution with 0.1M sodium carbonate bicarbonate buffer and incubated over night at 4^{o}C. After incubation, the plate was washed two times with 1X PBS + 0.05% Tween-20 and three times with ddH_2O. Blocking solution was prepared by adding 3% (w/v) BSA to 1X PBS and added to the plate and incubated for 2 hours at room temperature or overnight at 4^{o}C. The wash steps were repeated after completion of blocking. Purified Human IgG – h+l (Bethyl Laboratories; Montgomery, TX) and RPMI complete media were used to formulate the standard dilutions ranging from 0.104 ng/ml to 6 ng/ml for the standard curve. Supernatants from experimental set-ups and standard dilutions were added to the designated wells on the ELISA plate and incubated at 37^{o}C for 1.5 hours. The plate was then washed three times with 1X PBS + 0.05% Tween-20 and four times with ddH_2O. Secondary antibody, Goat anti-human IgG (Fc fragment)-HRP conjugated (Bethyl Laboratories; Montgomery, TX) was diluted at 1:10,000 dilution using 3% BSA:1x PBS:0.05% Tween20. Secondary antibody dilution was added to the wells and incubated at 37^{o}C for 1.5 hours. The wash steps were repeated after the incubation. Under dark conditions, TMB substrate (3,3′,5,5′-tetramethybenzidine) purchased from Millipore Sigma (St. Louis, MO) was added to the plates after wash and incubated for 30 mins in dark. 4N H_2SO_4 was added to stop the reaction after 30 mins. For detection, the Spectramax Plus 384 UV/VIS (Molecular devices, Sunnyvale, CA) was set to read the OD values of the wells at 450 nm. The SOFTmaxPRO

software (Molecular Devices) was used to interpret the OD values and generate the standard curve and subsequent calculation of IgM and IgG levels in supernatants.

RNA Isolation and Reverse Transcription

For RNA isolations, the cell pellets were suspended in TRI reagent® (Sigma-Aldrich) and incubated at room temperature for 5 mins. The suspension was centrifuged to collect the cell debris and DNA and the supernatant was transferred to the Phase Lock Gel™ tubes (Quantabio) and then kit protocol was followed to isolate RNA: 1-bromo-3-chloropropane (Thermo Fisher Scientific) was used to separate the aqueous and organic phase on the phase lock gel tubes. RNA in the aqueous phase was precipitated using isopropanol and centrifuged to pellet the RNA. The RNA pellets were washed using 75% ethanol. The RNA pellet was resuspended in nuclease-free H_2O by incubating at 56^oC for 10-15 mins. The RNA content was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Products).

RNA obtained from the cells after treatments was utilized for cDNA conversion via reverse transcription PCR. All the RNA samples were diluted to 50ng/µl (or lower) using nuclease-free H_2O. A total of 500 ng RNA was converted to cDNA using the Hi-Capacity cDNA reverse transcription kit (appliedbiosystems; Foster City, CA). The master mix was prepared using the contents of the kit as per recommended concentrations. The master mix was then mixed with RNA samples at a ratio of 1:1 and the PCR was run at following thermocycling conditions:

	Step 1	Step 2	Step 3	Step 6
Temperature	25ºC	37ºC	85ºC	4ºC
Time	10 min	120 min	5 min sec	hold

Table 1. Thermocycling conditions for reverse transcription PCR

Qualitative PCR Analysis

PCR was used for qualitative analysis of expression of AhR in different cell types. The master mix for the PCR reaction was prepared using Taq DNA polymerase (New England Biolabs; Ipswich, MA), Taq DNA polymerase buffer, dNTPs and primers following the guidelines of the manufacturer. The sequence of the forward primer and reverse primer for the AhR transcripts were GCACCGATGGGAAATGATAC and TTGACTGATCCATGTAAGTCTG respectively. A total of 5 µl cDNA was added to 45 µl master mix. The thermocycler was set to the cycling conditions as per the table 1. The PCR product was analyzed by electrophoresis on 1% agarose gel.

	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
Temperature	95ºC	95ºC	57ºC	68ºC	68ºC	4ºC
Time	15 sec	35 sec	60 sec	35 sec	5 min	hold

Table 2. Thermocycling conditions for qualitative PCR for amplification of AhR mRNA transcripts

Realtime Qt-PCR Analysis

cDNA obtained from reverse transcription was subjected to real-time quantitative PCR to measure the levels of expression of various Ig transcripts. The Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific); a total of 50ng cDNA and appropriate primers designed for each Ig transcript were mixed together and subjected to qt-PCR. The primers and expected product size for all the isotypes are descried in the table 2.

Housekeeping transcript		
Transcript	**Primer Sequence**	**Expected product size**
β-actin	RP – 5'ATCCACACGGAGTACTTGCGCTCA-3' FP – 5' CTACCACTGGCATCGTGATGGACT-3'	129 bp
Germline and functional transcripts		
ε	FP – 5' GACGGGCCACACCATCC – 3' RP – 5' CGGAGGTGGCATTGGAGG – 3'	125 bp
α1	FP-5' CGCCATGACAACAGACACAT-3' RP-5'GTATGCTGGTCACAGCGAAG-3'	166 bp
α2	FP-5'ACATTGATGTGGGTGGGTTT-3' RP-5'CCACCACCTACGCTGTAAC-3'	157 bp
γ1	FP-5'GATGTCGCTGGGATAGAAGC – 3' RP-5' TGTTGGAGACCTTGCACTTG – 3'	170 bp
γ2	FP – 5' GAGATGTCGCTGGGGTAGAA – 3' RP – 5' GGCAAGGAGTACAAGTGCAA – 3'	188 bp
γ3	FP – 5' TCACGTTGCAGGTGTAGGTC – 3' RP – 5' CTACTTCCCAGAACCGGTGA – 3'	164 bp
γ4	FP – GTTCTTGGTCATCTCCTCCT – 3' RP – GAACGGCAAGGAGTACAAGTG – 3'	154 bp

Table 3. List of the germline and functional transcripts with their respective primer sequences for real time PCR and expected product size.

Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) was used to sort out SKW AhR^+ (GFP+) cells from the rest of the population. The cells were treated with 0.2 μg/ml doxycycline 24 hours prior to sort. The cells were suspended in HBSS and filtered using 70 μM cell strainer insert (Falcon 352350) just before GFP-mediated cell sorting. The FACS was performed at Research Flow Cytometry Core in Cincinnati Children's Hospital using BD/FACS Aria II machine. After sorting, the cells were seeded in conditioned RPMI complete media in a 96 well plate at 1 cell/well. Conditioned RPMI media was prepared by mixing one part of cell supernatants after 48 hours of cell culture and two parts of fresh RPMI complete media.

Transient Transfection

Electroporation was used to transiently transfect luciferase reporter plasmid into the cell models. Six DRE sites were inserted upstream of the luciferase gene in pGL3 Basic vector (Promega Corporation; Madison, WI) to create the 6x DRE luciferase reporter plasmid. The schematic representation of construct of the plasmid is displayed in (Fig. 9)

For electroporation, a total of 10^7 cells were resuspended in fresh RPMI complete medium and 10ug plasmid was added to the suspension. The suspension was then transferred to a 2mm electroporation cuvette and subjected to a very short electric pulse (8ms to 13ms) using BTX electroporation system (Harvard Apparatus). The parameters for

the electroporation varied for each cell type. The CL-01 cells were subjected to 150 V voltage, 1500µF capacitance and 75 Ω resistance. For SKW 6.4 cells and its variants, the parameters were 200V voltage, 1500µF capacitance and 75 Ω resistance. Two electroporation cuvettes were prepared for each cell type and the cells from both tubes were pooled together after transfection. The cells were then diluted to a concentration of 2 x 10^5 cells/ml and treated with appropriate reagents and aliquoted out in triplicates (3 wells per treatment) in 12-well plates. The plates were incubated at 37°C with 5% CO_2 for 20 – 24 hours.

After incubation, the cells were carefully transferred to a microfuge tube and spun at 500g for 5 mins. Cell pellet were resuspended in reporter lysis buffer (Promega; Madison, WI) and the lysates were frozen at -80°C for at-least an hour to ensure complete lysis. The lysates were then thawed on ice and spun at maximum speed for two minutes to collect the cell debris. To measure the luciferase activity, 20 µl of supernatant was transferred to a glass tube and mixed with 100 µl of luciferase substrate (Promega; Madison, WI). The RLUs were measured to detect the luciferase activity using luminometer (Berthold detection system) and the data was analyzed using Prism GraphPad software (San Diego, CA).

Protein Isolation and Western Blotting

Protein was isolated from different cell types using mild lysis buffer. The mild lysis buffer was prepared (1% NP_4O, 150nM NaCl, 2mM EDTA and 10mM $NaPO_4$). The lysate was stored at -80°C overnight to ensure adequate cell lysis. The lysates were thawed on ice

and then spun at maximum speed for 2 mins to collect the cell debris. The supernatant was subjected to Bradford assay to determine the concentration of protein. Aliquots were made and stored at -80^{o}C until ready to use.

SDS-PAGE was used to separate the proteins on polyacrylamide gel (National diagnostics; Atlanta, GA). The protein samples were denatured at 95^{o}C for 5 mins in the presence of SDS and β-mercaptoethanol prior to loading on the gel. The electrophoresis was run at 100 V for about 45 mins to separate the proteins. The proteins were then transferred to a PVDF membrane (immobilon-P membranes; Millipore Sigma) from the gel. The transfer electrophoresis was run at 35 V for 15 hours in cold conditions.

Once on the membrane (blot) the AhR protein and the housekeeping protein β-actin was detected using immunoblotting technique. First, the blot was cut at appropriate sizes using the Spectra^{TM} Multicolour Broad Range Protein Ladder (ThermoScientific) as a marker. Next, the blots were blocked by incubating in 5% milk + TBS-Tween 20 buffer for three hours at room temperature on a rocker. The blots were washed thoroughly with TBS-tween 20 buffer thrice and then with ddH_2O four times. Then, the blots were incubated in respective primary antibodies diluted in 5% milk + TBS-Tween 20 buffer for three hours at room temperature on a rocker. Primary antibody, anti- human AhR antibody was purchased from Santa Cruz Biotechnology (Dallas, TX) and the anti-human βactin antibody. Wash steps were repeated after incubation. After that, the blots were incubated in secondary antibody solution for one hour on the rocker at room temperature. The wash steps were repeated, and the blots were stored in TBS-Tween20 buffer until ready for detection. Finally, the blots were incubated in the ECL substrate solution for 5 mins prior to detection.

RESULTS

Characterization of SKW WT and SKW AhR$^+$ cells

To determine the role of the AhR in human antibody production, our lab created variants of the CL-01 cells in which AhR expression was knocked down using shRNA constructs (CL-01 AhR-KD). To our surprise, IgG secretion by the CL-01 AhR-KD cells was prominently inhibited (Kashgari & Sulentic, 2015). Similar results were manifested when the *AhR* gene was targeted using CRISPR/Cas9 (Alhamdan & Sulentic, 2017). However, IgM secretion remained unaffected by loss of AhR expression. These studies imply that the AhR plays a critical role in class switching from IgM to IgG or that the AhR plays a physiological role in IgG expression or secretion.

The objective of the current study was to utilize a different cellular model derived from humans to validate the loss of IgG with AhR knockdown as observed in the CL-01 cells. The SKW 6.4 cell line is an EBV-transformed human B-cell line, also originated from a Burkitt's lymphoma patient like the Cl-01 cells (Saiki & Ralph, 1983). However, the two cell lines came from two different patients. A recent study with the SKW 6.4 cells suggests that they do not express the AhR (Kovalova et al., 2016). In this same study, a variant of the SKW 6.4 cells (SKW-AhR$^+$; originally named SKW E8) was created that stably expresses the human AhR.

To verify the lack of AhR expression in the SKW 6.4 (SKW WT) cells and AhR expression in the SKW-AhR$^+$ cells, we performed qualitative RT-PCR analysis for *Ahr* transcripts in the CL-01 (positive control), SKW WT and SKW-AhR$^+$ cells. As expected, we observed a band for the CL-01 and SKW-AhR$^+$ cells at the expected size for *Ahr* transcripts (549 bp) but no transcripts were detected in the SKW WT cells (Fig. 7a). Lack of AhR expression was also verified by western blot analysis. AhR protein at the expected size (~122 kDa) was detected in the control CL-01 cells but not in the SKW WT cells (Fig. 7b). However, the AhR protein band was also not detected in SKW-AhR$^+$ cells (data not shown). This might be because the primary antibody could not detect the AhR due to alteration of the sequence of few amino acids due to the fusion with GFP. Also, the cell population might be heterogenous in terms of AhR transgene expression.

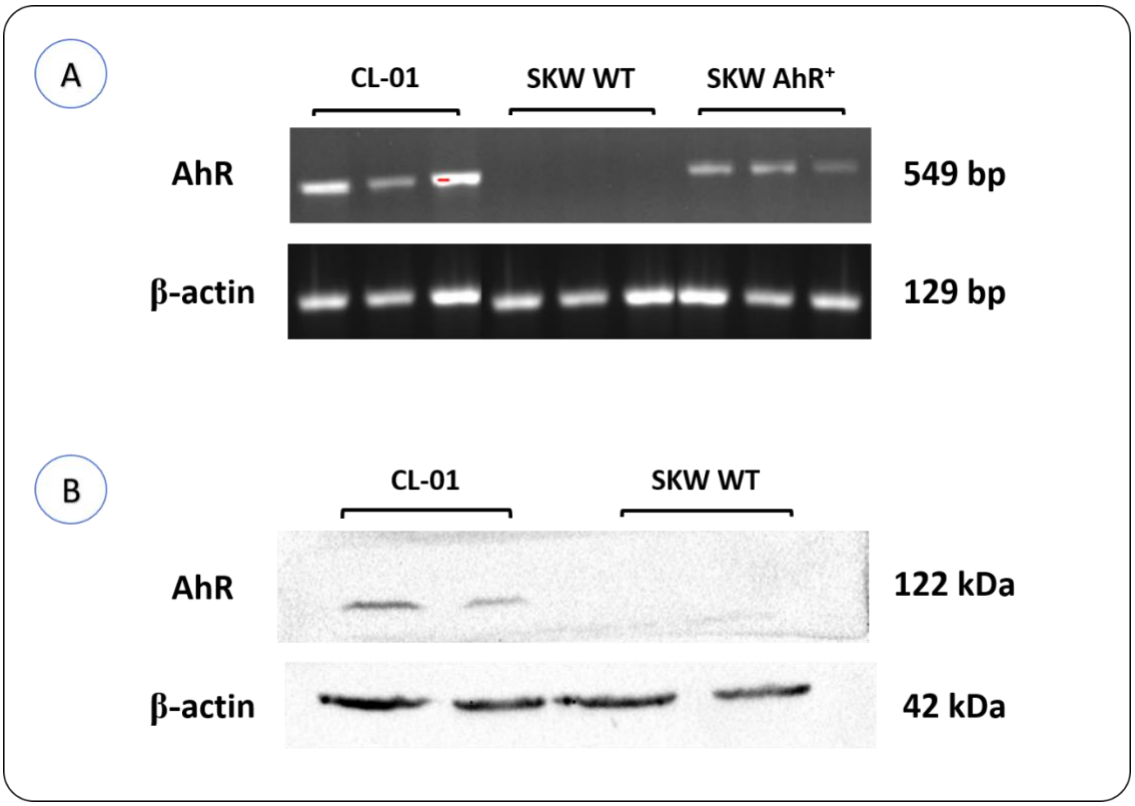

Figure 7. SKW WT cells do not express the AhR.

(A) RNA was isolated from a total of $1x10^6$ cells from either the CL-01 (positive control), SKW WT or SKW-AhR$^+$ cells and reverse transcribed to cDNA. cDNA (125 ng) was subjected to PCR analysis with primers specific for *Ahr* or *β-actin*. The PCR products were resolved on a 1% agarose gel. Expected PCR product sized for the *Ahr* and *β-actin* are 549 bp and 129 bp, respectively. The results are representative of two independent experiments. (B) Protein lysates (24 µg) from CL-01 cells and SKW WT cells were subjected to SDS-PAGE and then transferred to a PVDF membrane. AhR protein (122 kDa) was detected using a primary antibody for human AhR. The housekeeping protein, β-actin (42 kDa) was used as loading control. Results are representative of three independent experiments.

Analysis of AhR function in SKW WT cells

To further characterize the SKW 6.4 cells, we tested the ability of the cells to induce AhR-mediated expression of target genes, when treated with TCDD. We utilized the 6x DRE luciferase reporter plasmid in which the luciferase gene is under the regulation of six DRE sites. With this reporter, TCDD treatment will induce binding of the AhR/ARNT complex to the DRE sites and induce luciferase gene expression if the AhR signaling pathway is functional (Fig. 8A).

For the current study, we utilized the murine B-cell line CH12.LX that expresses a fully functional AhR, capable of inducing CYP1A1 expression (Suh, Kang, Yang, & Kaminski, 2003). We transiently transfected the CH12.LX cells (positive control), SKW 6.4 cells and SKW AhR^+ cells with the 6x DRE reporter plasmid using electroporation. The transfected cells were either incubated with no further treatment (naïve control), treated with the DMSO vehicle control, or with 30nM TCDD. As expected, a significant increase in luciferase activity was observed in TCDD-treated CH12.LX cells as compared to untreated cells; whereas, no change in luciferase activity was detected upon TCDD treatment in the AhR-deficient SKW 6.4 cells. SKW-AhR^+ cells were also unable to induce TCDD-mediated luciferase expression (Fig 8B). This observation was in-line with the fact that we could not detect AhR protein in SKW-AhR^+ cells by western blotting. Using cell imaging tools and flow cytometry, we observed only a few cells expressed AhR-GFP among the entire population of SKW-AhR^+ cells (data not shown). Since only a few cells expressed the AhR transgene, there was not enough DRE binding and transcriptional activity could not be detected in SKW-AhR^+ cells.

Characterization of the SKW AhR$^+$ cells was therefore challenging. To combat this, we performed GFP-mediated fluorescence-activated cell sorting (FACS) to enrich the GFP$^+$ SKW-AhR$^+$ cells. Meanwhile, we continued the characterization of SKW WT cells alone, performed the experiments to study the antibody (IgM and IgG) secretion levels and Ig isotype profiling which are discussed below. However, it was not possible to conduct the planned experiments on the GFP-sorted SKW AhR$^+$ cells because of the lockdown due to the COVID-19 pandemic.

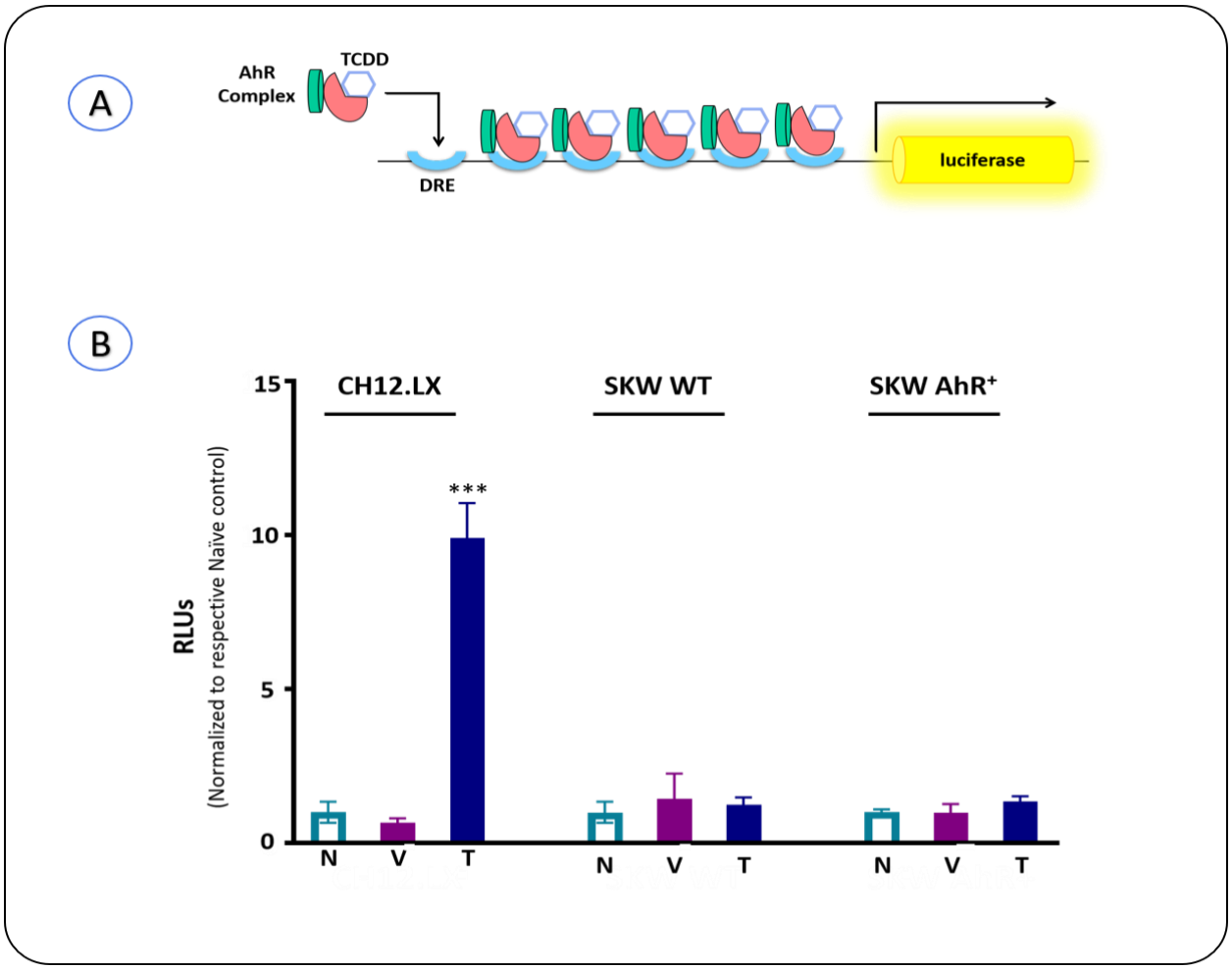

Figure 8. SKW cells are unable to induce DRE-mediated transcription

(A) Schematic of AhR complexes binding to the DRE sites on the 6x DRE luciferase reporter plasmid. The reporter plasmid was constructed using pGL3 Basic vector (Promega). The ligand-activated AhR complex binds to the DRE sites , which induces expression of the luciferase gene. (B) 6x DRE luciferase reporter plasmid was transfected in CH12.LX, SKW WT and SKW AhR^{+} cells using electroporation. Cells were then cultured for 24 hours with either no further treatment (naïve, N) or with 0.01% DMSO vehicle control (V) or 30nM TCDD (T). Lysates from the cells were tested for luciferase activity, which is represented on the y-axis (Mean±SEM, n=3) as relative light units (RLU) normalized to respective naive control. The data was analyzed using 1-way ANOVA analysis followed by Turkey post hoc test: *** $p < 0.0001$.

Stimulation of SKW WT cells promotes IgM secretion

CL-01 human B cells can be stimulated by CD40 ligand and IL-4 to secrete higher levels of Ig and undergo class switch recombination (Cerutti et al., 1998). In previous studies, B-cell differentiation factor (BCDF); macrophage derived cytokines (i.e. IL-1, IL-2, and IL-6); or pokeweed mitogen (PWM) were used to stimulate SKW WT cells to produce increased levels of IgM secretion (Goldstein & Kim, 1993; Jandl, Flanagan, & Schur, 1988; Kovalova et al., 2016). However, stimulation of SKW WT cells by T-cell derived factors like CD40 ligand and IL-4 has not been examined.

To determine if SKW WT cells can undergo T-cell dependent stimulation as shown in Fig. 3, we treated SKW WT cells and CL-01 cells (control) with CD40 ligand and IL-4 to mimic T-cell dependent stimulation. Secreted IgM levels were measured via ELISA analysis of supernatants after a 96-hour incubation. As seen previously, IgM secretion slightly, but not significantly. Whereas IgM secretion by SKW WT increased by 2.5 fold upon stimulation. These observations imply that when stimulated with CD40 ligand and IL-4, SKW WT cells secrete up to 2.5 times more IgM as compared to naïve control, suggesting that the SKW WT cells can increase IgM production with T-cell dependent stimulation. Fig 9A represents the fold change in IgM secretion by both cell types upon stimulation.

SKW WT cells do not secrete IgG

Previous studies show that knockdown of the AhR in human CL-01 cells abolishes IgG secretion with no effect on IgM secretion. Since the SKW WT cells do not endogenously express AhR but can be stimulated by CD40 ligand and IL-4, we evaluated IgG secretion in these cells.

For the current study, we utilized CL-01 AhR^{TA} cells with monoallelic expression of AhR with a functional TAD+ domain as a positive control (AhR expressing human B cell line). As expected, stimulation of the CL-01 AhR^{TA} cells resulted in significant increase in IgG secretion. However, like the AhR knockdown CL-01 cells, the SKW WT cells, which also lack AhR expression, did not secrete basal or stimulated IgG supporting a role of the AhR in IgG expression (Fig. 9B).

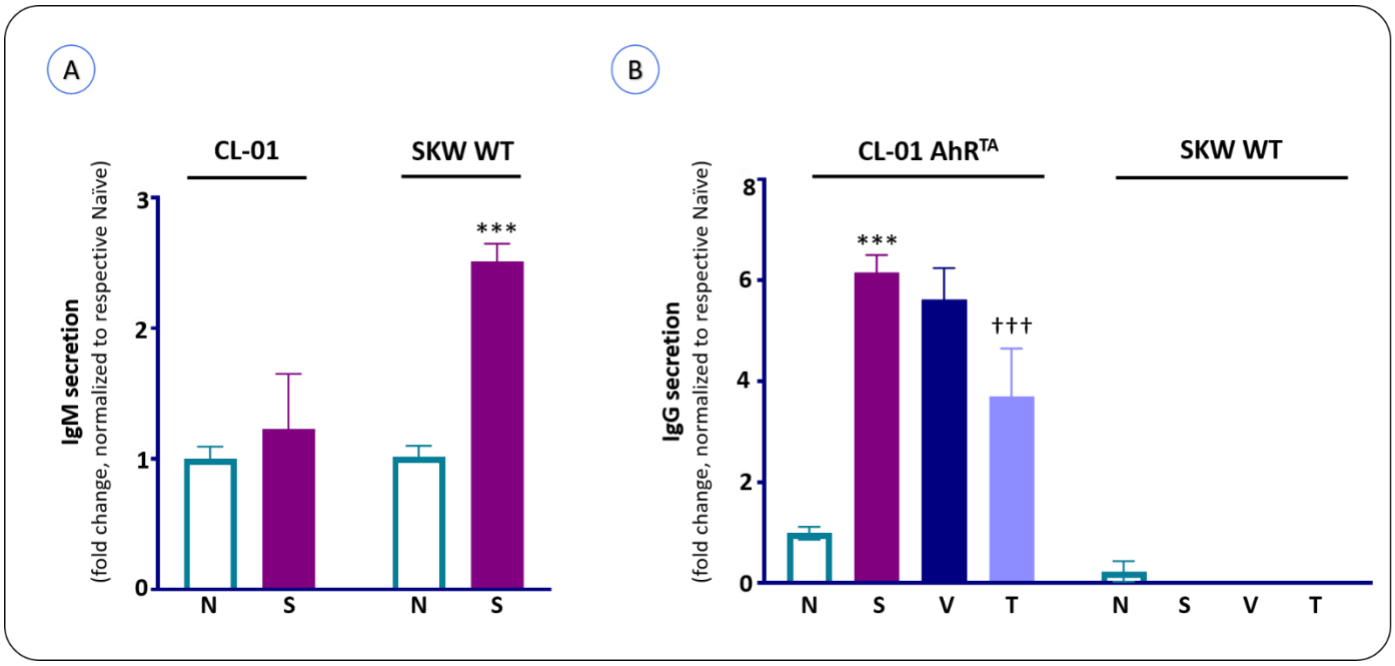

Figure 9. SKW WT cells secrete IgM but not IgG

(A) CL-01 cells ($1x10^5$ cells/ml) and SKW WT cells ($1x10^4$ cells/ml) were cultured for 96 hours, without stimulation (naïve, N) or stimulated with 6.25 ng/ml CD40 ligand and 50 ng/ml IL-4 (S). The y-axis represents IgM levels in supernatants normalized to the respective naïve control, (mean±SEM, n=3). (B) CL-01 AhR^{TA} (with expression of a TAD functional AhR) cells ($1x10^5$ cells/ml) and SKW WT cells ($1x10^4$ cells/ml) were cultured for 96 hours without stimulation (naïve, N) or CD40 ligand and IL-4 stimulation (S) with or without the 0.01% DMSO vehicle control (V) or 30nM TCDD (T). The y-axis represents IgG levels in supernatants normalized to the respective naïve control (mean±SEM, n=3). Results were analyzed using a 2-way ANOVA followed by a Dunnett's post hoc test. *** represents a significant difference when compared to the respective naïve (N) at $p<0.0001$. ††† represents a significant difference when compared to the stimulated group (S) at $p<0.0001$.

Stimulation of SKW WT cells results in increased γ2-4 transcript levels but not γ1

The CL-01 B-cell line undergoes class switch recombination to produce IgG upon stimulation with CD40 ligand and IL-4, which is reflected by both, increased total IgG secretion and increased γ1-4 transcript levels that code for IgG1-4 respectively (Burra & Sulentic, 2015; Cerutti et al., 1998). However, the SKW WT cells that do not express AhR endogenously, like AhR knockdown CL-01 cells, do not secrete IgG. To understand the mechanism by which the loss of AhR results in loss of IgG, we examined if stimulation of SKW WT cells induced Cγ1-4 germline transcripion, an early step in CSR to generate γ1-4. We designed primers for each of the γ1-4 transcripts and conducted quantitative real-time PCR analysis. The primers amplified both sterile (non-coding transcripts) and functional (coding transcripts) combined. We observed that the transcript levels of γ2, γ3 and γ4 were significantly increased with CD40L and IL-4 stimulation of SKW WT cells which was slightly decreased when treated with TCDD. The γ1 transcript levels were minimal in SKW WT cells (Fig. 10 A-D).

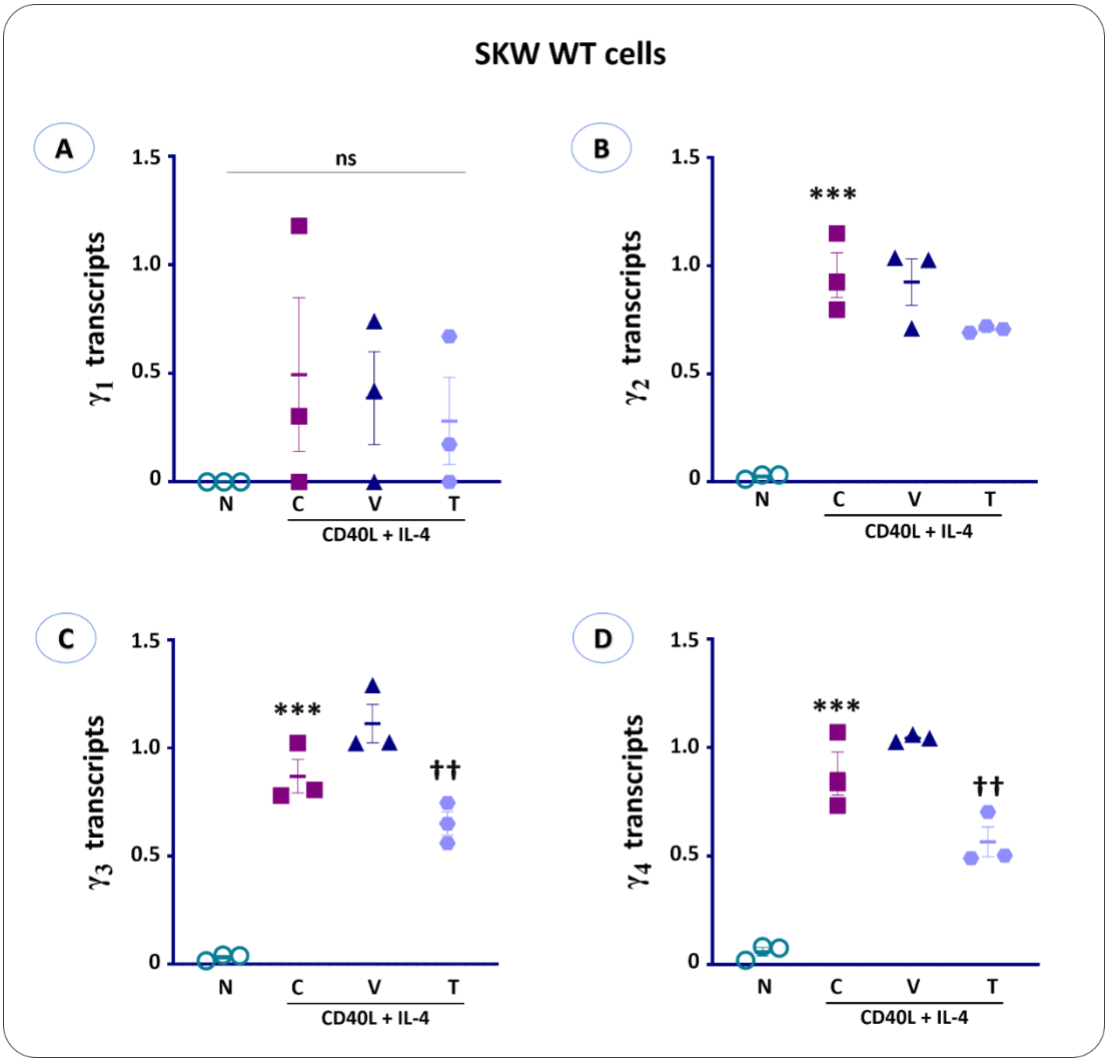
SKW WT cells
A
B
C
D
ns
***
***
***
††
††
1.5
1.0
0.5
0
γ_1 transcripts
γ_2 transcripts
γ_3 transcripts
γ_4 transcripts
N
C
V
T
CD40L + IL-4

Figure 10. Increase in γ2-4 transcript levels upon stimulation of SKW WT cells

SKW WT cells ($1x10^4$ cells/ml) were cultured without stimulation, naïve, N (○) or stimulated with 6.25 ng/ml CD40 ligand and 50 ng/ml IL-4 only, C (■) or stimulated and treated with 0.01% DMSO vehicle control, V (▲) or 30nM TCDD, T (⬣). Total RNA was isolated from cells after 96 hours of incubation and converted to cDNA. 50ng cDNA was subjected to real-time PCR to detect the amplification of γ1-4 (A-D, respectively) germline and/or functional transcripts. All y-axes represent fold change in amplification of transcripts normalized to the respective vehicle, V control (mean±SEM). The results are represented as means of three independent experiments (n=3 for each treatment group in an experiment). Results were analyzed by normalizing the transcript levels with the housekeeping gene β-actin of the respective group and then comparing the transcript levels to the control treatment group using a 1-way ANOVA followed by a Tukey's multiple comparison's test. "ns" = non-significant. *** represents a significant difference when compared to the respective naïve (○) at $p<0.0001$. † represents $p=0.016$, †† represents $p=0.0016$ and ††† represents $p<0.0001$ when compared to the respective vehicle control group (▲).

Lower Cγ1-4 expression by SKW cells as compared to CL-01 AhR^{+} cells

We observed that SKW WT cells do not secrete IgG however, there seems to be an increase in transcription of Cγ regions upon stimulation. However, the amplification of the γ transcripts occurred at a higher number or PCR cycle indicating low levels of transcripts in the SKW WT cells.

Therefore, we compared the γ1-4 transcript levels in the AhR expressing CL-01AhR^{TA} and SKW WT cells that do not express AhR endogenously. We observed that stimulation of CL-01 AhR^{TA} cells by CD40 ligand and IL-4 significantly increases the transcript levels of γ1-4 isotypes and TCDD treatment reduces the expression of Cγ1-4. However, the γ1-4 transcript levels in SKW WT cells are significantly lower as compared to CL-01 AhR^{TA} cells (Fig 11).

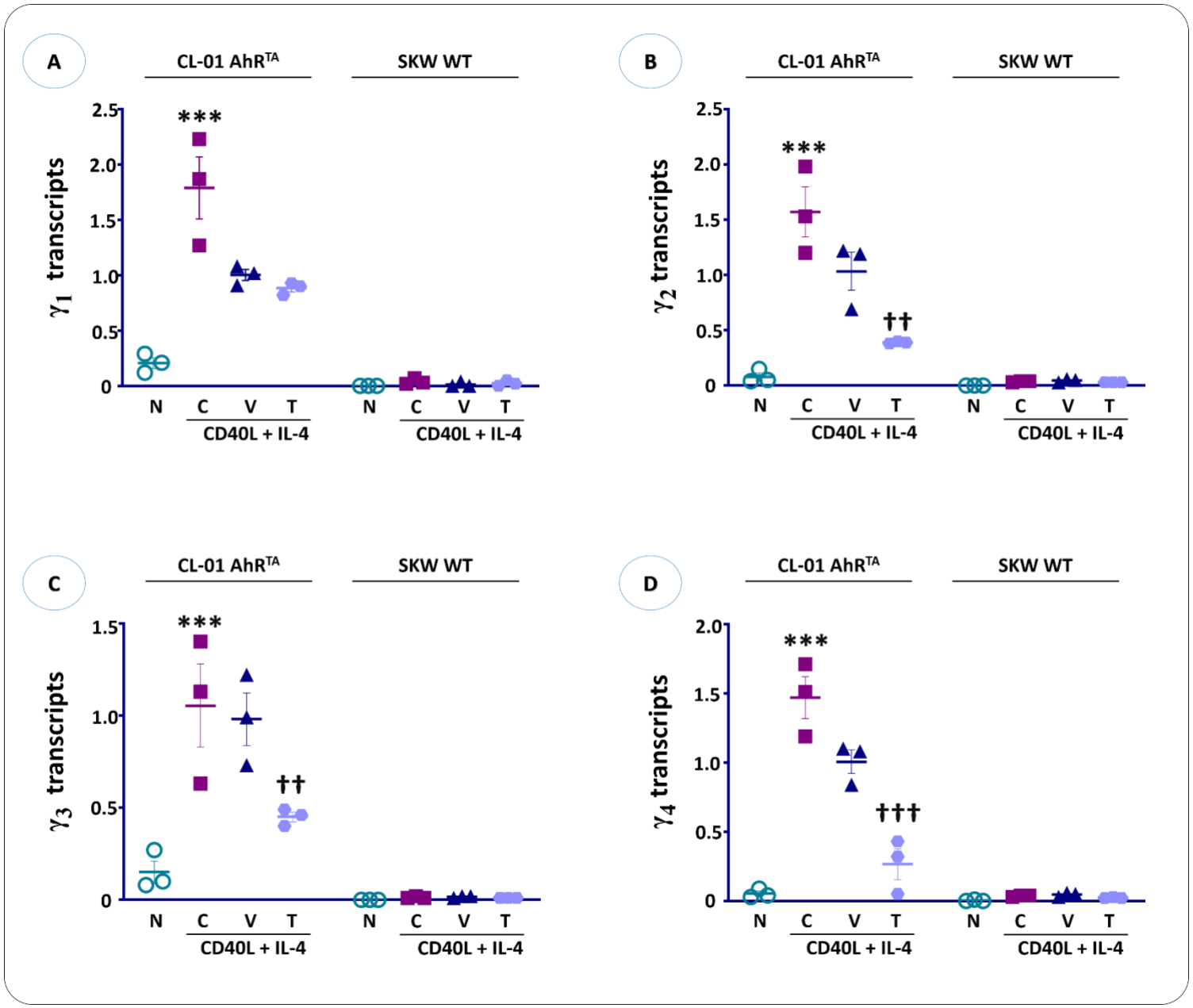
A
CL-01 AhR^TA
SKW WT
γ1 transcripts
***
N C V T
CD40L + IL-4
B
γ2 transcripts
***
††
C
γ3 transcripts
***
††
D
γ4 transcripts
***
†††

Figure 11. Transcription of Cγ1-4 is impaired in SKW WT cells

CL-01 AhR^{TA} cells ($1x10^5$ cells/ml) and SKW WT cells ($1x10^4$ cells/ml) were cultured either without stimulation, naïve, N (○) or stimulated with 6.25 ng/ml CD40 ligand and 50 ng/ml IL-4 only, C (■) or stimulated and treated with 0.01% DMSO vehicle control, V (▲) or 30nM TCDD, T (⬣). Total RNA was isolated from cells after 96 hours of incubation and converted to cDNA. 50ng cDNA was subjected to real-time PCR to detect the amplification of γ1-4 (A-D, respectively) germline and/or functional transcripts. All y-axes represent fold change in amplification of transcripts normalized to the CL-01 AhR^{TA} vehicle control (mean±SEM). The results are representation of three independent experiments. Results were analyzed by normalizing the transcript levels with the housekeeping gene β-actin of the respective group and then comparing the transcript levels to the control treatment group using a 2-way ANOVA followed by a Tukey's multiple comparison's test. *** represents a significant difference when compared to the respective naïve (○) at $p<0.0001$. † represents $p=0.016$, †† represents $p=0.0016$ and ††† represents $p<0.0001$ when compared to the respective vehicle control group (▲).

SKW WT cells express very low levels of ε transcripts but no α1-2 transcripts

To investigate if SKW WT cells can class switch to isotypes other than IgG1-4, we checked the expression of Cε and Cα1-2, which code for the heavy chain of IgE and IgA1-2, respectively. We measured the levels of ε and α1-2 germline and/or functional transcripts using quantitative real-time PCR. When compared within the cell type, we see that stimulation of SKW WT cells by CD40 ligand + IL-4 results in an increase in ε transcript levels but when compared to the ε transcript levels of the stimulated CL-01 cells, the ε expression in SKW WT is very low (Fig 12). Surprisingly, we did not see any amplification for α1 and α2 transcripts in the SKW WT cells (Fig 13).

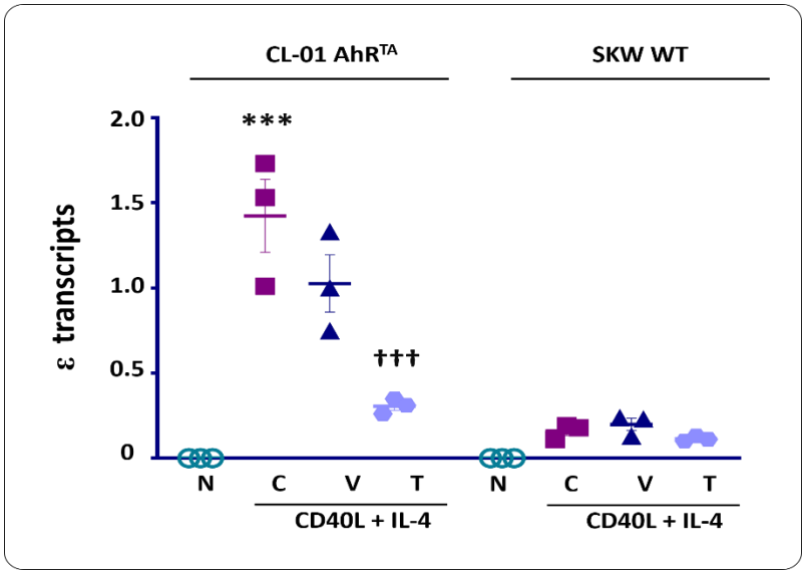

Figure 12. Low levels of ε transcripts in SKW WT as compared to CL-01 AhR^{TA} cells

CL-01 AhR^{TA} cells (1x10^5 cells/ml) and SKW WT cells (1x10^4 cells/ml) were cultured either without stimulation, naïve, N (○) or stimulated with 6.25 ng/ml CD40 ligand and 50 ng/ml IL-4 only, C (■) or stimulated and treated with 0.01% DMSO vehicle control, V (▲) or 30nM TCDD, T (⬣). Total RNA was isolated from cells after 96 hours of incubation and converted to cDNA. 50ng cDNA was subjected to real-time PCR to detect the amplification of ε germline and/or functional transcripts. All y-axes represent fold change in amplification of transcripts normalized to the CL-01 AhR^{TA} vehicle control (mean±SEM). The results are representative of three independent experiments. Results were analyzed by normalizing the transcript levels with the housekeeping gene β-actin of the respective group and then comparing the transcript levels to the control treatment group using a 2-way ANOVA followed by a Tukey's multiple comparison's test. *** represents $p<0.0001$ when compared to the respective naïve (○). ††† represents $p<0.0001$ when compared to the respective vehicle control group (▲).

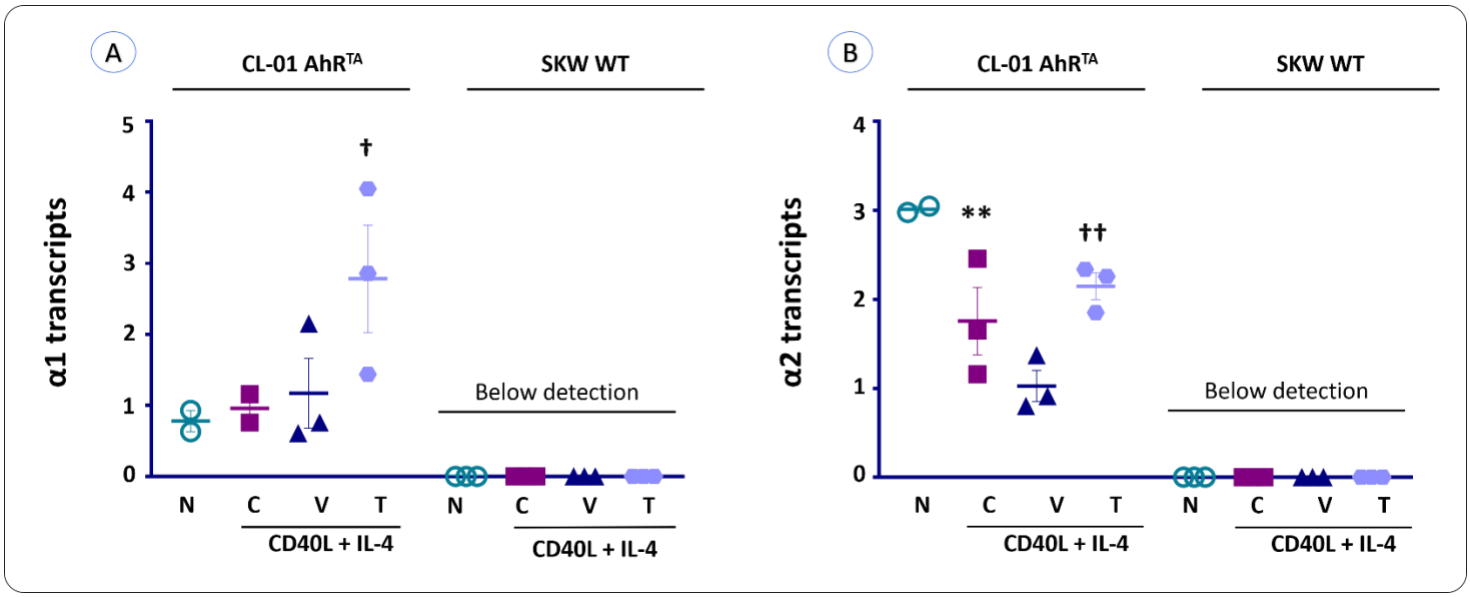

Figure 13. SKW WT cells do not express Cα1-2

CL-01 AhR^{TA} cells (1x10^5 cells/ml) and SKW WT cells (1x10^4 cells/ml) were cultured either without stimulation, naïve, N (○) or stimulated with 6.25 ng/ml CD40 ligand and 50 ng/ml IL-4 only, C (■) or stimulated and treated with 0.01% DMSO vehicle control, V (▲) or 30nM TCDD, T (⬣). Total RNA was isolated from cells after 96 hours of incubation and converted to cDNA. 50ng cDNA was subjected to real-time PCR to detect the amplification of α1 and α2 (A and B) germline and/or functional transcripts. All y-axes represent fold change in amplification of transcripts normalized to the CL-01 AhR^{TA} vehicle control (mean±SEM). Results were analyzed by normalizing the transcript levels with the housekeeping gene β-actin of the respective group and then comparing the transcript levels using a 2-way ANOVA followed by a Tukey's multiple comparison's test. ** represents p=0.001 when compared to the CL-01 AhR^{TA} naïve group (○). † represents p=0.0238 and †† represents p=0.001 when compared to the CL-01 AhR^{TA} vehicle control group (▲).

DISCUSSION

Regulation of the human *IGH* gene expression and CSR mechanism is poorly understood. In murine B cells, four enhancer regions (hs3a, hs1.2, hs3b and hs4), known as the 3'*Igh*RR, regulates *Igh* gene transcription and Ig isotype switching (Vincent-Fabert et al., 2010). The activity of the 3'*Igh*RR is decreased by AhR activation in murine B cell models, which mirrors the AhR-mediated inhibition of *Igh* expression and antibody secretion (Sulentic, Holsapple, & Kaminski, 2000; Sulentic, Kang, Na, & Kaminski, 2004; Wourms & Sulentic, 2015). However, there are structural differences in the 3' regulatory regions of human *IGH* and murine *Igh* genes (Hu et al., 2000; Mills et al., 1997; Pan et al., 2000; Pinaud et al., 2001; Vincent-Fabert et al., 2010). The AhR protein function and affinity for ligands are also different in humans as compared to mice. Therefore, studies related to the AhR and *IGH* gene using mice B cells might not entirely correspond to human B cells.

The previous studies in our lab utilized a human B-cell line (CL-01 cells) to study the effects of TCDD on human antibody production. TCDD differentially altered antibody production in that IgM production was not affected but IgG production was significantly reduced (Burra & Sulentic, 2015). The knockdown of the *AhR* gene in CL-01 cells by shRNA or CRISPR/Cas9 resulted in a profound inhibition of IgG production

(Alhamdan & Sulentic, 2017; Kashgari & Sulentic, 2015). These observations imply that the AhR has a physiological role in Ig class switching in human B cells CL-01 cells were isolated from a Burkitt's lymphoma patient. The primary mutation leading to the Burkitt's lymphoma was the translocation of the *MYC* oncogene to the *IGH* locus (Battey et al., 1983). However, several other mutations have been identified through genome sequencing of the Burkitt's lymphoma cells (Love et al., 2012; Schmitz et al., 2012). To verify that the loss of IgG production by AhR knockdown in the CL-01 cells is not due to an aberrant mutation specific to the CL-01 cells, the current study evaluated another human B-cell line SKW WT, which is also a Burkitt's lymphoma but isolated from a different patient, and it's AhR expressing variant, SKW AhR^+.

We confirmed that SKW WT cells do not express AhR endogenously using PCR analysis and western blotting. Induction of the cytochrome P450 family genes like CYP1A1 and CYP1B1 is considered a hallmark response of AhR activation (Nohara et al., 2006). The lack of *AhR* gene expression by SKW WT cells lead to the inability of the cells to induce transcription of our 6xDRE luciferase reporter and therefore AhR target genes. However, the SKW AhR^+ cell line also failed to induce expression of the AhR target gene when treated with TCDD.

The AhR transgene is fused with GFP for detection of AhR-expressing SKW AhR^+ cells (Kovalova et al., 2016). Results from flow cytometry and cell imaging tools suggested that only a small fraction of the cells from the entire population expressed the *AhR* transgene (data not shown). The simple explanation for the reduction in the number of cells that express the *AhR* transgene is that they are outgrown by the wild type cells, which in-turn might be due to improper isolation of the clone, i.e. presence of more than one cell

per well during limiting dilution cloning. The low number of cells expressing the *AhR* transgene made characterization the SKW AhR^+ cell line challenging. To enrich for AhR-expressing cells, we performed GFP-mediated fluorescence-activated cell sorting (FACS) to collect the GFP^+ SKW AhR^+ cells. Meanwhile, we continued the characterization of the SKW WT cell line and performed experiments to study the antibody (IgM and IgG) secretion levels and Ig isotype expression profile, which are discussed below. It was not possible to conduct the planned experiments on the GFP-sorted SKW AhR^+ cells because of the lockdown due to the COVID-19 pandemic.

I*n-vitro* stimulation of SKW WT cells by CD40 ligand and IL-4 significantly increased IgM secretion. Interestingly, the change in antibody secretion level was considerably greater than that of the CL-01 AhR^{TA} cells. T cell interaction via CD40 ligand on the T cell with CD40 on the B cell is critical for induction of CSR (Kim et al., 2005; Lane et al., 1992). In addition, T-cell derived cytokines (IL-4, IL-10 and IL-13) differentially activate B cells to produce a particular Ig isotype (Tangye et al., 2002). When human B-cell lines are stimulated with a combination of CD40 ligand and IL-4, they preferentially express IgG or IgE (Cerutti et al., 1998). Therefore, the fact that SKW WT cells can be stimulated by T-cell dependent factors (CD40 ligand and IL-4) *in-vitro* makes them a viable cellular model to study the role of the AhR in the regulation of IgG production. Moreover, it supports future studies using the enriched pool of SKW AhR^+ cells in comparison to the SKW WT cells to determine the role of the AhR in antibody secretion and the Ig isotype expression profile. The SKW WT cell line provides an excellent cell model to validate the hypothesis that the AhR is required for B cells to produce IgG. This statement is supported by the fact that SKW WT cells do not express

AhR endogenously, originated from a different Burkitt's lymphoma patient than CL-01 cells, and can be stimulated *in vitro* to produce antibodies.

We show that SKW WT cells lacking endogenous expression of the AhR gene, do not secrete IgG antibody. This corresponds to the loss of IgG secretion with AhR knockdown in the CL-01 cells and suggests a physiological role of the AhR in IgG production. A physiological role for the AhR in IgG expression is a novel finding and may have profound implications concerning overall regulation of antibody production in human B cells. Additionally, altered IgG levels could be one of the effects of AhR activation by exogenous ligands, which have been identified in pharmaceutical and dietary products and in the environment generally as pollutants. Indeed, a study observed that IgG levels in serum decreased for individuals exposed to the potent AhR ligand TCDD after a chemical plant explosion (Baccarelli et al., 2002).

The AhR-deficient SKW WT cells, show reduced expression of Cγ1 expression as compared to Cγ2-4 expression upon stimulation (fig. 10). This is congruent with our previous observation that the shRNA knockdown of AhR in CL-01 cells specifically targets and inhibits Cγ1 transcription (Kashgari & Sulentic, 2015). These observations are rather significant because we see a particular isotype targeted by lack of AhR expression in two human B-cell lines that originated from two different individuals, implying a specific influence of the AhR on γ1 production. Although the stimulation of SKW WT cells increases Cγ2-4 expression, normalization of the transcript levels to CL-01 AhR^{TA} revealed that the Cγ1-4 transcript levels in SKW WT cells are negligible when compared to AhR-expressing CL-01 AhR^{TA} cells, which also correlated with the IgG secretion levels in the two cell lines (fig. 11). These results support at least a partial contribution of the AhR in

regulating transcription of all Cγ regions, with more influence on Cγ1. One of the limitations of the current study is that the primers used to amplify the Cγ1-4 transcripts are unable to distinguish between the germline/sterile (non-coding) transcripts and the functional/mature (code for IgH) transcripts, meaning the primers will facilitate amplification of both transcripts. However, we did not detect any IgG protein secreted by SKW WT cells. Hence we can predict that the amplified transcripts were germline/sterile. On the other hand, for CL-01 AhR^{TA} cells, it is difficult to distinguish between functional/mature and germline/sterile transcripts since we do see IgG secretion. This is true for the primers used to detect Cα1-2 using quantitative real-time PCR as well; the results of which are discussed later.

Like Cγ transcription, the expression level of Cε is significantly less in stimulated SKW WT cells as compared to stimulated CL-01 AhR^{TA} cells (fig. 12). Although CD40L and IL-4 stimulation of human B cells preferentially induces class switch to IgE (Cerutti et al., 1998; Pène et al., 1988), we observe reduced expression of ε transcripts in SKW WT cells suggesting that lack of AhR also influences IgE production in human B cells. Studies have shown that germline transcription through the switch region corresponding to the target C_H region is essential for successful class switching. One of the supporting theories is that the continuous transcription of a particular C_H region aids in recruiting AID which acts on single stranded DNA (Chaudhuri et al., 2003; Ramiro, Stavropoulos, Jankovic, & Nussenzweig, 2003). Since the primers designed for Cε detect sterile transcripts only and we see amplification only in AhR-expressing CL-01-AhR^{TA} cells and significantly reduced levels in SKW WT cells, the AhR may play a regulatory role in germline transcription of the Cε region.

Expression of Cα1-2 is severely impacted in SKW WT cells (fig.13). The choice of the stimulating factors might not be optimal for of Cα1-2 transcription. Although the CD40L and IL-4 stimulation does not favor class switch to IgA isotype, CL-01 cells appeared to have spontaneously class switched as indicated by the basal Cα1-2 transcript levels (Burra & Sulentic, 2015; Cerutti et al., 1998) (fig. 13). In SKW WT cells, there were no detectable α1-2 transcripts. IgA is associated with immunity along the mucosal surfaces. Secreted IgA is dimeric and due to its unique structural properties, it can cross the epithelial barrier to enter the mucosal secretions and the lumen of the gut and confer immunity against pathogens at mucosal sites (Woof & Russell, 2011). IgA is also responsible for the sustainment of commensal microbiota in the gut and other mucosal areas (Pabst, Cerovic, & Hornef, 2016). AhR activation by dietary ligands has been found to modulate gut immunity. However, the target genes of AhR are not yet identified (Hooper, 2011). Modulation of IgA1 and IgA2 heavy chain gene expression might be one of the mechanisms by which AhR modulates humoral immunity in the gut. To determine if the lack of AhR is responsible for the suppression of the Cα1-2 expression, studies will be conducted using SKW AhR^+ cells. To evaluate AhR-mediated modulation of IgA, future studies will determine if the proper stimulation (TGF-β or TLRs) for IgA CSR induces Cα1-2 expression in the SKW AhR^+ but not SKW WT, which would be suggestive of an association between AhR agonists (dietary or microbial origin) and gut immunity.

In the current study, we observe that the expression of downstream C_H regions is very low to nil in the AhR-deficient SKW WT cells. Though the molecular mechanism for the regulation of C_H expression is not completely understood in humans, it is predicted that the enhancer regions in the 3' *IGH*RR differentially interact with intronic promoters

specific for each C_H region to regulate C_H expression (Hu et al., 2000; Pan et al., 2000). Another interesting finding is that some C_H (Cγ2-4 and Cε) regions are constitutively expressed as germline transcripts in naïve B cells suggesting that the chromatin structure is more euchromatin-like at *IGH* locus (Fear et al., 2004). However, in the current study, we observed that in naive SKW WT cells there is almost no basal transcription of γ1-4, ε and α and a slight increase in γ2-4 and ε transcripts in CD40L+IL-4 stimulated cells. Therefore, we speculate that the AhR may play a role in chromatin remodeling in naïve cells to keep the *IGH* locus available for constitutive transcription and CSR signals. Studies have revealed that ligand-activated AhR/ARNT bound to DRE sites in the promoter region of target genes has the ability to recruit "co-activators" that take part in 'loosening' the higher order chromatin structure (Hestermann & Brown, 2003; S. Wang & Hankinson, 2002). This supports the reasoning that the AhR could play a role in making C_H regions in the *IGH* gene locus available for constitutive germline transcription, which is thought to be a crucial step in CSR.

The canonical AhR signaling pathway requires the activation of AhR by an agonist. However, our previous studies and the current study demonstrate differences in the effects of agonist-activated versus constitutive AhR. When CL-01 AhR^{TA} cells are treated with the potent AhR agonist TCDD, IgG secretion is decreased, which also correlates to a decrease in transcript levels when compared to the stimulation control (fig. 9B and 11A-D). Thus, AhR activation by agonist has an inhibitory effect on IgG production. On the other hand, studies support a physiological role of the AhR in IgG production in two human B cell-lines, CL-01 (Alhamdan & Sulentic, 2017; Kashgari & Sulentic, 2015) and SKW WT (fig. 9B). These seemingly contradictory effects of the AhR in the presence vs. absence

of an exogenous agonist is not novel. Using AhR KO rats, studies support AhR regulation of IgM production and other physiological functions of B cells (Phadnis-Moghe, Chen, et al., 2016). Another study showed that the AhR can also signal through a non-canonical pathway involving other transcription factors to modulate the activity of the hs1.2 enhancer (Andrew D. Snyder, Ochs, Johnson, & Sulentic, 2020). Further, studies have suggested a non-consensus DRE binding site and a novel DNA binding co-factor of AhR, Kruppel-like Factor 6 (KLF6) (Joshi, Mustafa, Lichti, & Elferink, 2015; Wilson, Joshi, & Elferink, 2013). Collectively, these studies imply that there could be more than one signaling pathway mediating the effects of AhR.

AhR can crosstalk with other transcription factors, which are then thought to mediate the non-canonical signaling of AhR. Recently, a study confirmed that the mutations in binding sites of transcription factors such as POU and NF-1 differentially affected AhR-mediated transactivation of a luciferase reporter under the regulation of the hs1.2 enhancer upon TCDD treatment (Andrew D. Snyder et al., 2020). Further, the AhR/ARNT complex is thought to recruit estrogen receptor to the estrogen responsive promoter regions in addition to other co-activators (Ohtake et al., 2003; Safe & Wormke, 2003). Interestingly, ERα can effectively bind to the 3'*Igh*RR and switch regions upstream of C_H regions at murine *Igh* gene locus (Jones et al., 2016; Jones et al., 2020). It is not clear if the human *IGH* gene locus has binding sites for the ER. However, it has been shown that the AhR can recruit the ERα receptor to the promoter region of the CYP1A1 gene to sustain its transcription (Matthews, Wihlén, Thomsen, & Gustafsson, 2005). It would be intriguing to determine if the human *IGH* locus also contains binding sites for the ER and if the ER interacts with the AhR to regulate transcription of the *IGH* gene. The hs1.2 enhancer is

polymorphic among the human population, with variations in the number of repeats of an 'invariant sequence' containing DRE sites as well as other transcription factors (Giambra et al., 2005; Andrew D. Snyder et al., 2020). Meaning that each individual might have a different number of binding sites for AhR as well as for NF-1and other transcription factors that might be interacting with AhR in the hs1.2 enhancer. In humans, primary B cells from different patients respond to TCDD treatment differently. For example, TCDD treatment increases IgM secretion by B cells from one out of twelve patients; whereas IgM secretion was not affected in two out of twelve patients and in the remaining patients the IgM secretion was inhibited (Lu et al., 2010). These differences could be due to AhR polymorphisms or hs1.2 enhancer polymorphism or both. AhR KD studies in the CL-01 cell line and the current study with AhR null SKW WT cells suggest that AhR expression is a transcriptional regulator of the *IGH* gene. Therefore, we suppose that having different number of binding sites of AhR and other transcription factors (which possibly interact with AhR) due to hs1.2 polymorphism could impact the antibody isotype profile of an individual. Indeed, previous studies demonstrated that a variation in the number of invariant sequence repeats within the hs1.2 enhancer greatly impacts the Ig isotype profile (Andrew David Snyder & Sulentic, 2016). This could be directly due to the variation in number of the binding sites of other transcription factors that possibly interact with AhR to mediate the biological functions of AhR.

The Ig isotype profile of SKW WT cells shows that the cells secrete IgM but are unable to transcribe any other C_H region, which correlates with a IgM-memory cell or innate B cell stage. IgM-memory cells only produce IgM and are crucial for defense against enveloped bacteria during early stages of life (Capolunghi, Rosado, Sinibaldi, Aranburu,

& Carsetti, 2013). IgM memory cells can be categorized as cells that express both surface IgM and memory B cell marker, CD27 (Cameron et al., 2011). Physiologically, IgM memory cells are derived from germinal center independent pathway, generally they are activated in spleen and/or circulation via TLRs which binds to the polysaccharides on enveloped bacteria and CpG (Capolunghi et al., 2008; Weller et al., 2001). However, a study shows that stimulation of IgM memory cells by CD40 ligand in-vitro can induce CSR in these cells (Marasco et al., 2017), suggesting that the inadequacy of CSR in SKW WT cell could be due to lack of AhR expression and not because of any other physiological implications, like being an IgM memory cell. Moreover, the SKW WT cells are IgM and IgD positive B cells that also express CD20, which is considered to be a marker of peripheral mature B cell (Sirk, Olafsen, Barat, Bauer, & Wu, 2008; Suciu-Foca et al., 1988). Therefore, although unlikely, if SKW WT cells are derived from a IgM memory cell population, they would still be able to secrete IgG upon stimulation. Therefore, we still hypothesize that the loss of antibody production in the SKW WT cells is due to the lack of AhR expression.

For future studies, we plan to evaluate the Ig isotypic profile of the enriched pool of SKW AhR^+ cells. Based on our hypothesis and preliminary data, we anticipate that AhR expression will alter the Ig isotypic profile. Specifically, we expect to see IgG secretion and higher transcript levels of ε and α1-2 transcripts as seen in AhR-expressing CL-01 cells. The increase in transcription of C_H regions after introduction of AhR expression in the SKW WT cells would provide direct evidence that the AhR plays an important role in regulating transcription o the *IGH* gene locus.

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