THE ROLE OF LCK AND PD-1 IN TCDD-MEDIATED SUPPRESSION OF THE IgM RESPONSE BY HUMAN CD5^{+} INNATE-LIKE B CELLS

By

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ABSTRACT

THE ROLE OF LCK AND PD-1 IN TCDD-MEDIATED SUPPRESSION OF THE IgM RESPONSE BY HUMAN CD5^{+} INNATE-LIKE B CELLS

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The aryl hydrocarbon receptor (AHR) is a cytosolic ligand-activated transcription factor involved in xenobiotic sensing and cell regulation. The activation of AHR by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been shown to impair immunoglobulin M (IgM) responses in all laboratory animals. Previous studies in mouse splenocytes and purified mouse B cells revealed that AHR activation leads to a decrease of IgM production. It has been widely assumed that the molecular mechanisms responsible for AHR-mediated suppression of the IgM response would be similar across animal species. However, no direct comparison has been conducted between mice and humans. Therefore, the first part of this dissertation is focused on comparing AHR-mediated suppression of IgM responses in mouse and human B cells. Contrary to the observations in mouse B cells, TCDD treatment results in a significant suppression of the number of IgM secreting cells, but it is not due to a decrease in IgM molecules in human B cells. These results suggested that AHR-mediated suppression of the IgM response involves different mechanism between mice and humans.

The second part of this dissertation is focused on elucidating the role of lymphocyte-specific protein tyrosine kinase (LCK) in AHR-mediated suppression of the IgM response in human B cells. LCK is a well-characterized tyrosine kinase in T cell biology. In contrast, limited research has been done to understand the role of LCK in human B cells. An upregulation of LCK protein has been observed in AHR-activated

human B cells. Treatment with an AHR antagonist reversed the AHR-mediated increase of LCK. Furthermore, LCK specific inhibitors also reversed the AHR-mediated suppression of the IgM response by human B cells. Collectively, the studies demonstrate a novel role of LCK in IgM secretion and provide new insights into the mechanism for AHR-mediated impairment of immunoglobulin secretion by human B cells.

The third part of this dissertation is focused on understanding the role of LCK and program cell death protein-1 (PD-1), in $CD5^+$ innate-like B cells (ILBs). Human $CD5^+$ ILBs express high levels of LCK and PD-1 compared to $CD5^-$ B cells. Therefore, studies were conducted to determine the role of LCK and PD-1 in AHR-mediated suppression of the IgM response in $CD5^+$ ILBs. In the current studies, AHR activation significantly upregulated total LCK and PD-1 proteins in $CD5^+$ ILBs. LCK inhibitor treatment prevented the PD-1-mediated suppression of the IgM response in $CD5^+$ ILBs. Furthermore, PD-1 blocking antibody prevented the suppression of the IgM response in $CD5^+$ ILBs. Collectively, results from these studies support the critical role of LCK and PD-1 in AHR-mediated suppression of the IgM response by human $CD5^+$ ILBs.

Taken together, the results from these studies indicate that a) AHR-mediated suppression of the IgM responses is mechanistically different between mouse and human B cells; b) in humans, activation of AHR suppresses the IgM response through the modulation of LCK; c) LCK and PD-1 play a critical role in AHR-mediated suppression of the IgM response in $CD5^+$ ILBs.

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KEY TO ABBREVIATIONS

2,4-D	2,4-dichlorophenoxy acetic acid
2,4,5-T	2,4,5-trichlorophenoxy acetic acid
AHR	Aryl hydrocarbon receptor
ARA9	AHR-associated protein 9
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASC	Antibody secreting cell
Ca^{++}	Calcium
CD	Cluster of differentiation
CD40L	CD40 ligand
CTV	Cell trace violet
Cyp	Cytochrome P 450
CH	CH-223191 (AHR antagonist)
DLCs	Dioxin-like compounds
DMSO	Dimethyl sulfoxide
DRE	Dioxin response element
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorting
GC	Germinal center
h	Hour
HSC	hematopoietic stem cell
Hsp90	Heat shock protein 90

IgM	Immunoglobulin M
IgG	Immunoglobulin G
IL	Interleukin
IRF	Interferon regulatory factor
ILB	Innate-like B cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
JNK	Janus kinase
LPS	Lipopolysaccharide
LCK	Lymphocyte-specific protein tyrosine kinase
LYN	Lck/Yes novel tyrosine kinase
MAPK	Mitogen-activated protein kinase
MARE	Maf response elements
MFI	Mean Fluorescence Intensity
MHC	Major histocompatibility complex
min	Minute
mRNA	Messenger RNA
NFκB	Nuclear factor kappa B
NHL	Non-Hodgkin’s lymphoma
nAbs	Natural antibodies
nIgM	Natural IgM
PAX-5	Paired box-5

PBMC	Peripheral blood mononuclear cell
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo dioxins
pERK	Phosphorylated extracellular regulated kinase
PWM	Pokeweed mitogen
PI	Peptide
PKC	Protein kinase C
PFC	Plaque-forming cell
qRT-PCR	Quantitative Real time-polymerase chain reaction
RAG	Recombinase
SSG	Sodium stibogluconate
sRBC	Sheep red blood cell
SYN	Spleen tyrosine kinase
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
Tfh	T follicular helper
Th	T helper
TLR	Toll-like receptor
TRAF	TNF receptor associated factor
VH	Vehicle
ZAP70	Zeta-chain-associated protein kinase 70

CHAPTER 1: LITERATURE REVIEW

1.1. Purpose of this research

Aryl hydrocarbon receptor (AHR) is a cytoplasmic receptor involved in the regulation of cell functions and xenobiotic sensing (1-6). AHR also plays a role in immune modulation, especially in the expansion, maturation and differentiation of B cells (7-11). High affinity ligands, like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), have been widely used to study the physiological role of the AHR. B cells are a sensitive target for AHR activation as evidenced by significant impairment of B cell lineage commitment and the suppression of humoral immune responses (12-14). However, the underlying mechanism of how AHR modulates antibody responses in B cells is still largely unknown. Therefore, this dissertation research aims to understand AHR-mediated immune suppression, with a special focus on signal pathway in order to provide insights into mechanisms responsible for AHR-mediated suppression of human B cells.

1.2. History and general toxicity of dioxins

Dioxins and dioxin-like compounds (DLCs) are ubiquitous environmental contaminants. Dioxins belong to the family of halogenated aromatic hydrocarbons including dibenzo-*p*-dioxins, polychlorinated dibenzo-*p*-furans, polychlorinated biphenyls and polybrominated biphenyls. Within dioxins and DLCs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent congener with the highest binding affinity to the AHR. Dioxins and DLCs were mainly found as contaminant during herbicide manufacture and chlorinated bleach production in the industrial era. The production of chlorophenols used as fungicides also led to the release of dioxins and DLCs into the environment. Other

sources of dioxin and DLCs emission included industrial burning of steel and ceramic, landfill fires, backyard burning of trash and incineration of municipal solid waste.

Dioxins and DLCs are lipophilicity and they have high tendency of bioaccumulation in the environment (15). Therefore, consumption of a high fat diet is thought to account for more than 90% of the body burden of dioxin and DLCs in the human population (16). The toxicity of dioxin in humans has been subjected to numerous evaluations in the past decades. Even though the level of dioxins has been declined, public concern still continues with respect to the potential toxic effects of DLCs exposure in sensitive and highly exposed populations.

It has been demonstrated that TCDD elicits a broad spectrum of species and tissue-specific toxicity and biochemical effects. Hepatoxicity, immunotoxicity including lymphoid involution, dermal toxicity, teratogenicity, tumor promotion and wasting syndrome are some of the hallmark toxic effects observed in animal models (12, 17-19). Biochemical effects include alteration in proliferation and differentiation, endocrine homeostasis and the induction of cytochrome P450 enzymes (2). Animals are known to have different sensitivities toward TCDD exposure owning to the rate of metabolism of TCDD. The median lethal dose (LD50) for TCDD varies about 5000-fold between the sensitive guinea pig (LD50: 1 μg/kg) and the tolerant Syrian hamster (LD50: 5000 μg/kg) (20). In addition, variations in strain-sensitivity to TCDD were demonstrated by using inbred mouse strains. The C57BL/6 strain was classified as a “responsive” strain due to their ability for 3-methylcholanthrene (3-MC) to induce hepatic microsomal aryl hydroxylase (AHH) activity (21). In contrast, the DBA/2 strains were classified as “non-responsive” due to the inability to induce AHH activity by 3-MC. The decreased binding

to these chemicals was thought to depend on a mutation in the inducer binding site of the non-responsive mice (22). The genetic locus governing this trait was later identified to be the AH (Aromatic hydrocarbon) locus. The allele conferring responsiveness in the mouse strains is denoted as Ah^b for responsive strains (b-C57BL/6 mice) or Ah^d for non-responsive strains (d-DBA/2 mice). Similar effects were observed when C57BL/6 or DBA/2 mice were treated with TCDD; however, TCDD was bound to the same locus with a 30,000-fold higher affinity compared to 3-MC. The C57/BL6 mouse strain was found to elicit cytokine p450 (CYP) activity with 10-fold higher affinity compared to the DBA/2 mouse (23). The inheritance of AHR-mediated CYP induction was an autosomal dominant trait and is thought to be the basis for TCDD-induced toxic effects as well as biochemical effects (24). These findings led to the search for a cellular receptor that initiated the biological response, which is now known to be aryl hydrocarbon receptor (AHR).

Aryl hydrocarbon receptor (AHR)

AHR is a cytoplasmic receptor that can be activated by environmental contaminants and endogenous ligands (1, 2). Persistent AHR activation, which appears to be a characteristic associated with toxicity, is a consequence of exposure to high affinity AHR ligands such as members of the halogenated dibenzo-*p*-dioxins, dibenzofunrans and biphenyls. The amino acid sequence of AHR is highly conserved across different animal species, especially in vertebrates (25). In addition, AHR orthologs found in invertebrate species was lacking ligand binding but possessing physiological functions (26, 27). The identification of endogenous AHR ligands such as indoles, indigoids and tryptophan metabolites has changed the understanding and perception of AHR (4-6). It is

believed that endogenous AHR activation is critical for maintaining the homeostasis and upregulate the expression of cytochrome P450 enzymes that degrade endogenous ligands (28). Studies using endogenous and exogenous AHR ligands, AHR antagonists, and AHR knockout animal models have clarified the physiological roles for the AHR is in regulating various biological processes, including cell cycle (29, 30), apoptosis (31), tumor progression (32), neuronal development (33), vascular development (9), and ovarian development (34). From the standpoint of the immune system, AHR also plays a role in the expansion, maturation (10) and differentiation of immune cells (11). Within the immune system, AHR has been demonstrated to be involved in inflammatory responses (35, 36), autoimmune responses (5) and allergy (37) by regulating the development, homeostasis and function of immune cell populations (38). Additionally, the activation of AHR is also important for the differentiation of Th_{17} cells, induction of IL-22; generation of T_{reg} from $CD4^+$ T cells; differentiation of Th_{17} cells; maintenance of innate lymphoid cells; immunogenicity of dendritic cells; and function of mature B cells (39-42). The B lymphocyte is the most sensitive target for AHR activation as evidenced by significant suppression of humoral immune responses (12-14).

From a toxicological standpoint, TCDD serves as a prototypical ligand for AHR activation due to its high binding affinity. Thus, TCDD has been widely used to study the physiological role of AHR. TCDD-mediated activation of the AHR can produce a wide range of biological and toxicological responses including hepatotoxicity and immune suppression in different animal species (2, 12, 17-19). AHR null mice and rats (43) are resistant to hepatotoxicity, the induction of AHR gene battery and suppression of humoral immune responses upon exposure to DLCs or TCDD (44, 45). Previous studies have also

shown that exposure to inflammatory stimuli results in substantial defects in mucosal immunity in AHR null mice (46). The toxicity of AHR activation is primarily mediated through the AHR canonical pathway (Fig.1.1). In the absence of ligand, AHR remains quiescent as a multiprotein complex as it is bound by heat shock proteins (hsp90), Ah-associated protein-9 (Ara9) and co-chaperone protein (p23) in the cytosol (47). Upon ligand binding, the AHR undergoes a conformational change, exposing the nuclear localization sequence at its N-terminal, which facilitates translocation into the nucleus (48). In the nucleus, the ligand-receptor complex heterodimerizes with AHR nuclear translocator (ARNT) (49), thereafter, binds to Dioxin Response Elements (DREs) as a transcription factor throughout the genome. Negative regulation of AHR occurs after the dissociation of AHR from the DRE, followed by export to the cytoplasm and subsequent degradation through the ubiquitin-26S proteasome pathway or by a secondary pathway involving repression of AHR transcriptional activity by the AHR repressor (AHRR) (50) (Fig.1.2).

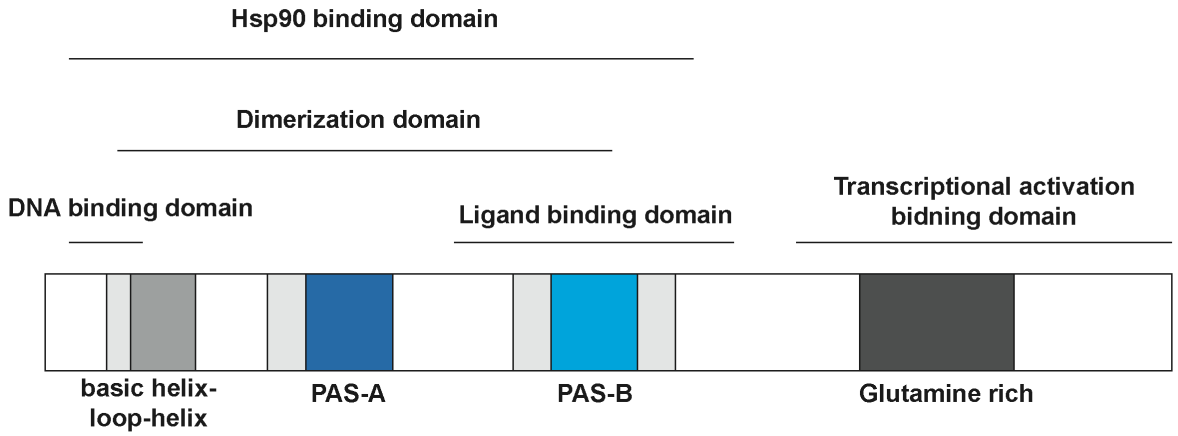


Figure 1.2.1. General organization of Aryl Hydrocarbon Receptor (AHR)

The figure above illustrates the different domains within the AHR. The DNA binding domain is located N terminus (27 – 39). The PAS-B domain is involved in ligand binding (230 – 397). Binding of chaperon protein (Hsp90) involves the basic helix-loop-helix domain and the PAS-A domain (27 – 79, 182 – 374). Transcriptional activation domain is located at the C terminal region.

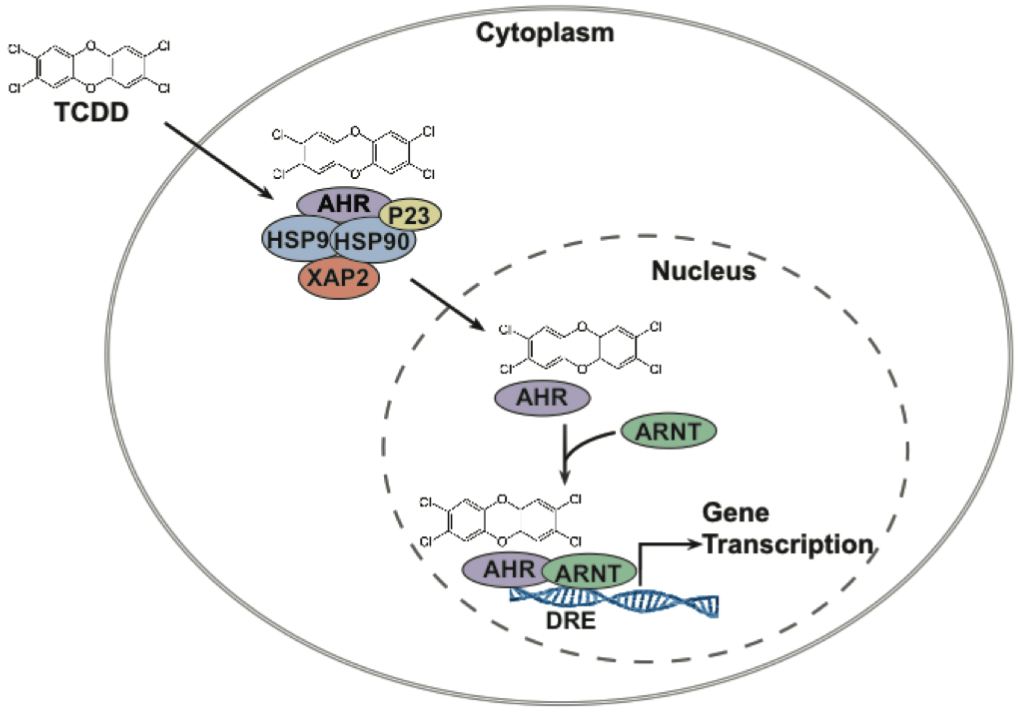


Figure 1.2.2. TCDD-mediated AHR activation.

Upon entering the cytoplasm, TCDD can bind to AHR. Without ligand binding, AHR remained bound to chaperon proteins (HSP90, XAP2 and P23). When TCDD binds to AHR, the TCDD-AHR complex then translocates into the nucleus. Upon entering the nucleus, AHR sheds the chaperon proteins and then binds to AHR nuclear translocator (ARNT). The TCDD-AHR-ARNT complex then acts as a transcription factor that can bind to dioxin responsive element (DRE) on the chromosome.

Immunotoxicity of TCDD

The immune system is particularly sensitive to toxicity of TCDD, and the effects has been observed in virtually all laboratory species (12). Some of the earliest studies have indicated the effects of TCDD on the immune system and demonstrated that TCDD could suppress cell-mediated immune responses in guinea pigs and mice (51). Thymic involution was also identified and later determined to be due to the suppression in differentiation of thymic epithelial cells, which in turn affected the maturation of T lymphocytes (52). Subsequently, it was shown that TCDD treatment significantly decreased the number of $CD4^+$ and $CD8^+$ T cells in the thymus (53). Administration of TCDD *in vivo* also led to a decrease in cytotoxic T lymphocyte (CTL) activity (54). Overall, the effects of TCDD on cell-mediated immunity occurred at high concentrations of TCDD, which significantly decreased cellular viability. Single doses of TCDD significantly impaired primary and secondary humoral immune responses to T-dependent and T-independent activation (55). In the early 1980s, one of the first studies showed selective suppression of the plaque-forming cell response following sheep red blood cell treatment (sRBC) or LPS-stimulation without affecting cellular viability. Suppression of *in vivo* IgM responses to sRBC, dinitrophenyl-Ficoll (DNP-Ficoll) or trinitrophenyl-LPS (TNP-LPS) with TCDD treatment had been shown in mice (56, 57). Moreover, direct addition of TCDD to naïve splenocyte cultures followed by *in vitro* activation with LPS or sRBC led to a concentration-dependent suppression of the IgM response (12). As the splenocytes contain several cell types, it was thought that the effect of TCDD on the IgM response was due to overall effects on leukocytes rather than direct effects on the B cells. To address this concern, separation-reconstitution experiments were performed on B cell, T

cell and macrophage fractions isolated from the spleens of mice treated with vehicle or TCDD treatment. Only those cell fractions containing B cells from TCDD-treated mice showed suppression of the IgM response thus suggesting that the B cells were indeed the main cell type affected by TCDD within the context of IgM suppression (56). Additionally, the direct effects of TCDD on B cells were also determined using magnetic purified mouse B cells, which were sensitive to the LPS-induced IgM response (58).

Additional studies were performed to understand whether the immunotoxicological effects of TCDD were dependent on the activation of AHR. The first study utilized the differences in binding affinity of AHR among the different PCDD, PCDF and PCB congeners; and the second study used congenic mice differing at the Ah locus. The structure-activity relationship study provided evidence for a correlation between significant suppression of antibody response to the affinity of AHR binding (57). The other study using congenic mice shows that the antibody response to sRBCs was differentially suppressed by TCDD in the mice harboring Ah^b allele as opposed to the Ah^d allele mice which required a 10-fold higher dosage of TCDD to elicit similar toxic effects (55). The most conclusive evidence for the role of AHR in suppression of the immune response was observed and verified through the generation of AHR null mice and cell lines. The highly sensitive mouse B cell line, CH12.LX, showed suppression of the primary IgM response at a concentration of 0.03 nM. In contrast, the BCL-1 mouse B cell line, which lacked AHR expression was unaffected by TCDD at 100 times higher concentrations (59). In addition, the presence of AHR was essential for TCDD-mediated suppression of the primary IgM response in mice (60). Studies performed in AHR null rats also confirmed the role of AHR in suppression of the antibody response (43). Interestingly, the basal level of

IgM in AHR null rats were significantly higher compared to the wild type (WT) rats, indicating the possible role of AHR in regulating IgM in rats.

From an immunological standpoint, activation is critical to determine the fate of the immune cell (61). For the immunotoxicological effects of TCDD, it is important to identify the critical window of sensitivity of TCDD, in order to determine the critical time for TCDD to elicit toxic effects in the immune system. Addition of TCDD on day 1 after cell activation led to no suppression of the IgM response (57). An additional study also showed that TCDD-mediated suppression of the primary IgM response could only be produced if TCDD was added to splenic cultures 3h prior to LPS activation (61). Recently, in a human B cell line and in mature B cells, the window of sensitivity of TCDD was determined to be within the first 24-hour of B cell activation (62, 63). The narrow window of susceptibility to TCDD strongly suggests that TCDD alters critical early B cell activation events, which subsequently lead to the alteration in antibody response. Subsequent studies have demonstrated that TCDD impaired the expression of B cell activation markers (CD69, CD80 and CD86) and BCL-6, a critical transcription factor that governs the transition of naïve B cell into plasma cells (64). With the development of flow cytometric analysis, studies have demonstrated that TCDD treatment significantly decreased CD69 in individual AHR^{hi} human B cell (by measuring *CYP1A1* mRNA as a biomarker), further suggesting that TCDD treatment altered the activation and differentiation processes of human B cells (65).

Along with the effects on mature B cells, TCDD has also been shown to be a potent immunotoxicant for hematopoietic stem cells (HSCs). Stem cells express high levels of AHR, suggesting a potential physiological role for the AHR in stem cell maturation and

development of immune cell precursors. AHR null mice exhibited an increase in the Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) population in the bone marrow (66), suggesting that AHR plays a role in maintaining quiescence of HSCs. Another important study has highlighted that AHR antagonists promote expansion of human hematopoietic stem cells (39). TCDD also impair the long-term ability to reconstitute stem cells in the bone marrow of irradiated host mice (67). Recently, an *in vitro* model using cord blood HSCs to mimic the development of stem cell to B cell lineage commitment has shown the role of TCDD-mediated AHR activation in regulating human B cell development and the transcriptional alteration of EBF1 and downstream transcriptional factors (PAX5, ETS1) by TCDD treatment. (13, 14).

TCDD immunotoxicity also extends to the innate immune system with effects seen on soluble mediators such as the complement protein C3 (68). TCDD exposure leads to increased susceptibility to several bacterial and viral infections (69). Recent studies have also determined that TCDD-treatment increased the expression of MHCII, CD80 and CD86 in dendritic cells in an AHR-dependent manner (70, 71).

Human health effects of TCDD

The effects of dioxins and DLCs on humans have mainly been obtained through epidemiological and occupational studies. There have been several instances of accidental exposure to dioxins and DLCs in the past several decades. One of the more well-documented incidents of dioxin exposure was the usage of Agent Orange during the Vietnam war as a defoliant in aerial spraying of herbicides (72). TCDD was present as a contaminant in 2,4,5-T at a mean level of 2 parts per million (NRC, 1994). However, the toxic effects of TCDD were only reported in the early 1970s. Around the same time,

researchers assessed the effects of TCDD in several other areas contaminated with dioxins. For example, the dioxin contaminated chemical waste disposed on Times Beach, Missouri was as high as 300 parts per billion that led to an evacuation of the residential area. One major event associated with occupational and environmental exposure to TCDD was during an accidental release of dioxin contaminated chemicals at Monsanto's chemical manufacturing plant in Nitro, West Virginia. Trichlorophenol contaminated with TCDD was accidentally released into the environment. Workers from this plant developed symptoms of 'chloracne' that involved hyperplastic and hyperkeratotic changes to the skin after expose to the contaminants (NAS, 1994). Chloracne has been referred to as a hallmark of acute TCDD toxicity in humans (72). Among international incidents of environmental exposures to TCDD, one of the largest exposures occurred in 1976, in Seveso, Italy. A chemical reactor containing TCDD-contaminated 2,4,5-trichlorophenol exploded and generated a chemical cloud which contaminated the nearby residential area. In Japan and Taiwan, rice oil contaminated with polychlorinated biphenyls and polychlorinated dibenzofurans led to serious food poisoning in the population (73). Apart from exposure through environmental disasters, one of the major ways by which humans are exposed to TCDD is through a high-fat diet. Average daily exposure to TCDD is about 0.1 to 0.3 pg TCDD/kg/day and adults in developed countries had approximately 6 ppt TCDD per mL serum (72, 74), which is several fold lower than the exposure due to the aforementioned environmental accidents. In 2011, a comprehensive reassessment of the concentrations of TCDD in human serum reported that the levels of dioxins have declined over the past 30 years (75). However, concerns still remain pertaining to the potential consequences of dioxin and DLCs exposure on human health due to the persistent effects

of this toxic contaminant. Some of the epidemiological studies performed in dioxin contaminated areas have examined relationships between occupational or environmental exposure to dioxins and their relative cancer risk. A cohort study conducted in two chemical factories in the Netherlands showed positive associations between the risk for developing non-Hodgkin's lymphoma (NHL) with TCDD exposure (76). A case-control cohort-based study in Sweden indicated that an increased risk for NHL upon exposure to herbicides containing phenoxyacetic acid and mixture of 2,4-D or 2,4,5-T containing chlorophenols (77). Other studies investigating the effects of TCDD exposure on industrial workers showed potential correlation between increased total cancer rates with increased exposure to TCDD (76, 78-80). Modulation in humoral immune responses, specifically in the decrease in the serum IgM and IgA levels and a suppression of cellular immunity was reported in the 'Yusho' rice-bran oil poisoning incident in Japan (73). Additionally, samples collected from exposed subjects during the Seveso incident found to have a decrease in IgG levels with increasing lipid-adjusted TCDD plasma concentrations (81). A follow-up study performed on Vietnam war veterans exposed to contaminated Agent Orange also displayed an increase in IgE levels with a modulation in the immune responses towards the Th_2 type, indicating an increase of sensitivity to allergic diseases (82). Evidence for NHL and soft-tissue sarcomas was also found in Beçanson, France, an area containing a municipal solid waste incinerator. A logistic regression analysis suggests that individuals living in the contaminated area had 2.3-fold higher risk to develop NHL as compared to individuals residing in areas with lower exposure (83). A study in a Netherlands cohort, measured effects of peri-natal exposure to dioxins reported a reduction of granulocyte numbers along with an increase in the incidence of infectious

diseases like otitis media and chicken pox (84, 85). The levels of dioxin in the same study indicated a significant correlation with increased peripheral $CD8^+$ T cell populations and decreased B cell markers. All these studies provided supportive experimental evidence for a positive correlation between environmental exposures to dioxin (TCDD) or DLCs and the incidence of lymphoma and immune modulation. However, a direct link between TCDD exposure and cancer in humans is hard to derive as several critical factors could influence epidemiological outcomes (86). Furthermore, human variability including confounders such as age, body weight, smoking, alcohol consumption, incidence of diseases and genetic composition, which underlies differences in the responses to environmental contaminants (87). Apart from human variability, differences in time frame of studies post-exposure, composition of the experimental cohort, levels of exposure and methods of assessment tend to also influence the results (81). Based on the experimental observations and data, it is rational to speculate that a possible correlation can exist between TCDD exposure and cancer development; however, the mechanism is still uncleared in the field. It is crucial to know if human cells exhibit polymorphisms at the Ah locus like in mouse species, especially for assessment of mechanisms of toxicity in human cells and estimating potential human risk posed by dioxins and DLCs. Most of the identified human polymorphisms are concentrated in the AHR transactivation domain in exon 10. Polymorphisms at codons 517 (1549 C > T), 554 (1661 G > A) and 570 (1708 G > A) were identified by single-strand conformation polymorphisms in Japanese subjects (88). A combination of three polymorphisms resulting in a haplotype coding for serine at 517, lysine at 554 and isoleucine 570. These three polymorphisms greatly affected CYP1A1 induction *in vitro* (89). Other polymorphisms have also been reported in the 5'

flanking sequence of AHR. However, their polymorphisms have not been associated with altering AHR function. Human AHR encoding valine at codon 381, which has a 10-fold lower binding affinity to TCDD as compared to the mouse AHR encoding alanine at codon 375 but no humans have been identified with any polymorphism at that locus (88, 90). It has been reported that the polymorphism at codon 554 (lysine to arginine) is associated with lower AHR, ARNT and CYP1B1 expression levels; however, the mechanistic basis of the differences between gene expression levels remains to be determined (62, 91). Recently, the polymorphisms at codon 517, 554 and 570 has been reported to be critical for the TCDD-mediated suppression of the IgM suppression in human SKW 6.4 B cell line (62). However, it is raw that all three of the polymorphisms would be present together in humans. Therefore, taking into consideration of the role of polymorphisms along with the toxic effects exhibited by TCDD upon binding to AHR can provide a comprehensive understanding of the diverse response of AHR activation in human cells.

Effect of TCDD on B cell function

With the advance in technologies, advancements have also been made in toxicological studies. Previous studies conducted in animal models have been validated with *in vitro* studies focusing on a better understanding of the molecular basis of toxicity. Development of cell line-based *in vitro* models has enabled detailed investigations into the transcriptional and translational processes taking place during B cell differentiation.

Mouse CH12.LX B cell line was isolated from a mouse CH12 lymphoma using cloning by limiting dilution (92). The CH12.LX cells can be induced to secrete large amount of IgM upon polyclonal activation (LPS or PWM) and were found to be sensitive

to TCDD treatment (59). The basal expression of AHR and ARNT in the CH12.LX cells has been reported to be much higher to the levels detected in primary lymphocytes and has showed an increase in AHR protein upon activation in agreement with the previous findings (93, 94). The TCDD-mediated suppression of the primary immune response has been documented and correlated with an inability of mature B cells to differentiate into antibody secreting plasma cells. At the molecular level, the TCDD-AHR complex was found to bind to the DREs in the Ig3'α enhancer region. TCDD-AHR has shown to bind to the regulatory region of Ig heavy (IgH) chain. The decrease of expression in heavy (H) chain led to a decrease in IgM protein secretion (95, 96). Additional studies further illustrated the mechanism underlying suppression of the IgM response by focusing on the upstream activators of IgM (Pax-5, Blimp-1, AP-1 and NFκB). LPS-induced DNA binding activity of AP-1 was significantly inhibited by TCDD in an AHR-dependent manner (97). Furthermore, LPS-activated mouse B cells showed elevated expression of Pax-5 with TCDD treatment further suggesting that the TCDD-AHR complex altered the IgM response through Pax-5 expression. Concordantly, *IgH*, *Igκ*, *IgJ* chain and *Xbp-1* genes were significantly suppressed in presence of TCDD (98). Blimp-1, a positive regulator of B cell differentiation was significantly suppressed in a TCDD concentration dependent manner with a decrease in its DNA-binding ability at the Pax-5 promoter region. The binding of AP-1 within Blimp-1 promoter was also decreased by TCDD treatment suggesting that TCDD altered IgM expression through Blimp-1 and Pax-5 (99). Primary splenocytes from TCDD-treated mice also displayed a TCDD dose-dependent suppression of Blimp-1, XBP-1, IgH, IgJ and Igκ along with a decrease in the total number of $CD19^+$ and $CD138^+$ plasma cells (100). An increase in BCL-6 levels was also observed

in TCDD treated primary mouse B cells (101). In addition, TCDD-mediated suppression of B cell differentiation involved the effects on proximal B cell signaling. Some of the earliest studies have shown TCDD treatment increased basal kinase activity (102) and altered phosphorylation of kinases in activated B cells (103). TCDD treatment has also been shown to increase membrane protein phosphorylation in B cells. This increase was proportional to the suppression of B cell antibody synthesis, therefore, suggesting that changes of early phosphorylation events could potentially affect B cell signaling and differentiation (104). Mobilization of Ca^{2+} is another important signaling event during proliferation of B cells when stimulated through the B cell receptor (105). TCDD was shown to attenuate B cell proliferation activated by ionomycin when phorbol ester and calcium ionophore (PMA/Io) was used to cross-link BCR signaling pathway (106). Disruption of Ca^{2+} was thought to be another mechanism by which TCDD affected IgM secretion (107). However, the exact mechanism by which these kinases could alter B cell function is not yet known with several hypotheses suggesting an abrogation of downstream cellular signaling phosphorylation or a signaling-mediated change in binding partners, which could eventually affect transcriptional control of B cells.

The ability of TCDD to suppress B cell activation and differentiation was further investigated by measuring LPS-induced kinase phosphorylation in presence of TCDD in mouse primary B cells (101). The results from this study suggested an involvement of signaling molecules (AKT, ERK and JNK) in the presence of TCDD. Moreover, TCDD decreased B cell activation by suppressing MHCII, CD69, CD80 and CD86 expression in mouse B cells (101). All these studies have indicated a multi-faceted mechanism controlling B cell activation and differentiation by TCDD. Genome-wide studies further

contributed towards the understanding of TCDD and AHR-mediated molecular alterations. A combination of Chromatin Immunoprecipitation (ChIP) on whole genome tiling arrays (ChIP-on-chip) analysis and gene expression microarray analysis identified genomic regions directly regulated by AHR, resulting in the changes in downstream gene expression. A total of 78 genes were directly bound by AHR and significantly altered in gene expression (upregulation or downregulation) upon TCDD-treatment in LPS activated CH12.LX cells (108). Signaling networks were reconstructed to connect the 78 new target genes to the existing transcriptional network genes, including Blimp-1, Pax-5 and Bcl-6, and multiple connections were identified. Overall this study highlighted that AHR-mediated suppression of B cell differentiation involved multiple pathways and mechanisms including the direct effects of AHR on regulatory elements of transcription factors and/or indirectly through disruption of signaling pathways and co-regulation of key target genes by TCDD and AHR (108). Additionally, studies have indicated that addition of IFNγ could restore the TCDD-mediated suppression of the IgM response in mouse B cells. IFNγ could also attenuate the increase of *CYP1A1* in TCDD treated mouse B cells. This phenomenon was only observed with IFNγ treatment, but not with type I IFN (IFNα and IFNβ) (100).

Mice have been in the focus for *in vivo* immunological experiments for several decades. In many ways, mice have been reliable models for research in human disease. Despite significant genomic conservation between mice and humans, there are significant differences in development, activation and response of the innate and adaptive immune systems between mice and humans (109). The validity of using mouse models for research has been questioned in several articles and reviews (110). From a toxicological

standpoint, it is informative to know the effects of toxicants on humans especially for risk assessment. A majority of the historical toxicological data has been obtained using animal models with a few exceptions of human epidemiological studies. Human risk assessments primarily based on extrapolation of data obtained from animal studies, which have been demonstrated in numerous uncertainties including the understanding of dose-response and adverse effects within species (111). One way to reduce the uncertainty would be to include experiments using primary cells obtained from healthy human donors or human tissues. Despite challenges associated with working with primary human cells, some pioneering studies were performed to detect and characterize AHR in the cytosols from homogenized human tonsils (112). From this study, TCDD treatment was shown to decrease the IgM responses of pokeweed mitogen (PWM)-activated human tonsillar lymphocytes (113). A TCDD dose-dependent increase in ethoxyresorufin-O-deethylase (EROD) activity was observed in PWM activated tonsillar lymphocytes. Resting lymphocytes were mostly refractory to TCDD treatment, but LPS and T cell substituting factor- activated human B cells demonstrated suppression of IgM secretion in a dose-dependent manner (114). Furthermore, studies indicated that the toxic shock syndrome toxin-superantigen-induced IgM response was sensitive to TCDD in human lymphocytes (114, 115). Taking together, these pioneering studies provided significant movement to understand the effects of TCDD on human B cells. In addition, the TCDD-AHR complex was found to bind to DREs in the human CD19 gene thereby suppressing the expression of CD19 upon TCDD treatment (116). These studies demonstrated AHR transcription altering human B cell function by down regulating a

mature B cell marker, CD19. However, a significant data gap existed in the field with regards of the sensitivity of human cells to TCDD.

The establishment of an IgM antibody-forming cell response model utilizing human primary B cells isolated from healthy human donors bridged this data gap. In this *in vitro* model, B cells were activated using CD40 ligand and cytokines in a T cell-dependent like manner (117). TCDD treatment in human primary B cells increased the expression of AHR-responsive genes such as *CYP1A1*, *AHRR* and *TIPARP* as expected. A suppression of the primary IgM response was observed in a TCDD-concentration dependent manner in TCDD-responsive human donors. One out of seven donors were refractory to the suppression of the IgM response (non-responders) possibly due to differences in AHR polymorphisms in human donors (27, 118). One of the interesting observations from this study was that the human cells showed a lower magnitude of increase in the AHR battery genes as compared to mouse B cells activated using a similar method. It is likely that the differences were a result of an approximately 10-fold difference in the binding affinity of TCDD between mouse and human AHR and epigenetic differences between mouse and human B cells. In addition, it is known that genes involved in the immune response were regulated differently in mice compared to humans (119). This potentially explains the differences observed between mouse and human responses to TCDD. It was also noted that TCDD did not alter the expression of plasmocytic differentiation genes such as Blimp-1 and Pax-5 in human cells as observed in mouse B cells (26). Instead, a significant suppression of B cell activation markers namely CD80, CD86, CD69 was observed in human cells. This result is intriguing as mouse B cells were seen to be activated and viable in contrast to the human B cells which

demonstrated decreased activation and consequently lower viability along with a decrease in the ability to differentiate into a plasma cell (26). Recent transcriptomic study comparing AHR-mediated gene regulations in both primary human and mouse B cells revealed few common genes, suggesting that the AHR-TCDD altered distinct transcriptomic makers in human and mouse B cells (120). It was also eluded that TCDD could potentially abrogate a proximal signaling event in human and mouse cells, which could lead to varied downstream effects owing to phenotypic differences between mouse and humans. In addition, previous studies have demonstrated that TCDD treatment increased expression level of BACH-2 and BCL-6, two key regulators in B cell activation, in human primary B cells (64, 121). TCDD treatment also increased the total expression level of SHP-1 in human B cell, strongly indicating that the role of TCDD and AHR in modulating signaling events (122). Furthermore, TCDD treatment altered the activation of human B cells through upregulation of both BCL-6 and SHP-1 in human B cells (43, 64). Recent studies also showed that TCDD treatment modulated the STAT-3 phosphorylation in mature human B cells (Manuscript under preparation). Furthermore, IFNγ treatment restored the TCDD-mediated alteration in STAT3 phosphorylation and the IgM response in human (Manuscript under preparation).

These differential effects of TCDD on mouse and human cells highlight important differences in their response to TCDD and reflect on potential mechanistic differences in the toxicity of TCDD in the two species. These studies are critical in comprehensively characterizing the immunotoxicity of TCDD in human cells.

1.3. Immune system and humoral immunity

Immune system

The Immune system plays a critical role in protecting an individual from infections and diseases. The three major functions of the immune system are: 1) recognition of antigens, 2) elimination of antigen with appropriate effector response and 3) establishment of immunological memory. There are two major components for the immune system; the innate and adaptive immune systems. From bone marrow, leukocytes migrate to secondary lymphoid organs after maturation. Mature leukocytes are then trafficked to site of infection through the lymphatic system. Mature leukocytes remain in circulation in the absence of bacterial or viral infections.

The innate immune system is a non-specific host defense mechanism that acts as the first line of defense against infectious pathogens. The innate immunity involves the phagocytic cells, i.e. macrophages and neutrophils, with the capability to directly destroy infective agents. Innate cells can recognize conserved pathogen associate molecular patterns and become activated to eliminate infections. The skin and epithelial surfaces, cytokines, chemokines and plasma proteins are known as the compliment constitute of the innate immune system. Another important function of the innate immunity is to assist the adaptive immunity through antigen presentation.

The adaptive immune system is responsible to effectively recognize and eliminate specific pathogens, and then provided memory from past infection to enhance earlier and more effective immune response should the pathogen be encountered again. Adaptive immunity requires lymphoid cells to recognize specific antigens on the infected cells. Antibodies produced by lymphoid cells provide long-lasting immunity to different

infectious pathogens. Adaptive immunity can be further classified into cell-mediated and humoral immunity with specific effector functions carried out by T and B lymphocytes respectively. Cell-mediated immune responses are mainly directly against intracellular pathogens and involves the lysis of infected cells. Humoral immunity is mediated by immunoglobulin-secreting B cells.

The activation, proliferation and differentiation of immunoglobulin-secreting B cells during infections is regulated at both the cellular and molecular levels. B cells can respond to various activation signals through cell surface receptors, including binding to the B cell receptor (BCR), CD40 receptor or signal molecules (cytokine and chemokines). Upon activation, B cells can differentiate into antibody-secreting plasma cells or memory cells. Specific antibodies are the effectors for humoral immunity to provide specific binding to antigens and recruit phagocytic cells to destroy infectious pathogens. Each antibody/immunoglobulin is comprised of a variable (V) region, also known as the antigen-binding region and the constant (C) region (Fig.1.3A). The variety of antigen specificities present in the V region arises from somatic rearrangement between gene segments (V, D and J segments). B cell-specific recombinase (RAG) proteins facilitate the rearrangement of the variable gene region. Somatic hypermutation further alters the genetic sequence of immunoglobulin variable gene by point mutation, and class switch recombination can bring further diversification to variable region specificity of antibodies. Class switch recombination can improve the functional diversity of immunoglobulin repertoire by generating different classes of antibodies (IgM, IgG, IgA, IgG and IgE). IgM is the first wave of antibody produced by mature naïve B cells upon activation. Secreted IgM has a pentameric structure consisting of five IgM monomers linked by a joining (J)

chain (Fig.1.3B). In the course of an immune response, effector B cells are selected on the basis of the antigen specificity. Cells having a high affinity for a given antigen BCR are selected and expanded clonally. This process of selection of high affinity BCR possessing B cells is called affinity maturation. All these processes greatly contribute to the antigen specifics of B cells in combating infectious agents and conferring appropriate immunity to the host.

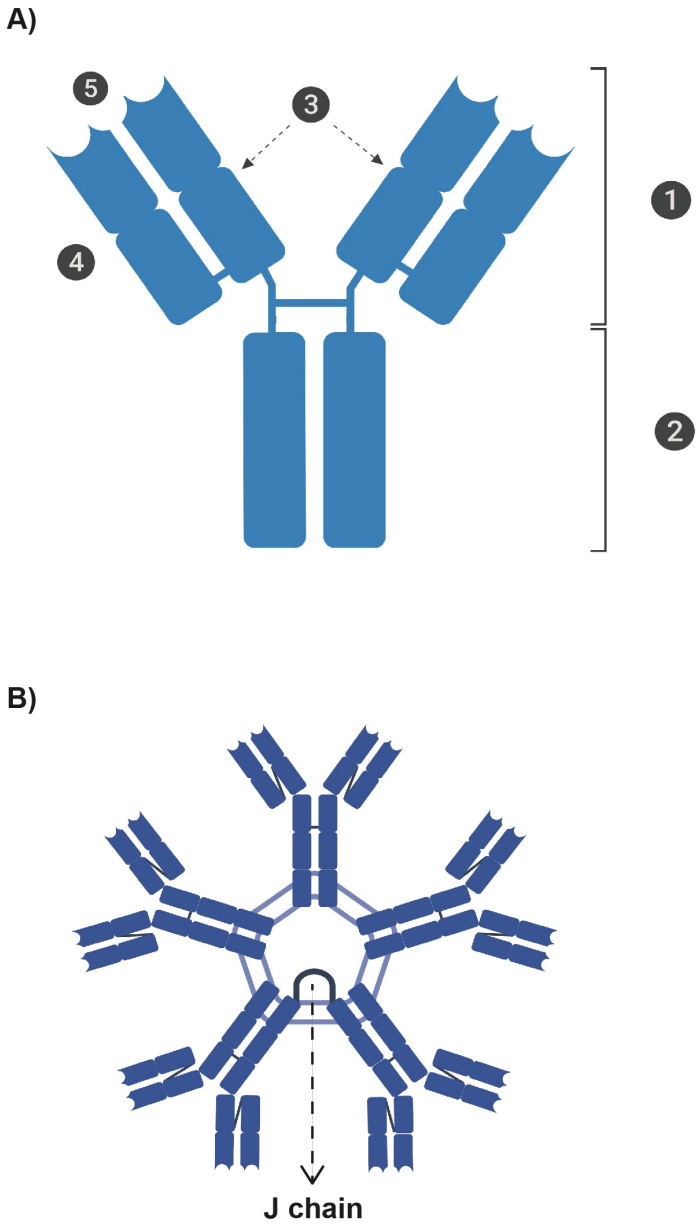


Figure 1.3.1. General immunoglobulin M (IgM) structure.

Structure of IgM is illustrated in the figure above. **A)** IgM monomer. The IgM molecule comprises of two identical heavy chains and light chains. 1: Fab region; 2: Fc region; 3: heavy chains; 4: light chains; 5: antigen binding region. **B)** IgM pentameric structure. IgM pentamer was formed by linking five IgM monomers via J chain.

B cell signaling and activation

The two-major functions of the BCR are to convey signals that regulate B cell activation/differentiation and to mediate antigen processing and presentation to T helper (T_h) cells (123). The BCR complex is composed of an immunoglobulin (Ig) heavy and light chain along with two additional signaling components (Igα and Igβ). Upon ligand binding to the BCR, the proximal protein kinases LYN and SYK are activated by phosphorylation at the SH-2 domain (124). LYN then phosphorylates the ITAMs domain on Igα and β, which in turn activate SYK and BTK kinases. PI3K, AKT and PLCγ2 are critical signal transducers enabling activation of secondary messengers such as Ca^{2+} and PKC. Subsequently, there is activation of the mitogen-activation protein kinase (MAPK) pathway, including the activation of extracellular signal regulated kinase (ERK), c-jun terminal kinase (JNK) and p38 (125, 126). The activation of ERK then enters the nucleus and activates nuclear factor kappa B (NFκB) and nuclear factor of activated T cells (NFAT) (Fig.1.2). Along with positive activation signal, negative feedback signals can also regulate the excessive activation and proliferation of B cell. The paired immunoglobulin-like receptor (PIR), FcγRIIB and CD5 mediate negative regulation of BCR signaling (Fig.1.4).

B cell activation can occur in two ways: 1) T helper (T_h) cell-dependent manner; or 2) T_h cell-independent manner. For instance, microbial elements and lipopolysaccharide (LPS) can activate TLR-4, single stranded RNA from viruses can activate TLR-3/7 and single stranded DNA (CpG) can activate TLR-9 ultimately leading to the direct activation of B cells. In contrast, to T cell-independent activation, activation of B cell, in a T cell-dependent manner requires antigen to be processed and presented on the B cell MHCII

complex (Fig 1.5). This complex is then recognized by T_h cells or T follicular helper (T_{fh}) cell in the germinal centers located in the spleen. The MHC complex is critical for the recognition of antigenic derived peptide, that can also lead to complete B cell activation. Co-stimulatory signals include the binding of CD28/B7 protein families to their ligands, CD80 (B7.1) and CD86 (B7.2), on the B cell surface. T cells can also secrete extracellular/soluble CD40 ligand that can bind to the CD40 receptor on the surface of B cells. CD40 receptor is constitutively expressed on B cell and it is involved in the proliferation and class switching in B cells. The binding of the CD40 to CD40 ligand (CD40L) results in the clustering of CD40 on the B cell surface thereby triggering the recruitment of TNFR-associated factors (TRAFs) to the cytoplasmic tail of CD40 (Fig. 1.2). TRAF proteins then activate NFκB, MAPK and PI3K/PLCγ pathways. CD40 can also activate downstream signal factors and transcription factors, for example, activator protein-1 (AP-1), NFκB and NFAT. The activation of these signaling pathways results in the upregulation of CD80, CD86 and CD69 along with MHCII and intracellular cell adhesion molecule-1 (ICAM-1). The importance of CD40-CD40L interaction has been demonstrated by using monoclonal CD40 antibody in CD40 null mice, which show defects in B cell activation, proliferation and development of hyper-IgM syndrome. Activated T_h cells can also secrete cytokines (IL-2, IL-4, IL-6, IL-10 and IL-21), which enhance B cell proliferation. Therefore, in an *in vitro* culturing system, human B cells can be activated via CD40 with either soluble recombinant CD40 ligand or CD40 ligand expressing L cells, and appropriate cytokines to induce B cell proliferation and differentiation into antibody secreting cells.

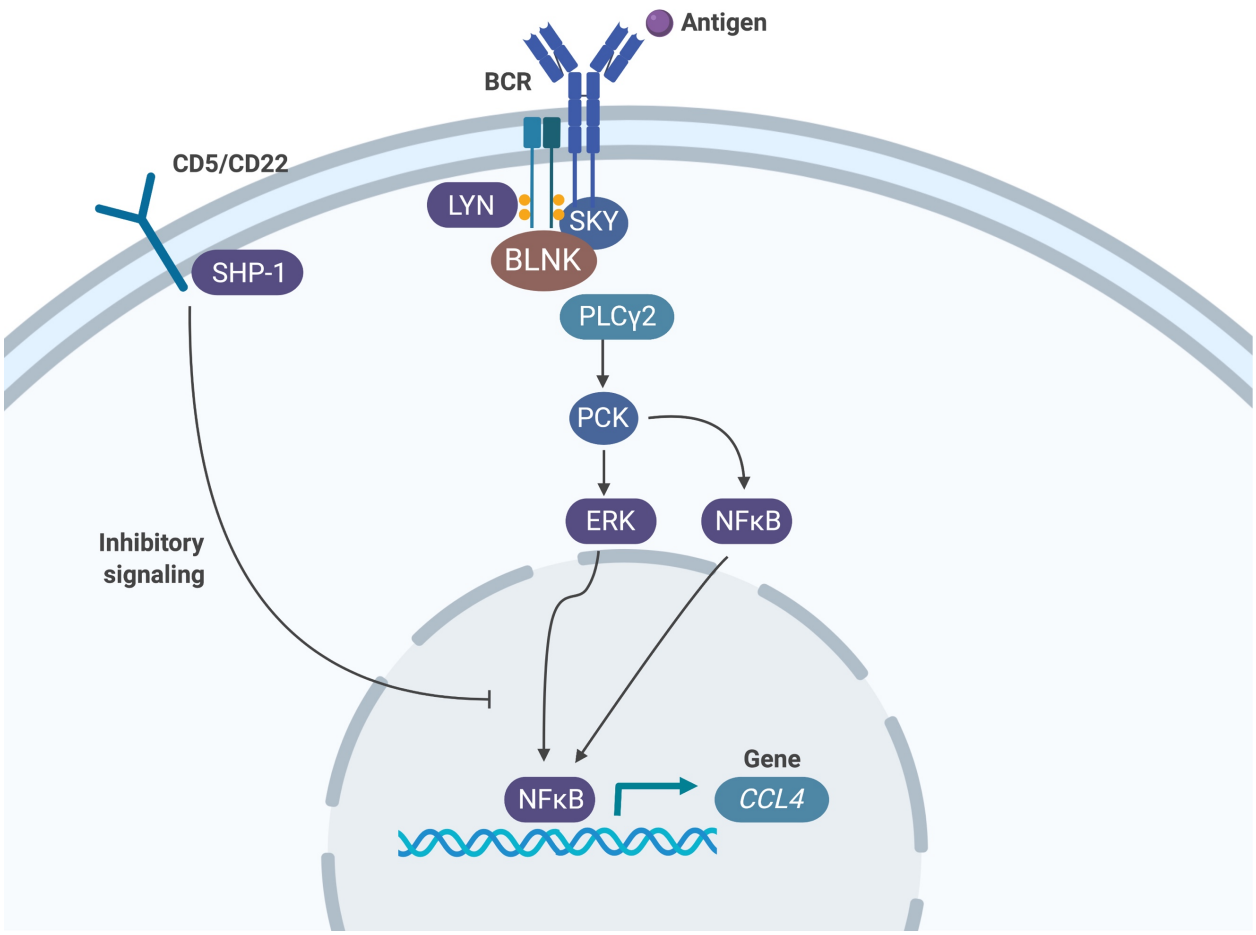


Figure 1.3.2. B cell receptor signaling.

Upon ligand binding to the BCR, the proximal protein kinases LYN and SYK are activated. Activated LYN and SYK could activate PLCγ2, which in turn activate ERK and NFκB. Activated ERK and NFκB serve as a transcription factors, which turn on critical gene transcription (i.e. CCL4). Inhibitory receptors, like CD5 and CD22, inhibit the BCR activation by providing inhibitory signaling to ERK and NFκB.

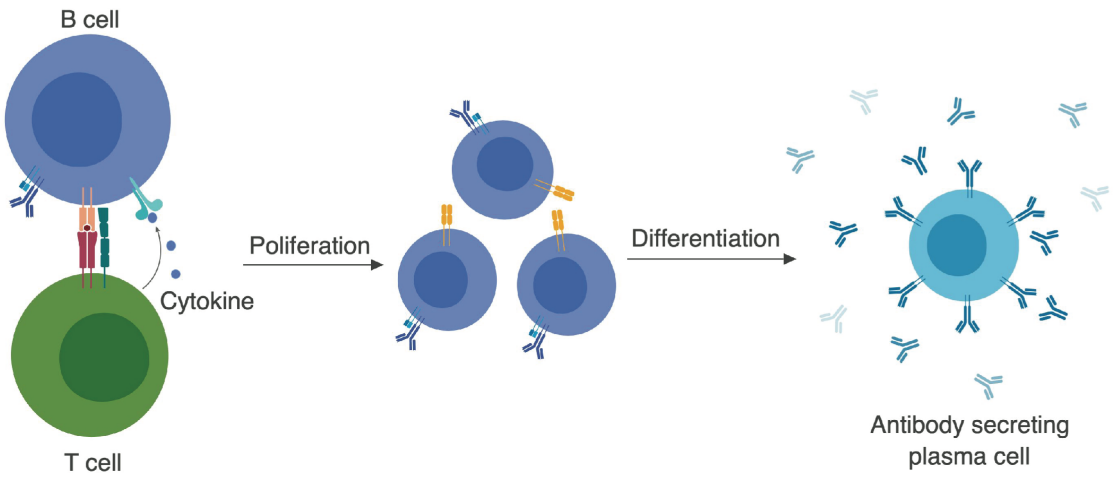


Figure 1.3.3. B cell differentiation.

Progression of the B cell differentiation. Interaction of MHCII – BCR, co-stimulatory receptors and cytokine receptors provide the signals required for B cell activation. After interacting with activated T cells, activated B cells then undergo proliferation followed by differentiation into antibody producing plasma cells.

B cell differentiation

Differentiated B cells are terminally committed to undergo an irreversible, tightly regulated differentiation process into antibody-producing plasma cells or long-lasting memory cells. Plasma cells can be generated from differentiated naïve B cells and memory cells. This process is associated with major changes in the cell structural morphology and gene expression governs the key events for cell differentiation. Terminal differentiation is preceded by robust proliferation of mature B cells. Plasma cells also undergo an increase in their cytoplasmic to nuclear ratio to accommodate increased immunoglobulin synthesis. On the cell surface, there is decreased of MHCII, CD45R (B220) and CD19. Expression of Syndecan-1 and CD38 marks the differential progression of mature B cells to plasma cells. Additionally, chemokine receptors (i.e. CXCR5 and CCL7) decrease expression in plasma cells, with the exception of CXCR4. This allows the migration of plasma cells from the follicles in the spleen to the bone marrow. Mature B cells residing in the spleen undergo differentiation into antibody-secreting plasma cells upon encountering antigen or stimulation through T cell-independent antigens. B cell differentiation is thought to be an all-or-none process established by the gene expression of critical transcription factors (BCL-6, BLIMP-1, PAX-5 and BACH2). BCL6, BACH2 and PAX5 are repressors of B cell differentiation and are expressed at high levels in mature B cells. The expression of PAX5 is critical for the B cell to maintain its identity and is expressed from the pro-B to plasma cell stage. PAX5 controls the expression of BCR components (BLNK, Igα, CD19 and IRF4) and it actively suppresses genes involved in the antibody secretion process (IgH, IgJ, Igκ and XBP1). Plasma cells are identified by the expression of CD27 and CD138 on the cell surface.

$CD5^+$ Innate-like B cells (ILBs)

With the increase of sensitivity in flow cytometric analysis, subsets of B cells with unique surface markers have been identified. One of these subsets is innate-like B cells, characterized by the expression of CD5 in both the mouse and humans; however, human ILBs have been less characterized compared to mouse ILBs (Fig1.6). $CD5^+$ ILBs constitute around 5 to 15% of the circulating B cells and they are the source for the vast majority of circulating IgM; playing a critical role in immunity against infectious agents, especially early in life during immune system development and late in life when the immune system is in decline (127). ILBs have been shown to provide long-lasting immunity to Lyme disease and relapsing fever spirochete.

ILBs have been well characterized in rodent models, commonly described as B-1 cells. Within B-1 cells, there are two sub-classes B-1a and B-1b in mouse innate-like B cells (128) (Fig. 1.4). B-1 cells can be derived from stem cells in the bone marrow and fetal liver (129, 130). B-1 cells are the major population in pleural and peritoneal cavities (131). B-1 cells within spleen and bone marrow secrete large amounts of IgM and are believed to contribute the majority of systemic circulating natural antibodies (132-134). The steady state levels of natural IgM (nIgM) in the circulation provides a critical first line of defense against pathogen replication before establishment of specific immunological responses (135-140). nIgM and B-2 cell-dependent pathogen-specific IgG response through the deposition of IgM antibody complexes on follicular dendritic cells (136, 141). In mice, due to the expression of CD5, B-1 cells are relatively insensitive BCR crosslinking (142). Recent studies demonstrated that the inhibition of BCR-mediated B-1 cell proliferation requires the expression of both CD5 and LCK (143, 144). Taken together, B-

1 cells have a selective unresponsiveness toward BCR-induced clonal expansion but increase responses with innate immune signaling (TLR signaling). Within the CD5 expressing B cells, recent studies also indicate that IL-10 producing B regulatory cells (B_{reg}) also express CD5; however, the link between B-1 cells and B_{reg} cells are currently unknown (145). $CD5^+$ ILBs express high basal levels of PD-1, an inhibitory receptor that suppresses immune response (146). LCK is one of the primary kinases responsible for the phosphorylation of the PD-1 cytoplasmic tail (147). These phosphorylated tyrosine residues allow for the docking of SH2-domain containing phosphatases such as SHP-1, SHP-2, and others (147, 148), which can then negatively regulate cellular functions (147, 148).

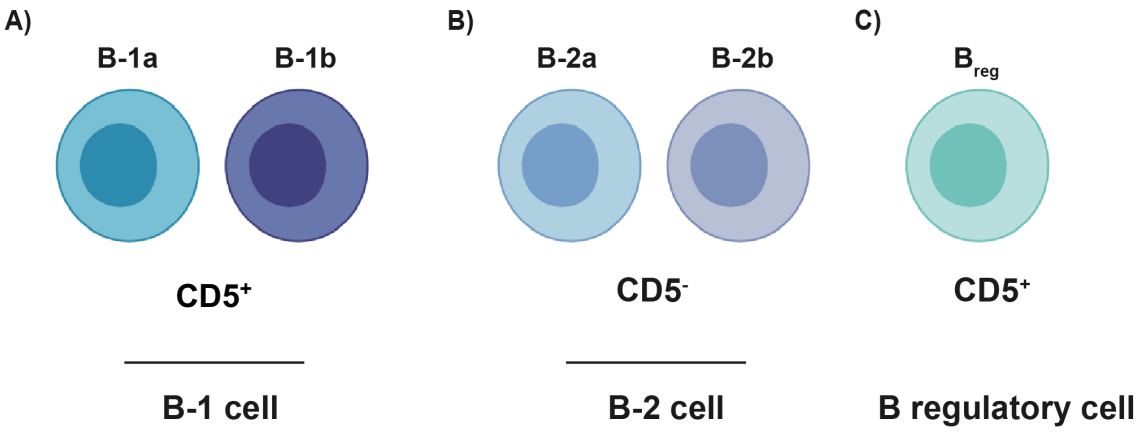


Figure 1.3.4. B cell subsets

5 major subsets of mature B cells (figure is modified based on the mature splenic B cell model). There were three major subsets of B cells, **A)** B-1 cells, **B)** B-2 cells and **C)** B regulatory (B_{reg}) cells. B-1 cell also include of $CD5^+$ B-1a cells and $CD5^+$ B-1b cells. B-2 cells were $CD5^-$ and comprised of B-2a and B-2b cells. B-1 cells are commonly known as innate-like B cells (ILBs), with the characteristic of secreting polyvalent IgM. B-2 cells are known as adaptive B cells, with the characteristic of secreting antigen specific antibodies.

Immunoglobulin M (IgM)

IgM is the first wave of antibodies produced by activated B cells. Membrane bounded IgM/B cell receptor (BCR) and secreted IgM (sIgM) are the same molecule, except that BCR has a membrane localization sequence on the C terminus. In place of the transmembrane domain, the sIgM has hydrophilic tail piece with one key cysteine for preventing premature secretion and for the assembly of mature secretory IgM. Recognition of antigen by the membrane bound BCR on naïve B cells can provide activation signals to B cells and initiate humoral immune responses. Activated B cells undergo cytoplasmic modification to increase the size of ER and Golgi in order to increase the capability to produce a large number of antibodies. IgM molecules are synthesized and assembled in the endoplasmic reticulum (ER). Expression of ERGIC-53 and ERp44 are critical for the transport and assembly of IgM molecules from the ER to the Golgi. IgM monomers are assembled to form the pentamers with the assistance of chaperones in the intermediate compartment between ER and Golgi (149, 150). Correctly assembled IgM pentamers then detach from chaperone proteins and continue through the secretory pathway in the Golgi (151) and secrete via vesicular transport to extracellular space.

Secreted IgM can be divided into nIgM and antigen-specific IgM. nIgM is polyreactive and fixes complement. As discussed earlier, nIgM plays a critical role in immunity against infectious agents, especially early in life during immune system development, and late in life when the immune system is in decline (127). Specific IgM is produced after B cells encounter antigens through antigen presenting cells (APCs). Studies have demonstrated that natural and specific IgM are critical for the protection against viral infection (152). The repertoire of natural IgM is not affected by external

antigens. Innate-like B cells (ILBs) have been shown to be the major producer for nIgM. Using allotype chimeric mice under germ-free condition, it was found that approximately 50% of IgM in circulation is produced by ILBs in the absence of infection or vaccination, though they can be induced to higher levels with activation (136, 153-155). nIgM is encoded by non-mutated germline variable gene segments providing the polyreactive binding specificities (156). Polyreactivity is defined as the ability to bind shared structures and epitopes on self or non-self-antigen. The polyreactivity of soluble IgM (sIgM) allows binding to different structures on the same pathogen, thereby, enhancing neutralization and opsonization of the pathogen. nIgM can commonly recognize epitopes on phosphorycholine (PC), which presents on the membrane of apoptotic cells and microbes (157, 158). nIgM has shown to enhance the clearance of apoptotic cells and to prevent the development of inflammation by activating complement (159-161). nIgM are a critical bridge between the innate and adaptive immune response.

Lymphocyte-specific protein tyrosine kinase (LCK)

The role of lymphocyte-specific protein tyrosine kinase (LCK) in B cells has not been well characterized. However, LCK has been well studied in the context of T cell activation and proliferation. LCK is a critical signaling molecule involved in signal transduction downstream of the T cell receptor and PD-1. Upon ligand binding to the TCR, phosphorylation at the immunoreceptor tyrosine-based activation motif (ITAM) of the TCR complex then transfers a phosphate to the Src homology 2 (SH2) domain on LCK (162). The phosphorylation of LCK is critical for recruitment and activation of ζ chain–associated protein kinase of 70 kD (ZAP70), which in turn phosphorylates signal adaptors which are

important for Ca^{2+} mobilization (163), and the activation of MAP kinase and NF-AT pathways (164) essential for cell proliferation. LCK also plays a role in protein secretion and cytoskeleton remodeling in T cells. Upon TCR ligation, the activated LCK can phosphorylate the membrane-associated adaptor protein LAT and VAV-1 to from distinct microclusters (165). The activation of vesicular associated proteins LAT and VAV-1 plays a critical role in vesicle transport and cytoskeleton remodeling. Little is known about the role of LCK in B lymphocytes. Recent studies have suggested a critical role for LCK in B cell receptor (BCR) signaling in chronic lymphocytic leukemia (CLL) cells, the increase of LCK has been used as a biomarker for the progression of CLL in patients (127, 166). In addition, $CD5^+$ B cells have been shown to express high level of LCK (167). LCK has also been reported to be one of the primary kinases responsible for the phosphorylation of the PD-1 cytoplasmic tail which contain ITIM sequences (147).

Programmed cell death protein 1 (PD-1)

PD-1 is an inhibitory receptor that primarily functions to suppress immune responses (146). High basal expression of PD-1 has been found by $CD5^+$ ILBs (168). There are two well-characterized PD-1 ligands, PDL1 and PDL2 (169). Both ligands are widely expressed on all immune cells; however, different immune cell types preferentially express either PDL1 or PDL2 (170). Binding of ligands to PD-1 initiate inhibitory signaling cascades that can suppress immune responses on PD-1 expressing cells. PD-1 signaling involves the phosphorylation of the immunoreceptor tyrosine switch motifs (ITSMs) and immunoreceptor tyrosine inhibitory motifs (ITIMs) (169). Phosphorylated PD-1 can then recruit Src homology 2 (SH2) domain containing phosphatases such as SHP-1, SHP-2,

and others, which can then negatively regulate immune activation and cellular functions (147, 148).

Interferon γ (IFNγ)

IFNγ is a well characterized cytokine that plays various roles in cellular regulation. IFNγ is classified as a type II IFN. IFNγ is primarily produced by lymphocytes and professional antigen-presenting cells (APCs) (171). T cells are the main producer of IFNγ, which induces innate cell-mediated immunity and macrophage activation (172-174). IFNγ has also been shown to modulate cell cycle, cell growth and apoptosis (175, 176). IFNγ mainly signals through the Jak-Stat pathway, including the activation of Janus family of kinases (Jak 1 – 3 and Tyk2) and Stats (Stat 1 – 6) (177). IFNGR1 and IFNGR2 are the two subunits of the IFNγ receptor (178). Upon ligand binding, intracellular domains of the receptor open to allow the binding of downstream signaling components. Binding of IFNγ to the receptor induces Jak2 autophosphorylation and activation, which allows Jak1 transphosphorylation by Jak2 (177). The activation of Jak1 then phosphorylates the SH-2 domain on the receptor subunits to provide the docking site for Stat1 (179). The phosphorylated Stat1-Stat1 homodimers then translocate into the nucleus and acts as a transcription factor to regulate gene expression (180). Previous studies indicated pretreatment with IFNγ can block AHR-mediated suppression of the IgM responses in mouse splenocytes (100). IFNγ secretion by Natural Killer (NK) cells and APCs are important in the early stage of host defense against infections.

Rationale

AHR is a cytoplasmic receptor involved in the regulation of cell functions and xenobiotic sensing (1-6). AHR also plays a role in immune modulation, especially in the expansion, maturation and differentiation of B cells (7-11). High affinity ligands, like TCDD, have been widely used to study the physiological role of the AHR in B cells. AHR activation has also been demonstrated to suppress humoral immunity (101). Therefore, the overarching goal of this dissertation is to elucidate the mechanism by which TCDD-mediated activation of AHR induces suppression of the IgM response in human B cells. This dissertation research is comprised of three components: 1) Comparison of TCDD-mediated impairment of the IgM response by mouse and human B cells; 2) Elucidation of the role of LCK in TCDD-mediated suppression of the IgM by human B cells; and 3) Ascertainment of the role of LCK and PD-1 in TCDD mediated suppression of the IgM response by human $CD5^+$ ILBs. For this dissertation research, I will specifically test the hypothesis: *TCDD-mediated impairment of the IgM response occurs through upregulation of lymphocyte-specific protein tyrosine kinase (LCK) and inhibitory receptors, PD-1 by human $CD5^+$ B cells.*

A recent transcriptomic study has indicated little overlap between mouse and human B cell in response to AHR activation by TCDD, indicating a divergent of mechanism for these two species. Therefore, in the first part of this dissertation, a comparison of AHR-mediated impairment of IgM responses in mouse and human provides detailed mechanistic differences between these two species. In the transcriptomic study, LCK was significantly upregulated with TCDD treatment in only human B cells, therefore, the second part of this dissertation focused on understanding

the role of LCK in the AHR-mediated suppression of the IgM response by human B cells. Previous studies have also indicated that human $CD5^+$ innate-like B cells (ILBs) express high levels of LCK and PD-1. Therefore, in the third part of this dissertation, studies were conducted to further understand the role of LCK and PD-1 in AHR-mediated suppression of the IgM response by $CD5^+$ ILBs. Taken together, this dissertation research provides a mechanistic framework describing AHR-mediated impairment of the IgM responses in humans.

CHAPTER 2: MATERIALS AND METHODS

2.1. Chemicals and Reagents

99.1% pure TCDD in dimethylsulfoxide (DMSO) was purchased from Accustandard Inc (New Haven, Connecticut). DMSO was purchased from Sigma-Aldrich (St. Louis, Missouri) and used to dilute the TCDD for all the studies. Approximate 0.02% DMSO was used for all treatments. The AHR antagonist (CH-223191) was purchased from Sigma-Aldrich (St. Louis, MO). LCK small peptide inhibitor (EGQYpEEIP) and control peptide (EGQYEEIP) were purchase from GenScript (Pascataway, NJ). LCK small molecule inhibitor (CAS213743-31-8) was purchased from Sigma-Aldrich. Human recombinant IFN-gamma (IFNγ) protein was purchased from Biolegend (San Diego, California). PD-1 blocking antibody (S228P) was purchased from Invivogen (San Diego, California).

2.2. Cell culturing and cell line

CD40 ligand-L cells were obtained as a generous gift from Dr. David Sherr (Boston Univeristy). CD40 ligand-L cells were a mouse fibroblast cell line stably transfected with human CD40 ligand (CD40L). The cells were cultured in Dulbecoo's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% bovine calf serum (ThermoScientific, Lafayette, Colorado), 50 μM of 2-mercaptoethanol, and HT supplement (Invitrogen). CD40 ligand-L cells were thawed 4 days before irradiation with 3500 rad of x-rays using X-Rad 320 (Precision X-Ray, Inc, North Branford, Connecticut) a day before co-culturing with human primary B cells. CD40 ligand-L cells were seeded at a concentration of 1 x 10^4 cells/ml in 500 μl of media per well in 48-well tissue culture

plates (Costar, Corning) for the culture period. The expression of human CD40L on the surface of the cells was monitored routinely to select for high CD40L-expressing cells. Human peripheral blood B cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% human AB serum (Vally Biomedical, Virginia), and 50 μM of 2-mercaptoethanol. In all cases, cells were cultured in 5% CO_2 incubator at 37°C. The B cell activation schematic used in this dissertation research was illustrated in Fig. 2.1.

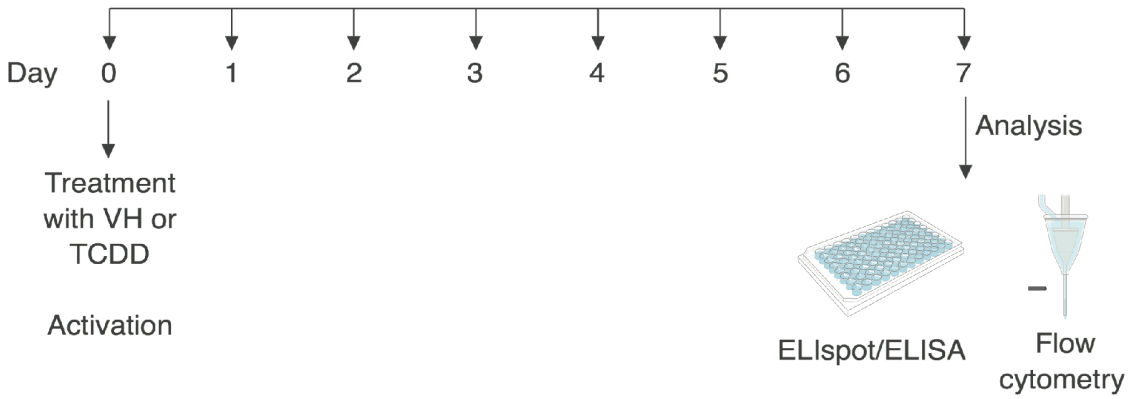


Figure 2.2.1. B cell activation schematic.

Cells were treated with either VH or TCDD and activated by co-culturing with CD40L expression L cells or soluble CD40 ligand and supplied with cytokines on day 0. The treated cells were then cultured for total 7 days. On day 7, cells were collected for analysis via IgM ELIspot/ELSA and flow cytometric analysis.

2.3. Flow cytometry

All antibodies used in the studies can be found in Table 2.1. For flow cytometry staining, approximately 0.5 x 10^6 cells were harvested at the indicated time points and viable cells were identified by Fixable Live/Dead Near-IR dye (Thermo Fisher, Massachusetts) following manufacturer's instructions prior to cell surface or intracellular staining. Surface Fc receptors were blocked using human AB serum before staining for surface and intracellular proteins. For surface staining, cells were resuspended in FACS buffer (1x phosphate-buffered saline, 1% bovine serum albumin [BSA] and 0.1% sodium azide, pH: 7.6) in the presence of 20% human AB serum and antibodies were added at the manufactural specified concentrations and incubated at 4°C for 15 min and then fixed by incubation in the BD Cytofix fixation buffer (BD Biosciences, California) for 10 min. For intracellular protein staining, cells that were previously fixed after surface staining were permeabilized with 1X BD PermWash buffer (BD Biosciences, California) for 30 min and then incubated with antibodies for 30 min. For phospho staining, cells were incubated with phospho antibodies as instructed by BD Biosciences. In brief, cells were fixed using BD Cytofix buffer for 10 min at 37°C then permeabilized using 1X of Perm buffer IV, stained for 1 h under continuous motion. Cells were then washed three times with 0.5X perm buffer and analyzed by flow cytometry. In all cases, cells were analyzed on BD FACSCanto II using FACS Diva software (BD Biosciences) and subsequently analyzed using FlowJo analytical software (Version 10, Treestar Software Ashland, Oregon). Unless stated, cells were gated on singlets, live (as determined by Live/Dead dye) followed by gating on lymphocyte populations. Gates were drawn based on the

unstimulated cells (resting human B cells, without CD40L and cytokine activation) or unstained cells as appropriate.

Name	Clone	Fluorophore	Company
Anti-human LCK	LCK-01	Alexa Fluor 647	Biolegend
Anti-human IgM	MHM-88	PE	Biolegend
Anti-human Ig light chain κ	MHK-09	PE	Biolegend
Anti-human Ig J chain		PE	Assaypro
Anti-human CD19	HIB19	PE/Cy7	Biolegend
Anti-human LCK-pY505	Clone 4	PE	BD Biosciences
Anti-human ZAP70-pY319	Clone 17A	Alexa Fluor 647	BD Biosciences
Anti-human ZAP70-pY292	J34-602	Alexa Fluor 647	BD Biosciences
Anti-human CD5	UCHT2	APC	Biolegend
Anti-human CD5	UCHT2	Biotin	Biolegend
Anti-human CD279 (PD-1)	EH122H7	APC	Biolegend
Anti-human CD273 (PD-L2)	24F.10C12	PE	Biolegend
Anti-human CD274 (PD-L1)	29E.2A3	Per/Cy5.5	Biolegend
Anti-mouse IgM	II/41	FITC	Biolegend
Anti-mouse Igk	RMK-45	PE	Biolegend
Anti-mouse IgJ	PA5-13486	Unconjugated	Biolegend
Anti-rabbit IgG	RJ243415	Alexa Fluor Plus 647	Thermo Fisher

Table 2.2.1. List of antibodies used in the studies.

All the antibodies were used as suggested by the manufactures. Specific concentration of each antibody could be found on the manufacture's website. The process of staining was described above in the Material and Method section.

2.4. Human leukocyte packs and human B cells purification

Peripheral blood mononuclear cells (PBMC) collected from anonymous platelet donors were obtained from Gulf Coast Regional Laboratories (Houston, Texas). All human leukocyte packs were tested to be negative for HIV, HBV, HCV and HTLV before shipment. For each experiment, blood packs were diluted with HBSS and overlaid on Ficoll-Paque Plus density gradient (GE Healthcare, Piscataway, New Jersey) and centrifuged at 1300g for 25 minutes with low acceleration and brake. The PMBCs were isolated post-centrifugation, washed, counted and subjected to magnetic column-based isolation that enriched $CD19^{+}CD27^{-}$ naïve human B cells (more than 95% purity). This negative selection was conducted using the MojoSort^{MT} human naïve B cell isolation kit (Biolegend, San Diego) following manufacturer's instructions. Purified human B cells at the concentration of 1 x 10^6 cells/ml were then treated with either 0.02% DMSO (VH) or various concentrations of TCDD. The treated B cells were then activated by co-cultured with sublethally irradiated CD40 ligand-L cells (1 x 10^4 cell /ml) in a 48-well cell culturing plate. Cells were cultured with recombinant human cytokines IL-2 (1 ng/ml), IL-6 (1ng/ml) (Roche Applied Science, Indianapolis, Indiana), and IL-10 (4 ng/ml) (Biovision Inc, Milpitas, California) for total 7 days (Fig.2.1). In order to obtain optimal B cell activation, the VH or TCDD treated B cells were activated by soluble human CD40 ligand (10 ng/mL) and supplying with recombinant human cytokines IL-2 (1 ng/ml) (Roche Applied Science, Indianapolis, Indiana), and IL-21 (50 ng/mL) (R&D system, Minnesota) for total 7 days. In order to obtain human $CD5^{+}$ ILBs, a second positive isolation was conducted following $CD19^{+}$ naïve B cell isolation to obtain $CD5^{+}$ ILBs. In brief, 10 μL of biotin anti-human CD5 antibodies per 10^6 cells was incubated with $CD19^{+}$ naïve B cells for 15 min on ice,

following with 10 μL of anti-biotin microbeads per 10^6 cells for additional 15 min. The detail of the isolation and enriching could be found in Blevins. *et al* (Manuscript under prepartion). Purified $CD5^+$ ILBs at the concentration of 0.25 x 10^6 cells/ml were then treated with either 0.02% DMSO (VH) or various concentration of TCDD and follow the culture schematic in Fig. 2.1.

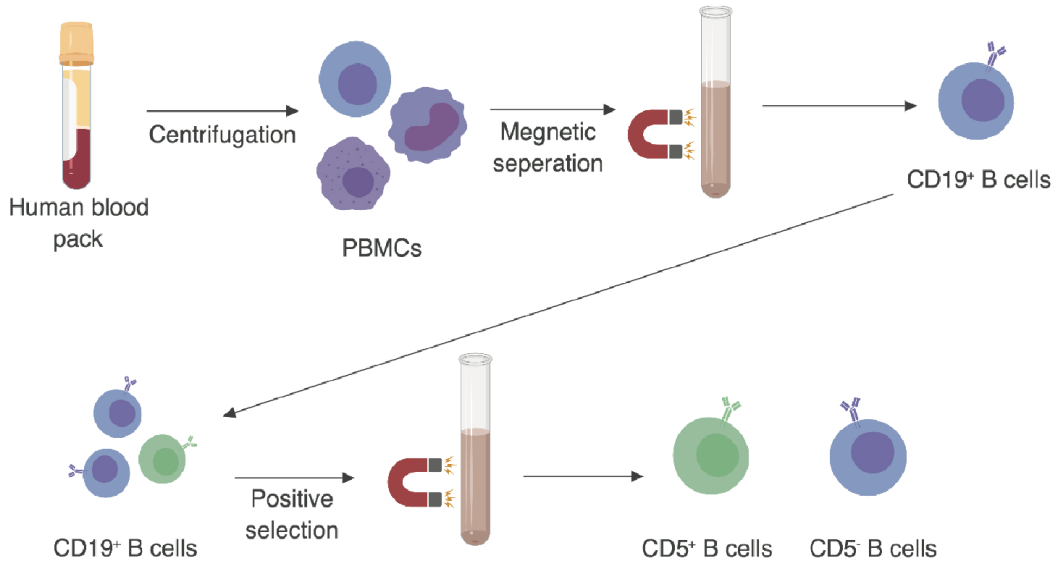


Figure 2.4.1. $CD5^+$ ILBs enrichment via magnetic separation.

Human PBMCs obtains from human blood pack were subjected to negative magnetic separation obtained from naïve $CD19^+CD27^-$ B cells. Naïve human B cells were then further enriched using a positive magnetic selection to separate $CD5^+$ cells and $CD5^-$ cells.

2.5. Mouse B cell purification

Pathogen-free female C57BL/6 mice (6 weeks of age) were purchased from Charles River (Portage, Michigan). Mice were randomized, transferred to plastic cages containing sawdust bedding (five mice per cage), and quarantined for 1 week. Mice were provided food (Purina certified laboratory chow) and water *ad libitum* and were not used for experimentation until their body weight was 17–20 g. Animal holding rooms were maintained at 21°C –24°C and 40–60% humidity with a 12-h light/dark cycle. The Michigan State University Institutional Animal Care and Use Committee approved all animal procedures used in this investigation. Mouse splenocytes were made into single-cell suspension by passage through a 40 μm cell strainer (BD Bioscience, San Jose, California). Negative selection of mouse B cells was conducted using MojoSort™ isolation kits following manufacturer's instruction (Biolegend, San Diego, California). Purity was above 95% in all isolations. Purified mouse B cells were cultured at a density of 1 X 10^6 cells/ml in RPMI-1640 supplemented with 10% bovine calf serum, 100 U/ml penicillin/streptomycin and 50 μM 2-mercaptoethanol at 37 C with 5% CO_2, 95% air and 98% humidity. In all cases, mouse B cells were treated with 0.02% DMSO (VH) or various concentration of TCDD and activated by co-culturing with CD40L expression L cells supplemented with cytokines (IL2, IL6 and IL10).

2.6. Gene Expression Analysis

The probe used in the studies with human B cells were LCK (Hs00178427_m1) IGHM (Hs00385741_m1), IgJ (Hs00376160_m1) and Igk (Hs02384840_gH). The probes used for mouse B cells were Ighm (Mm01718956_m1), IgJ (Mm00461780_m1). SYBR

Green system was used to quantify the level of *Igκ* mRNA in mouse B cells. The primers for *Igκ* were designed based on Schneider., *et a*l. 2008. The control used for SYBR Green reactions was mouse HPRT. RNA was isolated using Qiagen RNeasy kits (Germantown, Maryland) per the manufacturer's instructions. The RNA concentrations were determined by Nanodrop ND-1000 Scientific spectrophotometer (Thermo-Fisher Scientific, Wilmington, Delaware) and 500 ng of RNA was use for the reverse-transcription using High Capacity cDNA RT-PCR kit by Applied Biosystems (Foster City, California). The cDNA was amplified using Applied Biosystems Taqman Gene Expression Assays. All quantitative real-time PCR reactions were performed on an Applied Biosystems model ABI Prism 7900 Sequence Detection System. Human 18S ribosomal RNA (Applied Biosystems, Foster City, California) was used as an internal control. Fold change in gene expression was calculated using the $\Delta\Delta C_t$ method.

2.7. Enzyme-linked Immunospot Assay (ELIspot)

The number of IgM-secreting cells was quantified by ELIspot. Briefly, multiscreen 96-well filter plates (Millipore, Billerica, Massachusetts) were coated with anti-human IgM antibody (5 μg/ml) (Sigma Aldrich, St. Louis, MO) for overnight and, subsequently, blocked with 5% bovine serum albumin (Sigma Aldrich, St. Louis, MO) for 2 h. B cells were washed with RPMI 1640 twice, resuspended in RPMI 1640 containing 10% bovine calf serum (Thermo Scientific, Lafayette, Colorado) and incubated on the primary antibody-coated plates overnight at 37°C with 5% CO_2. Biotin-conjugated anti-human IgM antibody (Sigma Aldrich, St. Louis, Missouri) and, subsequently, streptavidinhorseradish peroxidase (HRP) (Sigma Aldrich, St. Louis, Missouri) were added for 1 h incubation at

37°C with 5% CO_2. All incubations were followed with three washes with phosphate-buffered saline (pH 7.4) containing 0.1% Tween-20 (Sigma Aldrich, St. Louis, Missouri) and three washes with nanopure water. The spots were developed with an aminoethylcarbazole staining kit (Sigma Aldrich, St. Louis, Missouri). The number of spots per well between 0.0001mm^2 and 9.6372mm^2 were quantified via the Immunospot Software (Cellular Technology, Ltd, Shaker Heights, Ohio) and normalized to the number of viable cells plated in each well.

2.8. Enzyme-linked Immunosorbent Assay (ELISA)

The amount of IgM secreted into the culture supernatant was quantified by sandwich ELISA. Briefly, Immulon 4 HBX 96-well microtiter plates (VWR International, Radnor, Pennsylvania) were coated with anti-human IgM antibody (1 μg/ml; Sigma Aldrich, St. Louis, Missouri) overnight. Culture media collected from human B cells was incubated over primary antibody-coated plates for 90 min at 37°C with 5% CO_2 and was followed by overlaying to an anti-human IgM-HRP conjugate antibody (Sigma Aldrich, St. Louis, MO). Incubations were followed by washes with phosphate-buffered saline (pH 7.4) containing 0.05% Tween-20 (Sigma Aldrich, St. Louis, Missouri) and nanopure water. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Roche Diagnostics) was then added as a colorimetric substrate for HRP. The rate of colorimetric change was quantified with a Synergy HT microplate reader (BioTek, Winooski, Vermont) at 405 nm for 1 h. Concentrations of IgM in media were calculated based on a standard curve created in each plate.

2.9. Proliferation Assay

Isolated human primary B cells were washed and resuspended in 1X HBSS to remove traces of serum and were incubated with 2mM Cell Trace Violet Dye (Cell Trace Violet Cell proliferation kit, Thermo Fisher) at 1 X 10^6 cells/ml for 20 min in the dark at 37°C. The labeled cells were then washed twice with complete RPMI and then the cell density was adjusted as desired prior to treatment of cells with TCDD or VH (0.02% DMSO) and activation.

2.10. Western Blotting and Native PAGE

Total cell lysates were prepared by lysing cells with Radioimmunoprecipitation assay (RIPA) containing protease inhibitor (Roche Applied Science, Indianapolis, Indiana). Cell lysates were stored at -20°C. The denatured samples were obtained by heating the cell lysates in 95°C for 10 min and were followed by loading the sample on an 10% SDS-PAGE gel at the Mini-PROTEAN Precast System (Biorad, Hercules, California). The gel was then transferred to a nitrocellulose blotting membrane (GE healthcare, Little Chalfont, United Kingdom) on a semi-dry transfer system TE70 DWR (GE healthcare, Little Chalfont, United Kingdom) for 1 h. The membrane was then blotted with antibodies for 1 h followed by washes with Tris-buffered saline (TBS) containing 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO). SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Lafayette, Colorado) was used with the HRP. The membrane was developed in an image developer to visualize the protein distribution. The amount of protein was quantified by densitometry and the total target protein levels were normalized to actin protein levels in each corresponding lane. For Native PAGE, it was

conducted using ThermoFisher™ mini gel tank. A total of 10 μg of protein from each sample was loaded per well. Protein sample must remain in cold to retain the native structure. The gel was then transferred to Amersham™Hybond™ 0.2 μm PVDF membrane and blotted with specific anti-human IgM antibodies. SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Lafayette, Colorado) was used with the HRP. The membrane was developed in an image developer to visualize the protein distribution.

2.11. Statistical Analysis

Linear regression was used in the correlation study. Student's t-test was used to compare VH control to TCDD treatment group. For multiple comparisons, one-way ANOVA followed by Fisher's LSD post hoc test or two-way ANOVA followed by Fisher's LSD post hoc test was used. Significant differences were indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The error-bars represent standard deviation.

CHAPTER 3: EXPERIMENTAL RESULTS

Suppression of the humoral immunity after exposure to TCDD has been demonstrated in different animal species with the largest body of work conducted in mouse models. To gain further insight into the molecular mechanism by which AHR activation suppresses the IgM response, a side-by-side comparison was performed in human and mouse primary B cells using an *in vitro* CD40 ligand activation system. Previous study has indicated upregulation of LCK in only human B cells, therefore, the second part of the result section was focused on understanding the role of LCK in the suppression of the IgM response by human B cells. Finally, the role of LCK and PD-1 were investigated in the suppression of the IgM response by $CD5^+$ ILBs.

3.1. Comparison of AHR-mediated suppression of the IgM response in mouse and human B cells

AHR activation suppressed IgM response in mouse and human primary B cells.

In the current studies, the IgM concentration was quantified by ELISA and the number of IgM secreting cells was quantified by ELIspot in both mouse and human B cells. Mouse B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1 and 10 nM) and, subsequently activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 6 days (Fig. 3.1.1). The IgM concentration in supernatants from mouse B cells ranged from 16,000 – 25,000 ng/mL in the VH control group. The number of IgM secreting mouse B cells ranged from 12,000 – 20,000 per 10^6 cells in the VH control group. The number of IgM secreting B cells and the concentration

of supernatant IgM in mouse B cells were significantly suppressed by TCDD-mediated AHR activation (Fig. 3.1.1A and B).

Human primary B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1 and 10 nM) and, subsequently activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 7 days. The IgM concentration in the supernatants ranged from 12,000 – 27,000 ng/mL in VH control group. The number of IgM secreting B cells ranged from 10,000 – 20,000 per 10^6 cells in the in the VH control group. Similar to mouse B cells, the number of IgM secreting B cells and the concentration of supernatant IgM in human B cells were significantly suppressed by AHR activation (Fig. 3.1.1C and D).

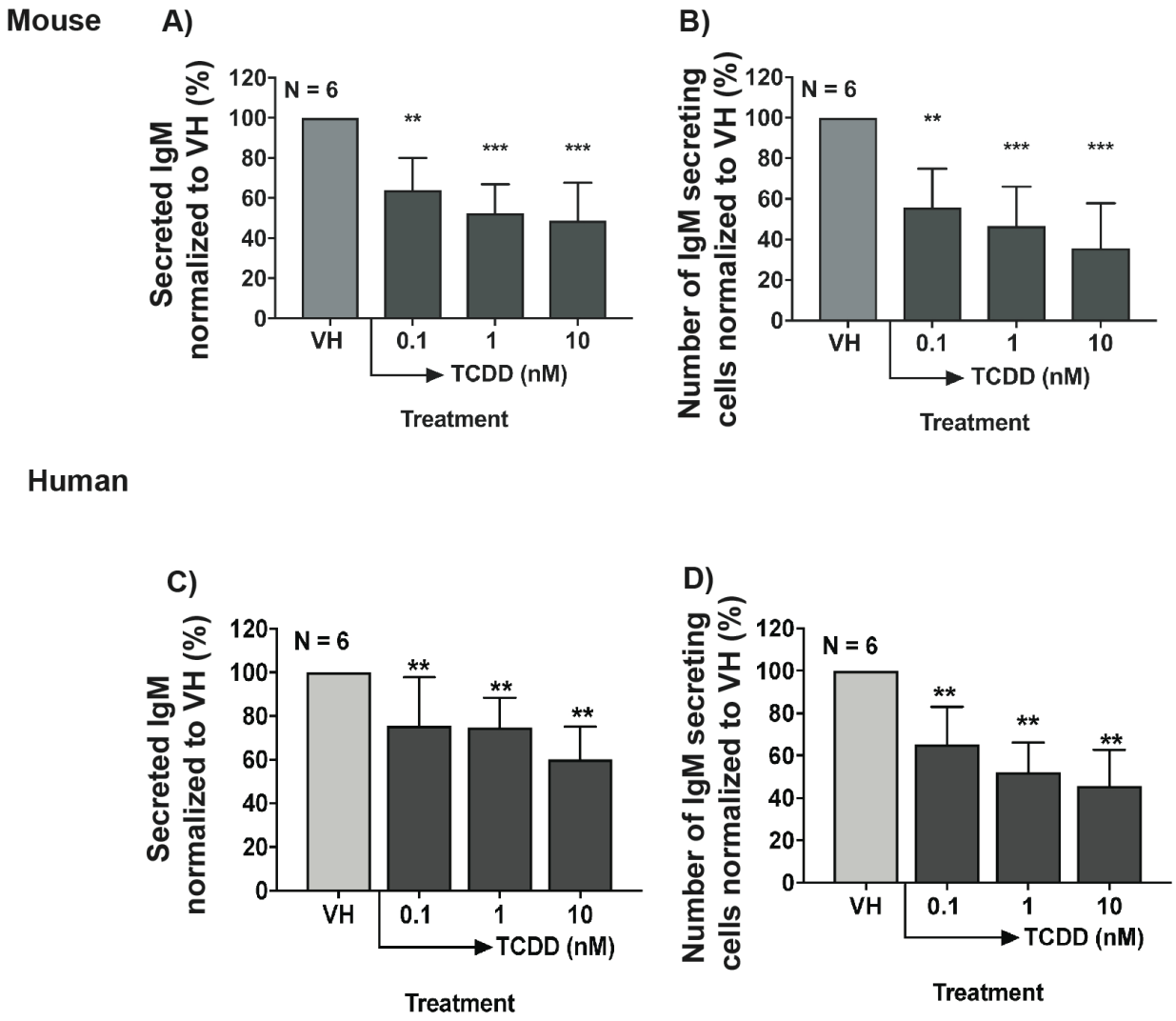


Figure 3.1.1. AHR-mediated suppression of the IgM response in mouse primary B cells.

Mouse and human primary B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1 and 10 nM) and, subsequently activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 6 days (mouse) or 7 day (human). **A)** Mouse primary B cells were collected to quantify the concentration of secreted IgM by ELISA on day 6. **B)** Mouse primary B cells were collected to quantify the number of IgM producing cells by ELIspot on day 6. The number of IgM secreting mouse B cells ranged from

Figure 3.1.1. (cont'd)

12,000 – 20,000 per 10^6 cells in the VH control group. **C)** Supernatant from human primary B cell cultures were collected and quantified for IgM by ELISA on day 7. **D)** Human primary B cells were collected to quantify the number of IgM secreting cells by ELISPOT on day 7. Data were normalized to the VH control for each human donor. Results were the normalized percentage to the VH group. N number indicated the number of animals or human donors in the study. Significant differences from VH control were indicated by ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA following by Fisher's post hoc test.

AHR antagonist reversed the AHR-mediated suppression of IgM response in human B cells.

Previous studies using AHR null mice showed an absolute requirement for the AHR in suppression of humoral immune responses by TCDD, demonstrating the receptor is required for the suppression of the IgM response (44, 45). To further determine the involvement of the AHR in the decrease of IgM secretion by human primary B cells, the AHR antagonist (CH-223191) was employed to block the interaction between TCDD and the AHR. Naive human primary B cells were treated with AHR antagonist, CH-223191 (CH) for 30 min. After treatment with antagonist, B cells were then treated with either VH (0.02% DMSO) or TCDD (10 nM) and activated as described in the Materials and Methods section. Treatment of CD40L-activated human B cells with the AHR antagonist alone increased the number of IgM secreting cells and enhanced the supernatant IgM concentration, compared to vehicle control (Fig. 3.1.2A and B). Treatment of human B cells with TCDD in combination with increasing concentrations of AHR antagonist produced a reversal of the IgM response. (Fig. 3.1.2A and B).

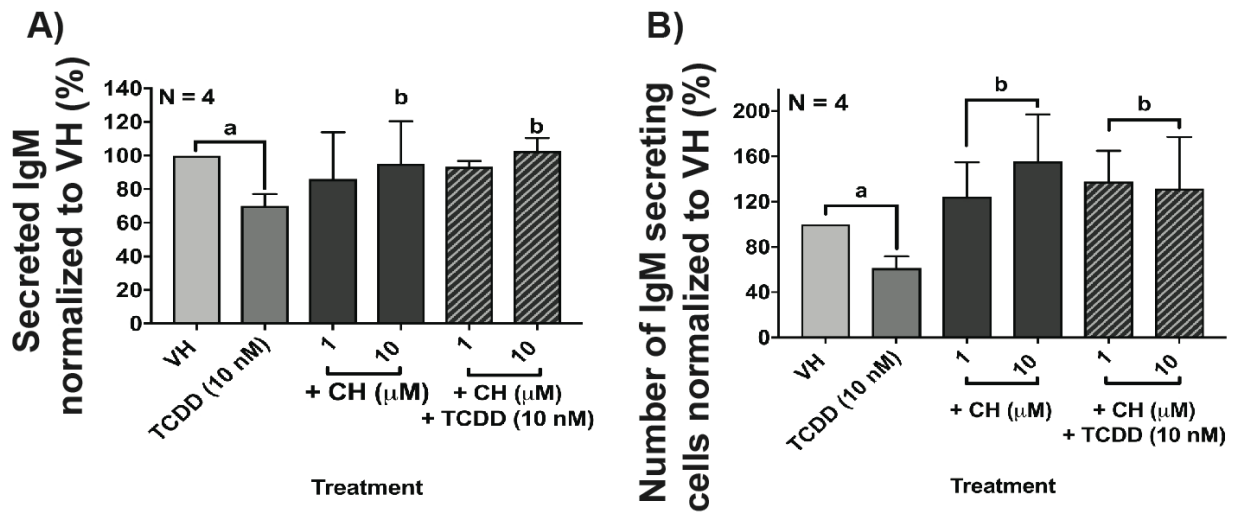


Figure 3.1.2. AHR antagonist reversed the AHR-mediated suppression of the IgM response in human primary B cells.

Human B cells were pre-treated with AHR antagonist (CH-223191) for 30 min before treatment with VH (0.02% DMSO) or TCDD (10 nM) and, subsequently activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for or 7 days. **A)** Concentration of secreted IgM in culture supernatants was quantified by ELISA on day 7. **B)** The number of IgM secreting B cells was quantified by ELIspot on day 7. **a** indicated a significant difference at $p < 0.05$ compared to the respective VH groups as determined by two-way ANOVA followed by Fisher's LSD post hoc test. **b** indicated a significant difference at $p < 0.05$ compared to the respective TCDD (10 nM) groups by two-way ANOVA as determined by Fisher's LSD post hoc test. N number indicated the number of human donors in the study. Results were the normalized percentage to the VH group.

AHR activation by TCDD suppressed IgH, IgJ and Igκ chains in mouse primary B cells.

Previous studies have shown that induction of mRNA levels of *IgH*, *IgJ* and *Igκ* chains in activated mouse splenocytes where impaired after AHR activation by TCDD (181). Therefore, studies were conducted to compare the effect of TCDD treatment on intracellular protein levels of IgH, IgJ and Igκ in mouse and human B cells. First and foremost, mouse B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1 and 10 nM) and then activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 6 days. On day 6, cells were collected to measure the intracellular expression of IgH, IgJ and Igκ**.** The intracellular protein levels of IgH and IgJ were significantly suppressed by TCDD in a concentration-dependent manner in mouse primary B cells; whereas, Igκ was less sensitive to suppression and was only significantly decreased at the highest TCDD concentration (Fig. 3.1.3).

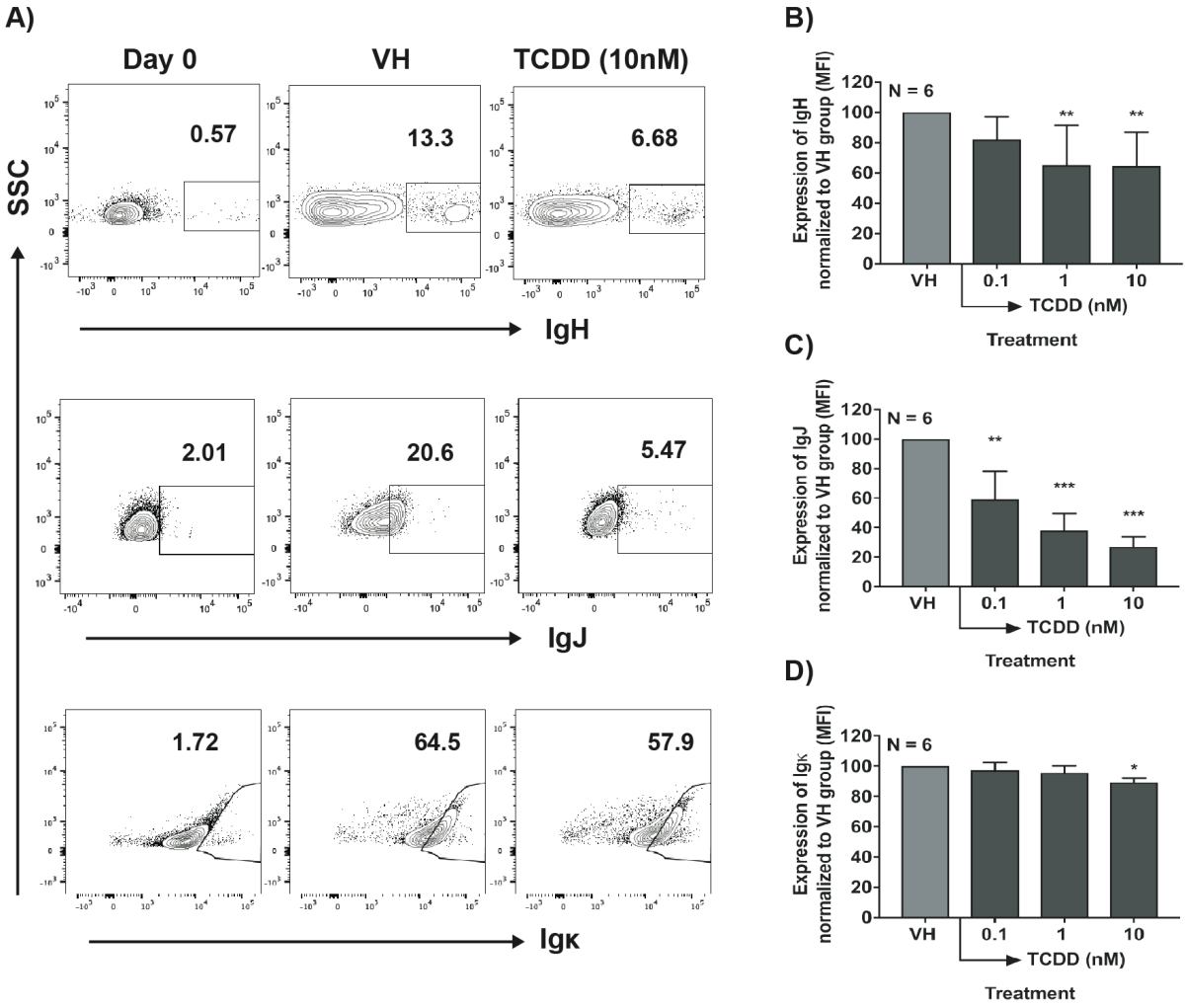


Figure 3.1.3. AHR activation by TCDD suppressed IgH, IgJ and Igκ chains in mouse B cells.

Mouse B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1 and 10 nM) and then activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 6 days. **A)** Flow cytometry dot plots of intracellular IgH, IgJ and Igκ chains with VH or TCDD treatment in mouse B cells. **B)** Level of intracellular IgH protein. **C)** Level of intracellular IgJ protein. **D)** Level of intracellular Igκ protein. N indicates the number of mice use in the study. Results were the normalized percentage to the VH group.

Figure 3.1.3. (cont'd)

Significant differences from VH control were indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as determined by a one-way ANOVA followed by Fisher's LSD post hoc test.

AHR activation did not alter the intracellular IgH, IgJ and Igκ chains in human primary B cells.

To compare with mouse B cells, human primary B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1 and 10 nM) and then activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 7 days. On day 7, cells were collected to measure the intracellular expression of IgH, IgJ and Igκ. Contrary to the observation in mouse B cells (Fig. 3.1.3), the intracellular protein levels of IgH, IgJ and Igκ were not suppressed by TCDD treatment in human B cells (Fig. 3.1.4).

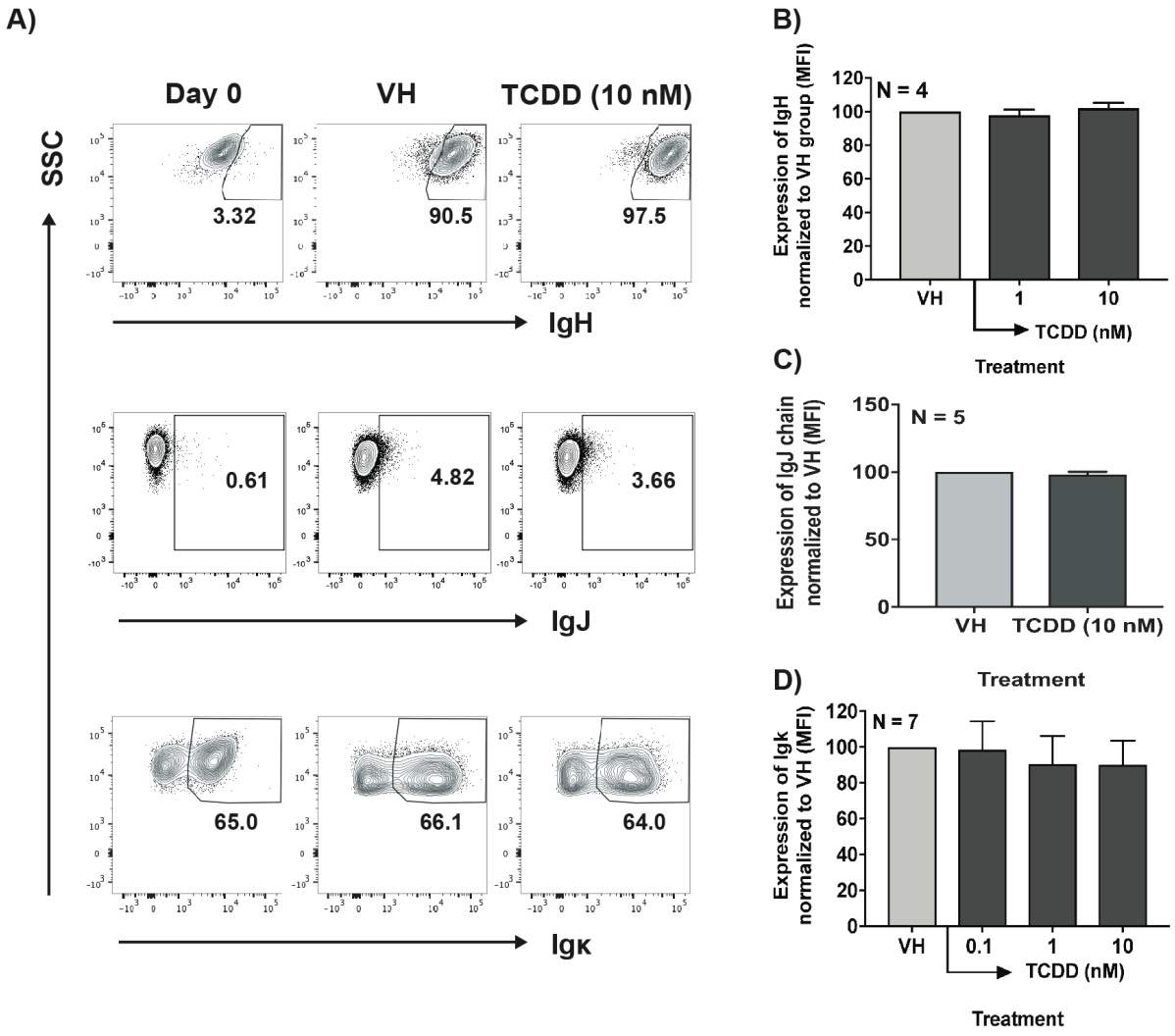


Figure 3.1.4. AHR activation did not alter the intracellular IgH, IgJ and Igκ chains in human B cells.

Human B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1 and 10 nM) and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 7 days. **A)** Flow cytometry dot plots of intracellular IgH, IgJ and Igκ in human B cells. **B)** Level of intracellular IgH protein. **C)** Level of intracellular IgJ protein. **D)** Level of intracellular Igκ protein. Results are the normalized percentage for each donor's VH control response. N indicates the number of human donors. Results were the normalized percentage to the VH group. Significant differences from VH control were

Figure 3.1.4. (cont'd)

indicated by ** $p < 0.01$ as determined by a one-way ANOVA followed by Fisher's LSD post hoc test.

AHR activation decreased the intracellular IgH in mouse but not human B cells quantified by Western Blotting.

To further verify the flow cytometry results, Western blotting was performed to assess the total expression level of IgH in both mouse and human B cells. For mouse B cells, similar to the intracellular measurement done via flow cytometry, the expression of IgH was impaired by TCDD treatment (Fig. 3.1.5A and B). In contrast, Western blotting revealed that TCDD treatment (10 nM), in fact, significantly increased intracellular IgM compared to the respective VH control group in human B cells (Fig. 3.1.5C and D). To further confirm the observation that AHR activation did not impair IgM production by human primary B cells, the effect of TCDD was assessed using a different activation stimulus, pokeweed mitogen (PWM). As observed with CD40L, PWM-activated human primary B cells showed no decrease in intracellular IgM protein levels at any of the TCDD concentrations tested (Fig. 3.1.5E).

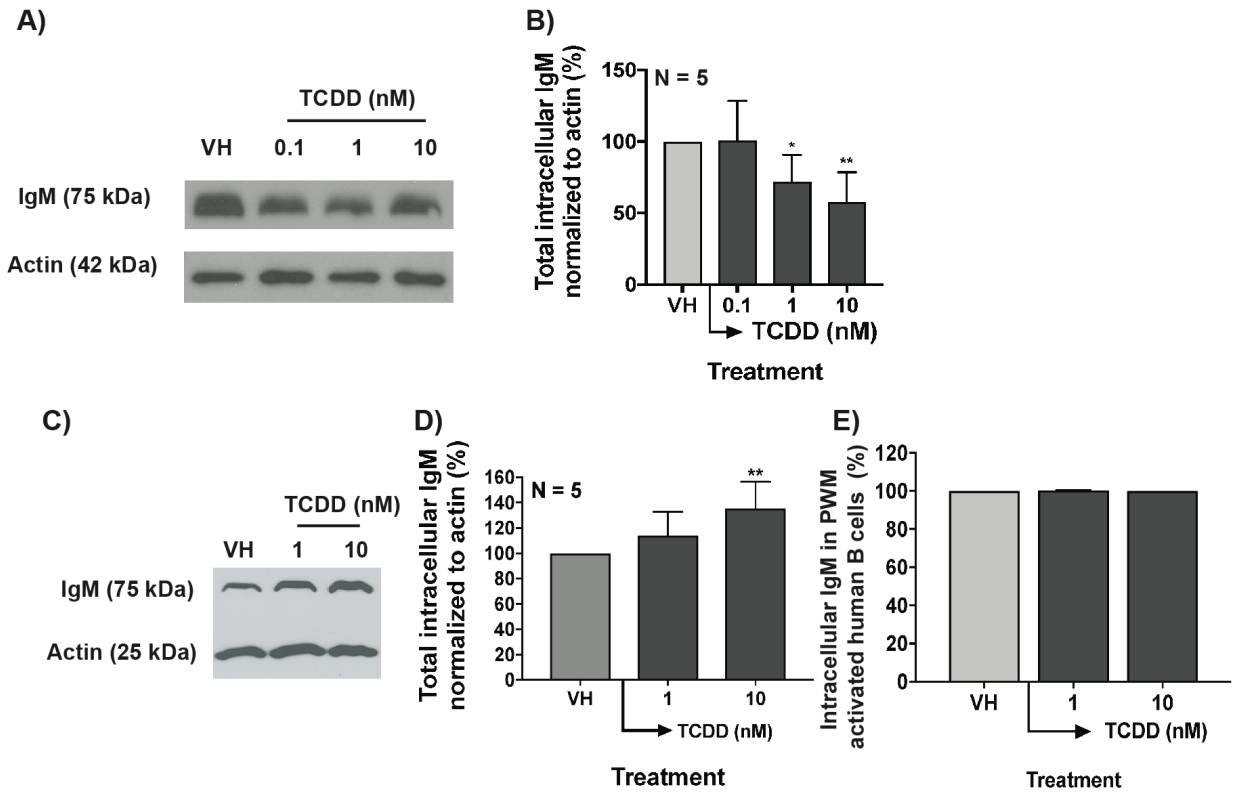


Figure 3.1.5. Comparison of IgH in mouse and human B cells via West blotting.

A) Western blotting for IgM in whole cell lysates for mouse B cells. **B)** Level of total IgM protein from A) normalized to actin protein quantified by Western blotting. **C)** Western blotting for IgM in whole cell lysates for human B cells. **D)** Level of total IgM protein from C) normalized to actin protein quantified by Western blotting. **E)** Human B cells were treated with VH (0.02% DMSO), or TCDD (1 and 10 nM) and activated with pokeweed mitogen (PWM) for 5 days. On day 5, human B cells were collected and quantified for intracellular IgH protein by flow cytometry. N indicates the number of human donors. Results were the normalized percentage to the VH group for each individual donor. Significant differences from VH control werw indicated by ** $p < 0.01$ as determined by a one-way ANOVA followed by Fisher's LSD post hoc test.

AHR activation suppressed the mRNA levels of IgH, IgJ and Igκ in mouse but not human primary B cells.

Previous studies have demonstrated that AHR activation decreased the mRNA expression levels of *IgH*, *IgJ* and *Igκ* chains in mouse B cells after LPS activation (181). In the current studies, the mRNA level of *IgH*, *IgJ* and *Igκ* chains were assessed in both mouse and human B cells. A concentration-dependent decrease of the mRNA levels of *IgH, IgJ* and *Igκ* in CD40L-activated mouse B cells when treated with TCDD (Fig. 3.1.6A – C). In contrast, the mRNA levels of *IgH*, *Igκ* and *IgJ* were not altered in CD40 ligand-activated human B cells with AHR activation at TCDD concentrations as high as 10 nM (Fig. 3.1.6D – E).

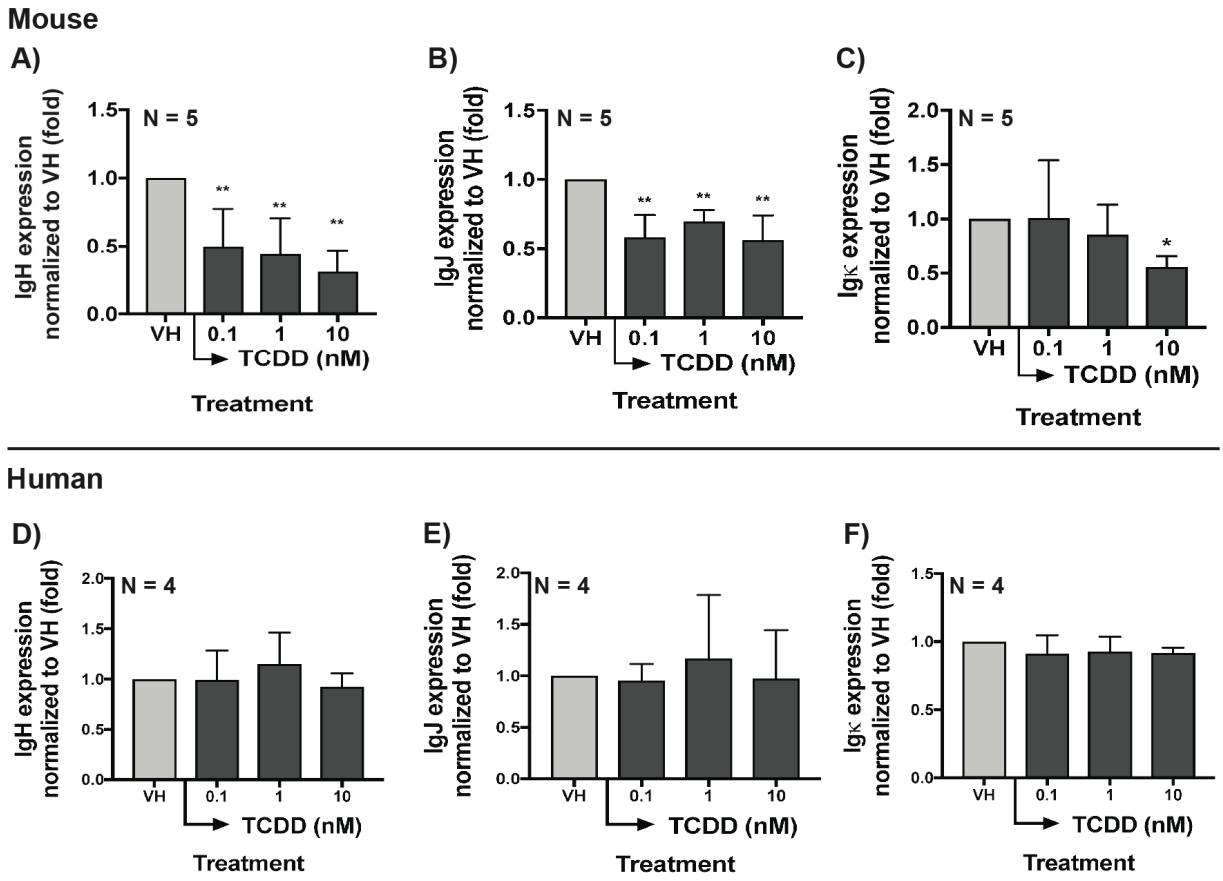


Figure 3.1.6. AHR activation suppressed the mRNA levels of *IgH*, *IgJ* and *Igκ* in mouse B cells, but not in human B cells.

Mouse and human B cells were treated with VH (0.02% DMSO), or TCDD (0.1, 1 and 10 nM) and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 6 days. **A)** Mouse B cells treated with VH or TCDD were collected on day 6 and quantified for mRNA levels of *IgH*, **B)** *IgJ* or **C)** *Igκ*. **D)** Human B cells were quantified for the mRNA levels of *IgH*, **E)** *IgJ* or **F)** *Igκ*. Results were presented as the normalized fold change for each treatment to VH control for both human donors and mice. N indicates the number of mice or donors use in the study. Significant differences from VH control were indicated by * $p < 0.05$ and ** $p < 0.01$ as determined by a one-way ANOVA followed by Fisher's LSD post hoc test.

AHR activation by TCDD caused intracellular accumulation of the assembled IgM pentamers.

The secreted IgM pentamer consists of five IgM monomers that are joined together by a single J chain (182). Due to the observation by Western blotting, that intracellular IgM was accumulating in human B cell treated with TCDD (Fig. 3.1.5C), studies were conducted to explore the possibility that AHR activation interfered with the assembly of the IgM pentamer. Native-PAGE assays were used to assess the amount of intracellular IgM pentamers in activated human B cells. Human B cells were treated with either VH (0.02% DMSO) or TCDD (10 nM) and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10). Cells were collected on day 7 and whole cell lysates were prepared for identification of IgM isoforms using Native-PAGE assay. The analysis was performed on B cells isolated from three separate human donors with all three of the donor's B cells exhibiting suppression of the IgM response by TCDD treatment as quantified by ELISA and ELIspot. The top band represents the 1024 kD an IgM pentamer (Fig. 3.1.7A). As shown in Fig.3.1.7, the large dark band located at the bottom showed a combination of IgM trimers (720 kDa) and dimers (480 kDa). TCDD-treatment increased the intracellular levels of IgM pentamers, trimers and dimers compared to the VH control (Fig. 3.1.7A). Longer exposure of the same Native PAGE showed noticeably more intracellular IgM pentamer in the TCDD treatment groups compared to VH group controls (Fig.3.1.7B)

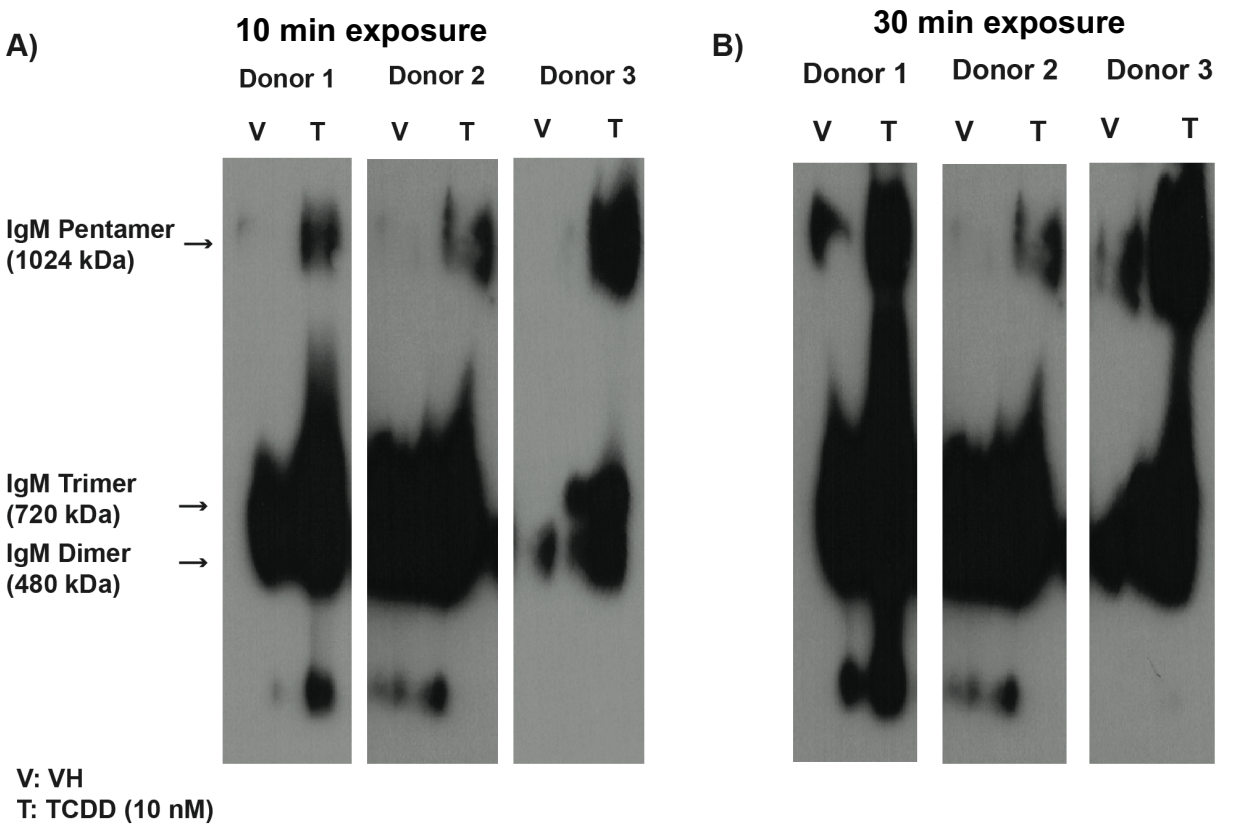


Figure 3.1.7. AHR activation by TCDD caused intracellular accumulation of the assembled IgM pentamers.

Human primary B cells were treated with VH (0.02% DMSO) or TCDD (10 nM) and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10). Cells were collected on day 7 and whole cell lysates were prepared for identification of IgM isoforms using Native-PAGE assay. The top band represents the 1024 kD IgM pentamer. The bottom large dark band indicates the combination of IgM trimers (720 kDa) and dimers (480 kDa). The analysis was performed on B cells isolated from three separate human donors with all three of the donor's B cells exhibiting suppression of the IgM response by TCDD treatment as quantified by ELISA and ELIspot. The Native PAGE was conducted using ThermoFisher^{TM} mini gel tank. A total of 10 μg of protein from each sample was loaded per well. The gel was then transferred

Figure 3.1.7. (cont'd)

to Amersham^{TM}Hybond^{TM} 0.2 μm PVDF membrane and probed with specific anti-human IgM antibodies. **A)** The membrane was exposed for 10 min prior to being developed. From left to right, lane 1: Donor 1 VH group; lane 2: Donor 1 TCDD group; lane 3: Donor 2 VH group; lane 4: Donor 2 TCDD group; lane 5: Donor 3 VH group; lane 6: Donor 3 TCDD group. **B)** Membrane exposed for 30 min prior to being developed.

TCDD-mediated activation of AHR decreased the secreted IgG in human primary B cells.

IgG was secreted by B cells after undergoing class switching and was the mature form of immunoglobulin secreted by B cell (183). Activation by cytokines has also shown to promote class switch in human B cells (184). Therefore, further studies were conducted to determine if impairment of immunoglobulin secretion was limited to IgM by AHR activation. Human primary B cells were treated with VH (0.02% DMSO) or TCDD (1 and 10 nM) and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10). On day 7, The culture medium was collected and quantified for IgG and IgM in the same human donor by ELISA. These studies showed that the levels of supernatant IgG in culture medium decreased as TCDD concentrations increased, with a significant decrease at 10 nM TCDD (Fig. 6).

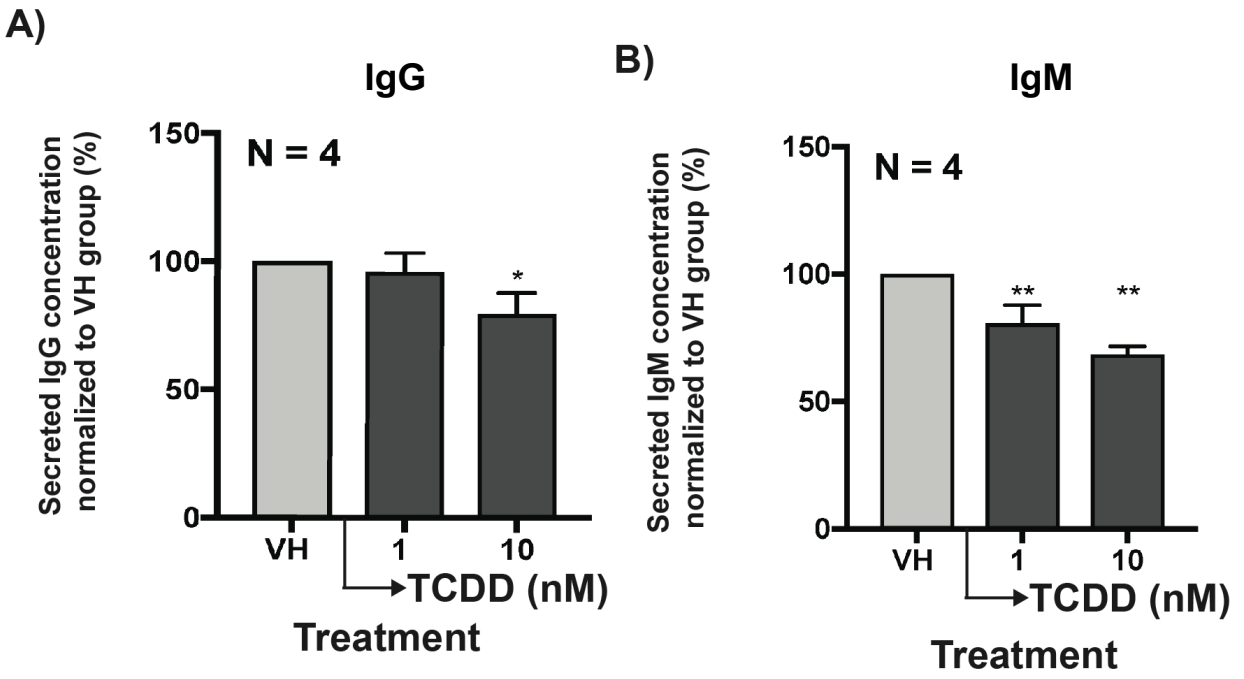


Figure 3.1.8. TCDD-mediated activation of AHR decreased the secreted IgG in human primary B cells.

Human B cell were treated with VH (0.02% DMSO) or TCDD (1 and 10 nM) and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10). On day 7, The culture medium was collected and quantified for **A)** IgG; and **B)** IgM by ELISA. Results were normalized to the individual VH group for each donor. The level of IgG ranged from 9,000 - 10,000 ng/mL and the level of IgM ranged from 15,000 – 25,000 ng/mL in the VH treated control B cells. "N" indicates the number of donors. Results were the normalized percentage to the VH group for each individual donor. Significant differences from VH control were indicated by * $p < 0.05$, ** $p < 0.01$ as determined by a one-way NOVA following by Fisher's LSD post hoc test.

3.2. Determine the role of LCK in the suppression of IgM response by human B cells

Previous transcriptomic analysis identified the upregulation of *LCK* in AHR activated human primary B cells, but not in mouse B cells (120). While little is known concerning the role of LCK in B cells, studies have implicated an important role for LCK in B cell receptor (BCR) signaling in chronic lymphocytic leukemia (CLL) cells, such that the increase of LCK has been used as a biomarker in CLL patients (127, 166). This observation prompted the further investigation of the role LCK in the context of AHR-mediated impairment of the IgM response in human B cells.

AHR activation increased LCK expression in naïve human primary B cells.

In the current study, mRNA and protein levels of LCK were quantified in activated human primary B cells. *LCK* mRNA significantly increased with AHR activation in a concentration dependent manner on day 3 (Fig. 3.2.1A). Likewise, the protein level of LCK increased significantly with AHR activation from day 3 to day 7 (Fig. 3.2.1C and D).

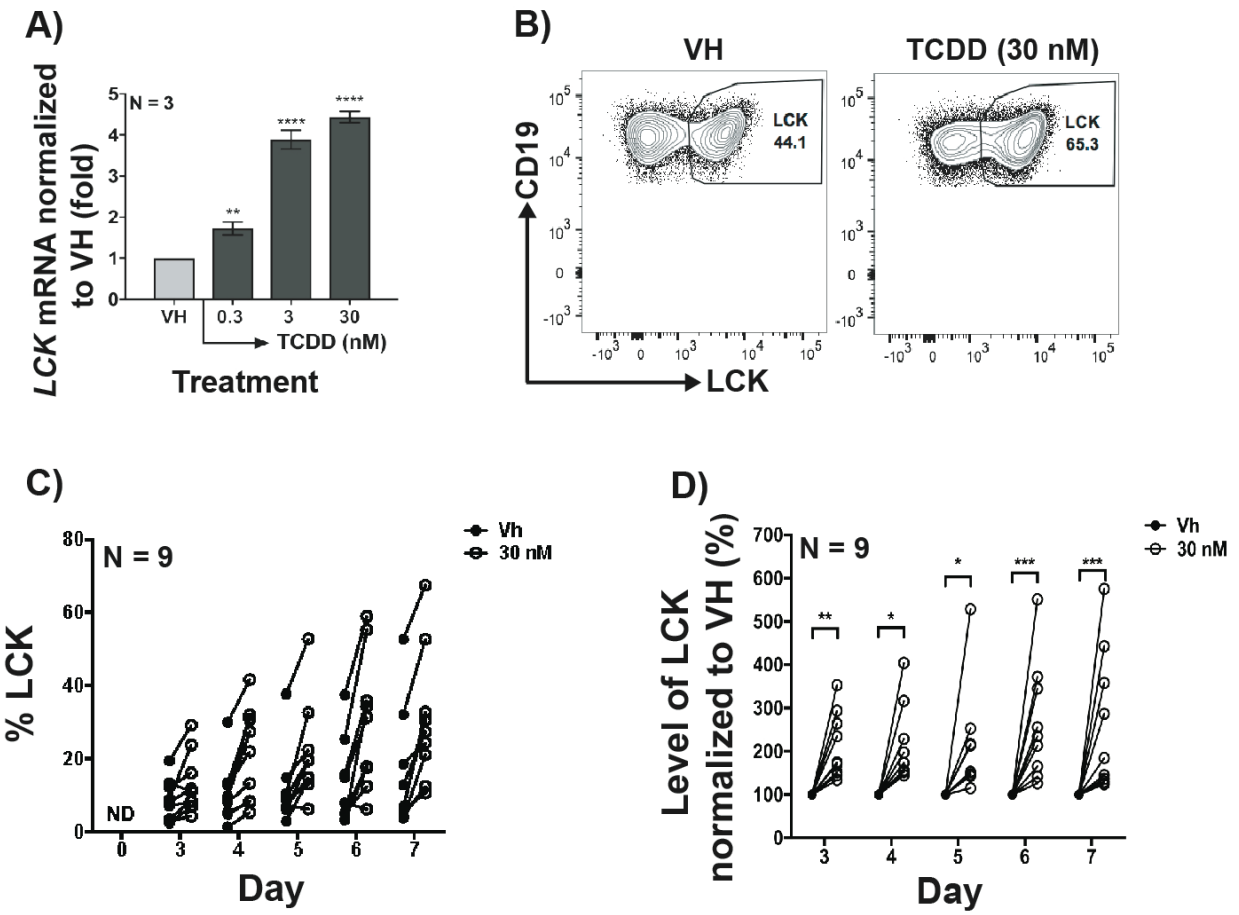


Figure 3.2.1. AHR activation increased LCK expression in naïve human primary B cells.

Human B cells were treated with VH (0.02% DMSO), or TCDD (0.3, 3 and 30 nM) on day 0 and cultured for 3 days, mRNA samples were collected on day 3. **A)** mRNA levels of *LCK* as determined by real-time qPCR in B cells. B cells were treated with VH (0.02% DMSO), or TCDD (30 nM) and cultured for 7 days. **B)** Flow cytometry dot plot of intracellular LCK in B cells with VH or TCDD treatment on day 7. Cells were collected on all 7 days to analyze the LCK protein level. **C)** Un-normalized percent positive LCK in B cells on day 0 (background) and day 3 to 7 post B cell activation with no treatment. **D)** Normalized percent LCK positive B cells with VH or TCDD (30 nM) treatment from day 3

Figure 3.2.1. (cont'd)

to day 7. Day 1 and 2 were excluded from the graph due to undetectable levels of LCK in human B cells. The circular dot indicates one individual human donor. N indicated the number of donors in the study. Results were normalized to the individual VH group for each donor. Significant differences from VH control were indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA following by Fisher's LSD post hoc test.

Concentration-dependent increase of LCK^{+} cells with TCDD treatment in human B cells.

Human B cells were treated with VH or TCDD (0.1, 1 10 nM) and activated by co-cultured with CD40L-expressing L cells with cytokines (IL-2, IL-6 and IL-10) for 7 days. On day 3 and 7, cells were harvested to measure intracellular LCK protein via flow cytometry. TCDD-mediated AHR activation significantly increased LCK protein, and it was concentration dependent with TCDD in human B cells on both day 3 and 7 (Fig. 3.2.2).

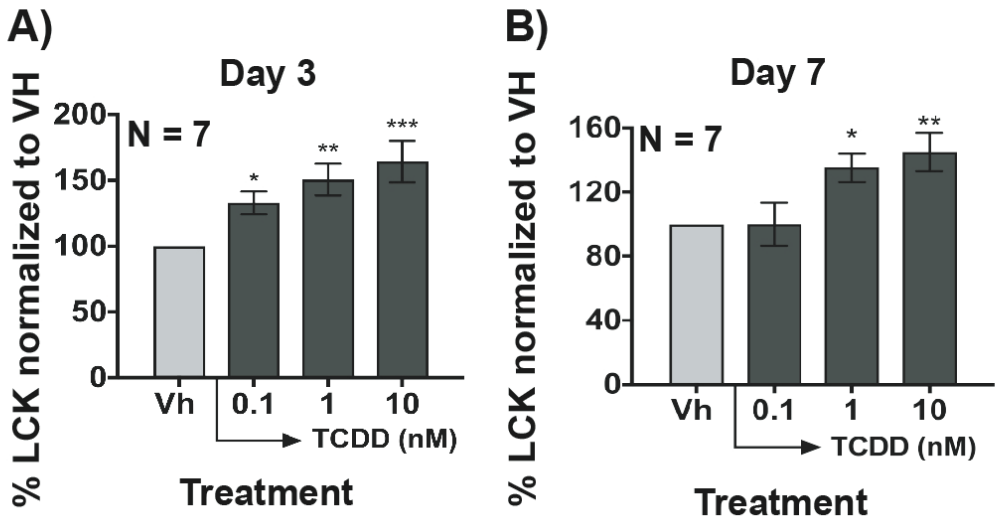


Figure 3.2.2. Concentration-dependent increase of LCK$^+$ cells with TCDD treatment in human B cells.

Human B cells were treated with VH (0.02% DMSO) or TCDD (10 nM) for 7 days. Cells were collected on days 3 and day 7 to analyze the percent positive of LCK in human B cells. Percent LCK positive B cells on **A)** day 3 and on **B)** day 7 measured by flow cytometry. Results were normalized to the individual VH group for each donor. N indicated the number of donors in the study. Significant differences from VH control were indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA following by Fisher's LSD post hoc test.

AHR antagonist treatment reversed the AHR-mediated increase of percent positive LCK in human B cells.

To further determine if the increase in LCK was dependent on AHR activation, the AHR antagonist (CH-223191) was employed to block the activation of AHR in human B cells. First, the specificity of the antagonist (CH-223191) was verified by measuring the *CYP1A1* mRNA induction with TCDD treatment. With the addition of AHR antagonist, the expression of *CYP1A1* mRNA was attenuated. (Fig. 3.2.3A). After verifying the specificity of the antagonist, human B cells were treated with AHR antagonist, following with VH or TCDD (10 nM) treatment and activated as described in the Materials and Methods Section. Interestingly, the percent positive of LCK was suppressed with 10 μM AHR antagonist treatment. With both antagonist and TCDD treatments, the LCK protein was reduced to similar to the VH control (Fig. 3.2.3B).

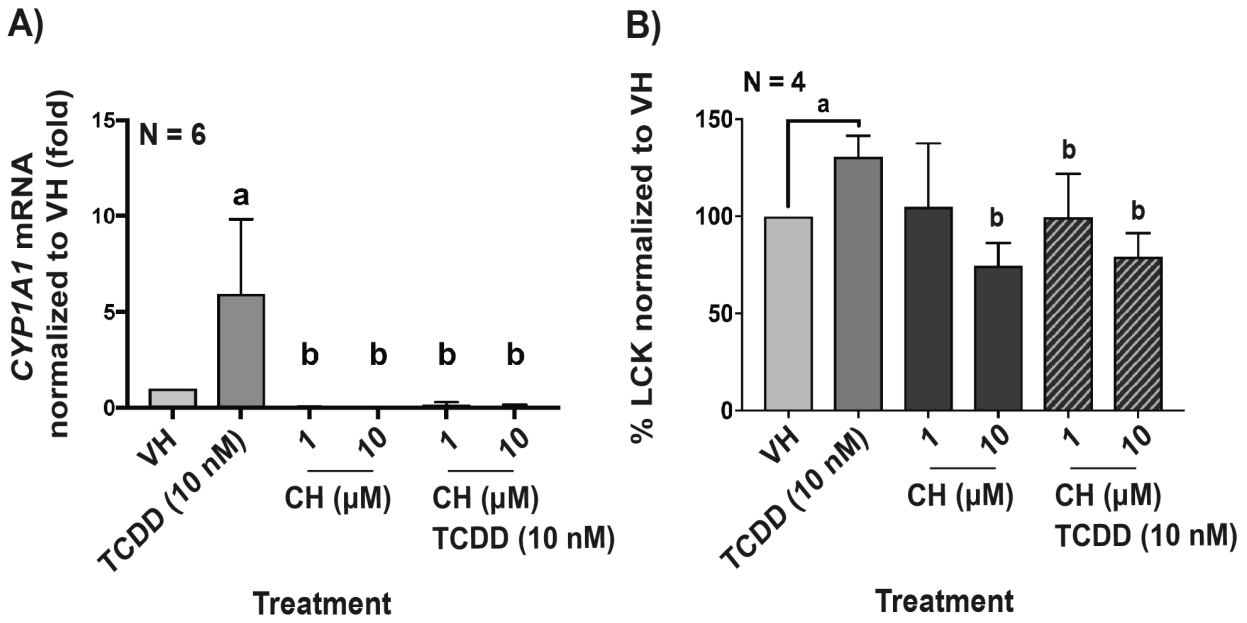


Figure 3.2.3. AHR antagonist treatment prevented the increase of percent positive LCK in human B cells.

Naive human primary B cells were treated with AHR antagonist, CH-223191 (CH) on day 0. Following antagonist treatment, cells were treated with VH (0.02% DMSO) or TCDD (10 nM) for 7 days. **A)** *CYP1A1* mRNA induction with TCDD, TCDD and CH-223191 (CH) treatments on day 1. **B)** Percent LCK positive B cells on day 7. N indicated the number of donors in the study. Two-way ANOVA followed by Fisher's LSD post hoc test has been performed to compare all treatment groups. Significant differences from **VH** control were indicated by **a** ($p < 0.05$); significant differences from the **TCDD** group were indicated by **b** ($p < 0.05$). Results were presented as the normalized percentage compared to the VH group for each donor. "N" indicates the number of donors used in the study.

AHR activation increased the percentage of LCK^{+} cells in naïve human primary B cells activated with different stimuli.

To ascertain whether upregulation of LCK by AHR activation was specific to the mode of B cell activation, B cells were activated in several different methods (CD40L fibroblast plus IL-2 and IL-21; by soluble CD40L plus IL-2 and IL-21 and by pokeweed mitogen [PWM]). Irrespective of the manner in which the cells were activated AHR activation resulted in upregulation of LCK in human primary B cells (Fig. 3.2.4A – C).

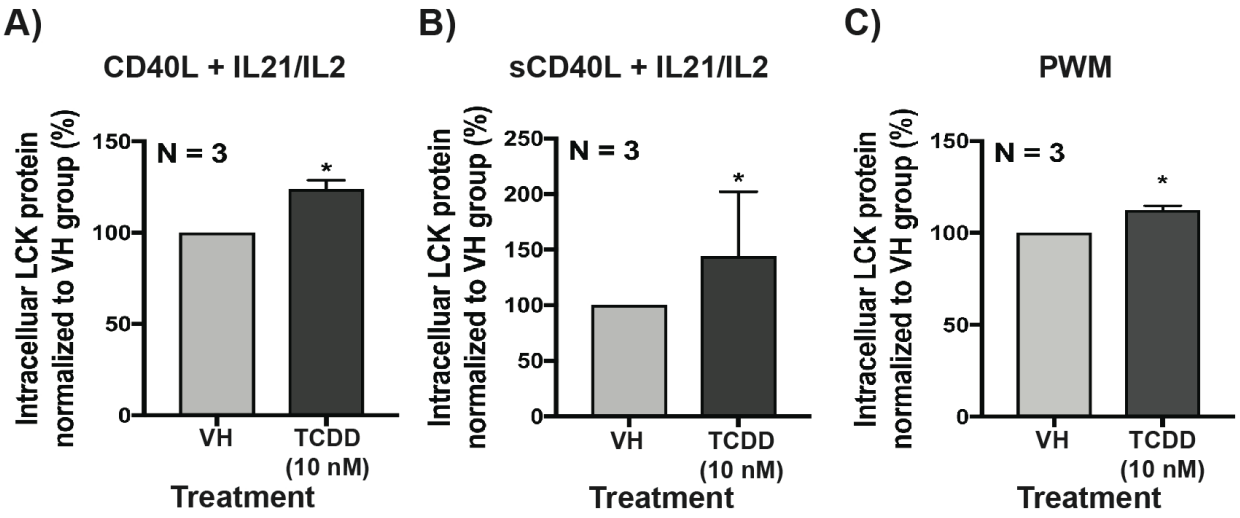


Figure 3.2.4. AHR activation increased the percentage of LCK$^+$ cells in naïve human primary B cells activated with different stimuli.

B cells were treated with VH (0.02% DMSO) or TCDD (10 nM) and activated by **A)** CD40 ligand expressing L cells plus cytokines (IL-2 and IL-21); **B)** soluble CD40 ligand plus cytokines (IL-2 and IL-21); or **C)** PWM. The percent positive of LCK was quantified by flow cytometry on day 7. N indicates the number of donors used in the study. Significant differences from VH control were indicated by * $p < 0.05$ by Student's t-test. Results were presented as the normalized percentage compared to the VH group for each human donor.

Upregulation of LCK and suppression of IgM secretion were dependent on AHR activation within the first 24 h post B cell activation.

Murine B cells are sensitive to suppression of IgM responses only when exposed to TCDD within the first 24 h post B cell activation, after which time they become refractory to TCDD-mediated suppression (12). Likewise, SKW-AhR$^+$ 6.4 cells, a mature human B cell line transduced with AHR, exhibited the same temporal sensitivity to TCDD (i.e., within first 24 h post B cell activation) (62). In the present study, human naïve B cells were activated with CD40L expressing fibroblasts plus cytokines (IL-2, IL-6 and IL-10) and treated with TCDD (1 and 10 nM) on the day of activation (D0), day 1 (D1), day 2 (D2) or day 3 (D3) post activation. On day 7, cells were collected and assayed for intracellular LCK protein, the number of IgM secreting cells, and the IgM concentration in culture supernatants (Fig. 3.2.5). A temporal relationship was observed with respect to the TCDD-mediated increase in LCK protein level, with the maximum percentage of LCK$^+$ B cells being observed when TCDD was added to B cells on day 0 (Fig. 3.2.5A). Similarly, the number of IgM secreting cells and the concentration of supernatant IgM was not affected by the TCDD treatment when TCDD was added to cultures on day-2 after B cell activation or later (Fig. 3.2.5B and C), which is consistent with a number of prior reports (57, 62).

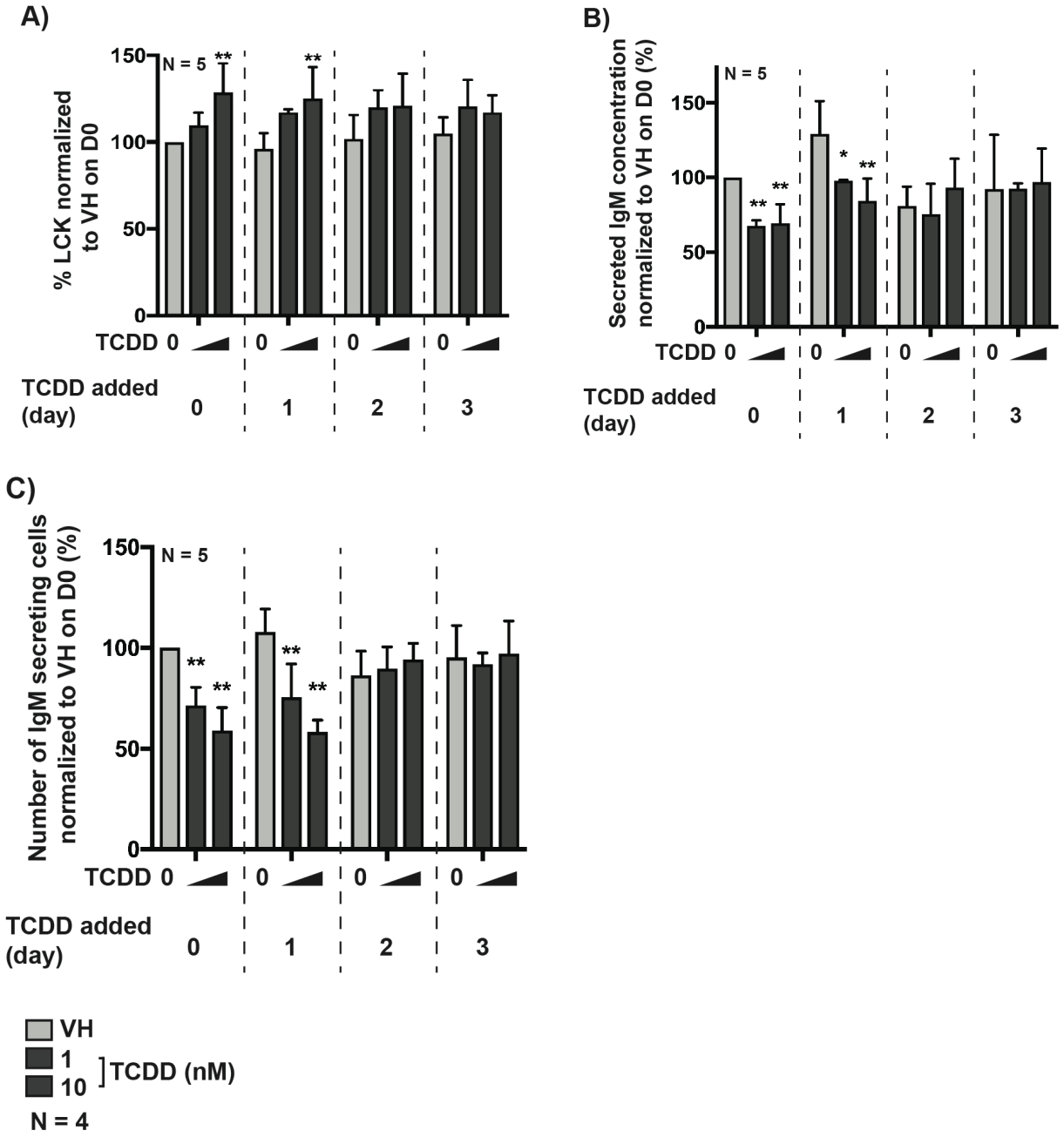


Figure 3.2.5. Upregulation of LCK and suppression of IgM secretion were dependent on AHR activation within the first 24 h post B cell activation.

B cells were activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) on day 0. Following activation, B cells were treated with VH (0.02% DMSO), or TCDD (1 and 10 nM) on day 0, 1, 2 or 3 and cultured for a total of 7 days. **A)** Percent LCK positive B cells measured by flow cytometry on day 7; **B)** IgM concentration

Figure 3.2.5. (cont'd)

in supernatants as quantified by ELISA and **C)** The number of IgM secreting cells as quantified by ELIspot on day 7. N indicates the number of donors used in the study. Significant differences from VH control were indicated by * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA following by Fisher's LSD post hoc test. Results were presented as the normalized percentage compared to the VH group.

Specific small peptide LCK inhibitors restored IgM secretion in the present of TCDD

The upregulation of LCK has been observed in chronic lymphocytic leukemia (CLL) patients, and LCK has been suggested as a biomarker for the progression of CLL in patients (166). However, little is known about the role of LCK in B cell function. To more directly explore whether LCK played a role in AHR-mediated suppression of IgM secretion, an LCK-specific inhibitor was used as a molecular probe to block the activity of LCK in human B cells. Based on our prior observation that TCDD treatment impaired IgM secretion on day 7 coupled with the kinetics of LCK induction (peak levels occurring around day 3 to day 5), day 5 was selected for treatment with the LCK inhibitors. The LCK inhibitor is a well-characterized small peptide (PI) (EGQYpEEIP) that directly binds to the SH-2 domain of LCK. A control peptide (EGQYEEIP) was created to ensure the peptide was not affecting cell function. The percentage of LCK^+ cells increased with the peptide control (Fig. 3.2.6A). No protection from the suppressive effects of TCDD on the number of IgM secreting cell or the amount of IgM secreted was observed with the control peptide (Fig. 3.2.6). By contrast, in the presence of the PI, the percentage of LCK^+ cells were increased by AHR activation but to a lesser extent than in the absence of the inhibitor (Fig. 3.2.6). The number of IgM secreting cells and the IgM concentration in supernatant was restored with the addition of PI (Fig. 3.2.6B and C).

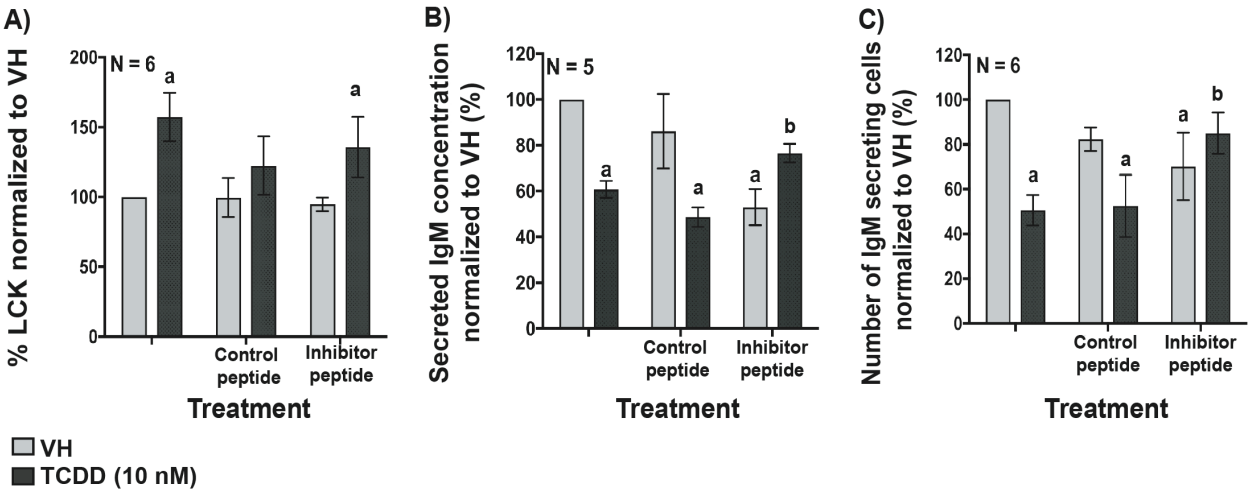


Figure 3.2.6. Specific small peptide LCK inhibitor restored IgM secretion in the presence of TCDD.

B cells were treated with VH (0.02% DMSO), or TCDD (10 nM) and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 7 days. On day 5, the LCK inhibitor (EGQYpEEIP) or control peptide (EGQYEEIP) was added to the cell cultures. **A)** Percent LCK positive human B cells measured by flow cytometry on day 7; **B)** IgM concentration in supernatant as quantified by ELISA; and **C)** The number of IgM secreting B cells as quantified by ELIspot on day 7. N indicates the number of donors used in the study. Significant differences from VH without LCK inhibitor were indicated by **a** ($p < 0.05$) by two-way ANOVA following by Fisher's LSD post hoc test; significant differences from TCDD (10 nM) group without LCK inhibitor were indicated by **b** ($p < 0.05$) by two-way ANOVA following by Fisher's LSD post hoc test. Results are presented as the normalized percentage compared to the VH group.

Small molecule LCK inhibitor (RK24466) restored IgM secretion in the presence of TCDD.

To further verify our finding, we employed a second and more potent small molecule LCK inhibitor (RK24466) with high LCK binding affinity to attenuate the LCK activity in human B cells. Similar to PI, RK24466 was added in the cell culture on day 5 to allow the upregulation of LCK to reach its peak level. RK24466 possessed an IC50 for 1 – 2 nM for LCK in human cells, with minimal effects on other kinases. The results with RK24466 were similar to the PI LCK inhibitor such that the TCDD-mediated suppression of IgM secretion was restored to the VH control level with RK24466 treatment (Fig. 3.2.7A – C). Collectively, these findings show that LCK activity influences IgM secretion in response to AHR activation in B cells.

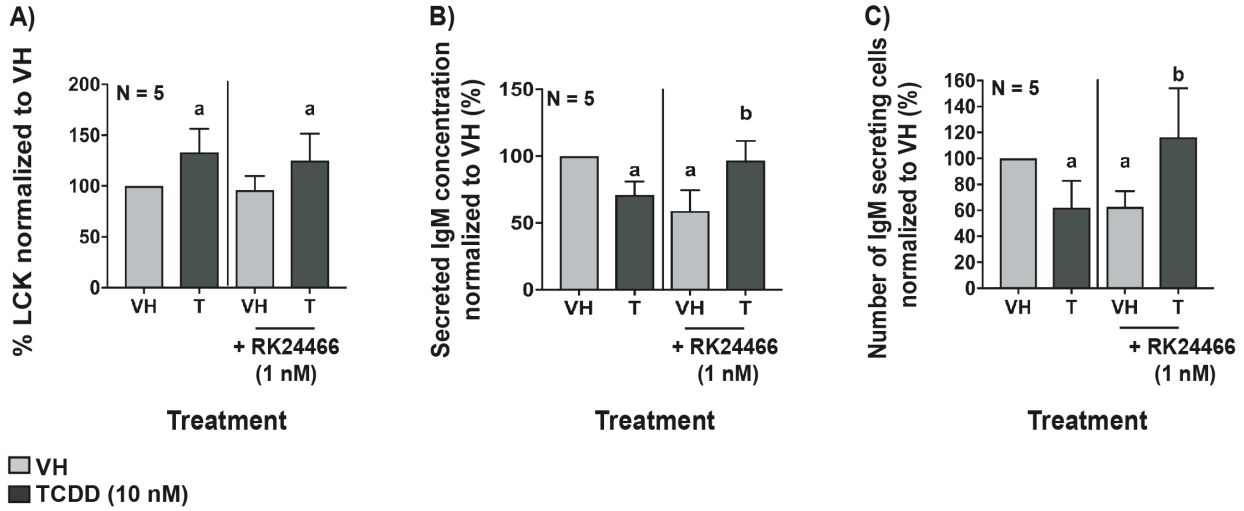


Figure 3.2.7. Small molecule LCK inhibitor (RK24466) restored IgM secretion in the presence of TCDD.

B cells were treated with VH (0.02% DMSO), or TCDD (10 nM) and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 7 days. On day 5, the LCK inhibitor (RK24466) was added to the cell cultures and the cells were harvested on day 7. **A)** Percent LCK positive B cells measured by flow cytometry on day 7. **B)** IgM concentration as quantified by ELISA on day 7. **C)** The number of IgM secreting cells as quantified by ELIspot on day 7. N indicates the number of donors used in the study. Significant differences from VH without LCK inhibitor were indicated by **a** ($p < 0.05$) by two-way ANOVA following by Fisher's LSD post hoc test; significant differences from TCDD (10 nM) group without LCK inhibitor were indicated by **b** ($p < 0.05$) by two-way ANOVA following by Fisher's LSD post hoc test. Results are presented as the normalized percentage compared to the VH group.

Matrix analysis of TCDD treatment and LCK inhibitor treatment on human B cells.

As we observed in Fig. 3.2.6 and Fig. 3.2.7, the LCK inhibitors reversed the AHR-mediated suppression of the IgM response. More interestingly, the addition of LCK inhibitor to human B cells without TCDD treatment also showed a significant decrease of the IgM response. In order to further understand the interplay between AHR activation and LCK, we designed a matrix study with varying combinations of RK24466 and TCDD concentrations. The decision of using RK24466 over small peptide inhibitor was RK24466 had high potency and binding affinity toward LCK compared to PI. In the absence of RK24466, there was a concentration-dependent suppression in IgM secretion (Fig. 3.2.8A and B). Similarly, in the absence of AHR activation, there was also a concentration-dependent suppression of the IgM response with increasing concentrations of LCK inhibitor. Interestingly, for cells treated with both TCDD and LCK inhibitor simultaneously, their individual suppressive effects on the IgM response, rather than being additive, were opposing, i.e., the LCK inhibitor can restore the IgM response in the presence of TCDD. When assessed using the number of IgM secreting cells, a similar profile of activity was observed (Fig. 3.2.8).

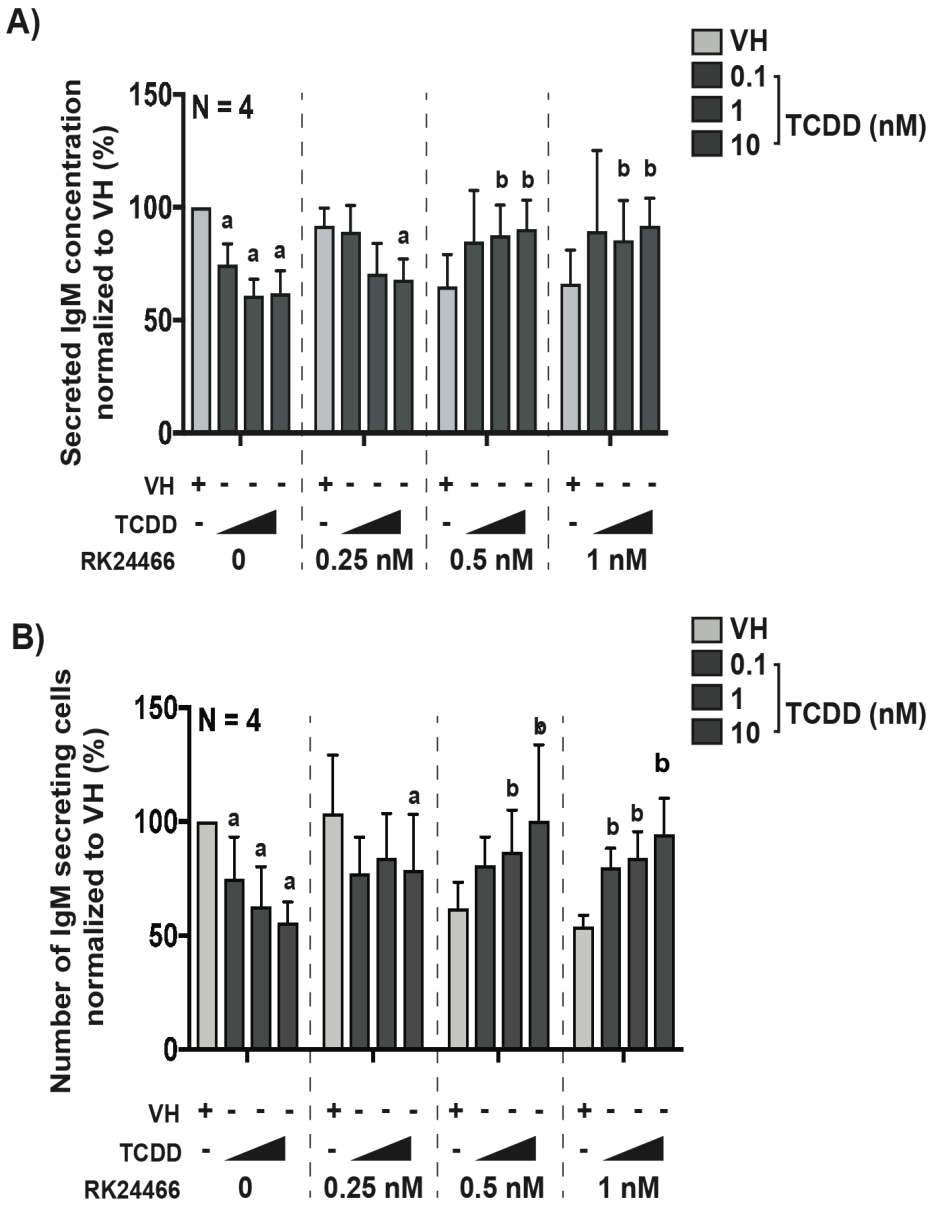


Figure 3.2.8. Matrix study on the interplay between LCK inhibitor treatment and TCDD treatments on human B cells.

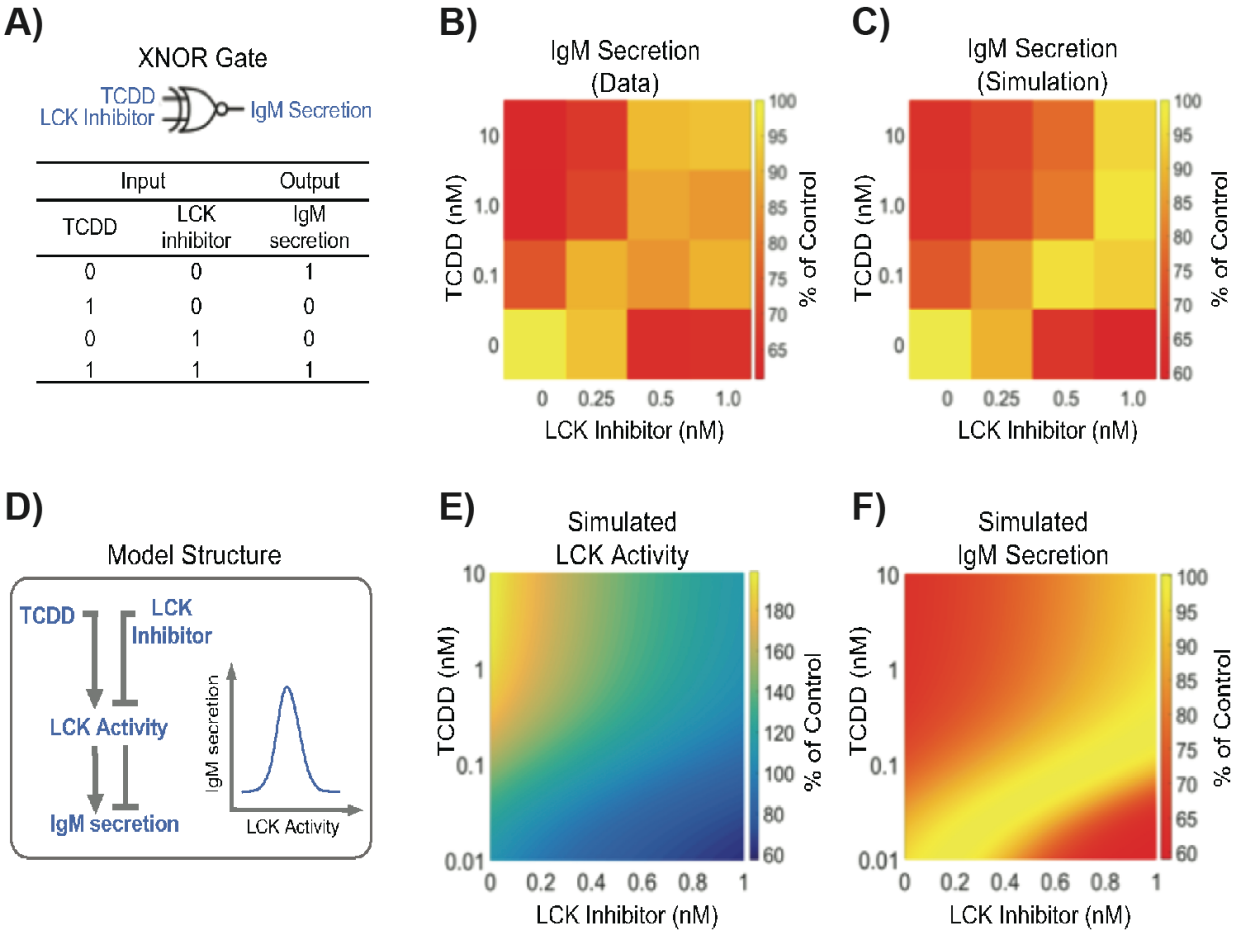
B cells were treated on day 0 with TCDD (0.1, 1 and 10 nM) and LCK inhibitor (0, 0.25, 0.5 and 1 nM) and cultured for 7 days. On day 5, the LCK inhibitor (RK24466) was added to the cell cultures and the cells were harvested on day 7. Cells were collected on day 7 to quantify **A)** IgM concentration in supernatant; and **B)** The number of IgM secreting cells. N indicates the number of donors used in the study. Significant differences from VH

Figure 3.2.8. (cont'd)

without LCK inhibitor were indicated by **a** ($p < 0.05$) by two-way ANOVA following by Fisher's LSD post hoc test; significant differences from TCDD (10 nM) group without LCK inhibitor were indicated by **b** ($p < 0.05$) by two-way ANOVA following by Fisher's LSD post hoc test. Results are presented as the normalized percentage compared to the VH group without RK24466 treatment for each human donor.

Modeling the XNOR gate effect of TCDD and LCK inhibitor on IgM secretion.

The counterintuitive effects of TCDD and LCK inhibitor on IgM secretion are reminiscent of an XNOR logic gate, where the output is 1 (unsuppressed IgM secretion in this case) when both inputs are absent or present, whereas the output is 0 (suppressed IgM secretion) when only one of the two inputs is present (Fig. 3.2.9A). The heat map in Fig. 3.2.9B, which summarized the findings in Fig. 3.2.8, is clearly consistent with an XNOR gate I/O relationship. To interpret this interesting result, we formulated a simple XNOR gate model (see Methods for model details) by hypothesizing that the activity of LCK has a dual effect on IgM secretion and needs to reach an optimal level during the B cell activation and differentiation process; deviation from this optimal level, either too high or too low, will lead to impaired IgM secretion (Fig. 3.2.9D). Specifically, based on the effects of TCDD on LCK expression described above, we postulate that TCDD exposure alone results in an increase in total LCK activity from its optimal level. Conversely, LCK inhibitor alone results in a decrease in total LCK activity from its optimal level; however, when both are present in the appropriate ratio, their effects are opposing leading to near-optimal LCK activity (Fig. 3.2.9E). As a result, IgM secretion exhibits an XNOR gate response profile (Fig. 3.2.9F). After parameter optimization, our model quantitatively recapitulated the concentration response matrix effects observed experimentally (compare Fig. 3.2.9B and C).



Input		Output
TCDD	LCK inhibitor	IgM secretion
0	0	1
1	0	0
0	1	0
1	1	1

Figure 3.2.9. Modeling the XNOR gate effect of TCDD and LCK inhibitor on IgM secretion.

A) The combined effects of TCDD and LCK inhibitor follow an XNOR logic gate phenomenon. **B)** Heat map of data showing percent suppression of IgM secretion by combinations of TCDD and LCK inhibitor concentrations in human B cells. **C)** Heat map of simulated % inhibition of IgM secretion using the model in D. **D)** The model structure used to emulate an XNOR gate; see text for model details. LCK activity has a biphasic effect on IgM secretion: below an optimal level, as LCK activity increases IgM secretion increases; above the optimal level, as LCK activity increases IgM secretion decreases. **E)** Simulated LCK activity under different combinations of TCDD and LCK inhibitor

Figure 3.2.9. (cont'd)

concentrations. **F)** Simulated IgM secretion under different combinations of TCDD and LCK inhibitor concentrations.

AHR activation increased the level of active LCK.

LCK activation is critical for T cell receptor (TCR) signaling and cytokine secretion (185). Upon TCR activation, the phosphorylation of the inhibitory site (Tyr505) on LCK is dephosphorylated, which in turn allows the activation of LCK (185). Once activated, LCK phosphorylates ZAP70 at Tyr319 and Tyr292 (186, 187), which then phosphorylates downstream signal adaptors. In the current studies, the activity of LCK was examined using phospho-intracellular staining targeting the inhibitory phosphorylated site (Try505) on LCK measured by flow cytometry (185). The activation of AHR decreased the percentage of inhibitory pLCK (Tyr505), thereby increasing the ratio of active LCK in human B cells (Fig. 3.2.10A and B). In addition, we quantified the percent of $ZAP70^{+}$ B cells following treatment with VH or TCDD. The total ZAP70 in human B cells did not change with TCDD treatment. The activation of AHR slightly increased intracellular ZAP70 only at the highest TCDD concentration (Fig. 3.2.10C).

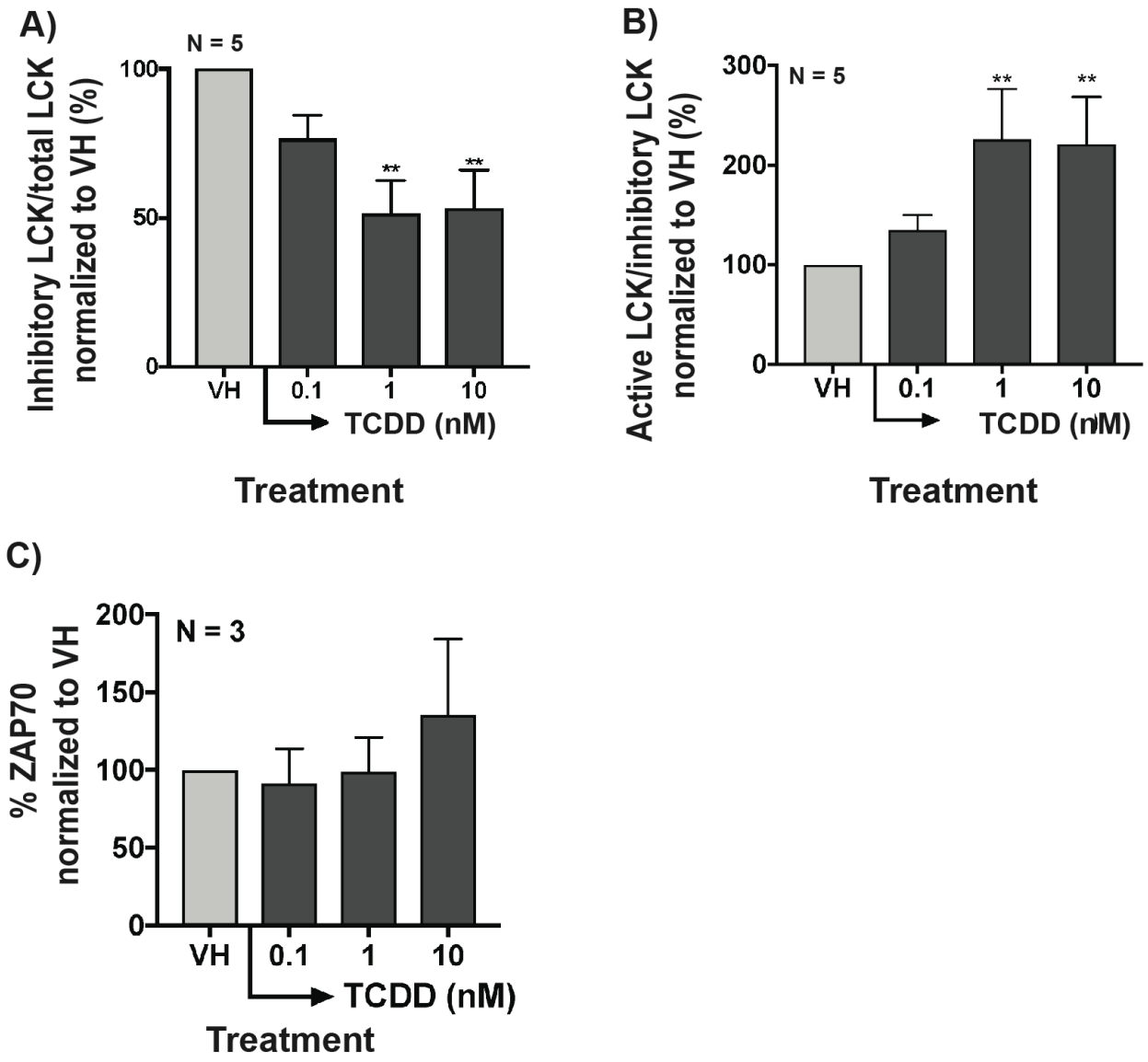


Figure 3.2.10. AHR activation increased the level of active LCK.

B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1 and 10 nM) and activated by soluble human recombinant CD40 ligand and cytokines (IL-2 and IL-21) for 7 days. **A)** Ratio of inhibitory pLCK (Tyr505) versus total LCK; and **B)** Ratio of active pLCK (Tyr505) versus inhibitory pLCK. The ratio was calculated based on the flow cytometry measurement of total LCK and pLCK (Tyr505) on each donor. **C)** Percent total ZAP70^{+} B cells measured by flow cytometry on day 7. N indicates the number of donors used in the

Figure 3.2.10. (cont'd)

study. Significant differences from VH control were indicated by ** $p < 0.01$ by one-way ANOVA following by Fisher's LSD post hoc test. Results are presented as the normalized percentage compared to the VH group for each donor.

LCK inhibitor did not change the phosphorylation of LCK.

The activation of AHR decreased the percentage of inhibitory pLCK (Tyr505), thereby increasing the ratio of active LCK in human B cells (Fig. 3.2.10A and B). Similar to previous observation, the percent positive of pLCK and the expression level of pLCK (Tyr505) decreased significantly with TCDD treatment (Fig. 3.2.11A and B), indicating that there was more active LCK with TCDD treatment in human B cells. However, the addition of high affinity LCK inhibitor (RK24466) did not alter the pLCK positive cells or the level of pLCK (Tyr505) (Fig. 3.2.11A and B);

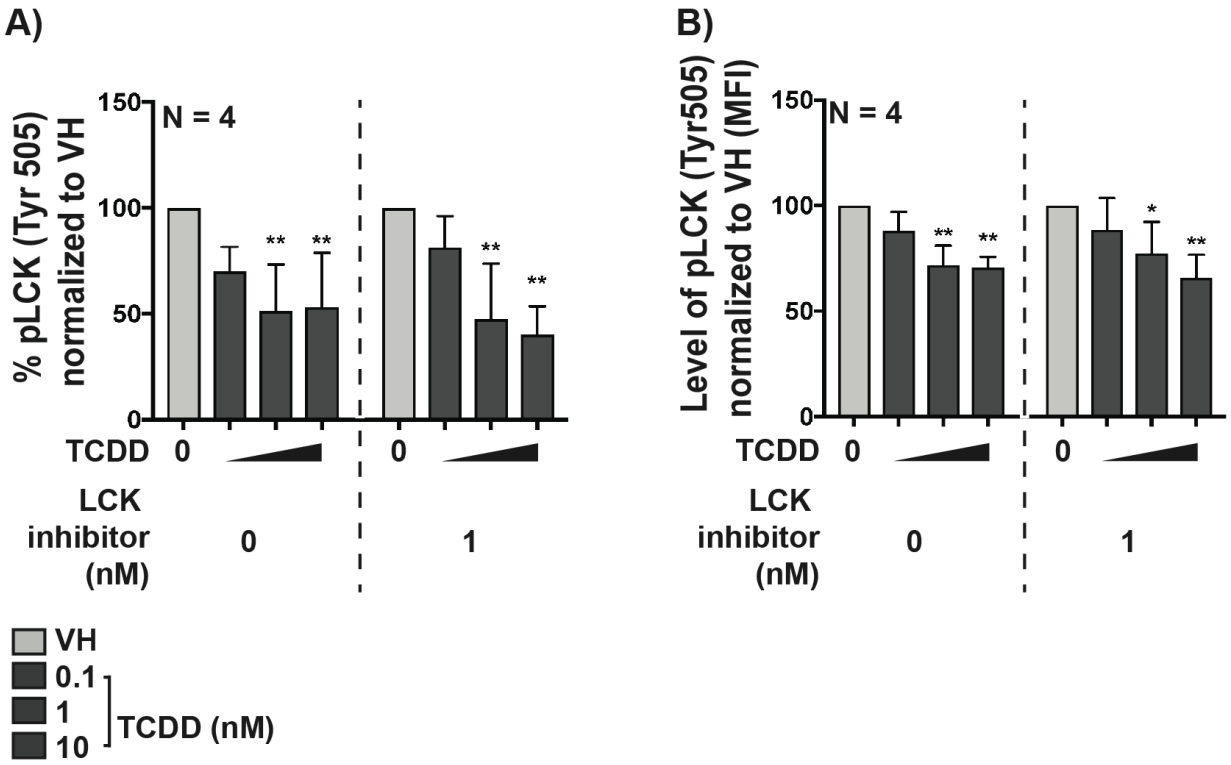


Figure 3.2.11. LCK inhibitor did not change the phosphorylation of LCK.

B cells were treated with VH (0.02% DMSO) or TCDD (10 nM) and activated by soluble human recombinant CD40 ligand and cytokines (IL-2 and IL-21) for 7 days. All samples were measured by flow cytometry. **A)** Percent pLCK-Tyr505 positive and **B)** Level of pLCK-Tyr505^{+} B cells with or without the addition of LCK inhibitor (RK24466). N indicates the number of donors used in the study. Significant differences from VH control were indicated by * $p < 0.05$ by one-way ANOVA following by Fisher's LSD post hoc test. Results are presented as the normalized percentage compared to the VH without RK24466 treatment.

LCK inhibitor attenuated downstream phosphorylation in pZAP70

As observed in Fig. 3.2.10, AHR activation increased the proportion of active LCK in human B cells. To further verify this finding, we measured the known downstream target of LCK, ZAP70. With TCDD treatment, the overall level and the percent positive of pZAP70 at both Tyr319 and Tyr292 increased, indicating that TCDD treatment upregulate the activity of LCK (Fig. 3.2.12A and B). The addition of LCK inhibitor (RK24466) attenuated the overall level of downstream phosphorylation of ZAP70 (Fig. 3.2.12C and D). Overall, the effects of TCDD and LCK inhibitor on the activity of LCK, as monitored by ZAP70 phosphorylation, was consistent with that predicted by the XNOR gate model (Fig. 3.2.9).

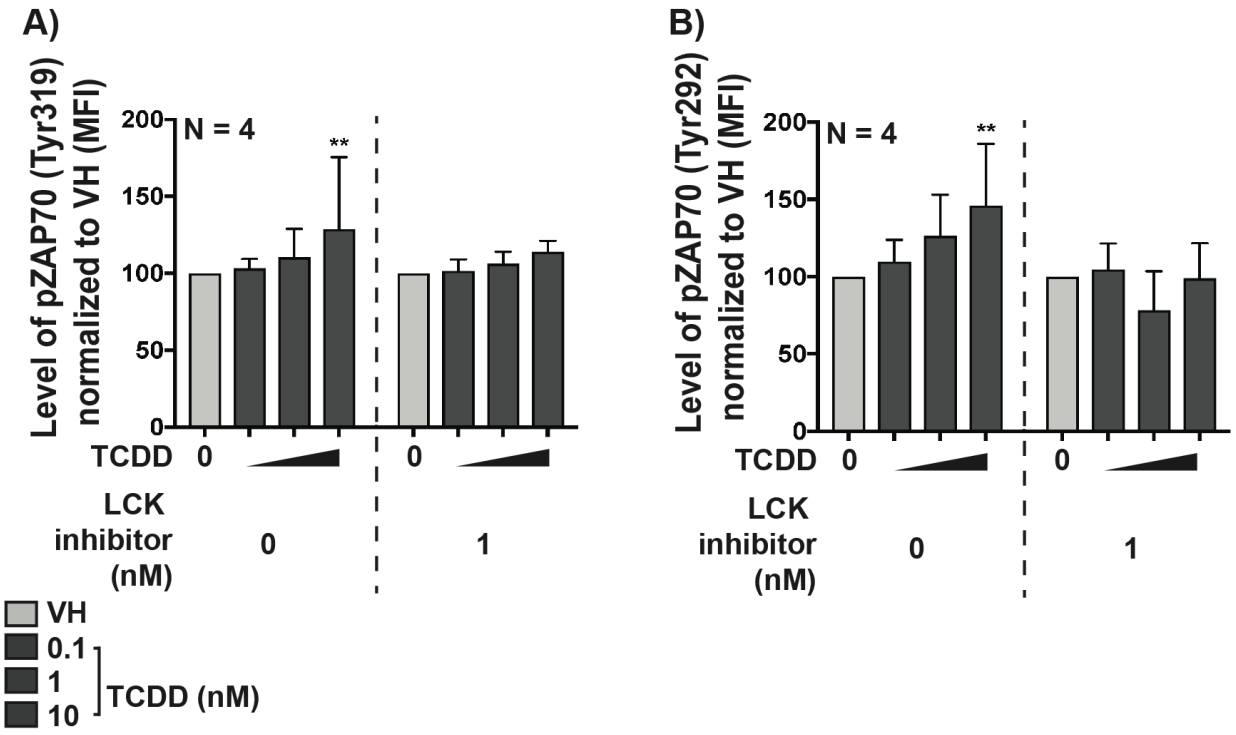


Figure 3.2.12. LCK inhibitor attenuated downstream phosphorylation in pZAP70.

B cells were treated with VH (0.02% DMSO) or TCDD (10 nM) and activated by soluble human recombinant CD40 ligand and cytokines (IL-2 and IL-21) for 7 days. All samples were measured via flow cytometry. **A)** Level of pZAP70-Tyr319; and **B)** Level of pZAP70-Tyr292 in B cells with or without the additional of LCK inhibitor. N indicates the number of donors used in the study. Significant differences from VH control were indicated by * $p < 0.05$ by one-way ANOVA following by Fisher's LSD post hoc test. Results are presented as the normalized percentage compared to the VH without RK24466 treatment in each human donor.

Comparison of total LCK expression levels in responsive and non-responsive donors.

Approximately one in seven human donors are “non-responders” to TCDD treatment as defined by the absence of TCDD-mediated suppression of IgM secretion by B cells from these human donors (26, 118). Specifically, responders were classified by a greater than 20% suppression of IgM in the presence of 10 nM TCDD treatment compared to VH control as measured by ELISA and ELIspot. The explanation for why an individual is non-responsive to TCDD treatment is still largely unknown. Interestingly, comparing the total LCK expression level between responsive and non-responsive human donors, with TCDD treatment (10nM), the level of total LCK significantly increased in responders (Fig. 3.2.13A, C and E) while not affected in non-responders (Fig. 3.2.13B, D and F).

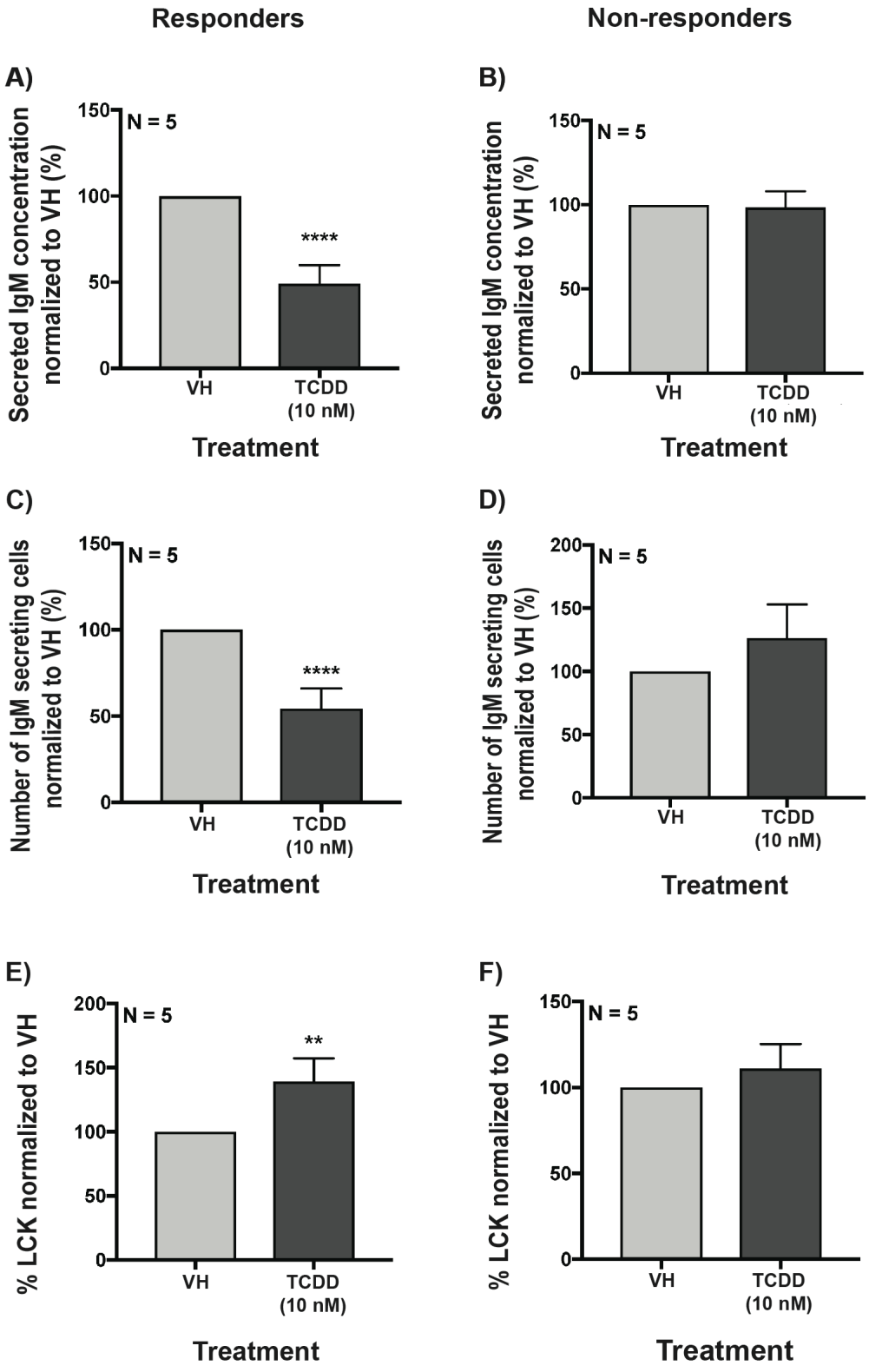


Figure 3.2.13. Comparison of total LCK expression levels in responsive and non-responsive donors.

Figure 3.2.13. (cont'd)

B cells were treated with VH (0.02% DMSO) or TCDD (10 nM) and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 7 days. The IgM concentration in culture supernatants in: **A)** responders; and **B)** non-responders. Number of IgM secreting B cells in: **C)** responders; and **D)** non-responders. Percent LCK positive human B cells in: **E)** responders; and **F)** non-responders. N indicates the number of donors used in the study. Significant differences from VH control were indicated by ** $p < 0.01$ and **** $p < 0.0001$ by Student's T-test. Results are presented as the normalized percentage compared to the VH control group.

Activation of AHR by TCDD did not affect cell division

B cells were treated with CellTrace™ Violet Cell Proliferation dye for 30 min followed by VH (0.02% DMSO) or TCDD (30 nM) treatment and activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 7 days. On day 7, cells were collected to quantify the CellTrace™ dye in individual B cells via flow cytometry. AHR activation did not change the overall division of B cell, evidenced by the same cell number within each division generation (Fig. 3.2.14A). Interestingly, the highly dividing cells express high percentage of LCK (Fig. 3.2.14B).

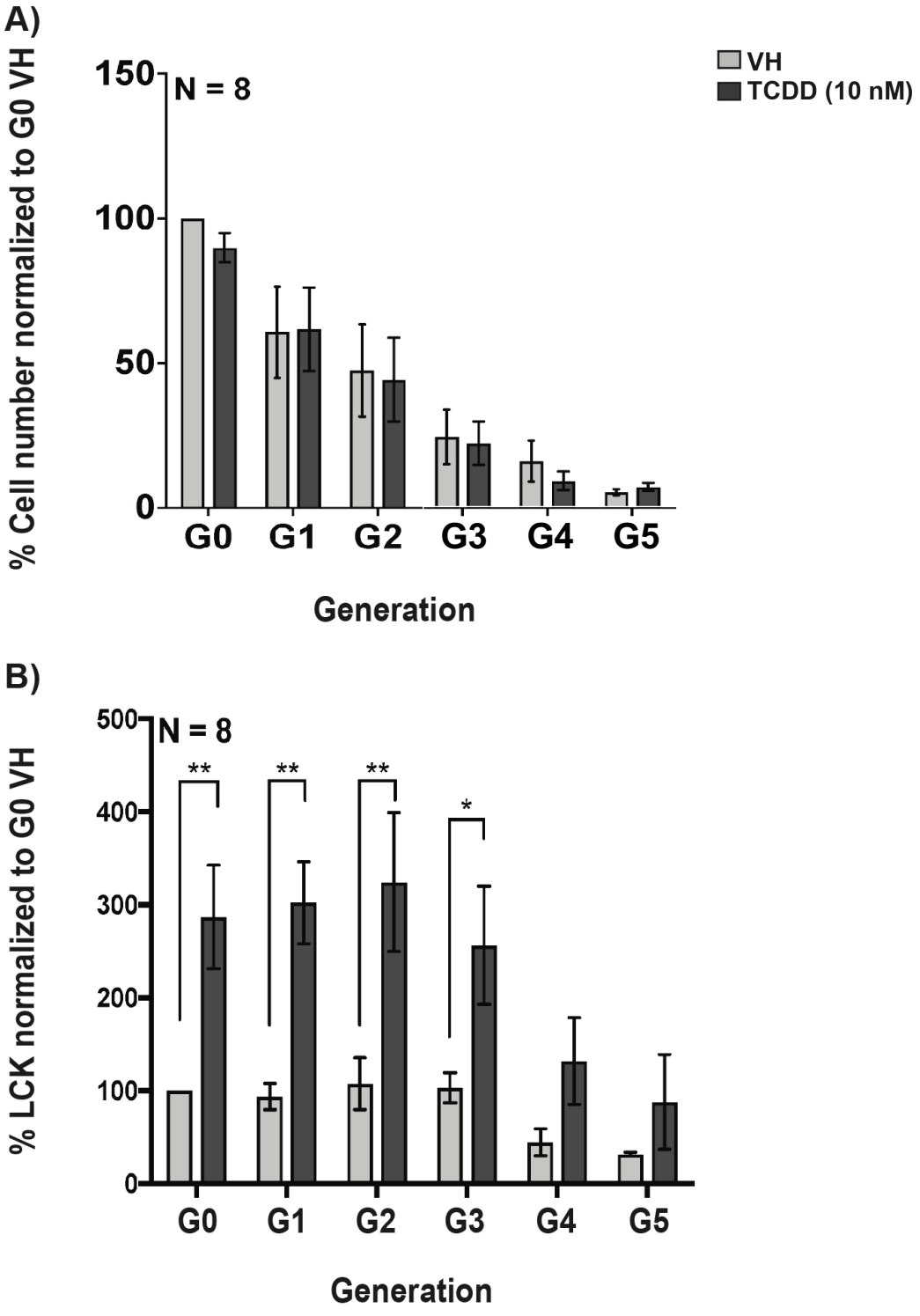


Figure 3.2.14. No change in cell division with AHR activation by TCDD.

B cells were treated with CellTrace™ Violet Cell Proliferation dye for 30 min followed by VH (0.02% DMSO) or TCDD (10 nM) treatment and activated by co-culture with CD40

Figure 3.2.14. (cont'd)

ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for 7 days. On day 7, cells were collected to quantify the CellTrace^{TM} dye in individual B cells via flow cytometry. **A)** Cell number; and **B)** Percent positive LCK in each cell division with VH or TCDD treatment in B cells. N indicates the number of donors used in the study. Significant differences from VH control were indicated by ** $p < 0.01$ by one-way ANOVA following by Fisher's LSD post hoc test. Results are presented as the normalized percentage compared to the VH group on G0.

The percentage of LCK^{+} cells did not change upon B cell activation.

Upon B cell activation, we did not observe LCK expression from day 0 to day 2 in our culture system. From day 3 to 7, LCK protein was detectable via flow cytometry in our system. Interestingly, after B cells activation, the overall expression of intercellular LCK did not change in human B cells (Fig. 3.2.15).

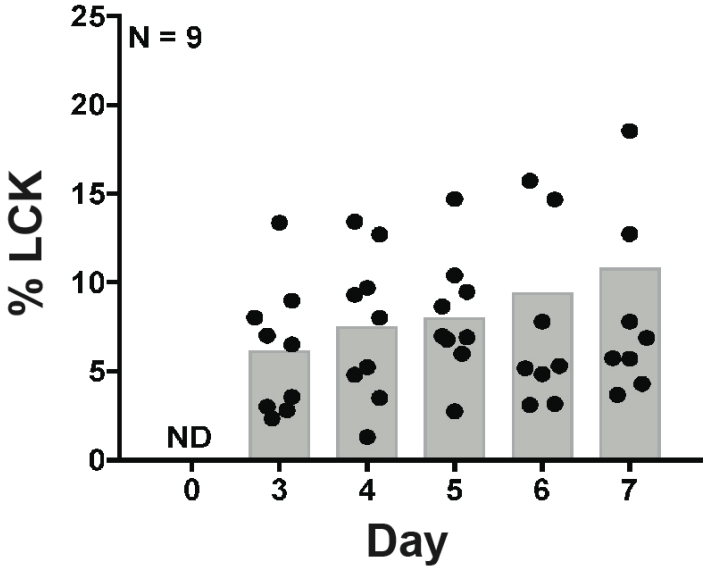


Figure 3.2.15. The percentage of LCK$^+$ cells from day 3 to 7 post human B cell activation.

B cells were activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6 and IL-10) for a total 7 days. Cells were collected from day 0 to day 7 to measure the level of total intracellular LCK protein expression. Day 1 and 2 were excluded from the graph due to undetectable levels of LCK in human B cells. Each dot indicated one human donor.

3.3. Ascertain the role of LCK in TCDD-mediated suppression of the IgM response in human CD5$^+$ innate-like B cells

A recent study reported high LCK expression in CD5$^+$ B cells in human (167). Our previous studies also demonstrated that AHR activation significantly upregulated total LCK in human B cell. A previous study by Till *et al*. demonstrated high LCK expression in human CD5$^+$ B cells in circulation (167). Therefore, studies are conducted to further investigate the role of LCK in CD5$^+$ ILBs. In order to understand the role of LCK in CD5$^+$ ILBs, a double magnetic enrichment was used as described in the Materials and Methods section. By using this strategy, we were able to achieve approximately 90% enrichment of CD5$^+$ ILBs (Fig. 3.3.1). In the third part of the thesis research, studies were designed to understand the role LCK and inhibitory receptor in CD5$^+$ ILBs.

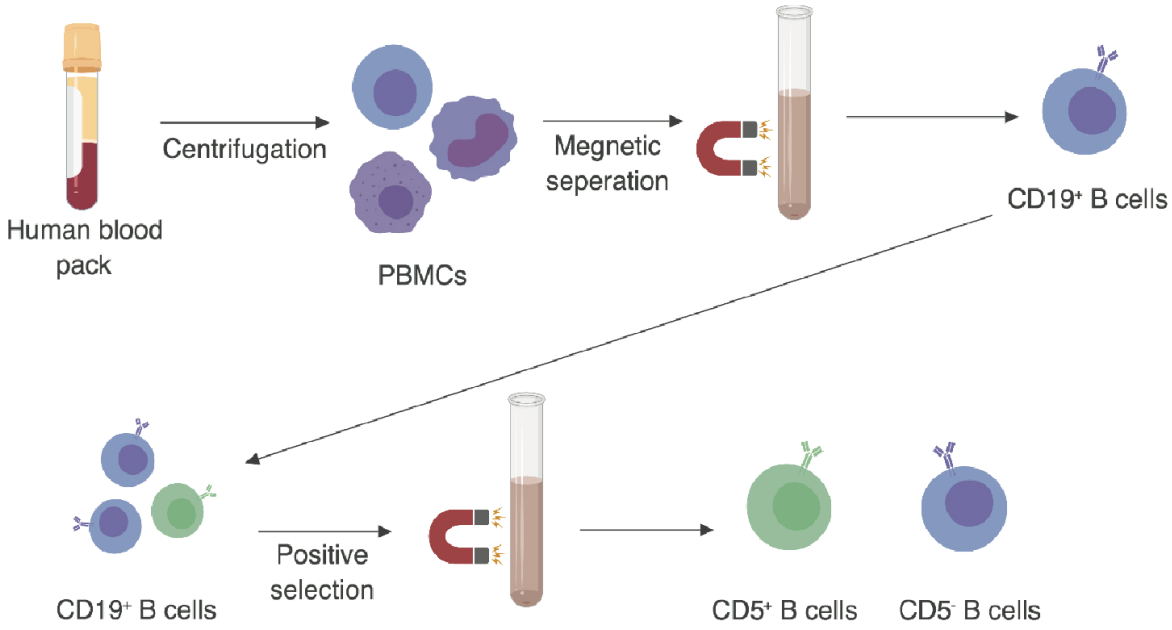


Figure 3.3.1. CD5$^+$ ILBs enrichment via magnetic separation.

Human PBMCs obtains from human blood pack were subjected to negative magnetic separation to obtain naïve human B cells. Naïve human B cells were then further enriched using positive magnetic selection to separate CD5$^+$ cells and CD5$^-$ cells. In brief, biotin anti-human CD5$^+$ antibodies were added to the CD19$^+$ B cells, followed by addition of anti-biotin microbeads. After antibody binding, CD5$^+$ B cells were eluded from the total B cell pool using MojoSort^{TM} system.

AHR-mediated increase of total LCK and suppression of the IgM responses in CD5$^+$ ILBs.

Previous studies showed that AHR activation significantly upregulated LCK in total human B cells. Furthermore, LCK inhibitor treatment reversed the AHR-mediated suppression of the IgM response in total B cells (188). Recent studies by Blevins *et al*. also showed that CD5$^+$ ILBs were extremely sensitive toward AHR activation as evidenced by the significant impairment of the IgM response (Manuscript under preparation). Therefore, in the current study, the role of LCK was investigated in CD5$^+$ ILBs. A significant correlation of CD5$^+$ cells and high LCK$^+$ cells was observed in total B cells (Fig. 3.3.2A). In order to further understand the role of LCK in CD5$^+$ ILBs, studies were conducted to measure the LCK expression in CD5$^+$ ILBs. Interestingly, after enrichment, the percent positive LCK was significantly higher in CD5$^+$ ILBs compared to CD5$^-$ B cells on day 0 (Fig. 3.3.2B and C). With AHR activation, an increase of total LCK protein was observed at all time points measured, with the most marked increase on both day 1 and 7 (Fig. 3.3.2D and E). In contrast, the percentage of LCK positive cells was not altered in CD5$^-$ B cells with AHR activation (Fig. 3.3.2E). In addition, CD5$^+$ ILBs exhibited marked IgM suppression which occurred concordantly with the increase in LCK (Fig. 3.3.2F – E). Conversely, CD5$^-$ B cells were refractory IgM suppression by AHR activation (Fig. 3.3.2F – E).

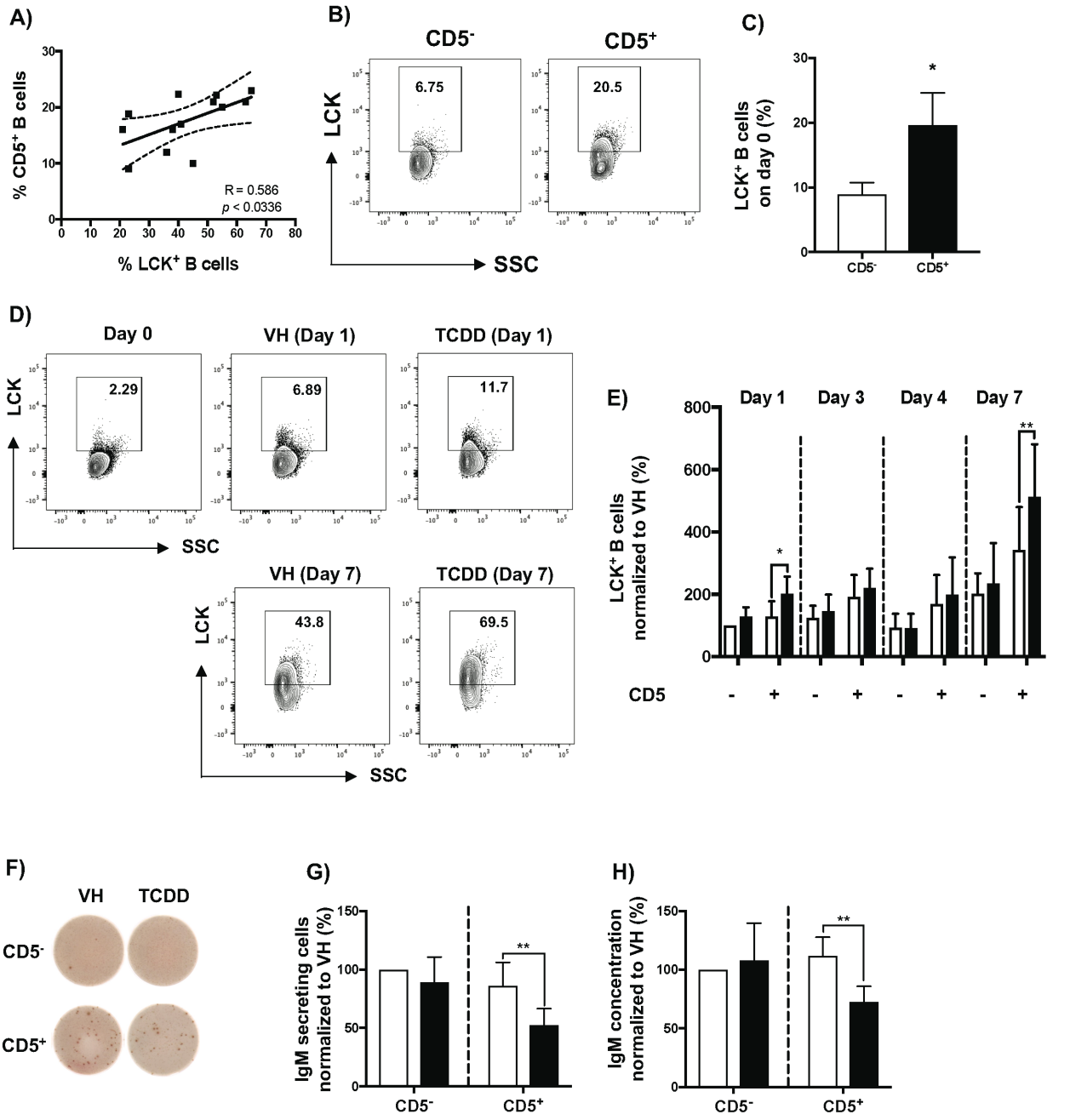


Figure 3.3.2. AHR-mediated increase of the percentage of LCK$^+$ cells and suppression of the IgM responses in CD5$^+$ B cells.

Human CD5$^{+/-}$ B cells were activated and treated with VH (0.02% DMSO), or TCDD (10 nM) on day 0 and cultured for 7 days. **A)** Correlation of percent CD5$^+$ B cells and percent LCK$^+$ B cells; **B)** Flow cytometry dot plot of CD5$^+$ and LCK$^+$ cells; **C)** Percent LCK$^+$ B cells

Figure 3.3.2. (cont'd)

within the $CD5^{+/-}$ populations on day 0; **D)** Flow cytometry dot plot of LCK^{+} cells within $CD5^{+/-}$ populations; **E)** Percent LCK^{+} B cells within $CD5^{+/-}$ populations on day 1, 3, 4 and 7 with VH or TCDD treatment; **F)** IgM secreting cells measured via ELIspot within $CD5^{+/-}$ populations with VH or TCDD (10 nM) treatment on day 7; **G)** Number of IgM secreting cells; and **H)** IgM concentration in $CD5^{+/-}$ populations with VH or TCDD treatment. Determinations were made using B cells from 6 human donors (N = 6). For E), data were normalized to $CD5^{-}$ VH on day 1. For G) and H), data were normalized to $CD5^{-}$ VH. Significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$ (Student's T test or two-way ANOVA following with Fisher's LSD post hoc test).

AHR antagonist treatment prevented the AHR-mediated upregulation of LCK and the suppression of the IgM response in CD5$^+$ ILBs.

To further confirm the role of the AHR in induction of LCK and suppression of the IgM response, the specific AHR antagonist (CH-223191) was employed to block the activation of AHR. Addition of AHR antagonist alone and the combination of AHR antagonist and TCDD treatment both caused a reduction in total LCK compared to the TCDD treatment group in CD5$^+$ ILBs (Fig. 3.3.3A – C). Furthermore, AHR antagonist treatment alone did not decrease the IgM response by CD5$^+$ ILBs when compared to the TCDD-treated group (Fig. 3.3.3D and E).

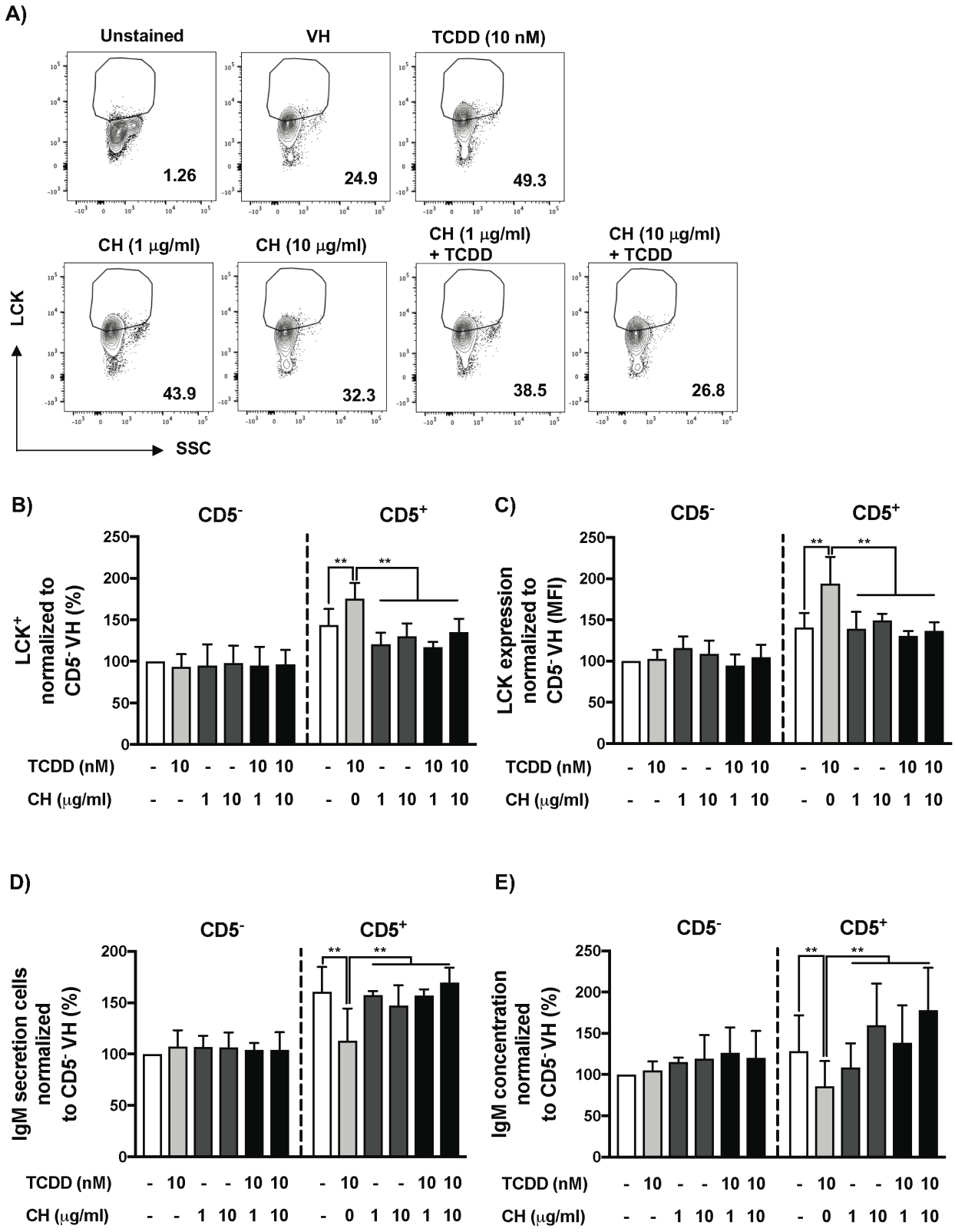


Figure 3.3.3. AHR antagonist treatment prevented the AHR-mediated upregulation of LCK and the suppression of the IgM response in CD5$^+$ ILBs.

Figure 3.3.3 (cont'd)

Human $CD5^{+/-}$ B cells were pre-treated with 1 or 10 μg/ml of AHR antagonist (CH-223191, abbreviated as CH, for 30 minutes and then followed by activation and treatment with VH (0.02% DMSO) or TCDD (10 nM) on day 0 and cultured for 7 days**. A)** Flow cytometry dot plot of $CD5^{+}$ ILBs with or without AHR antagonist treatment; **B)** Percentage of LCK^{+} cells; **C)** Mean florescence intensity (MFI) of LCK in $CD5^{+/-}$ populations; **D)** Number of IgM secreting cells; and **E)** IgM concentration within $CD5^{+/-}$ populations with VH or TCDD treatment. Determinations were made using B cells from 6 human donors (N = 6). Data were normalized to $CD5^{-}$ VH group. Significant differences are indicated by ** $p < 0.01$ (two-way ANOVA following with Fisher's LSD post hoc test).

LCK inhibitor reversed the AHR-mediated suppression of the IgM responses in CD5$^+$ ILBs.

LCK inhibitor (RK24466) was shown to have high binding affinity to LCK and could reverse the AHR-mediated suppression of IgM response (188, 189). Therefore, RK24466 was used as a probe in the current studies to further understand the role of LCK in CD5$^+$ ILBs. With LCK inhibitor (RK24466) treatment, AHR-mediated suppression of the IgM response was restored in CD5$^+$ ILBs but not in the CD5$^-$ B cells (Fig. 3.3.4A and B). Consistent with previous observations, the percentage of LCK positive cells continue to increase with AHR activation even in the presence of LCK inhibitor (Fig. 3.3.4C).

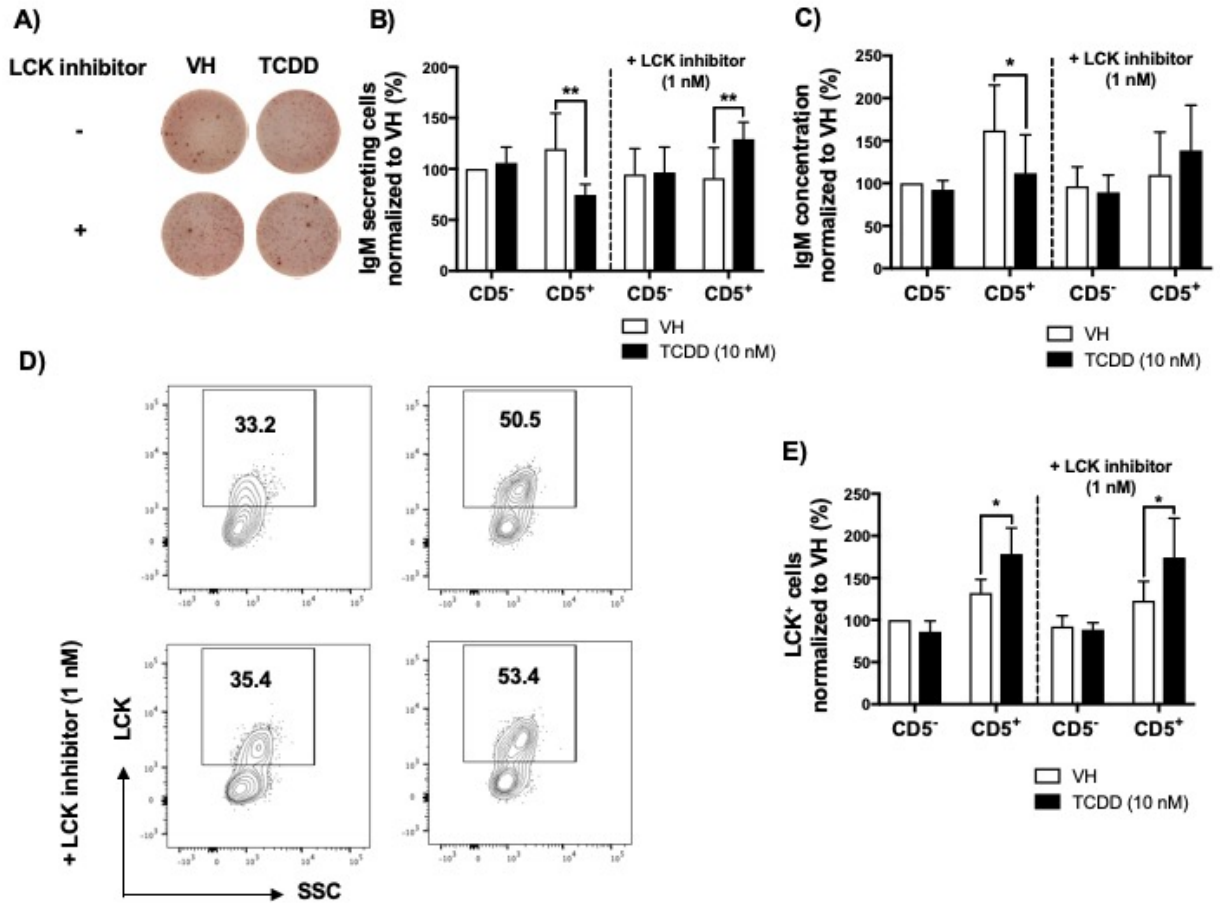


Figure 3.3.4. LCK inhibitor reversed the AHR-mediated suppression of the IgM responses in CD5$^+$ ILBs.

Human CD5$^{+/-}$ B cells were treated with VH (0.02% DMSO), or TCDD (10 nM) on day 0 and cultured for 7 days. LCK inhibitor (RK24466) was added on day 5 to the cell culture. **A)** IgM secreting cells measured via ELIspot within CD5$^{+/-}$ B populations with VH or TCDD (10 nM) and the addition of LCK inhibitor (1 nM) treatment on day 7; **B)** Number of IgM secreting cells; **C)** IgM concentration; and **D)** Percentage of LCK$^+$ cells within CD5$^{+/-}$ populations with or without LCK inhibitor treatment. Determinations were made using B cells from 6 human donors (N = 6). Data presented in the figure were normalized to CD5$^-$

Figure 3.3.4. (cont'd)

VH. Significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$ (two-way ANOVA following with Fisher's LSD post hoc test).

Treatments of soluble PD-1 ligands (sPD-L1 and sPD-L2) suppressed the IgM response in CD5$^+$ ILBs.

LCK has been shown to have at least two important roles in initiating signaling events in T cells: 1) upon engagement of the TCR (190); and 2) PD-1 signaling (191). Based on the previous studies, the level of LCK increased significantly with AHR activated in CD5$^+$ ILBs. Due to ILBs have limited diversity within the BCR and the expression of CD5, ILBs do not receive activating signals through the BCR. Hence, it is unlikely that LCK is involved in BCR signaling in CD5$^+$ B cells. In addition, previous studies have indicated that CD5$^+$ ILBs expressed higher basal levels of PD-1 and its ligands compared to CD5$^-$ B cells (Manuscript under preparation). Therefore, the involvement of LCK in PD-1 signaling in CD5$^+$ ILBs was investigated in the current studies. Soluble PD-1 ligands (sPDL1 and sPDL2) were used to activate PD-1 in CD5$^+$ ILBs. The ligand concentrations used in the current studies were determined to provide equivalent binding affinity to PD-1 (sPDL1: 1 μg/mL and sPDL2: 50 ng/mL). Interestingly, ligand treatment resulted in significant suppression of the IgM response in CD5$^+$ ILBs (Fig. 3.3.5A and B). However, greater suppression was observed with PDL2 treatment or the combination of both ligands compared to PDL1 treatment alone (Fig. 3.3.5A and B). In addition, PD-1 ligand treatment did not change the percent positive LCK in CD5$^{+/-}$ B cells (Fig. 3.3.5C). Similar to previous observations, CD5$^-$ B cells were refractory to treatment with PD-1 ligands (Fig. 3.3.5A – C).

LCK inhibitor reversed the PD1-mediated suppression of the IgM response in CD5$^+$ ILBs.

Previous studies have demonstrated that LCK could phosphorylate the ITSM domain on PD-1, which in turn activate PD-1 for the docking of SHP-1 or SHP-2 (191). Therefore, to further investigate the involvement of LCK in PD-1 signaling in the context of CD5$^+$ ILBs, LCK inhibitor (RK24466) (1 nM) was added in addition to sPDL2 (50 ng/mL) treated CD5$^{+/-}$ B cells. PDL2 was selected based on the previous observation that sPDL2 treatment had greater suppression of IgM response in CD5$^+$ ILBs compared to sPDL1 (Fig. 7). In this study, LCK inhibitor was supplied to the cells on day 0 (D0) or day 5 (D5) post activation. Treatment of LCK inhibitor significantly reversed the PD-1 mediated suppression of the IgM response in only CD5$^+$ ILBs (Fig. 3.3.5D and E).

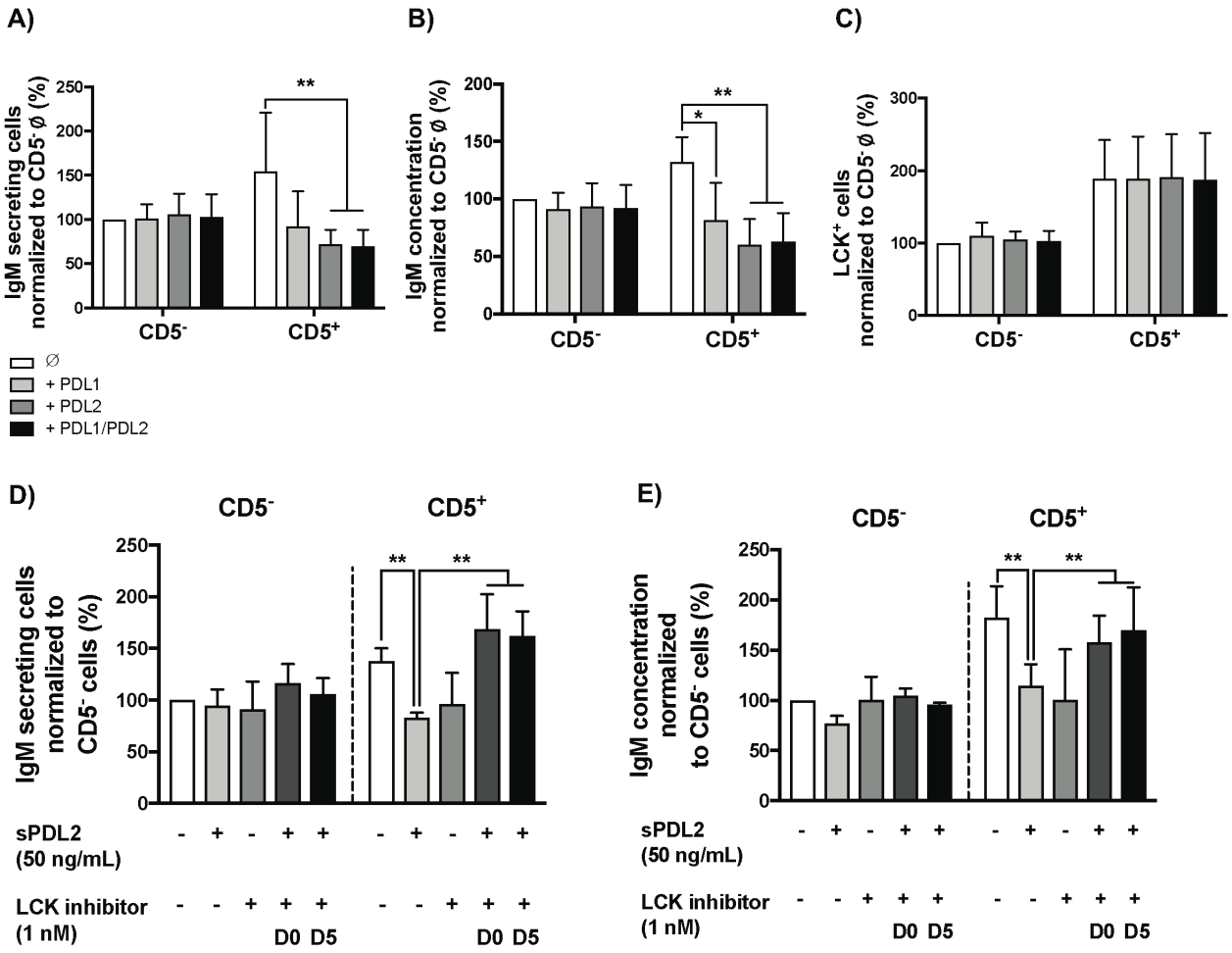


Figure 3.3.5. Treatment with soluble PD-1 ligands (PDL1 and PDL2) suppressed the IgM response and LCK inhibitor reversed the PD1-mediated suppression of the IgM response in CD5$^+$ ILBs.

Human CD5$^{+/-}$ B cells were treated with soluble PDL1, PDL2 or the combination of both on day 0 and cultured for 7 days. LCK inhibitor (RK24466) was added on day 0 or day 5 to cell culture. **A)** Number of IgM secreting cells; **B)** IgM concentration; **C)** Percentage of LCK$^+$ cells within CD5$^{+/-}$ populations on day 7; **D)** Number of IgM secreting cells; and **E)** IgM concentration within CD5$^{+/-}$ populations with PDL2 and LCK inhibitor treatments. Determinations were made using B cells from 6 human donors (N = 6). For A – C data presented in the figure were normalized to CD5$^-$ B cell without PD-1 ligand treatment.

Figure 3.3.5. (cont'd)

For D and E, data were normalized to $CD5^-$ B cells without PDL2 or LCK inhibitor treatment. Significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$ (two-way ANOVA following with Fisher's LSD post hoc test).

Treatment with PDL2 did not further suppress the IgM response than AHR activated alone in CD5^{+} ILBs.

In order to further understand whether there was a synergistic effect between PD-1 and AHR activations, treatment with soluble PDL2 and TCDD were administrated to CD5$^{+/-}$ B cells and the IgM response was measured on day 7. The combination of PDL2 and TCDD treatment did not suppress the IgM response further compared to the TCDD only group in CD5^{+} ILBs (Fig. 3.3.6A and B). Similar to previous observations, CD5^{-} B cells were refractory to AHR or PD-1 activation (Fig. 3.3.6A and B).

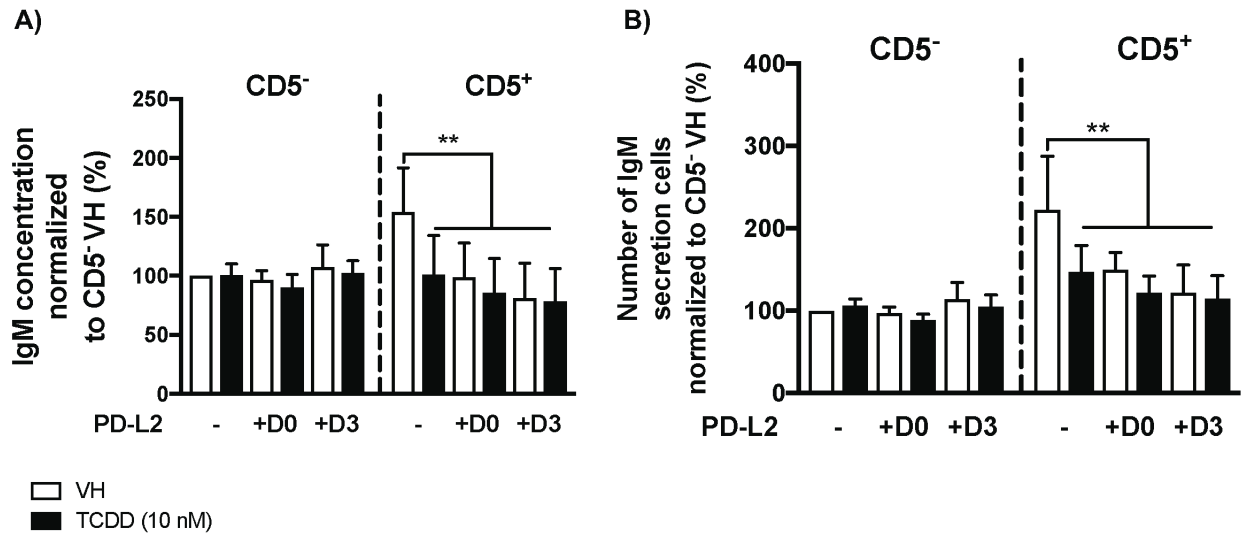


Figure 3.3.6. Treatment with PDL2 did not further suppress the IgM response than the AHR activated alone in CD5$^+$ ILBs.

Human CD5$^{+/-}$ B cells were treated with VH (0.02% DMSO), or TCDD (10 nM) on day 0. In addition, cells were treated with PDL2 on day 0 or day 3 and cultured for 7 days. **A)** Number of IgM secreting cells; and **B)** IgM concentration within CD5$^{+/-}$ populations on day 7. Determinations were made using B cells from 6 human donors (N = 6). Data presented in the figure were normalized to CD5$^-$ B cell without PDL2 treatment. Significant differences are indicated by ** $p < 0.01$ (two-way ANOVA following with Fisher's LSD post hoc test).

PD-1 blocking antibody prevented the AHR-mediated suppression of the IgM response in CD5$^+$ ILBs.

In the current studies, we have observed upregulation of PD-1 with AHR activation and PD-1 activation significantly suppressed IgM response in CD5$^+$ ILBs. Therefore, we hypothesized that increase of PD-1 plays a critical in the AHR-mediated suppression of IgM response in CD5$^+$ ILBs. To test this hypothesis, we used a PD-1 blocking antibody (10 ng/mL, S228P), commonly known as nivoumab, to block the PD-1 signal in CD5$^+$ ILBs. In order to obtain maximum blockage of PD-1, the blocking antibodies were added on day 3, therefore, cells could upregulate PD-1 during day 1 to 2 post activation. With PD-1 blocking antibody treatment, the AHR-mediated suppression of the IgM response was abrogated in CD5$^+$ ILBs (Fig. 3.3.7A – C). Similar to previous observations, CD5$^-$ B cells did not respond to either TCDD or anti-PD-1 treatment (Fig. 3.3.7).

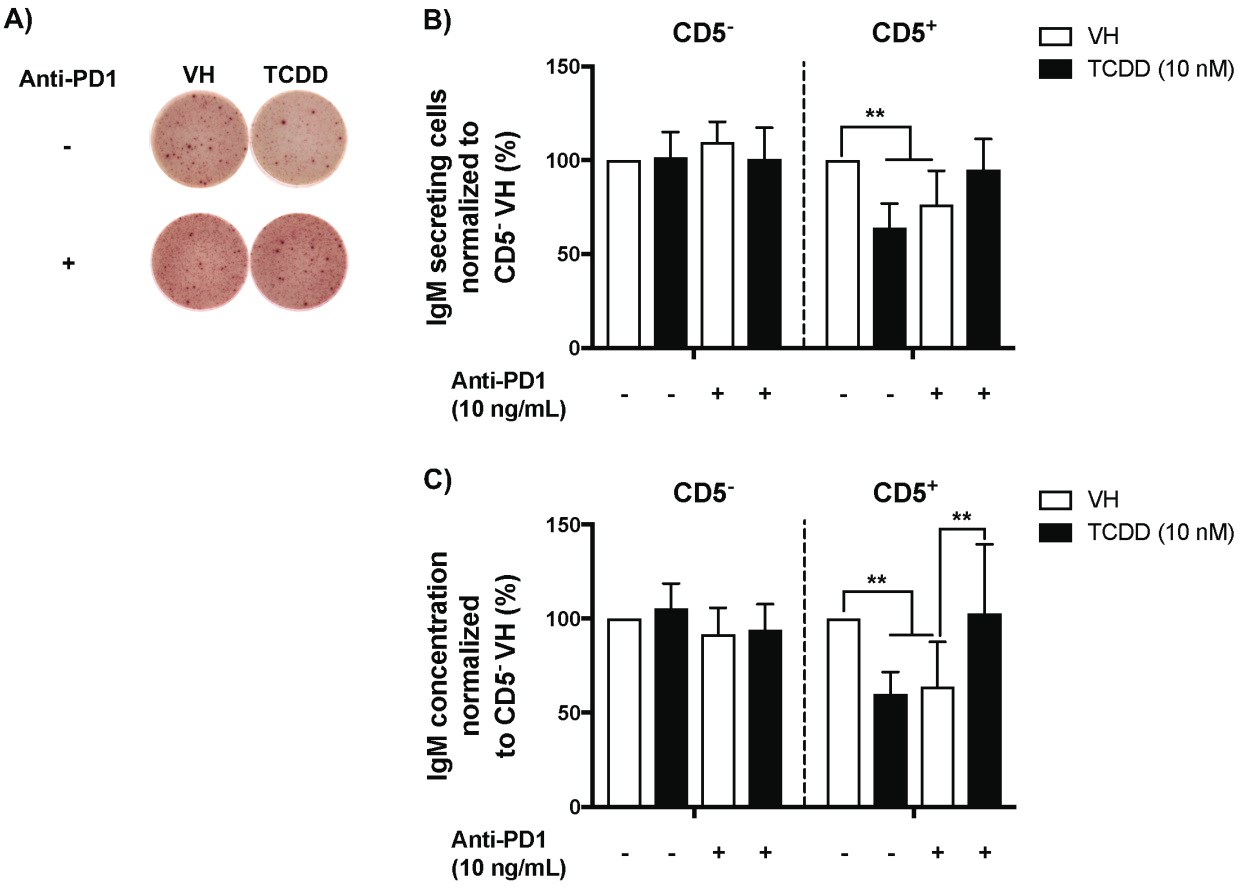


Figure 3.3.7. PD-1 blocking antibody prevented the AHR mediated suppression of the IgM response in CD5$^+$ ILBs

Anti-PD-1 blocking antibody (S228P) was added to CD5$^{+/-}$ B cells after activation and treatment with VH (0.02% DMSO) or TCDD (10 nM) and cultured for 7 days. **A)** IgM secreting cells measured via ELIspot within CD5$^+$ population with or without anti-PD-1 treatment; **B)** Number of IgM secreting cells; and **C)** IgM concentration within CD5$^{+/-}$ populations. Determinations were made using B cells from 7 human donors (N = 7). Data presented in the figure were normalized to CD5$^-$ VH without anti-PD-1 treatment. Significant differences are indicated by ** $p < 0.01$ (two-way ANOVA following with Fisher's LSD post hoc test).

IFNγ reversed the AHR-mediated suppression of the IgM response by reducing the total LCK in CD5$^+$ ILBs.

Our previous studies have shown that an optimum level of LCK has to be achieved in order to have optimum IgM production (188). Additionally, Blevins *et al*. has demonstrated that IFNγ treatment reversed the AHR-mediated IgM suppression in human B cells via modulation of STAT3 (Manuscript under preparation). Therefore, in the current studies, we also employed IFNγ as a molecular probe to further understand the role of AHR activation in CD5$^+$ ILBs. First and foremost, a matrix study with increasing concentrations of both IFNγ and TCDD was conducted to investigate the interplay between LCK and IFNγ. Interestingly, with increase concentration of IFNγ, the percentage of LCK positive cells decreased in a concentration-dependent manor, with significant suppression from 0.1 to 10 U/mL of IFNγ (Fig. 3.3.8A). AHR activation continued to increase total LCK, however, the increase was remarkably lower with IFNγ treatment compared to non-treated B cells (Fig. 3.3.8A). IFNγ treatment also reversed the AHR-mediated IgM response in total B cells (Fig. 3.3.8B). Previous studies have shown that CD5$^+$ ILBs were particularly sensitive toward AHR activation, evidenced by the significant suppression of the IgM response (Fig. 3.3.2G and H). Therefore, the level of IFNγ receptors were assessed in both CD5$^+$ and CD5$^-$ B cell population. Interestingly, the level of IFNγ receptor chains (IFNγR1 and IFNγR2) were significantly higher in the CD5$^+$ ILBs compared to CD5$^-$ B cells (Fig. 3.3.8B and C). The high expression level of IFNγ receptors on CD5$^+$ ILBs suggests that this subset of B cells might elicit higher response compared to CD5$^-$ B cells when treated with IFNγ. Therefore, treatments with TCDD (10 nM) in combination with IFNγ (1U/mL) were used to determine the role of LCK in CD5$^+$ ILBs.

Similar to the observation in total B cells (Manuscript under review), IFNγ treatment also abrogated AHR-mediate IgM suppression in $CD5^+$ ILBs (Fig. 3.3.8H and I). The protein level of LCK significantly decreased with IFNγ treatment in $CD5^+$ ILBs (Fig. 3.3.8F and G), similarly to the observation in total B cells (Fig. 3.3.8A). However, IFNγ treatment did not have any effect on the level of LCK and IgM response in $CD5^-$ B cells (Fig. 3.3.8).

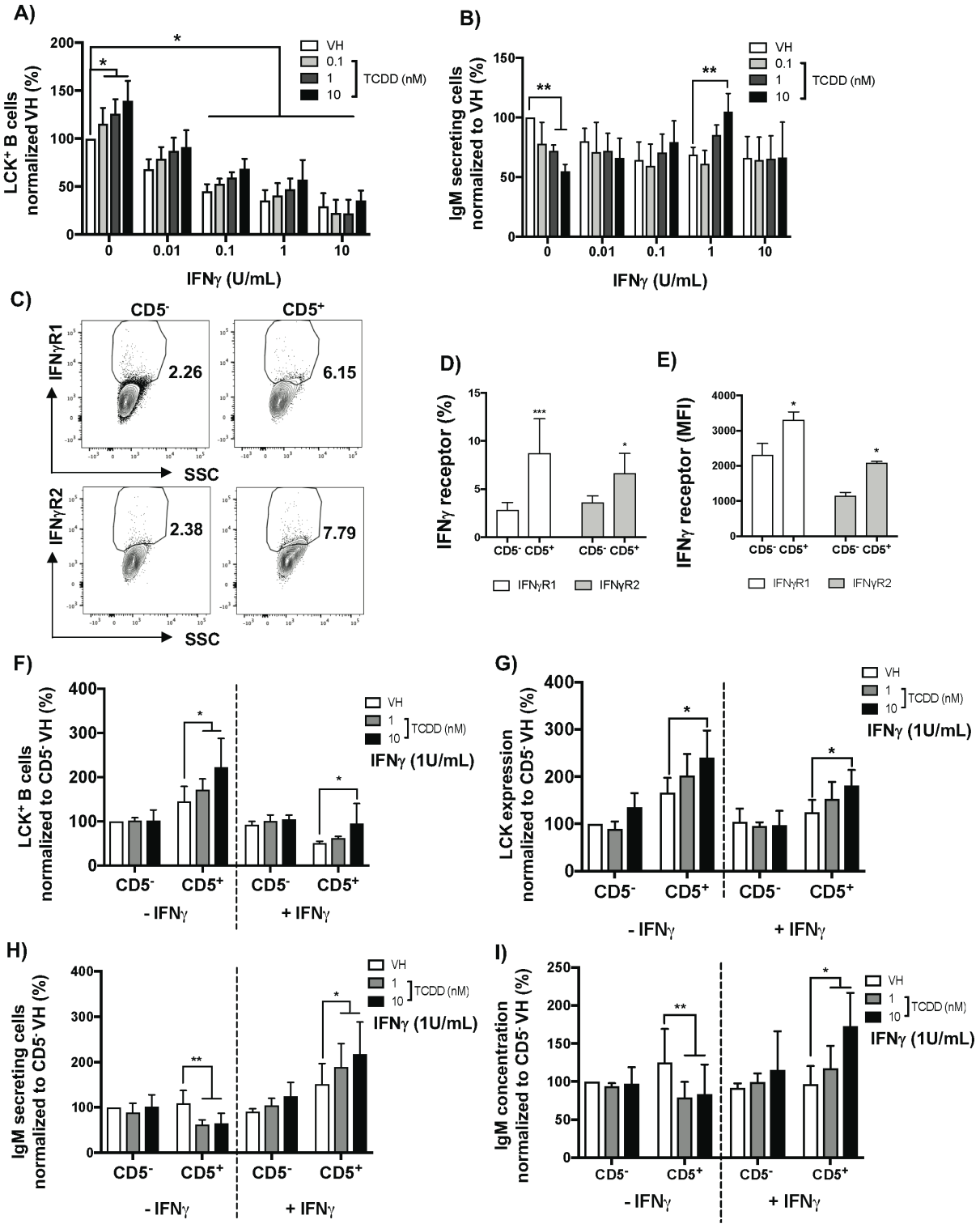


Figure 3.3.8. IFNγ treatment reversed the AHR-mediated suppression of the IgM response through a decrease of LCK in CD5⁺ ILBs

Figure 3.3.8. (cont'd)

Human $CD5^{+/-}$ B cells were activated/treated with soluble PDL1 or PDL2 on day 0 and cultured for 7 days. In addition, IFNγ treatment (1 U/mL) was provided on day 0. **A)** Percentage of LCK^{+} cells; **B)** Number of IgM secreting cells in $CD19^{+}$ B cells; **C)** Flow cytometry dot plot of $IFN\gamma R1^{+}$ and $IFN\gamma R2^{+}$ cells; **D)** Percent $IFN\gamma R1^{+}$ and $IFN\gamma R2^{+}$; **E)** Mean MFI of IFNγR1 and IFNγR2 within $CD5^{+/-}$ populations; **F)** Percentage of LCK^{+} cells; **G)** MFI of LCK within $CD5^{+/-}$ populations treated with IFNγ; **H)** Number of IgM secreting cells; and **I)** IgM concentration within $CD5^{+/-}$ populations treated with IFNγ. Determinations were made using B cells from 6 human donors (N = 6). For A and B, results presented in the figure were normalized to VH group without IFNγ treatment. For F to G, results presented in the figure were normalized to $CD5^{-}$ VH without IFNγ treatment. Significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$ (two-way ANOVA following with Fisher's LSD post hoc test).

IFNγ treatment reversed the PD1-mediated suppression of the IgM response in CD5$^+$ ILBs.

In the current studies, IFNγ treatment reversed the AHR-mediated IgM suppression through the reduction in LCK in CD5$^+$ ILBs (Fig. 7). In addition, previous studies have shown that IFNγ signaling regulated the expression of PD-1 and PDL1 (192, 193). Therefore, to further understand the role of PD-1 signaling in CD5$^+$ ILBs, 1 U/mL of IFNγ was used to treat CD5$^+$ ILBs in the presence of sPDL1 (1 μg/mL) or sPDL2 (50 ng/mL). IFNγ treatment restored the PD-1 mediated IgM suppression in CD5$^+$ ILBs (Fig. 3.3.9A – D). IFNγ treatment reversed the PD-1-mediated IgM suppression in CD5$^+$ ILBs (Fig. 3.3.9A - D). IFNγ treatment only enhanced the percent positive PD-1 cells, but TCDD-mediated AHR activation with IFNγ treatment did not change the percentage of PD-1 and PDL1 positive cells in CD5$^+$ ILB population (Fig. 3.3.9E - F). Interestingly, IFNγ treatment decrease the percent positive PDL2 cells in the presence of TCDD. Also, the decrease of PDL2 was still higher compared to the VH group without IFNγ treatment (Fig. 3.3.9G).

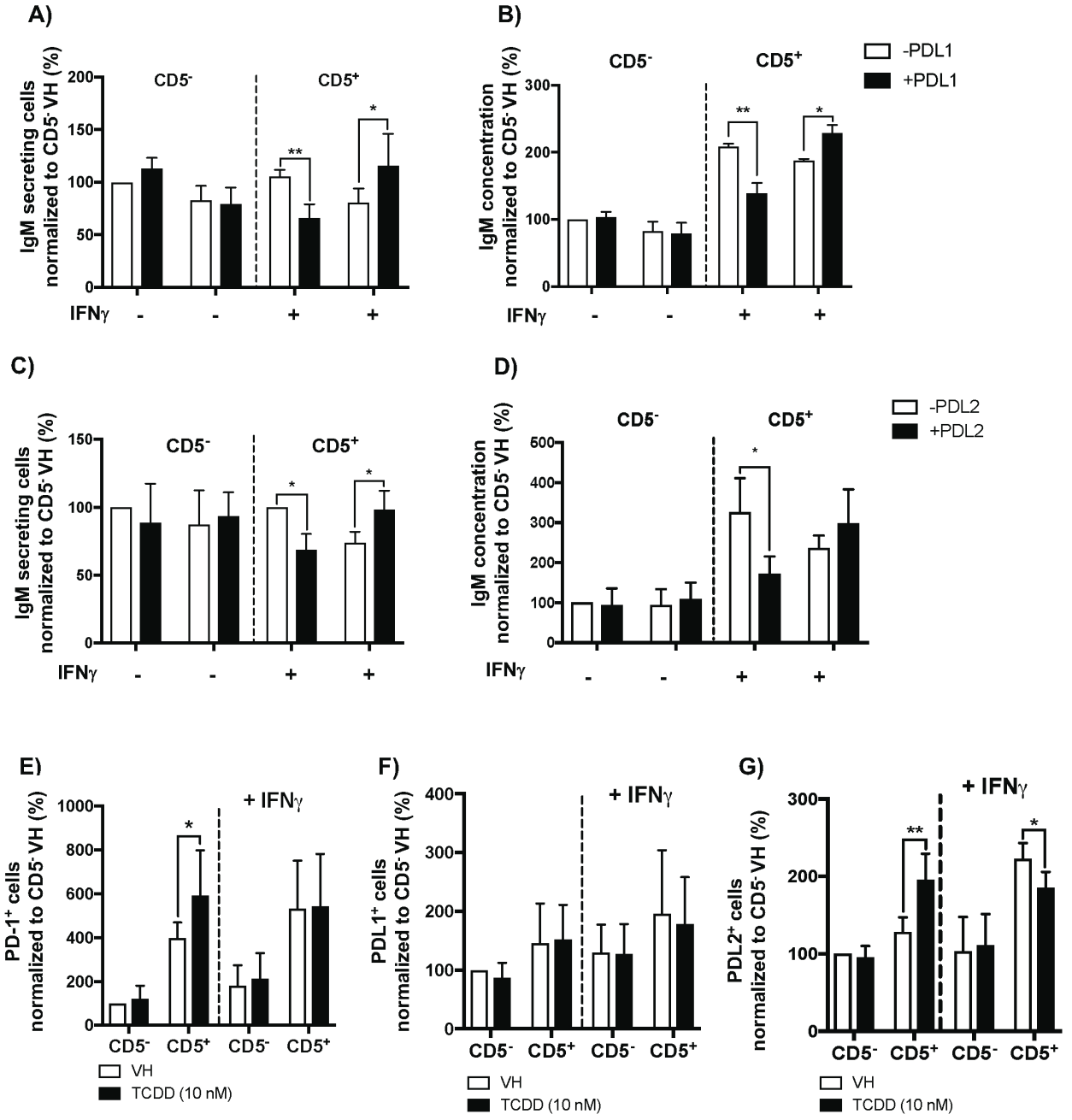


Figure 3.3.9. IFNγ treatment reversed the PD-1-mediated suppression of the IgM response in CD5$^+$ ILBs.

Human CD5$^{+/-}$ B cells were activated, treated with soluble PDL1 or PDL2 on day 0 and cultured for 7 days. In addition, IFNγ treatment (1 U/mL) was provided on day 0. **A)** Number of IgM secreting cells and; **B)** IgM concentration with soluble PDL1 treatment

Figure 3.3.9. (cont'd)

within $CD5^{+/-}$ populations in the presence of IFNγ treatment; **C)** Number of IgM secreting cells and; **D)** IgM concentration with soluble PDL2 treatment within $CD5^{+/-}$ populations in the presence of IFNγ treatment; **E)** Percentage of PD-1^{+} cells; **F)** Percentage of $PDL1^{+}$ cells; and **G)** Percentage of $PDL2^{+}$ in $CD5^{+/-}$ populations with or without IFNγ treatment. Determinations were made using B cells from 6 human donors (N = 6). For A – D, data were normalized to $CD5^{-}$ B cells without PD-1 ligand treatment. For E – G, data were normalized to the VH control group in $CD5^{-}$ B cells. Significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$ (two-way ANOVA following with Fisher's LSD post hoc test).

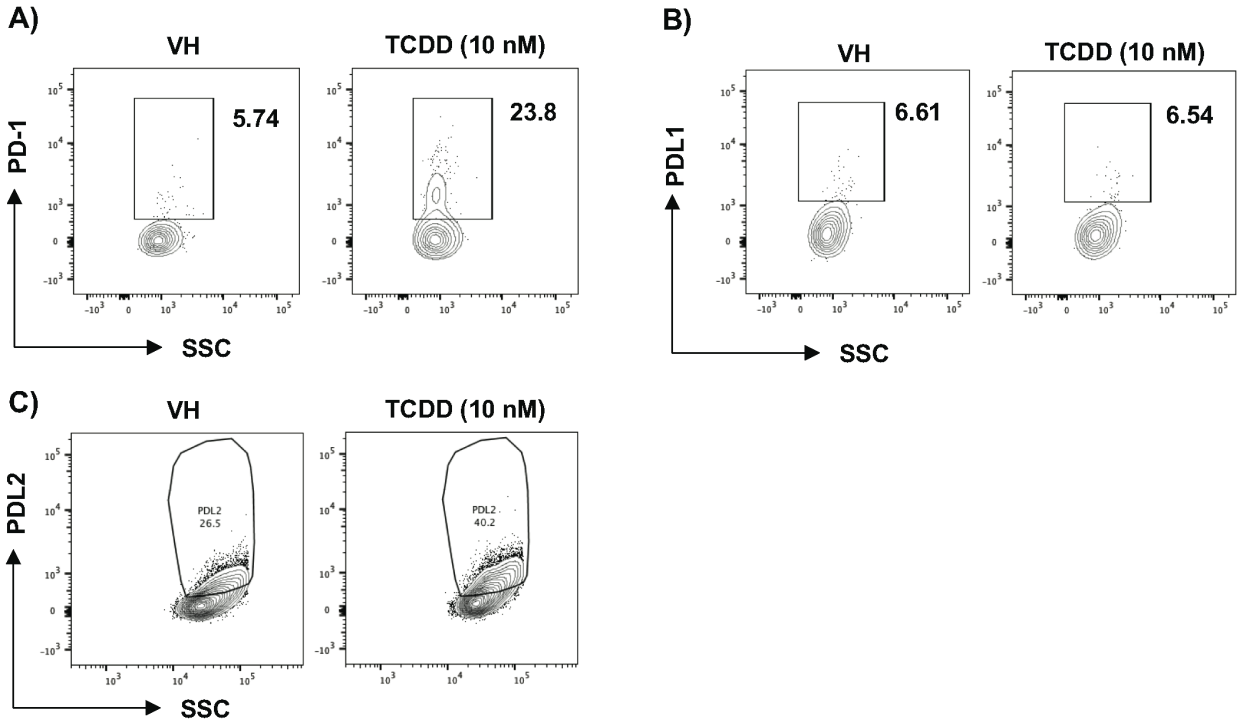


Figure 3.3.10. Flow cytometric dot plot for PD-1, PDL1 and PDL2.

Human $CD5^{+/-}$ B cells were treated with VH (0.02% DMSO) or TCDD (10 nM) on day 0 and cultured for 7 days. Flow cytometry dot plot of A) PD-1, B) PDL1 and C) PDL2. The gates were drawn based on the day 0 sample.

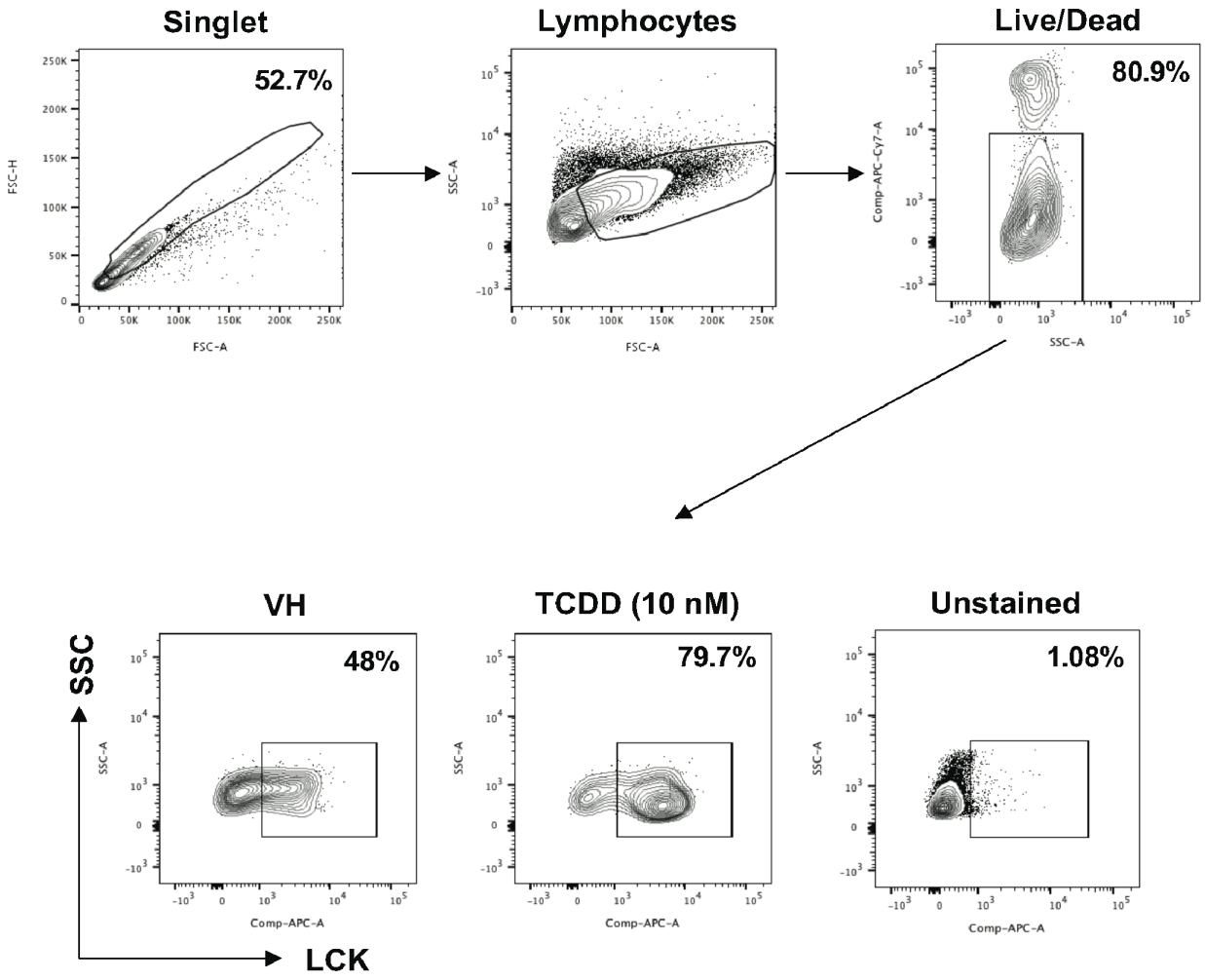


Figure 3.3.11. Flow cytometry gating scheme.

The gating scheme used to identify $LCK^{+}CD5^{+}$ by flow cytometry. The percent positive gate for LCK on day 7 was identified based on the unstained control and the same gate was overlaid to the other experimental groups and overlaid to treatment groups.

CHAPTER 4: DISCUSSION

The main objective of this dissertation is to elucidate the mechanism by which TCDD impairs the IgM response in human B cells. Toward this end, firstly, a side-by-side comparison of AHR-mediated suppression of the IgM response in mouse and human B cells is discussed in section 4.1. Secondly, the role of LCK in the IgM response is discussed in the context of AHR activation in section 4.2. Finally, the role of LCK and inhibitory receptor, PD-1 in the AHR-mediated suppression of the IgM response in $CD5^+$ ILBs is discussed in section 4.3.

4.1. Comparison of AHR-mediated suppression of the IgM response in primary mouse and human B cells.

AHR-mediated suppression of the antibody responses has been observed in many laboratory animal species (2, 194). Investigations using animal models, especially mouse models, have been particularly useful to elucidate the molecular mechanism for the impairment of humoral immunity in response to AHR activation by TCDD (101). Previous studies in mice have shown that mouse B cells were particularly sensitive toward AHR activation by TCDD, as evidenced by the significant suppression of the IgM response linked to a decrease in mRNA and protein levels for the immunoglobulin chains (IgH, IgJ and Igκ chains) (181). Studies using the CH12.LX mouse cell line further verified the findings and provide more mechanistic insights into the role of AHR activation by TCDD on IgM suppression in mice (95). Furthermore, AHR null mice did not exhibit a decrease in the antibody response with TCDD treatment, indicating a requisite role of AHR activation in the suppression of the antibody response in mouse B cells (195). Previous

studies have also demonstrated that AHR activation impaired the IgM response by human B cells to different stimuli including: PWM; co-stimulation by CD40L with cytokines; and toxic shock syndrome toxin (TSST) superantigen (26, 62, 64, 114). The TCDD-mediated activation of AHR also impaired suppression of the critical B cell activation markers, CD69, CD80 and CD86, and signaling proteins, thereby, down-regulating antibody production (26). One of our working hypotheses has been that the molecular mechanisms responsible for immune suppression by AHR ligands, including suppression of the IgM antibody response, would be similar across animal species. Therefore, the first aim of this dissertation research was focused on comparing AHR-mediated IgM suppression between mouse and human primary B cells.

In aim 1 of the dissertation research, we compared the IgM response, mRNA and protein expression of IgM in both mouse and human B cells. First and foremost, we observed significant suppression of the IgM response with TCDD treatment in mouse B cells (Fig.3.1.1). Furthermore, we observed an overall decrease of immunoglobulin mRNA and protein (IgH, IgJ and Igκ) following TCDD treatment in mouse B cells, as determined by qRT-PCR, intracellular staining and Western blotting (Fig. 3.1.3 and 3.1.5A and 3.1.6A – C). This finding is consistent with prior reports of decreased mRNA levels for *IgH*, *IgJ* and *Igκ*, resulting from an impairment of B cells to develop into antibody-secreting plasma cell through changes in the regulation of paired box 5 (Pax5) and B lymphocyte-induced maturation protein-1 (Blimp-1) (95, 101, 196). These observations further suggest that TCDD treatment impairs all processes, from antibody synthesis to cell differentiation, in mouse B cells. Additionally, these observations in mouse B cells show that the activation of AHR by TCDD impairs the key transcriptional and translational

regulators in immunoglobulin production. The decrease of Pax5 and Blimp-1 also indicate that the differentiation of mouse B cells was interrupted by TCDD treatment. Therefore, we conclude that TCDD treatment impairs IgM production in mice. By contrast, TCDD-mediated AHR activation in human B cells did not alter the transcription and translation (Fig. 3.1.4, 3.1.5 and 3.1.6D – F), which is also consistent with an absence in altered Pax5 or Blimp-1 regulation in human B cells (26). Moreover, the accumulation of IgM pentamers with TCDD treatment suggested that TCDD-mediated activation of AHR does not impair the synthesis and assembly of IgM pentamers, trimers and dimers in human B cells (Fig. 3.1.7). The assembly of IgM dimers, trimers and pentamers occurs between the ER and Golgi (197), further suggesting that it is possible the trafficking of IgM proteins is likely being affected after Golgi-associated processes are completed. However, current technologies are limited to be able to measure the IgM secretion in human B cells. This is partly due to the high glycosylation of IgM molecules in human. An alternative explanation for the differences we observed in mouse and human B cells with AHR activation is that the locations which we obtain B cells from are different from these two species. Therefore, the heterogenicity of the human B cell populations could also contribute to the differences we observed in the first part of the dissertation research. Nonetheless, the results from the first aim suggested a divergence in the mechanism by which AHR activation suppresses the IgM response in human and mouse primary B cells.

Transcriptomic analysis investigating the effect of AHR activation in PWM-activated mouse and human primary B cells revealed remarkably few common differentially expressed genes across the two species, which provided the first insights suggesting a divergence in mechanism by which AHR activation influences human and

mouse B cell function (120). An alternative possibility that can explain our results is that TCDD treatment is only suppressing a small subset of human B cells, which would decrease the detection sensitivity of any analysis when the entire peripheral blood B cell pool is evaluated. Although results from aim 1 of the dissertation research suggest that AHR activation impairs immunoglobulin secretion, it was unclear which specific processes within the immunoglobulin secretory pathway might be affected. Studies by Anelli *et al*. have shown that the upregulation of critical secretory genes and the enlargement of the ER and Golgi are critical in the secretion of immunoglobulins by human B cells (198). B cells then undergo structural modification in preparation to become an antibody secreting cell (198). This is also supported by the transcriptomic study, since the most commonly downregulated pathways were cytoskeletal pathways after TCDD treatment in human B cells (120). ER associated proteins and secretory signal cascades, all of which have the potential of decreasing immunoglobulin secretion, which is supported by the observation in the Native PAGE (Fig. 3.1.7) (197). Likewise, activation of AHR may alter immunoglobulin trafficking within human B cells, therefore leading to diminution of antibody transport and secretion. Therefore, it was tempted to speculate that AHR activation by TCDD alter critical trafficking proteins for immunoglobulin secretion in human B cells. In these initial studies, not only did we observe a decrease in secreted IgM but also IgG antibodies by human B cells with AHR activation suggesting the effects are not limited to the IgM isotype (Fig. 3.1.8). Importantly, the pathway(s) of immunoglobulin secretion remain poorly characterized.

One particular interesting phenomenon that we have consistently observed when studying AHR-mediated suppression of the IgM responses in human B cells is that even

when high concentrations of TCDD (>10 nM) are used to treat B cells, the magnitude of suppression rarely exceeds 50% of the vehicle control response, regardless of whether it is quantified by ELIspot or ELISA. The ELIspot results, which enumerate the number of IgM secreting cells, further suggested the possibility a sub-population of B cells is refractory to suppression by TCDD. One explanation for the lack of suppression in a subset of B cells, although unlikely, is that TCDD does not distribute homogenously to all of the B cells in culture, thus resulting in AHR activation in only a fraction of the cultured B cells. A second possible explanation is that an insufficient number of AHRs are activated in some of the cells, hence, impairment of IgM secretory processes only occurs in a subset of B cells. Although plausible and difficult to test, until the molecular targets, responsible for impaired IgM secretion have been elucidated, this hypothesis is worthy of further study. A third possibility, and what we believe may be the most plausible explanation, is that only a specific subpopulation of human B cells is, in fact, sensitive to suppression by high affinity AHR ligands such as TCDD. This would explain why close to a 100% suppression of the IgM response cannot be attained unless cytotoxic concentrations of an AHR ligands are used to treat B cells. It is well known that there are different sub-populations of B cells (B1a, B1b and B2) in mice. Similarly, humans also have different sub-populations of B cells; however, they are less well characterized compared to those in mice.

Indeed, in addition to “classical” B cells, the best characterized B cell subset has been termed innate-like B cells. These innate-like B cells are the major source of natural IgM antibodies and they play a critical role in early immune responses (127). Innate-like B cells can produce a large amount of circulating IgM following activation through Toll-

like receptors (TLRs) or CD40L activation to provide critical early defense against infections (153, 199). This newly generated circulating IgM plays an important role in neutralizing pathogens and enhancing complement activation to protect the host prior to the generation of an adaptive immune response (136). It is tempting to speculate that AHR activation by TCDD is affecting IgM production in innate-like B cells since they are characterized by producing IgM. The possibility that a subset of B cells is more sensitive toward TCDD treatment would be explored in the third part of this dissertation research.

Another interesting phenomenon which we historically observed is that approximately one in seven donors lack the sensitivity to IgM suppression by TCDD (2). We have previously classified these donors as “non-responders”. This differential sensitivity across human donors would further support the possibility that AHR activation, or at least, TCDD targets a specific sub-population of B cells, for example, non-responders may possess a very low number of B cells that are responsive to TCDD treatment. This possibility was, in fact, explored in the third part of this dissertation research.

Our current studies also demonstrate that the AHR is directly involved with the impairment of IgM response by TCDD-treated human B cells as evidenced by pretreatment of B cells with an AHR antagonist restored IgM secretion. Interestingly, we have also observed increased IgM secretion in B cells treated only with AHR antagonist in the absence of treatment with an AHR ligand. These data suggest the AHR plays a role in the regulation of the antibody response through its activation by one or more endogenous ligands. A similar phenomenon has also been observed in AHR-null rats, which exhibited an increase in secreted IgM compared to wild type rats (43). Furthermore,

recent study has also shown that AHR plays a critical role in B cell development from hematopoietic stem cells (14).

Collectively, results from aim 1 indicate divergent mechanisms governing impaired B cell function by AHR activation in mouse and human primary B cells. In mouse B cells, AHR activation impairs transcription of immunoglobulin IgH, IgJ and Igκ chains and is therefore the likely cause for the decrease of synthesis and production of IgM. By contrast, in total human B cells, AHR activation has no effect on transcription, translation or IgM assembly. Due to this observation, studies were conducted to further investigate the mechanism in AHR-mediated suppression of the IgM response by human B cell in aim 2. Nonetheless, the findings presented in the first part of this dissertation are the first to show key differences in AHR-mediated suppression of IgM responses between human and mouse primary B cells.

4.2. The role of LCK in the AHR-mediated suppression of IgM secretion by human B cells

Recent transcriptomic analysis by our laboratory revealed a significant increase in *LCK* mRNA in human B cells after AHR activation but not in mouse or rat B cells (120). This observation is particularly interesting to us, since the data from aim one indicated that human B cells have distinct mechanisms in the suppression of the IgM response by AHR activation. This observation served as the basis to further investigate the role of LCK in AHR-mediated impairment of humoral immunity.

In the second aim, we demonstrate a significant increase of LCK mRNA and protein that coincide with AHR activation in B cells. This finding has been confirmed using different B cell activators (Fig. 3.2.4). The increase of total LCK with TCDD-mediated AHR activation using various B cell stimuli suggests that AHR ligation is critical to further increase LCK compared to control and does not appear to be dependent on the mode of B cell activation. In addition, we have observed little LCK expression in naïve resting human B cells (Fig. 3.2.1); however, the level of LCK increases upon B cell activation. This observation is particularly interesting since LCK is mainly associated with T cell activation. In the absence of activation, T cells express high level of LCK and the level of LCK is only modestly increased upon T cell activation (200). Also interesting is the observation that the increase of LCK in B cells has little effect on proliferation (Fig.3.2.12). These observations differ from the known role of LCK in T cells. Specifically, expression of LCK is critical for T cell proliferation and activation with LCK null mice exhibiting thymic atrophy (201). Likewise, knockout LCK T cell lines show altered proliferation after activation when compared to the wild-type control expressing LCK T cells (202). The late

expression of LCK post activation in human B cells suggests a different role for the protein tyrosine kinase than in T cells. In addition, the modest increase of LCK when TCDD was added to cultures 24 h post B cell activation suggests that LCK may not be regulated directly by the AHR at the level of transcription (i.e., via dioxin response elements [DREs] binding in LCK regulatory regions) but rather through indirect mechanisms. Interestingly, we also observe a decrease in LCK by treatment of B cells with an AHR antagonist alone, suggesting that endogenous AHR ligand(s) also regulates the expression level of LCK in human B cells, but again likely through an indirect mechanism. This conclusion is further supported by the observation that LCK upregulation by TCDD, when added at day 0, is most marked on day 3 and continues to increase over the 7-day culture period (Fig. 3.2.1). Another possible explanation for this observation is that a small subset of human B cells expresses LCK, therefore, it is impossible to identify those cells within the total B cell pool without activation. Collectively, the results from the aim 2 studies suggest an important role for LCK in the IgM response by human B cells.

LCK is a well characterized kinase with T cell receptor (TCR) signaling. Both LCK and LYN belong to the Src kinase family, LYN has been well studied in B cells, as it closely associates with the B cell receptor (BCR) (203). Phosphorylation of LYN is crucial for B cell activation (204). Therefore, a specific pathway, involving LYN and SYK phosphorylation has been well characterized in B cells. ZAP70 is known to act similarly as SYK in T cells. It is clear that there is a redundancy between the LYN-SYK pathway and the LCK-ZAP70 pathway (205); but each pathway is only associated to one particular cell type; LYN-SYK pathway with B cell and LCK-ZAP70 pathway with T cell. However, there are few studies describing a role for LCK-ZAP70 in B cells. Based on the

transcriptomic study, the significant increase of LCK appears to occur independently of LYN or any Src kinase proteins (LYN, SRC, BLK and HCK) (120). In addition, previous studies have shown no changes in the expression level of PAX-5 and BLIMP-1 in TCDD-treated human B cells, suggesting that activation of the AHR does not alter B cell differentiation but rather the secretion of immunoglobulin by human B cells (26, 63). Alternatively, TCDD is exerting effects on a subpopulation of B cells and we lack the sensitivity to detect changes in PAX-5, BLIMP-1 or intracellular IgM in those cells when they are present in the heterogenous pool of $CD19^{+}$ B cells. Previous studies indicated that TCDD treatment attenuate the phosphorylation of pERK, which could potentially also alternate the phosphorylation of LCK, due to the crosstalk between ERK and LCK (26, 206). In addition, TCDD treatment also increases the expression level of SHP1, which could dephosphorylate LCK in human B cells (122). This scenario could also explain the small changes in phosphorylation in signaling proteins, due to the low sensitivity when dealing with mix populations.

An interesting observation from the studies in aim 2 was that LCK inhibitors restore the AHR-mediated decrease in IgM secretion (Fig. 3.2.5 and 3.2.6). This observation indicates the activity of LCK as a kinase plays a critical role in the IgM response in human B cells. Interestingly, treatment with LCK inhibitors alone impaired the level of IgM secretion, indicating that there is an optimal level of LCK activity required for immunoglobulin secretion. The different level of LCK activity has also been reported in human T cells (207). However, the studies in aim 2 are the first to report the non-monotonic response of LCK in human B cells. Upon observing this phenomenon, we have further investigated the inter-play between LCK and IgM secretion by treating the cells

with increasing concentrations of LCK inhibitor and TCDD. Using this matrix system, we have observed a restoration of the IgM response with increasing concentrations of LCK inhibitor (Fig. 3.2.8). This observation sparked a collaboration with Dr. Qiang Zhang from Emory University, led to the development of a XNOR logic gate model. In this model, suppression of the IgM response was mediated in the presence of either TCDD or the LCK inhibitors. However, in the presence of both TCDD and the LCK inhibitors, no IgM suppression was observed. The simulation from the XNOR model successfully recapitulated the experimental observation (Fig. 3.2.9). Taking into consideration of the observations from both experimental and computational models, the activity of LCK and IgM secretion exhibit a non-monotonic response, indicating that, in fact, an optimal level of LCK activity is required for the IgM response. Similar biphasic response can also be observed in the expression of PHD2 to tumor formation and SFRP ato Wnt signaling (208, 209). This observation is particularly interesting, since there are no publications to our knowledge reporting on the involvement of LCK in immunoglobulin secretion. Since LCK is a tyrosine kinase, it is likely that it has multiple downstream targets. Studies have shown that LCK can phosphorylate PD-1, a checkpoint inhibitor, to activate inhibitory signaling in order to dampen immune responses. Therefore, in the third aim of this dissertation research, a mechanism between LCK and PD-1 was investigated.

The activity of LCK is governed by phosphorylation of critical tyrosine residues located at the SH-2 domain at the C terminal of LCK (185). Since we observed an increase in total LCK levels with AHR activation, it is also important to identify the LCK phosphorylation profile. In the present study, we investigated the phosphorylation of tyrosine 505 (Tyr505), the dominant inhibitory site for LCK (185). Our studies show that

AHR activation decreased the level of phospho-Tyr505 LCK, which is consistent with an overall increase of active LCK (Fig. 3.2.10A and 3.2.11). The downstream increase of phosphorylated ZAP70 (Tyr319 and Tyr292) further confirms the increase of LCK activity (Fig. 3.2.12). In addition, the change in LCK phosphorylation status further shows that AHR activation is modulating the phosphorylation events in human B cells. This observation is in agreement with previous findings indicating that AHR activation changes the phosphorylation of AKT and ERK (26). The change of phosphorylation status by AHR activation could have detrimental effects on B cell activation or immunoglobulin production/secretion, since changes in phosphorylation could eventually impact downstream targets. However, the pathways presented in the current studies only offer a limited snapshot of a few proteins. A more global analysis using phosphoproteomics will be required to provide additional insights on the influence of AHR activation in mediating changes in phosphorylation of critical target proteins in human B cells transitioning from a resting status to antibody producing cells.

As mentioned earlier, it has been well documented that B cells from approximately 1 out of 7 human donors were refractory to suppression of IgM secretion by TCDD-mediated AHR activation (26, 118). In our studies, the responses of AHR activation in a particular individual was defined by whether suppression of the IgM response is being observed with AHR activation. In the last part of the study, we compared the total amount of LCK in both responders and non-responders and observed significant upregulation of LCK in only responsive human donors (Fig. 3.2.13). This observation further suggesting a role of LCK in the impairment of immunoglobulin secretion. This observation is particularly interesting, since chronic lymphocytic leukemia (CLL) patients have shown to

have high level of LCK, which has been used as a biomarker to measure the progression of disease (167). In addition, epidemiological studies on TCDD that exposure indicate a TCDD exposure could lead to the development of NHL, which includes CLL. With this observation, it is likely that the AHR-mediated increase of LCK maybe as an early marker for the development of CLL (NHL) in humans. With continued upregulation of LCK, individuals exposed to TCDD likely develop lymphoma. Taken together, it is tempting to speculate that LCK could be used as a biomarker to assess sensitivity of a given individual to TCDD and dioxin-like environmental contaminants.

Recently, studies have shown that human peripheral $CD5^+$ B cells expressed high levels of LCK as evidenced in chronic lymphocytic leukemia patients (167). $CD5^+$ B cells are characterized as innate-like B cells in both mice and humans. Not only do $CD5^+$ B cell express high levels of LCK, these cells are also a major producer of IgM in circulation (128). $CD5^+$ B cells although well defined in the mouse, are poorly characterized in the human, however, they are known to express CD5 as a surface marker. The high level of LCK expression in $CD5^+$ human B cells leads us to speculate that LCK might play a role in the functionality of $CD5^+$ B cells. Previous data have also shown that the AHR-mediated suppression of the IgM response cannot exceed 50% compared to the control group in human B cells. Likely, AHR activation by TCDD preferentially suppress the IgM response in certain populations of B cells within the total B cell pool. With the high level of LCK in $CD5^+$ B cells, it exists a strong possibility that $CD5^+$ B cells are preferentially affected by TCDD treatment. Therefore, in aim 3 of this dissertation research, studies were preformed to understand the role of LCK in $CD5^+$ B cells.

4.3. Ascertain the role of LCK in TCDD-mediated suppression of IgM response in human CD5$^+$ innate-like B cells

In aim 2, our data showed that AHR activation increases total LCK and impairs the IgM response in human B cells. The upregulation of total LCK with AHR activation is an interesting observation, since LCK is not commonly associated with B cell activation and differentiation. Instead, LCK is commonly known for involvement in TCR signaling. Therefore, the AHR-mediated increase of LCK in human B cells is both interesting and puzzling. However, a recent publication by Till *et al*. showed that CD5$^+$ B cells also have high LCK expression (167). CD5$^+$ B cells are characterized as innate-like B cells (ILBs) with the ability to produce large amount of polyvalent IgM in circulation (136). Collectively, it is possible that different B cells could have various responses toward TCDD treatment. Interestingly, recent studies done by Blevins *et al*. demonstrated a high basal level of AHR, PD-1, PDL1 and PDL2 in human CD5$^+$ ILBs. Most importantly, AHR activation in CD5$^+$ ILBs suppressed the IgM response, as evidenced by the marked decrease of IgM secreted and by the number of IgM secreting cells (Manuscript Under Preparation). Therefore, in the third part of this dissertation research, we examined the role of LCK and PD-1 in the context of AHR-mediated suppression of the IgM response by CD5$^+$ ILBs.

Firstly, a positive correlation was observed between the percentage of CD5$^+$ cells and the percentage of LCK$^+$ cells by human B cells (Fig. 3.3.2A), suggesting that the CD5$^+$ B cells in peripheral blood were also high LCK$^+$ cells. Secondly, the basal level of the percentage of LCK$^+$ cells was significantly higher in CD5$^+$ ILBs compared to CD5$^-$ B cells (Fig.3.3.2B and C). With AHR activation, the percentage of LCK$^+$ cells increased in CD5$^+$ ILBs (Fig. 3.3.2D and E). However, CD5$^-$ B cells were refractory toward AHR

activation (Fig. 3.3.2E). This finding combined with the correlation that $CD5^+$ B cell express high LCK further suggests that $CD5^+$ ILBs also express a high percentage of LCK and are particularly sensitive toward AHR activation. AHR antagonist treatment prevented the AHR-mediate increase of LCK^+ cells and the AHR-mediated suppression of the IgM response in $CD5^+$ ILBs (Fig. 3.3.3). This observation further indicates that the increase of LCK^+ cells within $CD5^+$ ILBs is due to AHR activation. In aim 2, we also have demonstrated that specific LCK inhibitors blocked the AHR-mediated suppression of the IgM response in human B cells (188). Therefore, to further understand the role of LCK in $CD5^+$ ILBs, the same specific LCK inhibitor was used to treat $CD5^+$ ILBs. Similar to the observation in aim 2, treatments with LCK inhibitor blocked the AHR-mediated suppression of the IgM response in $CD5^+$ ILBs, further demonstrating that LCK played a role in the IgM response by $CD5^+$ ILBs (Fig. 3.3.4).

Initial characterization of $CD5^+$ ILBs has shown that ILBs express high levels of PD-1 and its ligands (PDL1 and PDL2) (Manuscript under preparation). PD-1 is a check-point inhibitor that can negatively regulate immune responses. Considering the initial characterization of CD5+ ILBs, we investigated the role of PD-1 and AHR activation in the suppression of the IgM response by $CD5^+$ ILBs in aim 3 of this dissertation. The percentage of $PD\text{-}1^+$ cells and $PDL2^+$ cells increased significantly with TCDD treament, indicating that TCDD treatment increased PD-1 on the surface of ILBs, which in turn increased the possibility of PD-1 signaling by $CD5^+$ ILBs (Manuscript Under Preparation). To further investigate the role of PD-1 in the context of $CD5^+$ ILBs, soluble PD-1 ligands were used to activate PD-1 in $CD5^+$ ILBs. Therefore, $CD5^{+/-}$ populations were treated with either soluble PDL1 (sPDL1), PDL2 (sPDL2) or both in combination, and the IgM

response was evaluated. Treatment with PD-1 ligands significantly suppressed the IgM response in only $CD5^+$ ILBs (Fig. 3.3.5A – C), indicating that PD-1 activation could suppress the IgM response. Therefore, an increase of PD-1 in AHR-activated $CD5^+$ ILBs could have a higher chance of interacting with PD-1 ligand bearing cells, which is responsible for the suppression of the IgM response in $CD5^+$ ILBs. Intriguingly, treatments with TCDD and sPDL2 did not further suppress the IgM response in $CD5^+$ ILBs (Fig. 3.3.6). This finding shows that there is likely no synergistic effect between AHR and PD-1 activation. A second explanation could be that it is impossible to achieve 100% IgM suppression in $CD5^+$ ILBs. Lastly, it is possible that ILBs are a relatively heterogenous population with different sub-population exhibiting differential sensitivity to AHR activation.

LCK has been shown to phosphorylate PD-1 with ligand engagement (210). To understand the interplay between PD-1 and LCK, a specific LCK inhibitor was used in combination with sPDL2 to treat $CD5^+$ ILBs. PDL2 was selected since previous observation suggested sPDL2 treatment resulted a higher magnitude of suppression of the IgM response when compared to PDL1 treatment. Interestingly, LCK inhibitor treatment restored the PD1-mediated suppression of the IgM response, indicating that by blocking LCK activity, the PD-1 inhibitory signaling was terminated in $CD5^+$ ILBs (Fig. 3.3.5D and E). This observation further suggests LCK plays a role in PD-1 signaling and its activity governs the IgM response in $CD5^+$ ILBs. Moreover, epidemiological studies have shown a positive correlation of TCDD exposure and the development of Non-Hodgkin's Lymphoma (NHL) (167, 211). Considering our data, it is likely that the increased of LCK and PD-1 in $CD5^+$ ILBs with AHR activation could potentially play a role

in the development of CLL in humans. However, further investigation is required to understand whether the upregulation of PD-1 and LCK are involved in the development of CLL.

IFNγ treatment has been shown to regulate the expression of PD-1 and PDL1 (193). Previous studies from our laboratory have showed that IFNγ treatment can restore the IgM response in mouse and human B cells with TCDD treatment (100) This observation suggests that the addition of IFNγ potentially disrupted the AHR-mediated signaling in B cells. Therefore, IFNγ was used as a molecular probe to further determine the role of LCK and PD-1 in $CD5^+$ ILBs in aim 3. An extended IFNγ and TCDD concentration response was performed to determine in total human B cells to further elucidate the mechanism of IFNγ in the context of AHR-mediated suppression of the IgM response. With increasing concentrations of IFNγ, the AHR-mediated suppression of the IgM response was blocked in total B cells, especially with 0.1 and 1 ng/ml of IFNγ treatment (Fig. 3.3.8B). In addition, a significant reduction of the percentage of LCK^+ cells were observed with IFNγ treatment of human B cells (Fig. 3.3.8A). Interestingly, the reduction of LCK with IFNγ treatment correlated with the restoration of the IgM response in B cells. Further analysis revealed that $CD5^+$ ILBs expressed a higher level of IFNγ receptors compared to $CD5^-$ B cells (Fig. 3.3.8C – E). Moreover, when $CD5^{+/-}$ B cells were treated with IFNγ, the blockage of AHR-mediated suppression of the IgM response was observed in $CD5^+$ ILBs. $CD5^-$ B cells were refractory to both IFNγ and TCDD treatments (Fig. 3.3.8H and I). Similar to the observation in total B cells, IFNγ treatment also significantly decreased the expression of LCK in $CD5^+$ ILBs (Fig. 3.3.8F and G). These observations suggest that IFNγ treatment can block AHR-mediated suppression of the

IgM response by modulating LCK in $CD5^+$ ILBs. Further investigations on how IFNγ modulate PD-1 signaling revealed that IFNγ treatments blocked the PD1-mediated impairment of the IgM response in $CD5^+$ ILBs (Fig. 3.3.9A – D). These observations further suggest that IFNγ treatment likely interferes with the PD-1 signaling cascade. Overall, IFNγ treatment did not modulate the expression level of PD-1 and PDL1, with the exception of a slight reduction of PDL2 in $CD5^+$ ILBs (Fig. 3.3.9E - G). However, the decrease of PDL2 with both IFNγ and TCDD treatments was similar to the TCDD treatment alone. Taken together, the decrease in LCK with IFNγ treatment could potentially lead to a decrease in phosphorylation of PD-1. Based on the collective results from aim 3, a proposed model was established in Fig 4.1. In addition, PD-1 is just one of the many inhibitory receptors (CTLA-4, TIM-3, CD22, BTLA and LAG-3) expressed on $CD5^+$ ILBs (212). Further delineration of the susceptibility of different inhibitory receptors are required to better understand the mechanisms of inhibitory receptors in $CD5^+$ ILBs.

Interestingly, we did not observe an upregulation of LCK until day 3 post B cell activation in aim 2 (Fig. 3.2.1). This was in contradiction to the observation in $CD5^+$ ILBs in aim 3 showing LCK expression in ILBs on day 0 (Fig. 3.3.1). One possible explanation is that the results from aim 2 were obtained using total B cells. Therefore, the sensitivity of LCK detection in such small population of cells ($CD5^+$ ILBs) within the total B cell pool is not possible using our current approaches. Physical separation of $CD5^+$ ILBs from the total B cells enhance our ability to detect LCK and therefore we observe the high LCK in this small subset of B cells.

For the first time, the studies from aim 3 provide a detailed mechanism by which AHR activation causes upregulation of LCK and PD-1 (Fig. 4.1A, arrow 1). The increase

of LCK could potentially activate PD-1 signaling in $CD5^+$ ILBs (Fig. 4.1A, arrow 2). The activation of PD-1 eventually leads to the suppression of the IgM response in $CD5^+$ ILBs (Fig. 4.1A arrow 3). With IFNγ treatment, the expression level of LCK decreases (Fig. 4.1B, arrow 1), which results in the decrease of PD-1 activation (Fig. 4.1B, arrow 2). The decrease of PD-1 signaling leads to the restoration of the IgM response in $CD5^+$ ILBs (Fig. 4.1B, arrow 3). The current studies provide a mechanistic insight into the upregulation of PD-1 and LCK in the AHR-mediated suppression of the IgM response in $CD5^+$ ILBs. Based on the current results, the percentage of $CD5^+$ cells in circulation can potentially be used as a biomarker to assess the susceptibility of an individual toward AHR-mediated immunomodulation. Additionally, endogenous AHR ligands, like indole and tryptophan metabolites, have been discovered to play a critical role in the regulation of gut homeostasis (213). It is likely that these endogenous ligands also preferentially activating AHR in $CD5^+$ ILBs, which in turn modulate the secretion of nIgM in the circulation. Further characterization of the role of AHR activation with endogenous ligands in $CD5^+$ ILBs will provide insight into the physiological role of AHR in immune regulations, especially in the context of B cell function.

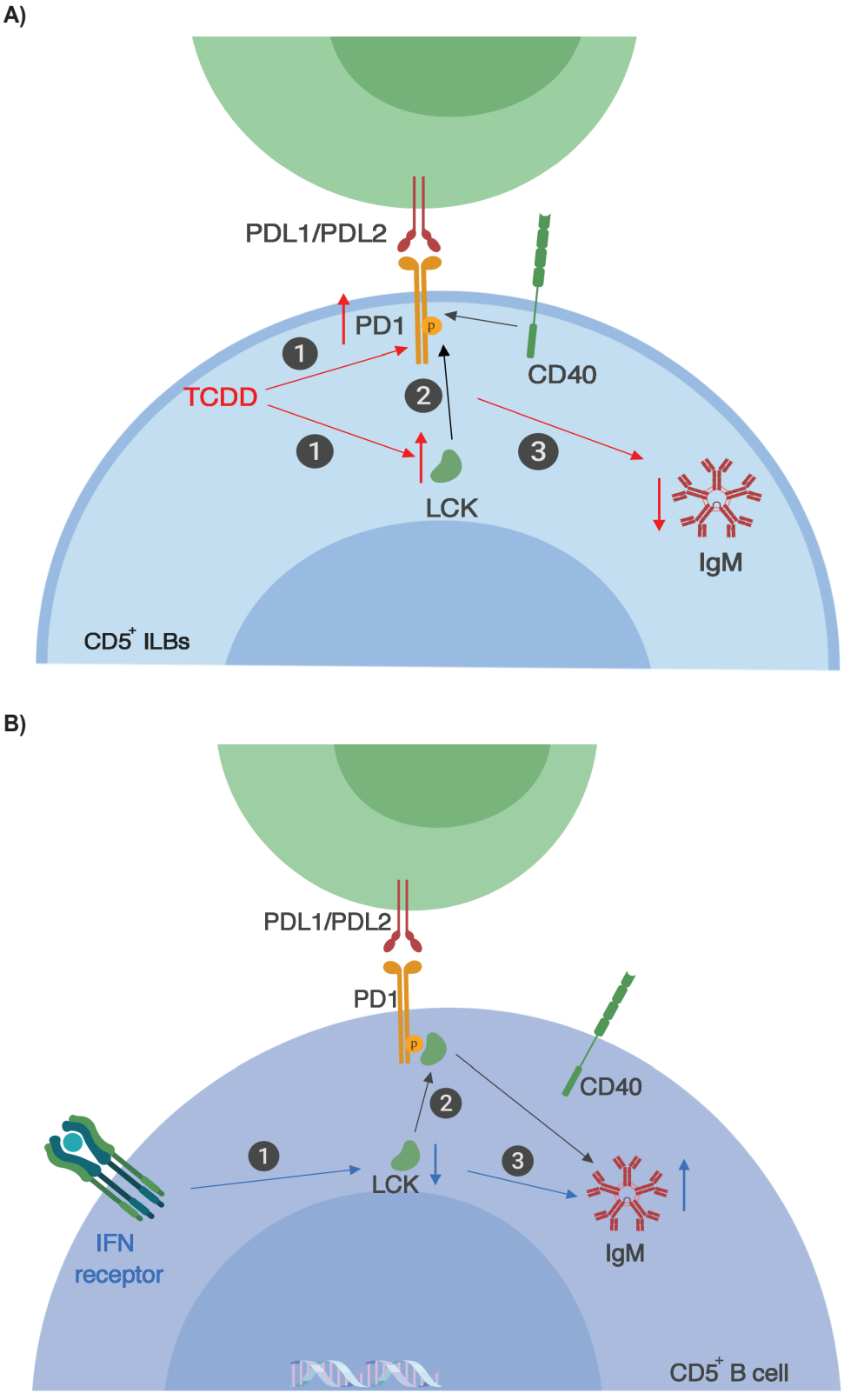


Figure 4.3.1. Schematic of proposed mechanisms.

Figure 4.1 (cont'd)

A) Proposed mechanism of TCDD effects in $CD5^+$ ILBs. With the TCDD-mediated increase of LCK and PD-1, which eventually led to the decrease of the IgM response in $CD5^+$ ILBs. **B)** Proposed model of IFNγ signaling in $CD5^+$ ILBs. IFNγ treatment decreased the total LCK, which in turn decreased the activation of PD-1.

CHAPTER 5: FINAL CONCLUDING REMARKS

Activation of AHR has been shown to have diverse effects on immune cells. The results from this dissertation provide a novel insight into the mechanism underlying the AHR-mediated suppression of the IgM response in human B cells. The first part of the dissertation focuses on comparing the mechanistic changes in both mouse and human B cells with TCDD treatment. The studies showed that AHR activation significantly impairs the IgH, IgJ and Igκ, which led to the over decrease of IgM production in mouse B cells. This observation was in agreement with previous findings from our laboratory using mouse splenocytes and mouse cell line, CH12.LX (95, 159). In contrast, AHR activation did not alter the mRNA or protein levels of IgH, IgJ and Igκ in total human B cells, but the overall IgM response was suppressed. One possible explanation is that the underlying mechanism of AHR-mediated suppression of the IgM response is different between mouse and human B cells. Therefore, investigations only using mouse models may not yield applicable toxicological effects in humans. However, it is also likely that the heterogeneity of the B cell populations in humans are masking the AHR-mediated impairment of the IgM response in subsets of B cells. In addition, the identification of the critical cell populations must be taken into consideration when evaluating immunotoxicological effects in humans.

In aim 2 of this dissertation, studies were conducted on further elucidating the mechanism of AHR-mediated suppression of the IgM response in human B cells. One potential target, LCK, was identified through the transcriptomic study, which demonstrated an AHR-mediated increase of *LCK* mRNA in only human B cells. The results from aim 2 showed a significant increase in LCK mRNA and protein with AHR

activation in human B cells with CD40L activation. Specific LCK inhibitor treatments prevented the TCDD-mediated suppression of the IgM in human B cells, suggesting that LCK plays a role in mediating the IgM response. The data generated from the studies was used to collaborate with Dr. Zhang from Emory University to develop a computational model (XNOR model). Similar observations can also be found in other kinases involve in cell activation and differentiation. The computational and experimental data further supported the notion that an optimal level of LCK was required to achieve IgM secretion in humans. Interestingly, AHR activation significantly increased the activity of LCK in human B cells, further suggesting that the optimum level of LCK has to be achieved in order to have a high IgM response in human B cells. Recent studies have demonstrated that $CD5^+$ B cells express high level of LCK. It is possible that $CD5^+$ B cells more sensitive toward TCDD treatment compared to $CD5^-$ B cells. Further investigation of the role of LCK in $CD5^+$ B cells was conducted in aim 3.

In aim 3, studies were conducted using $CD5^+$ ILBs, which demonstrate a high level of LCK compared to $CD5^-$ B cells. Similar to the observations in aim 2, TCDD treatment significantly upregulated LCK protein in $CD5^+$ ILBs; however, $CD5^-$ B cells were refractory toward AHR activation, possibly due to the low AHR expression. Furthermore, the LCK inhibitor prevented the AHR-mediated suppression of the IgM response in only $CD5^+$ ILBs, demonstrating a role of LCK in the IgM response by $CD5^+$ ILBs. Interestingly, one of the targets of LCK phosphorylation is PD-1, which is highly expressed on the surface of $CD5^+$ ILBs. Therefore, studies were conducted to investigate the interplay of LCK and PD-1 in $CD5^+$ ILBs. Firstly, we determined that PD-1 activation by its ligands (PDL1 and PDL2) was able to suppress the IgM response in $CD5^+$ ILBs. Secondly, PD-1 blocking

antibody also prevented the AHR-mediated suppression of the IgM response in $CD5^+$ ILBs, indicating that PD-1 signaling plays a crucial role in the regulation of the IgM response. Additionally, a specific LCK inhibitor blocked the PD-1-mediated suppression of the IgM response in $CD5^+$ ILBs, further suggesting that LCK plays a role in PD-1 signaling. Taken together, AHR activation increased both LCK and PD-1 expression in $CD5^+$ ILBs. The upregulation of LCK can phosphorylate PD-1, which in turn suppresses the IgM response in $CD5^+$ ILBs. IFNγ has been previously reported to modulate the expression of PD-1 and prevent AHR-mediated suppression of the IgM response in mouse and human B cells. Therefore, IFNγ was used as a molecular probe in aim 3 to further elucidate the role of LCK and PD-1 in $CD5^+$ ILBs. Interestingly, IFNγ treatment inhibited the AHR-mediate suppression of IgM via the decrease of the expression of LCK in human $CD5^+$ ILBs. This decrease leads to a downregulation of PD-1 signaling, therefore, restoring the IgM response in $CD5^+$ ILBs. The proposed model system with TCDD and IFNγ treatments is illustrated in Fig. 5.1. Overall, this dissertation provides a detailed mechanism of the AHR-mediated impairment of the IgM response due to PD-1 signaling involving LCK in $CD5^+$ ILBs.

The inter-individual variability in humans plays a crucial part in understanding the mechanistic changes among toxic responses. The variability is taken into consideration during experimental design, with experiments being performed using B cells from at least 6 individuals (N = 6) with three replicates per study. Despite the variability between humans, consistent and significant effects of TCDD-mediated AHR activation were observed in all studies. Studies conducted using human cells have become simpler and

easier with the development of materials to study the immune system and increase sensitivity of detection of the subtle changes with AHR activation.

The first and second parts of this dissertation research utilized the human CD40 ligand expressing L cells with the addition of cytokines (IL-2, IL-6 and IL-10) to activate human B cells has proved to be useful toward understanding the mechanism by which AHR activation impairs B cell function. However, this system is only capable of sub-optimal activation of human B cells. Therefore, in the third part of the study, a modified version of this activation system was employed consisting of soluble CD40 ligand and cytokines (IL-2 and IL-21). The usage of IL-21 provides stronger activation evidenced by the increase of the number of IgM secreting cells compared to the previous model.

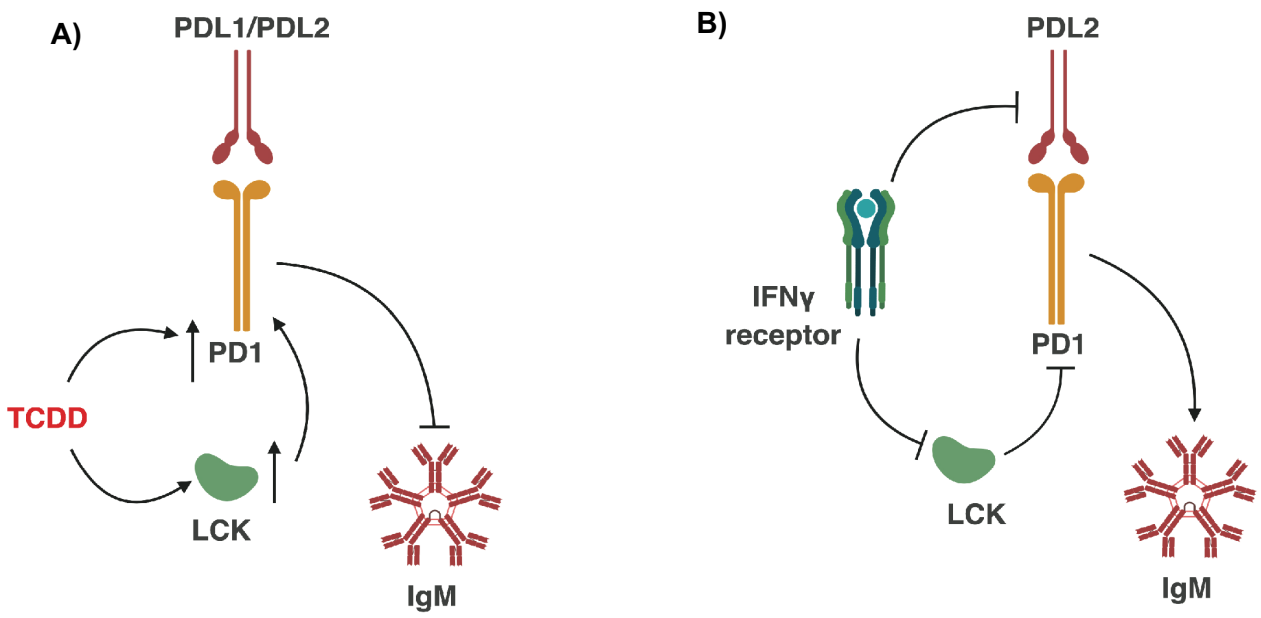


Figure 5.1.1. Proposed mechanisms.

The proposed mechanisms in $CD5^+$ ILBs. **A)** With TCDD treatment, the level of LCK and PD-1 increases, which provides a negative signal to down-regulate IgM response in $CD5^+$ ILBs. **B)** With the addition of IFNγ, the level of LCK and PDL2 decreases, which in turn decreased the activation of PD-1. Therefore, positive signal was presented to upregulate IgM response in $CD5^+$ ILBs.

In conclusion, this dissertation research has addressed the question of how AHR activation suppresses the IgM response in human B cells. The mechanistic findings in this dissertation have highlighted the role of LCK and PD-1 in AHR-mediated suppression of the IgM response. To date, this dissertation is the first to highlight the role of LCK and PD-1 in the IgM response and the potential contribution of LCK in the development of CLL. This dissertation research also demonstrated that a relatively small subset of B cells ($CD5^+$ ILBs) are a sensitive target to TCDD treatment and susceptible to IgM suppression through the involvement of the PD-1 signaling. Furthermore, this dissertation not only provides an insight into the physiological role of AHR in $CD5^+$ ILBs, a previously less characterized cell population, but it also elucidates the immunotoxicological effects of AHR activation in $CD5^+$ ILBs.

In this dissertation, I have highlighted the important role of PD-1 signaling in the TCDD-mediated suppression of the IgM response in human $CD5^+$ ILBs. One possible future experiment will be to determine the activation of PD-1 via phosphorylation in the context of TCDD treatment. Furthermore, we have identified LCK as a key kinase in the PD-1 signaling in $CD5^+$ ILBs. It is likely that there are other kinases or phosphatases also involve in the PD-1 signaling. Therefore, it is likely that TCDD treatment will alter the phosphorylation of PD-1, which changes PD-1 signaling. One prime candidate is SHP-1, since SHP-1 has been suggested to hydrolyze a phosphate group on PD-1. Therefore, studies can be conducted to understand the interplay between PD-1 signaling and SHP-1 activity. In addition, even though PD-1 signaling has been suggested to be critical to IgM suppression in $CD5^+$ ILBs, it is also crucial to investigate other inhibitory receptors (i.e. CTLA-4 and CD22). Thus, experiments involving the characterization of CTLA-4 and

CD22 can be performed to further understand the involvement of inhibitory receptors in the suppression of IgM response in $CD5^+$ ILBs.

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