MECHANISMS UNDERLYING 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN-MEDIATED SUPPRESSION OF B CELL ACTIVATION AND DIFFERENTIATION

By

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ABSTRACT

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Exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is known to alter B cell function, resulting in marked suppression of the primary immune response. The immunotoxic effects of TCDD involve transcriptional regulation through the aryl hydrocarbon receptor (AHR) but the exact molecular mechanisms are still unknown. To identify novel genes directly modulated by the ligand-activated AHR during B cell differentiation, a genome-wide study was performed in mouse B cells through which Bach2; a direct target of AHR was identified. Bach2 is known to repress expression of Blimp-1, a master regulator of B cell differentiation by binding to Maf elements (MAREs) in the regulatory regions of the gene. Electrophoretic mobility shift assays confirmed the binding of AHR to intron1 of Bach2. TCDD induced expression of Bach2 and decreased expression of Blimp-1 in B cells. Increased binding of Bach2 was observed in presence of TCDD to the intron 5 MARE in the Blimp-1 gene. These studies suggest transcriptional regulation of Bach2 by AHR as one of the mechanisms involved in suppression of B cell differentiation by TCDD.

B cell differentiation can also be affected by the strength of B cell activation, a process initiated upon ligation of the CD40 receptor and by signaling through cytokines IL-2, IL-6 and IL-10. In a previous study, it was shown that TCDD markedly affected B cell activation by decreasing the expression of B cell activation markers CD80, CD86

and CD69. Hence, the second part of this study investigated the mechanisms underlying suppression of human B cell activation by TCDD. BCL-6 was identified as a likely candidate owing to its role as a transcriptional repressor of B cell activation and differentiation. In the presence of TCDD, BCL-6 protein levels were elevated in human B cells in an AHR-dependent manner. A decrease in B cell activation was also evident through the attenuation of surface CD80 and CD69. BCL-6 repressed CD80 in presence of TCDD by binding to the enhancer region of CD80. Moreover, the suppressed activation marker expression was reversed by treatment of cells with a specific BCL-6 inhibitor thus suggesting a role for BCL-6 in decreasing B cell activation in presence of TCDD. Part of the mechanism underlying TCDD-mediated suppression of B cell activation also involves SHP-1, a protein tyrosine phosphatase inhibiting signaling in activated B cells which was identified through the same genome-wide analysis of AHR binding in presence of TCDD. SHP-1 mRNA and protein levels were elevated in presence of TCDD. An increase in SHP-1^{+} BCL-6^{+} cells was observed upon TCDD-treatment thereby suggesting cross talk between SHP-1 and BCL-6 pathways. Addition of SHP-1 inhibitor to naïve B cells affected BCL-6 protein levels suggesting possible regulation of BCL-6 by SHP-1 for the first time.

Taken together, the results of this investigation suggest that a) TCDD: AHR-mediated inhibition of B cell activation occurs through de-regulation of BCL-6 and SHP-1 and that b) the inhibition of B cell differentiation occurs through elevated Bach2 levels in B cells. These studies contribute to the field of TCDD immunotoxicity by presenting novel insights into the mechanisms by which TCDD affects B cell activation and effector function.

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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
Ah	Aromatic hydrocarbon
AHH	Aryl hydrocarbon hydroxylase
AHR	Aryl hydrocarbon receptor
AP-1	Activator protein-1
ARA9	AHR-associated protein
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASC	Antibody secreting cell
Bach2	BTB and CNC homology 2
BCL-6	Whole Genome Duplication
BCR	B cell receptor
BLIMP-1	B lymphocyte-induced maturation protein-1
Ca^{++}	Calcium
CD	Cluster of differentiation
CD40L	CD40 ligand
ChIP	Chromatin immunoprecipitation
CTV	Cell trace violet
Cyp	Cytochrome P 450
DLBCL	Diffuse large B cell lymphomas
DLC	Dioxin-like compounds
DMSO	dimethyl sulfoxide

DNP	dinitrophenol
DRE	Dioxin response element
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal regulated kinase
EROD	ethoxyresourfin-o-deethylase
FACS	Fluorescence activated cell sorting
GC	Germinal center
h	hour
HSC	hematopoietic stem cell
Hsp90	heat shock protein 90
ICAM-1	Intracellular cell adhesion molecule-1
IgM	Immunoglobulin M
IL	Interleukin
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	Janus kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MARE	Maf response elements
MCDD	monochlorinated dibenzodioxin
MFI	Mean Fluorescence Intensity
MHC	Major histocompatibility complex

min	minute
mRNA	messenger RNA
MTA3	Metastasis-associated 1 family member 3
NA	naïve
NFκB	Nuclear factor kappa B
NHL	non-Hodgkin's lymphoma
Pac Blue	Pacific blue
PAX-5	Paired box-5
PBMC	peripheral blood mononuclear cell
PCB	polychlorinated biphenyls
PCDD	polychlorinated dibenzo dioxins
pERK	phosphorylated extracellular regulated kinase
PFC	plaque forming cell
PI3K	Phosphoinositide-3 kinase
PLCγ	Phospholipase-C gamma
PRDM1	positive regulatory domain motif-1
PWM	pokeweed mitogen
qRT-PCR	quantitative Real time-polymerase chain reaction
RAG	Recombinase
SHP-1	Src homology protein-
sRBC	sheep red blood cell
SSG	sodium stibogluconate
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Tfh	T follicular helper
Th	T helper
TLR	Toll-like receptor
TNP	trinitrophenol
TRAF	TNF receptor associated factor
VH	vehicle
XBP-1	X-box protein-1

CHAPTER 1: LITERATURE REVIEW

1.1. Purpose of this research

Dioxins are ubiquitous environmental contaminants belonging to the group of halogenated aromatic hydrocarbons. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener among dioxins causing profound suppression of the primary humoral immune response. So far, decades of research have contributed to our understanding of the toxic effects of TCDD in rodent species but the effects of TCDD on human health still represent a major data gap in the field. Recent observations elude that TCDD suppresses human B cell activation and differentiation but the mechanism underlying this effect is currently unknown. This dissertation research aims to bridge the gap by providing a deeper molecular insight into the mechanisms by which TCDD affects human B cell activation and differentiation.

1.2. Immune system and the humoral immune response

The primary objective of the immune system is to protect the individual effectively from infection or disease. In order to perform this role, the immune system must be able to perform four major functions including: recognition of the antigen, elimination of antigen involving an appropriate effector response, immune regulation including the ability to coordinate activities of multiple cell types and finally establishment of immunological memory for protection against subsequent exposures. There are two major arms of the immune system – the innate and the adaptive. The innate arm of the immune system is a non-specific host defense mechanism responsible for the first line of defense against pathogens that the system encounters and occurs rapidly after infection. The innate immune system involves the phagocytic cells such as macrophages and the natural killer cells, which are involved in direct killing of the infected cell. The anatomical barriers of the skin, the mucosal epithelial cells lining the airways and the intestines, cytokines, chemokines and plasma proteins known as 'complement' constitute the innate immune system. The innate immune response can distinguish between self and non-self though it lacks the specificity of the adaptive immune response. The pattern recognition receptors of the innate immune system are designed to recognize repetitive structures on viruses and bacteria called pathogen-associated molecular patterns. Another important function of the innate immune system is the activation of the adaptive immune response. The adaptive immune response results in elimination of the pathogen in a specific and efficient manner. It exploits the property of lymphoid cells to exquisitely recognize specific antigens by virtue of the cell surface receptors. Antibodies produced by activated lymphocytes persist after the

original response has diminished and are responsible for long-lasting immunity to a variety of pathogens. The innate and adaptive immune responses depend on activation of white blood cells or leukocytes that originate in the bone marrow. These leukocytes often migrate to secondary lymphoid organs after maturation where they reside. They are then transported to the site of infection via lymphatic system soon after the infection ensues. In absence of an infection, naïve lymphocytes that have not encountered an antigen remain circulating in the blood. Once the lymphocytes have met their cognate antigen, they become activated and differentiate into effector lymphocytes. The adaptive immune response carried out by T and B lymphocytes can be further classified into cell-mediated and humoral immune response. Cell-mediated immune responses are primarily directed towards intracellular pathogens and involve the lysis of infected or tumor cells. In contrast, humoral immune responses are mediated by immunoglobulin-secreting differentiated B cells that neutralize the pathogen thus clearing the infection.

Numerous signaling pathways govern B lymphocyte behavior. Some of the activation signals are delivered via the B cell receptor (BCR) or the CD40 receptor or cytokines secreted by other immune cells. B cell development, activation and longevity are determined by the strength and the kind of stimulus received. Upon activation, B cells differentiate into plasma cells, which secrete antibodies. Antibodies are effector molecules of the humoral immune response and serve two major functions: first to specifically bind antigens and second to recruit other molecules to destroy the pathogen. Each antibody molecule is comprised of a variable region or V region, also known as the antigen-binding region that varies extensively between antibody molecules in a given species. The constant region or C region is involved in the effector

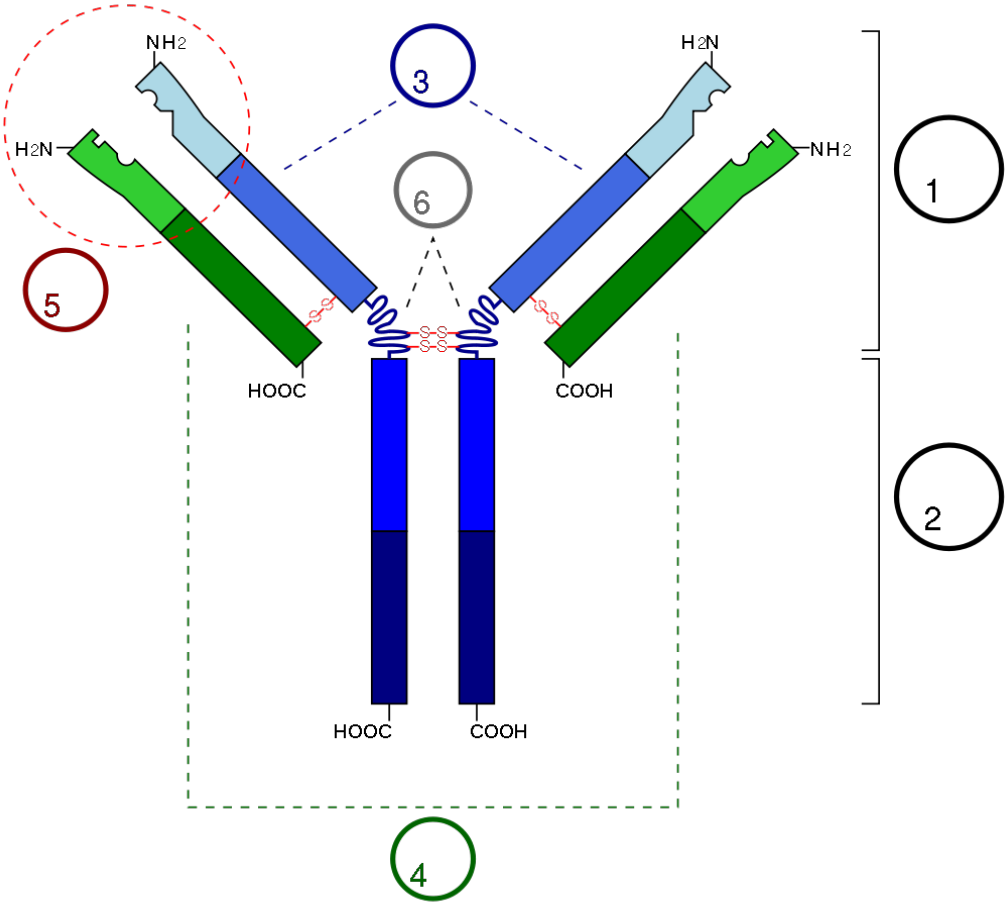


Figure 1.1: General immunoglobulin structure

Structure of a typical immunoglobulin molecule is illustrated in the figure above. The immunoglobulin molecule comprises of two identical heavy chains and light chains. Numbers in the figure indicate 1- Fab region, 2- Fc region, 3- Heavy chains (light blue- variable region, dark blue- constant region), 4- Light chain (light green- variable region, dark green-constant region), 5- antigen binding regions, 6- hinge regions. “For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis (or dissertation).”

function. The wide variety of antigen specificities in the V region of the receptor arises due to a unique molecular event involving rearrangement between different gene segments (V, D and J segments) at the germinal level. B cell- specific recombinase (RAG) proteins catalyze this rearrangement generating antibody diversity. Further diversification is brought about in two ways: 1) Through somatic hypermutation, which alters the sequence of the immunoglobulin by introducing point mutations 2) through class switch recombination, which enhances the functional diversity of the immunoglobulin repertoire or constant region diversity by generating different classes of antibodies. There are five major classes of immunoglobulins - IgM, IgD, IgG, IgA and IgE, which have distinct biological functions. Each immunoglobulin molecule has a lambda (λ) or kappa (κ) light chain. IgM has a pentameric structure consisting of a J-chain to join the five monomers and is the first antibody to be secreted during an effector response. In the course of an immune response, effector B cells are selected on the basis of their ability to combat an antigen. Cells that harbor a specific BCR are then selected and expanded clonally. This process of selection of high affi nity-BCR possessing B cells is called affinity maturation. All these processes contribute to the robustness of the B cells in countering an infection and delivering an appropriate immune response to the antigen.

B cell signaling and activation

Mature B cells are constantly instructed by the BCR to make critical cell-fate decisions (Niiro and Clark, 2002). The BCR has two major roles: first to transmit signals that regulate B cell activation and differentiation and second to mediate antigen

processing and presentation to T helper (T_h) cells thereby leading to B cell activation. The BCR complex is composed of an Ig heavy and light chain along with two additional signaling components the Igα and Igβ chain. Upon ligation of the BCR by the cognate antigen, the proximal protein kinases LYN and SYK are activated. LYN phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) in the Igα and β, which then activate SYK and BTK kinases. LYN and SYK are the major kinases associated with the BCR as mutations in LYN and SYK are known disrupt downstream signaling and B cell development in mice (Xu et al., 2005). The PI3K, AKT and PLCγ2 are important signal transducers enabling activation of secondary messengers such as Ca^{++} and protein kinase C (PKC). These subsequently induce activation of mitogen-activated protein kinase (MAPK) pathway including the extracellular signal regulated kinase (ERK), c-jun terminal kinase (JNK) and p38. Activation of ERK causes translocation into the nucleus and activation of nuclear factor-kappa B (NFκB) and nuclear factor of activated T cells (NFAT) and degradation of BCL-6 (Niu et al., 1998a) thereby regulating B cell fate. Along with these positive regulators of B cell signaling, negative feedback regulation controls excessive B cell activation and proliferation. The paired immunoglobulin-like receptors (PIR) and FcγRIIB mediate negative regulation of BCR signaling. These receptors harbor immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails. Association of ITIMs with phosphatases such as Src-homology phosphatase-1 (SHP-1) and CD22 leads to inhibition of B cell receptor signaling.

Activation of B cells can also occur dependently as well as independently of T_h cells. Antigens including microbial constituents such as bacterial lipopolysaccharide (LPS) activate Toll-like receptor-4 (TLR-4), single stranded RNA from viruses can activate TLR-3/7 and CpG sequences from bacterial DNA can activate TLR- 9 ultimately leading to B cell activation and differentiation. In contrast, activation of B cells via T-dependent antigens requires the antigen to be processed and presented by the B cell in context of MHCII. This is then recognized by the cognate T_h cell or T follicular helper (T_{fh}) cell in the germinal centers of the spleen. Although recognition of the antigenic peptide: MHC complex is essential it is insufficient for antigen-specific T cell activation. A secondary stimulus in the form of co-stimulatory signals is required for proper response. The co-stimulatory signals involve engagement of the CD28/B7 family such as CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4), which bind their ligands CD80 (B7.1) and CD86 (B7.2) expressed on the surface of B cells. The T cells also express the extracellular CD40 ligand that binds to the CD40 receptor on the surface of B cells. The CD40 receptor is constitutively expressed on B cells through all developmental stages (Banchereau et al., 1994) and is involved in B cell proliferation and class switch recombination (Bishop and Hostager, 2003). The engagement of CD40 by CD40 ligand results in clustering of CD40 on the B cell surface thereby triggering recruitment of TNFR-associated factors (TRAFs) to the cytoplasmic tail of CD40. TRAF proteins activate NFκB, MAPK and PI3K/PLCγ pathways (Elgueta et al., 2009; Kehry, 1996). CD40 ligation can also activate downstream transcription factors such as Activator protein- 1 (AP-1), NFκB and NFAT (Bishop and Hostager, 2001; Francis et al., 1995;

Hsing et al., 1997). The activation of these signaling pathways ultimately results in extensive signaling in the B cell culminating in an activated B cell phenotype characterized by increased expression of CD80, CD86 and CD69 along with MHCII and intracellular cell adhesion molecule-1 (ICAM-1) (Bishop and Hostager, 2001). Apart from functioning as co-stimulatory molecules for T cells, CD80 and CD86 regulate B cell activation, curb proliferation of B cell lymphomas (Suvas et al., 2002), regulate anti-viral B cell responses and enhance IgG secretion (Rau et al., 2009). Recently, expression of CD80 on B cells has been shown to regulate T follicular helper (T_{fh}) cell development and enhance development of germinal center (GC) and antibody-forming cells (Good-Jacobson et al., 2012). With the advent of monoclonal antibodies in the mid-1970s, a monoclonal antibody generated against CD40 was used to demonstrate the importance of the CD40-CD40L pathway in the initial stages of activation of normal human peripheral blood B cells (Splawski and Lipsky, 1994). Moreover, the importance of CD40/CD40L has been demonstrated by the generation of CD40 null mice, which show severe defects in B cell function and the development of an autoimmune disease called the hyper-IgM syndrome (Bishop and Hostager, 2001). Activated T_h cells also secrete cytokines such as IL-2, IL-4, IL-6, IL-10 and IL-21 that enhance B cell proliferation. Hence, *in vitro* cultures of human B lymphocytes activated with recombinant CD40 ligand or CD40 ligand transduced cells can be supplemented with cytokines IL-2 and IL-6 or IL-2 and IL-10 to induce B cell proliferation and IgM secretion (Armitage et al., 1993; Arpin et al., 1995; Rousset et al., 1991). Recently, IL-21 has been shown to act synergistically with CD40 ligand to promote human B cell differentiation into antibody-secreting plasma cells (Ding et al., 2013; Ettinger et al., 2005).

Regulation of B cell differentiation

Terminal differentiation of B cells is an irreversible, tightly regulated process as it determines the fate and functional response of the B cells. Plasma cells generated as a result of differentiation are capable of secreting copious amounts of antibody for mediating B cell effector function. This process is associated with major changes in the morphology and gene expression profile of the differentiated cells as compared to their naïve counterparts. Terminal differentiation is preceded by robust proliferation of mature B cells and followed by an abeyance of cell division. During this time, the steady-state mRNA levels increase due to increased transcription and mRNA stability (Jack and Wabl, 1988). Along with this, plasma cells also display an increased cytoplasmic to nuclear ratio to accommodate for the increased immunoglobulin synthesis (Calame et al., 2003). The constellation of cell surface proteins expressed on the B cell also changes with plasmacytic differentiation. The expression of MHCII, B220 and CD19 decreases whereas expression of Syndecan-1 and CD38 increases on the surface of plasma cells. In addition, the expression of chemokine receptors CXCR5 and CCL7 also decreases in plasma cells with the exception of CXCR4, which enables transition of plasma cells from the follicles in the spleen to the bone marrow (Calame et al., 2003). Mature B subsets residing in the spleen undergo differentiation into antibody-secreting plasma cells upon antigen encounter or upon stimulation via T-independent antigens. The intricacies of the B cell differentiation process have been illuminated by using *in vitro* culture systems adopting T cell-dependent stimuli such as ligation of CD40 antigen or by TLR ligands such as LPS and CpG to stimulate mature B cells. Gene expression programs established in mature B cells are strikingly different from the programs in

plasma cells as B cell differentiation is thought to be an all-or-none process guided by a bi-stable gene regulatory network (Bhattacharya et al., 2010; Muto et al., 1998; Zhang et al., 2013). The bi-stable network is comprised of critical transcription factors such as: B cell lymphoma-6 (BCL-6), B lymphocyte-induced maturation protein 1 (BLIMP-1), Paired box protein 5 (Pax-5) and BTB and CNC homology 2 (Bach2). BCL-6, Bach2 and Pax-5 are repressors of B cell differentiation and are expressed at high levels in mature B cells. Pax-5, aptly named the guardian of B cell identity is expressed in B cells (Cobaleda et al., 2007) from the pro-B cell stage until its expression is extinguished by Blimp-1 in the plasma cells. Pax-5 controls the expression of BCR components such as Blnk, Igα, CD19 and transcription factors such as Interferon regulatory factor-4 (IRF4), IRF8 and Bach2 by activating or repressing their expression in a context-dependent manner (Pridans et al., 2008; Schebesta et al., 2007). In addition, Pax-5 actively represses genes involved in the antibody secretion process in plasma cells such as IgH, J chain, κ light chain and XBP-1. Blimp-1, the master regulator of B cell differentiation is also reciprocally repressed by Pax-5 in plasma cells (Lin et al., 2002). Pax-5 deficient DT-40 cells showed decreased IgM secretion and loss of BCR signaling along with higher Blimp-1 and X-box protein-1 (XBP-1) expression levels. This illustrates that downregulation of Pax5 is essential for promoting B cell differentiation (Nera et al., 2006; Nera and Lassila, 2006). BCL-6 and Bach2 also help in keeping the B cell differentiation program repressed by preventing expression of Blimp-1 as illustrated in Figure 1.1. BCL-6 and Bach2 are maintained at high expression levels in the naïve GC B cells. Metastasis-associated 1 family, member 3 (MTA3) (Jaye et al., 2007), the cytokine IL-21 (Linterman et al., 2010; Zotos et al., 2010) and IRF8 (Lee et al., 2006)

are known to maintain BCL-6 expression in the GC. A B cell-specific deletion of Bach2 demonstrated the importance of Bach2 in repressing Blimp-1 and XBP-1 thereby preventing plasmacytic differentiation. The role of Bach2 and BCL-6 in regulation of B cell differentiation and activation is discussed in greater detail in the next section. Expression of Blimp-1 ensues the B cell differentiation program. This is facilitated by B cell activation through the TLR, BCR, CD40 receptor or cytokines, which activate downstream signaling pathways leading to an increase in AP-1 transcription. AP-1 and IRF4 positively regulate Blimp-1 (Vasanwala et al., 2002). Induction of Blimp-1 ensures irreversibility of plasmacytic differentiation by repressing BCL6 and Pax-5 through double negative feedback loops (Calame et al., 2003). In the next section, the role of specific transcription factors involved in B cell differentiation and activation will be discussed.

BTB and CNC homology 2 (Bach2)

Bach2 is a B cell specific transcription factor and a member of the basic region-leucine zipper (bZip) family (Muto et al., 1998). Bach2 is expressed at high levels in naïve B cells and the expression is turned off in terminally differentiated plasma cells. It is thought that phosphorylation by PI3K activates Bach2 subsequent to its translocation into the nucleus (Kallies and Nutt, 2010) to repress target genes. In activated B cells, Bach2 represses the transcription factor Blimp-1 (Ochiai et al., 2006). The mechanism of repression involves heterodimerization with small oncogenic Maf proteins such asMafK, MafG, MafF, followed by, binding to Maf recognition elements (MAREs) in the regulatory regions of the genes it controls. Two MARE elements are found in Blimp-1:

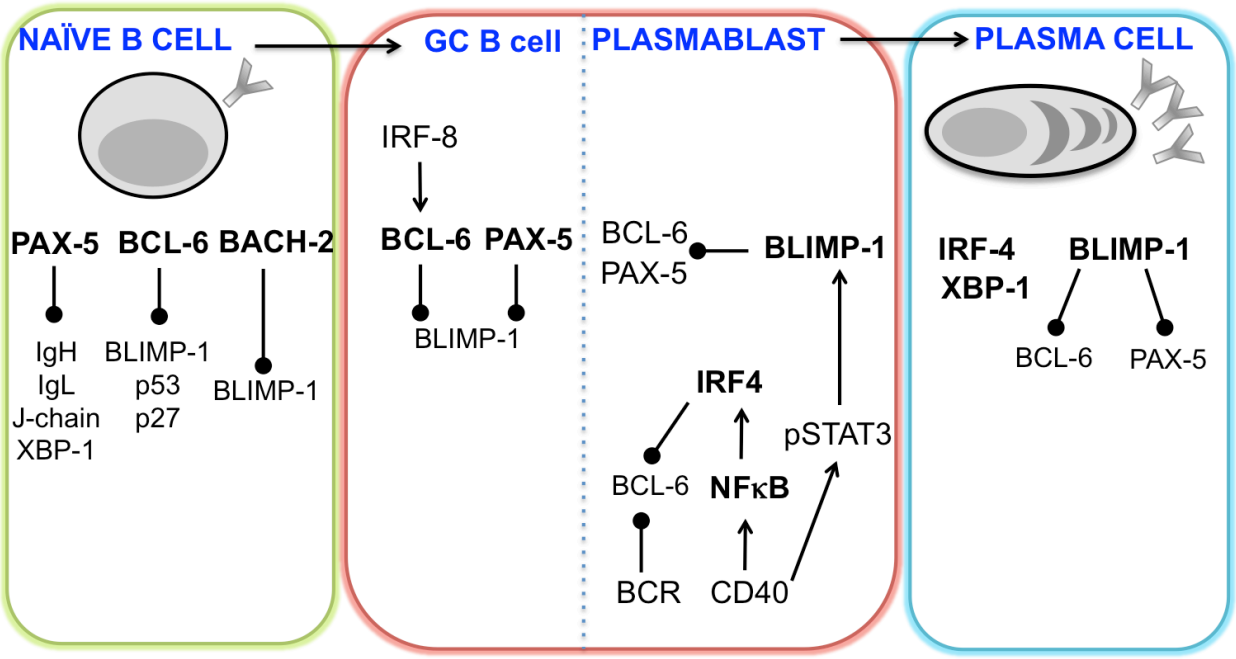


Figure 1.2: Schematic representing the different stages of B cell differentiation

Naïve B cells are activated through BCR or T-dependent stimulation to differentiate into antibody-secreting plasma cells. The network of repressors BCL-6, PAX-5 and Bach2 keep the B cell in a naïve state but their expression is extinguished upon B cell activation and up-regulation of Blimp-1. In the plasma cells, IRF-4, BLIMP-1 repress BCL-6 and PAX-5 permitting plasmacytic differentiation and antibody production. Black arrows indicate activation and black lines with a solid dot indicate repression.

one in the promoter and another in intron 5 of the gene (Muto et al., 2010; Ochiai et al., 2008a). In addition to this, putative MAREs are also found in the 3'α regulatory region of the IgH gene through which Bach2 negatively regulates IgH expression in B cells (Muto et al., 1998). In regulating the B cell transcriptional network, Bach2 is thought to act downstream of Pax5 since a study by Schebesta et.al, showed that Pax5 activated Bach2 in B cells (Schebesta et al., 2007). Bach2 further delays B cell differentiation by suppressing Blimp-1 function and allowing the B cell to undergo class switch recombination (Muto et al., 2010). The critical role of Bach2 in mature B cells was demonstrated by the generation of $Bach2^{-/-}$ mice, which show defects in GC formation and an inability to produce IgM antibodies after antigen challenge (Muto et al., 2004).

B cell lymphoma-6 (BCL-6)

BCL-6 regulates the processes of B cell differentiation, inflammation and cell cycle control along with immune responses (Dalla-Favera et al., 1999; Shaffer et al., 2000; Staudt et al., 1999). The expression of BCL-6 is tightly regulated in normal B cells but deregulation of BCL-6 mostly through alterations in the BCL-6 promoter region are commonly seen in lymphomas. A third of Non-Hodgkin's lymphomas possess a mutation in BCL-6 (Dalla-Favera et al., 1999; Shaffer et al., 2000; Staudt et al., 1999) and a significant proportion of the diffuse large B cell lymphomas (DLBCLs) harbor translocations within the BCL-6 gene (Ye, 2000). BCL-6 is also one of the three cardinal transcription factors, along with Blimp-1 and Pax-5, controlling B cell differentiation. BCL-6 blocks B cell differentiation by repressing Blimp-1 while, Pax5 contributes to maintain BCL-6 expression. Other important target genes of BCL-6 include p53, p21

and p27kip1 that control the cell cycle and CD69, STAT1, CD80 and MIP-1α that control B cell activation (Dent et al., 2002; Niu, 2002; Shaffer et al., 2000).

Src homology protein-1 (SHP-1)

SHP-1 encoded by the gene PTPN6 in humans is an intracellular non-receptor type protein tyrosine phosphatase. The protein structure of SHP-1 is characterized by the presence of two Src-homology 2 (SH2) domains at the N-terminus, a central catalytic domain and a C-terminal domain with tyrosine phosphorylation sites responsible for phosphorylation of SHP-1 (Lorenz, 2009; Pao et al., 2007a). The C-terminus of SHP-1 contains a tightly regulated nuclear localization signal that also plays a role in regulating activity of SHP-1. The SH2 domains are critical for activation of the phosphatase (Lorenz, 2009; Pao et al., 2007a; Pao et al., 2007b) and serve as docking sites for several molecules such as receptor tyrosine kinases, cytokine receptors, scaffolding adapters (Grb, SLAM) and ITIMs. SHP-1 is expressed in hematopoietic stem cells and cells of the lymphoid and myeloid lineage. One of the main functions of SHP-1 is in regulation of B cell receptor signaling, specifically attenuation of signaling downstream of the BCR. Much of the biological function of SHP-1 has been gleaned after studying the genetic model of SHP-1 deficiency. A splicing mutation near the 5' end of SHP-1 coding sequence led to a frameshift forming truncated mRNA, eventually leading to loss of protein expression, and thus generating SHP-1 null mice (Shultz et al., 1993). These null mice display numerous immunological abnormalities characterized by an accumulation of myeloid cells due to loss of T and B cell populations. In the bone marrow, a reduced number of early B cell progenitors were observed along with an

accumulation of large number of abnormal plasmacytoid cells called Mott cells (Shultz et al., 1993). B cell specific deletion of SHP-1 leads to altered B cell development, increased B cell Ca^{++} fluxes, higher serum antibody concentrations and autoimmunity (Pao et al., 2007b). Activation of phosphatase activity of SHP-1 occurs upon tyrosine phosphorylation of its C-terminal tail (Uchida et al., 1994). Association of SHP-1 with the phosphorylated negative regulatory receptor CD22 leads to a decrease in Ca^{++} by promoting Ca^{++} efflux thereby controlling the extent of B cell activation (Nitschke, 2005). SHP-1 also serves as a global negative regulator of BCR function by inhibiting kinases such as LYN, SYK, Jak2 and ERK (Dustin et al., 1999; Jiao et al., 1996).

1.3. Dioxins and TCDD-AHR pathway

History and general toxicity of dioxins

Dioxins and dioxin-like compounds (DLCs) are ubiquitous environmental chemicals occurring as trace contaminants in the food chain. DLCs comprise the structurally related halogenated aromatic hydrocarbons including the dibenzo-*p*-dioxins, polychlorinated dibenzo-*p*-furans, polychlorinated biphenyls and polybrominated biphenyls. Among all the DLCs, TCDD is the most potent congener owing to its acutely toxic nature. Historically, dioxins were released into the environment in very small quantities as a result of combustion processes such as burning of fossil fuels and through geological activities such as volcanic eruptions (NRC, 2003). In the industrialization era, dioxins were majorly produced as a contaminant during the processes of herbicide manufacture for example; 2, 4,-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) used as a broad spectrum defoliants

(Poland and Knutson, 1982). The process of production of chlorophenols, which were used as fungicides during manufacture of paper pulp, also led to release of dioxins into the environment. Other sources of dioxin emission include industrial burning of steel and ceramic, landfill fires, backyard burning of trash and incineration of municipal solid waste (NRC, 2003).

Dioxins, which are released into the environment as a result of human activity, have been shown to be toxic to human and animal life. The toxicity of dioxins is higher due to their lipophilic nature and the tendency to bio-accumulate in the environment. Hence, consumption of fatty foods is thought to account for over 90 percent of the body burden of dioxin in the human population (Viel et al., 2000). Dioxin toxicity in humans has been the subject of numerous evaluations in the past several years. Though the levels of dioxins have declined, public concern still persists with respect to the potential adverse effects of TCDD exposure in sensitive and high exposed populations. TCDD elicits a broad spectrum of species and tissue-specific toxic and biochemical effects. Hepatotoxicity, immunotoxicity including lymphoid involution, dermal toxicity, teratogenicity, tumor promotion and wasting syndrome are some of the hallmark toxic effects seen in rodents and other laboratory animals. The biochemical effects include alterations in proliferation and differentiation, endocrine homeostasis and the induction of cytochrome P450 enzymes (Denison et al., 2011). Not all sequela are seen in a given species and neither do all species show the same toxic effect. Animals are known to have varying sensitivities to the toxic effects of TCDD owing to the rate of metabolism of TCDD. The median lethal dose or LD50 for TCDD varies over a 5000-fold range between the very sensitive guinea pig (LD50 ~ 1μg/kg) and the tolerant Syrian hamster

(LD50 ~ 5000μg/kg) (Poland and Knutson, 1982). The variation in strain-sensitivity of aromatic hydrocarbons was illustrated by using inbred mouse strains. The C57BL/6 strain was classified as a “responsive” strain owing to ability of 3-methylcholanthrene (3-MC) to induce hepatic microsomal aryl hydrocarbon hydroxylase (AHH) activity whereas; the DBA/2 strain was identified as a “non-responsive” strain due to an inability to induce AHH activity (Nebert et al., 1972; Poland and Glover, 1974). The decreased ability to bind to these chemicals was thought to depend on a defect in the inducer-binding site of the non-responsive mouse (Poland and Glover, 1974). The genetic locus governing this trait was later identified to be the *Ah* (Aromatic hydrocarbon) locus. The allele conferring responsiveness was denoted as Ah^b (*b*- C57BL/6 mice) or Ah^d (*d*-DBA/2 mice). Similar effects were observed when C57BL/6 or DBA/2 mice were treated with TCDD although TCDD bound to the same locus with a 30,000-fold higher affinity than 3-MC. The C57/BL6 mouse strain was found to elicit CYP activity with 10-fold higher affinity to TCDD than the DBA/2 mouse (Okey et al., 1989). The inheritance of AHR-mediated CYP inducibility is an autosomal dominant trait (Nebert et al., 1972) and is thought to be the basis for TCDD-induced toxic as well as biochemical effects. These findings led to the search for a receptor that initiated the biological response, which is known to be Ah receptor or the aryl hydrocarbon receptor.

The Ah receptor was first identified in hepatic cytosol by Poland, Glover and Kende in a seminal paper which showed that (^{3}H)-TCDD bound with the highest affinity (K_D of 0.27nM) in the C57BL/6 mice and with much less affinity to proteins present in the hepatic cytosol from the DBA/2 mice (Poland et al., 1976). Subsequently, using the photoaffinity ligand for Ah receptor (^{125}I)-2 azido-3-iodo-7, 8-dibromodibenzo-*p*-dioxin,

the Ah receptor was purified from the rat liver (Perdew and Poland, 1988). At that time, the location of Ah receptor within the cell was unknown but experiments performed using (^{3}H)-TCDD produced binding peaks in both the nuclear and cytosolic fractions (Okey, 2007a) thereby suggesting nuclear translocation of the ligand-bound Ah receptor. Through genetic analysis, it was implied that TCDD could elicit its toxic and biochemical responses by changing the expression of specific target genes. The differences in biochemical responses between different species were attributed to the polymorphisms in aryl hydrocarbon receptor (AHR). The mouse strain C57BL/6 carries the Ahr^{b} allele encoding Ala at codon 375 and the DBA/2 mouse strain carries the Ahr^{d} allele encoding Val at codon 375. Currently, it is accepted that AHR mediates most, if not all, of the toxic and biochemical effects following TCDD exposure. AHR is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors. The bHLH domain is responsible for DNA binding whereas the PAS-B domain is responsible for ligand binding. The glutamine-rich C-terminal domain is important for transactivation as the different coactivators and corepressors are found bound to that domain of AHR.

Historically, the molecular mechanism of action by which TCDD elicited its toxic effects was determined by studying the ability of TCDD to induce CYP1A1 expression. The current model of the mechanism of action of TCDD and the AHR pathway is shown in Figure.1.2. It is believed that TCDD and other AHR agonists can enter the cell via diffusion being highly lipophilic compounds. Upon entering the cytoplasm, TCDD binds to the cytosolic AHR. AHR is normally seen to be in an inactive multiprotein complex with two molecules of heat shock protein 90 (hsp90), Ah receptor-associated protein-9

(ARA-9) and the co-chaperone protein p23 in the cytosol (Bell and Poland, 2000; Carver et al., 1998; Hankinson, 1995; Petrulis and Perdew, 2002). Upon ligand binding, the AHR undergoes a conformation change, which exposes the N-terminal nuclear localization sequence facilitating translocation of the ligand-bound AHR to the nucleus. The chaperone proteins are thought to prevent premature translocation of AHR to the nucleus but the exact cellular compartment for dissociation of chaperone proteins is still a matter of debate. Once in the nucleus, the AHR heterodimerizes with its binding partner Aryl hydrocarbon receptor nuclear translocator protein (ARNT) thus transforming the AHR into a high affinity DNA binding protein. The TCDD: AHR: ARNT complex can then bind to specific DNA recognition sites called 'dioxin response elements' (DRE) which are located within the regulatory regions of dioxin-sensitive genes thereby regulating their expression (Hankinson, 1995). Binding of the AHR at DREs is dependent on different coactivators resulting in a wide array of genes that are regulated upon exposure to TCDD. AHR-mediated changes in gene expression are a result of DNA bending chromatin and nucleosome displacement and increased accessibility to transcriptional activators or corepressors (Beischlag et al., 2008; Hankinson, 2005; Whitlock, 1999). This gene regulation event is terminated by dissociation of the ligand-bound AHR: ARNT complex, followed by export of AHR into the cytoplasm and its subsequent degradation via the 26S ubiquitin-proteasome pathway (Roberts and Whitelaw, 1999). The AHR repressor (AHRR) is a ligand-independent AHR-like protein that attenuates the persistent activation of the AHR pathway by forming complexes to DREs and by decreasing the nuclear AHR concentration (Baba et al., 2001). Among the many adverse effects of TCDD, the

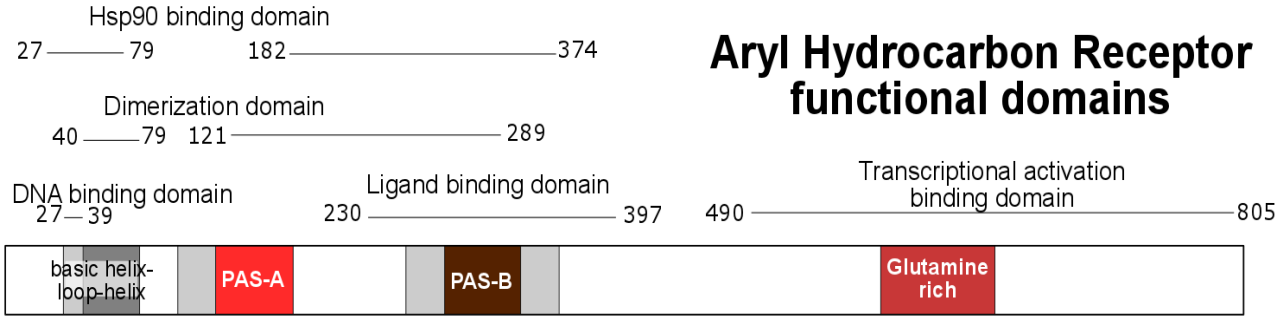


Figure. 1.3. General domain organization of the Aryl Hydrocarbon Receptor

Figure illustrates the different domains of AHR. The PAS-B domain is involved in binding to the ligand TCDD. Binding to hsp90 involved both bHLH domain and the PAS A and B domains. Transactivation function involves the C-terminal region. This domain shows the highest structural variation across different species.

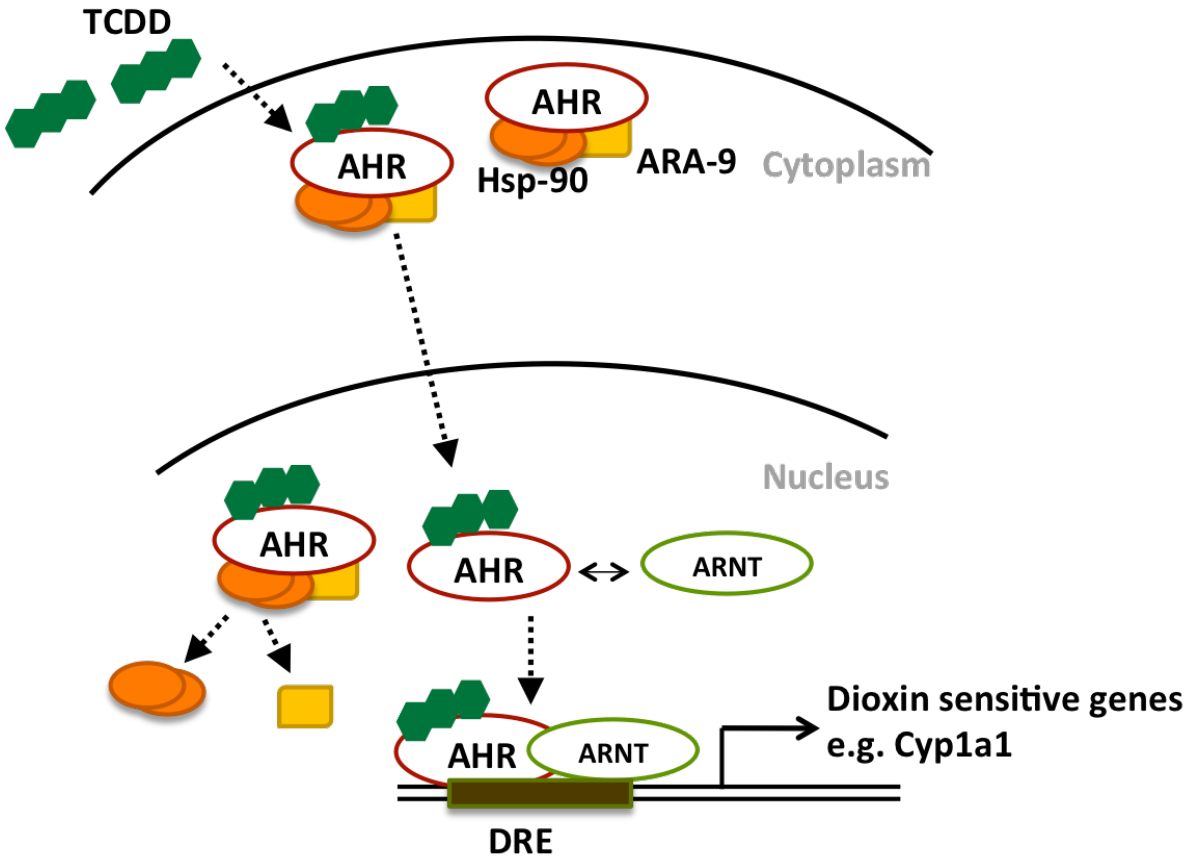


Figure.1.4: Schematic representation of the TCDD-AHR pathway

The figure illustrates the general TCDD-AHR pathway. TCDD diffuses across the cytoplasm and binds to the AHR. AHR is present in a cytosolic complex with 2 subunits of hsp90 and ARA9. Binding to TCDD induces a conformational change in AHR resulting in the translocation of AHR to the nucleus. In the nucleus, the TCDD:AHR complex binds with its binding partner ARNT and regulates expression of dioxin-sensitive genes. In the figure : DRE- Dioxin Response Element, AHR- Aryl Hydrocarbon Receptor, ARNT- Aryl Hydrocarbon Receptor Nuclear Translocator.

effects on the liver or immune system are observed several hours or days after exposure to TCDD. It is hypothesized that these toxic effects are elicited by the continuous alterations in gene expression generated by repeated activation of the AHR pathway. On the contrary, the low-affinity ligands of AHR result in transient activation of the AHR pathway. The importance of AHR in driving toxic responses has been demonstrated extensively by the generation of AHR null mice (Bunger et al., 2003; Gonzalez and Fernandez-Salguero, 1998; Mimura et al., 1997). These studies suggest that presence of AHR, its nuclear translocation and DRE binding is essential for TCDD to produce its major toxic effects.

There is increasing evidence for AHR-independent effects of TCDD mostly due to associations of the TCDD: AHR complex with other cytosolic proteins and activation of early signaling cascades (Matsumura, 2009). Also some recent studies have suggested an AHR-dependent but DRE-independent mechanism of action of TCDD (Dere et al., 2011; Lo and Matthews, 2012). These non-classical mechanisms have been observed in several cell lines and may potentially explain some of the adverse effects of TCDD. In addition to being a xenobiotic receptor, AHR also modulates responses to hormones such as estrogens, androgens, glucocorticoid and thyroid by multiple mechanisms (Denison et al., 2011). Ligand-bound AHR also alters cell cycle progression and proliferation in a cell-type specific manner by binding to hyperphosphorylated retinoblastoma protein and repressing transcription of E2F-dependent genes (Marlowe et al., 2008). An overwhelming number of studies have also demonstrated significant interaction between the AHR and intracellular signaling pathways including the protein kinase, receptor tyrosine kinase, MAPK, Src kinase,

NFκB, cytokine-growth factor among others (Haarmann-Stemmann et al., 2009; Matsumura, 2009; Puga et al., 2009; Tian et al., 2002). All these studies reflect on the complexity and the diversity in cellular responses to AHR agonists and activation of the AHR signaling pathway.

From an evolutionary perspective, the role of the dioxin receptor in normal physiological function has remained enigmatic. AHR is expressed in virtually all vertebrates indicating that AHR has important physiological functions (Hahn, 2002). It is speculated that TCDD toxicity results from an interference of the toxicological pathway with the physiological role of AHR (McMillan and Bradfield, 2007). In the recent years, several endogenous agents such as arachidonic acid, heme and tryptophan metabolites, bilirubin and related tetrapyroles, lipoxin4A and natural flavonoids such as quercetin have been shown to bind AHR at high concentrations (Nguyen and Bradfield, 2008; Okey, 2007b). The development of AHR null mouse models and more recently the AHR null rat have suggested that AHR has intrinsic roles in development (Harrill et al., 2013). With respect to the immune system, AHR promoted differentiation and expansion of Th_{17} cells and was essential for production of IL-22 (Quintana 2008, Veldhoen, 2008). AHR also plays an important role in the development of natural and induced T_{reg} cells, Tr1 cells and dendritic cells (Quintana and Sherr, 2013) and stem cells (Sherr and Monti, 2013). These studies emphasize the role of AHR as a critical regulator of normal cellular development.

Immunotoxicity of TCDD

The immune system is also particularly sensitive to TCDD-toxicity and the effects of TCDD on the immune system are seen in almost all species tested and at doses that do not produce overt cytoxicity *in vivo* (Holsapple et al., 1991). TCDD immunotoxicity has been studied since the 1970s. Vos et al, published some of the earliest reports of the effects of TCDD on the immune system and demonstrated that TCDD could suppress cell mediated-immune responses in guinea pigs and mice (Vos et al., 1973). Around the same time, it was seen that TCDD-treatment led to thymic atrophy and lymphopenia in sub-lethally dosed rats and mice (Harris et al., 1973). Thymic involution, a characteristic TCDD-mediated anomaly was also identified and later determined to be due to suppressed differentiation of thymic epithelial cells, which in turn affect the maturation of T lymphocytes (Greenlee et al., 1985). Subsequently, it was shown that TCDD treatment significantly decreased the number of $CD4^+$ $CD8^+$ T cells in the thymus (Kerkvliet and Brauner, 1990). *In vivo* administration of TCDD also led to a decrease in CTL activity (Kerkvliet, 2002). Overall, it seemed that the effects of TCDD on cell-mediated immunity occurred at high concentrations of TCDD that compromised cellular viability. Single doses of TCDD profoundly suppressed primary and secondary humoral responses to T-dependent and T-independent antigens (Vecchi et al., 1983). One of the first studies demonstrating suppression of humoral responses in cultured lymphocytes treated with TCDD was performed in the early 1980s. These studies showed a selective inhibition of plaque-forming cells following sheep red blood cell (sRBC) or LPS-stimulation without affecting cellular viability. Suppression of *in vivo* IgM responses to sRBC or dinitrophenyl-Ficoll (DNP-Ficoll) or trinitrophenyl-LPS (TNP-LPS)

in presence of TCDD was shown in mice (Dooley and Holsapple, 1988; Tucker et al., 1986). Moreover, direct addition of TCDD to naïve spleen cultures followed by *in vitro* activation with LPS or sRBC led to concentration-dependent suppression of the IgM response (Holsapple et al., 1986a). As the splenocyte preparations contain several cell types, it was thought that the effect of TCDD on the IgM response was due to bystander effects of leukocytes rather than direct effects on the B cells. To refute this concern, separation-reconstitution experiments were performed wherein splenocytes were fractionated into B cell, T cell and macrophage fractions from vehicle and TCDD treated mice and then reconstituted in various combinations in vitro. 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 major cell type affected by TCDD within the context of suppressed IgM responses (Dooley and Holsapple, 1988). Additionally, the direct effects of TCDD on B cells were also determined using purified mouse B cells, which were sensitive to the LPS-induced IgM response (Morris et al., 1993).

To understand if the immunotoxic effects of TCDD were dependent on AHR, two studies were performed; one utilizing the differences in binding affinity for AHR among the different PCDD, PCDF and PCB congeners and the other using congenic mice strains differing at the Ah locus. The structure-activity relationship study provided substantial evidence for a correlation between significant suppression of the *in vitro* antibody response and affinity to AHR (Tucker et al., 1986). The other study using congenic mice strains showed 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 times higher dose to elicit similar toxic effects (Vecchi et al., 1983). Conclusive evidence for the role of AHR in suppression of the primary immune response was obtained upon 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 cells, lacking AHR expression was unaffected by TCDD at 100 times higher concentration (Sulentic et al., 1998). Around the same time, Vorderstrasse et al, demonstrated that presence of AHR was essential for TCDD-mediated suppression of the primary IgM response in mice (Vorderstrasse et al., 2001).

Along with the effects on mature B cells, TCDD has also been shown to be a potent immunotoxicant for hematopoetic stem cells (HSCs). These cells express high levels of AHR, reflecting on a potential endogenous role for AHR in stem cell maturation and development of immune cell precursors. AHR null mice exhibited an increase in the number of Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$ (LSK) population in the bone marrow (Singh et al., 2011) thus suggesting that AHR plays a role in maintaining normal quiescence of HSCs. Another important study highlighted that AHR antagonists could promote expansion of human hematopoietic stem cells (Boitano et al., 2010). Recently, using an *in vitro* model of B cell development from HSCs, it has been shown that TCDD-treatment of human $CD34^{+}$ HSCs leads to a rapid decline in the percentage of $CD34^{+}$ cells and further a significant decrease in the amount of IL-7R expressed on the surface of pro-B cells thereby decreasing the total number of pro-B cells generated (unpublished results, Kaminski Lab). TCDD also impaired the long-term reconstitution ability of the stem cells in bone marrow of irradiated host mice (Singh et al., 2009).

Dioxin immunotoxicity also extends to the innate immune system with effects seen on soluble mediators such as the complement protein C3 (White et al., 1986). TCDD exposure leads to increased susceptibility to several bacterial and viral infections as seen using different host-resistance models (Burleson et al., 1996). Recent studies have also determined that TCDD-treatment increased the expression of MHCII, CD80 and CD86 in dendritic cells in an AHR-dependent manner (Bankoti et al., 2010; Simones and Shepherd, 2011).

For an immune response, activation of the immune cell is deterministic of its fate. Numerous intracellular signaling cascades are triggered immediately upon activation, which could be different if activation occurs several hours or days later. For TCDD immunotoxic effects, it was deemed important to identify the critical window of sensitivity to TCDD. Some reports suggested that the greatest magnitude of suppression was obtained if TCDD treatment occurred 60 min prior to or at the time of activation. Addition of TCDD on day 1 post-activation led to no suppression of IgM PFC response (Tucker et al., 1986). Another report by Holsapple et al. showed that TCDD-mediated suppression of the primary IgM response could be produced only if TCDD was added to splenic cultures 3h prior to LPS activation (Holsapple et al., 1986a). These experiments suggest that TCDD hampers an early B cell activation event thus affecting the differentiation of B cells into IgM secreting plasma cells. The narrow window of susceptibility to TCDD strongly suggests that TCDD alters critical early B cell activation events.

Human health effects of TCDD

Evidence for the effects of dioxins on humans has largely been obtained from epidemiological and occupational studies. There have been several incidences of accidental exposure to dioxin and dioxin-like compounds in the past. One of the more popularized instances of dioxin exposure has been in Operation Ranch hand during the Vietnam War of 1962. A 50:50 mixture of 2,4-D and 2,4,5-T was used as a defoliant in aerial spraying of herbicides. TCDD was a present as a contaminant in 2,4,5-T at a mean level of 2 parts per million (NRC, 1994). The toxic effects of TCDD were only reported by the early 1970s. Around the same time, researchers assessed the effects of TCDD in several other areas contaminated with dioxins such as soil in Times Beach, Missouri wherein dioxin contaminated waste oil was used to minimize the dust on dirt roads. The dioxin contamination in soils near Times Beach was as high as 300 parts per billion that led to an evacuation of the town. The first major event associated with occupational and environmental exposure to TCDD was during an accident at Monsanto's chemical manufacturing plant in Nitro, West Virginia. Trichlorophenol contaminated with TCDD was released as a result of an accident. The workers from this plant developed symptoms of 'chloracne' that involves hyperplastic and hyperkeratotic changes to the skin (NAS, 1994). Chloracne has often been referred as the 'hallmark of TCDD toxicity' in humans (Birnbaum, 1994). Among international incidents of environmental exposures to TCDD, one of the largest accidents occurred in July 1976, in Seveso, Italy where a chemical reactor containing 2,4,5-trichlorophenol contaminated with TCDD exploded generating a chemical cloud releasing half a pound of dioxin in a nearby residential area. In Japan and Taiwan rice oil contaminated with polychlorinated

biphenyls and polychlorinated dibenzofurans led to poisoning (Nakanishi et al., 1985). Apart from such environmental disasters, one of the major ways by which humans are exposed to TCDD is through food consumption. Daily exposure to TCDD is approximately 0.1 to 0.3 pg TCDD/kg/day (Birnbaum, 1994) and adults in the industrialized world have approximately 6 ppt TCDD per ml serum (Aylward and Hays, 2002), which are several folds lower than the exposure due to environmental accidents. A comprehensive reassessment of the concentrations of TCDD in human serum has reported that the levels of dioxins have declined over the past 30 years (Hites, 2011). Nevertheless, concerns surrounding the potential consequences of dioxin exposure on human health still exist due to the persistence of this toxic contaminant.

Some of the epidemiological studies performed in areas of dioxin contamination have examined relationships between occupational or environmental exposure to toxicants and their relative cancer risk. A cohort study conducted in two chemical factories in the Netherlands showed positive associations between a risk for non-Hodgkin's lymphoma (NHL) and TCDD exposure levels (Hooiveld et al., 1998). Four other studies investigating the effects of TCDD exposure on industrial workers showed an association between elevated total cancer rates and increased exposure to TCDD (Cheng et al., 2006; Flesch-Janys et al., 1998; Hooiveld et al., 1998; Ott and Zober, 1996). A case-control cohort based study in Sweden also found an increased risk for NHL upon exposure to herbicides containing phenoxyacetic acid and mixture of 2,4-D or 2,4,5-T and pesticides containing chlorophenols (Hardell and Eriksson, 1999). Alteration in immune status, specifically a 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 in 1968 (Nakanishi et al., 1985). In addition, samples collected from exposed subjects during the Seveso incident found a decrease in IgG levels with increasing lipid-adjusted TCDD plasma concentrations (Baccarelli et al., 2002). A study performed on Korean Vietnam war veterans exposed to Agent Orange also displayed an increase in IgE levels with a skewing of the immune responses towards the Th2 type indicative of increased sensitivity to allergic diseases (Kim et al., 2003). Evidence for incidence of NHL and soft-tissue sarcomas was found in Beçanson in France, an area containing a municipal solid waste incinerator. A logistic regression analysis suggested that individuals living in the highly exposed zones had 2.3-fold higher risk to contract NHL as compared to individuals residing in areas of low exposure (Floret et al., 2003). A study in a Netherlands cohort, measured effects of peri-natal exposure to dioxins and DLCs and reported reduced granulocyte numbers along with an increase in the incidence of infectious diseases like otitis media and chicken pox (Weisglas-Kuperus 1995, 2000). Total dioxin levels in the same study significantly correlated with increased peripheral and $CD8^+$ T cell populations and decreased B cell markers. All these studies provide empirical evidence for associations between environmental exposures to TCDD and DLCs and the incidence of lymphoma and immune defects. But a causal link between TCDD exposure and cancer risk in humans is hard to derive (Boffetta et al., 2011) as several factors could influence epidemiological outcomes. Human variability underlies differences in the responses to environmental chemicals including confounders such as age, body mass, smoking, alcohol consumption, incidence of diseases and genetic make-up (Neubert et al., 2000). 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 influence the results (Baccarelli et al., 2002). Based on these findings it is reasonable to speculate that a possible correlation could exist between TCDD exposure and cancer outcomes; however, the mechanistic underpinnings still pose a question to researchers in the field.

It is imperative to know if human cells exhibit polymorphisms at the Ah locus like described in mouse species, especially for assessment of mechanisms of toxicity in human cells and estimating potential human risk posed by dioxins. Most of the human polymorphisms identified thus far are concentrated in the exon 10 coding for the AHR transactivation domain. 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 (Harper et al., 2002). A combination of three polymorphisms resulting in a haplotype coding for serine at 517, lysine at 554 and isoleucine 570 affected CYP1A1 induction *in vitro* (Wong 2001). Some other polymorphisms have also been reported in the 5' flanking sequence but these have not been associated with alteration of AHR function. Several other polymorphisms remain to be identified and characterized. The human AHR harboring codon 381 encoding valine has a 10-fold lower affinity to bind 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 (Harper et al., 2002; Nebert et al., 2004). It has recently been shown that the polymorphism at codon 554 encoding lysine in place of the arginine is associated with lower AHR, ARNT and CYP1B1 levels; however, the mechanistic basis of the differences between gene expression levels remains to be determined (Helmig et al., 2011). Understanding the role of polymorphisms along with the myriad toxic effects

exhibited by TCDD upon binding to AHR can provide a comprehensive understanding of the biological role of AHR in human cells.

1.4. Effects of TCDD on B cell function

TCDD-mediated suppression of B cell signaling, activation and differentiation

In the past two decades, advancements in research technologies have paved the way for in-depth understanding of toxicity pathways. Classical studies performed in animals have been supplemented with *in vitro* studies focusing on understanding molecular basis of toxicity. Development of cell line- based *in vitro* models has enabled detailed investigations into the transcriptional processes taking place during B cell development.

The CH12.LX mouse B cell line was isolated from a mouse CH12 lymphoma using cloning by limiting dilution (Bishop and Haughton, 1986). The CH12.LX cells could effectively differentiate into plasma cells upon polyclonal activation and were sensitive to low nanomolar concentrations of TCDD (Sulentic et al., 1998). The basal expression of AHR and ARNT in these cells was slightly higher to the levels detected in primary lymphocytes (Williams et al., 1996). The CH12.LX cells also show an increase in AHR protein levels upon LPS-activation in agreement with the previous report by Crawford and co-authors, which showed increased AHR activation upon primary leukocyte stimulation (Crawford et al., 1997). By that time, it was known that the TCDD-mediated suppression of the primary immune response was 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. Binding of TCDD: AHR at the regulatory region of Ig heavy chain decreased the

expression of the μ chain leading to a decrease in IgM protein secretion (Sulentic et al., 2000; Sulentic et al., 2004a). The next couple of studies further illustrated the mechanism underlying suppression of IgM response by focusing on upstream activators of IgM such as Pax-5, Blimp-1, AP-1 and NFκB. LPS-induced DNA binding activity of AP-1 but not NFκB, was markedly inhibited by TCDD in an AHR-dependent manner (Suh et al., 2002). LPS-activated mouse B cells showed elevated expression of Pax-5 in the presence of TCDD thus suggesting that the TCDD: AHR complex affected IgM expression through Pax-5. Concordantly IgH, Igκ, J chain and XBP-1 genes were significantly suppressed in presence of TCDD (Yoo et al., 2004). Blimp-1, a positive regulator of B cell differentiation was significantly downregulated in presence of TCDD along with a decrease in its DNA-binding ability at the Pax-5 promoter. As expected, the binding of AP-1 within Blimp-1 promoter was decreased by TCDD thus suggesting that TCDD altered IgM expression through Blimp-1 and Pax-5 (Schneider et al., 2009). Primary splenocytes from TCDD-treated mice also displayed a TCDD dose-dependent suppression of Blimp-1, XBP-1, Igμ, IgJ chain, Igκ chain along with a decrease in the total frequency of $CD19^+$ and $CD138^+$ plasma cells (North et al., 2009). An increase in BCL-6 levels was also observed in presence of TCDD in primary mouse B cells (North et al., 2010). This *in vivo* study thus highlights the role of TCDD in impairing the LPS-induced primary IgM response and corroborates with the findings from the mouse B cell line. Another aspect of TCDD-mediated suppression of B cell differentiation involves the effect of TCDD on proximal B cell signaling. Some of the earliest studies in the signaling field had shown TCDD treatment increased basal kinase activity (Kramer et al., 1987) and enhanced phosphorylation of kinases in activated B cells (Snyder et al.,

1993). TCDD-treatment also increased membrane protein phosphorylation in B cells. This increase was directly proportional to the suppression of B cell antibody synthesis thereby suggesting that alteration of early kinases could potentially control B cell signaling and differentiation (Clark et al., 1991). Mobilization of calcium is another important signaling event driving proliferation of B cells especially when stimulated via the B cell receptor (Dennis 1987). The phorbol ester and calcium ionophore (PMA/Io) was used to pharmacologically activate BCR signaling pathway and TCDD was shown to attenuate B cell proliferation driven by ionomycin (Karras and Holsapple, 1994). Disruption of calcium homeostasis through delayed elevation of basal intracellular calcium was thought to be another mechanism by which TCDD affected IgM secretion (Karras et al., 1996). The exact mechanism by which these kinases could alter B cell function is not yet known but several hypothesis suggest an abrogation of downstream cellular signaling cascades, alteration in protein localization 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 more recently by North et al, by measuring LPS-induced kinase phosphorylation in presence of TCDD in mouse primary B cells. The results from this study suggested an involvement for AKT, ERK and JNK kinases in elevating BCL-6 expression in presence of TCDD. Moreover, TCDD decreased B cell activation by suppressing MHCII, CD69, CD80 and CD86 expression in mouse B cells (North et al., 2010).

All these studies provide evidence for a multi-faceted mechanism controlling B cell activation and differentiation by TCDD. Genome-wide studies further contributed towards the understanding of molecular pathways by which TCDD and AHR could affect B cell function. 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 gene expression changes downstream. A total of 78 genes were directly bound by AHR and significantly altered in gene expression (increased or decreased) upon TCDD-treatment of LPS-activated CH12.LX cells at 8 and 12h (De Abrew et al., 2010). Signaling networks were reconstructed to connect the 78 new target genes to existing transcriptional network genes 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 nodes and mechanisms including 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 (De Abrew et al., 2010).

Mice have been in the spotlight for *in vivo* immunological experiments for several decades and in many ways are reliable models for human disease research. Despite significant genomic conservation between mice and humans, there exist significant differences in development, activation and response of the innate and adaptive immune systems between mice and men (Mestas and Hughes, 2004). The validity of using mouse models for research has recently been questioned in several articles and reviews (Seok et al., 2013; Shanks et al., 2009). From a toxicological standpoint, it is imperative to know the effects of toxicants on humans especially for risk assessment. A

majority of the toxicological data has historically been obtained using animal studies with a few exceptions of human and epidemiological studies. Human risk assessment decisions based on extrapolation of data obtained from animal studies have been associated with numerous uncertainties including an understanding of dose-response and adverse effects within species (Selgrade, 1999). One way to reduce the uncertainty would involve 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 in the early 1990s. The detection and characterization of AHR in the cytosols from homogenized human tonsils paved the way for human cell-based studies (Lorenzen and Okey, 1991). TCDD-treatment decreased the background proliferation and IgM responses of pokeweed mitogen (PWM)-activated human tonsillar lymphocytes (Wood et al., 1992). A TCDD dose-dependent increase in ethoxyresorufin-O-deethylase (EROD) activity was observed in tonsillar lymphocytes activated with PWM. Resting lymphocytes were mostly refractory to TCDD treatment as seen before, but LPS and T cell replacing factor- activated human B cells demonstrated suppression of IgM secretion in a dose-dependent manner (Wood and Holsapple, 1993). Further studies showed that the toxic shock syndrome toxin- superantigen induced IgM response was sensitive to TCDD in human lymphocytes (Wood and Holsapple, 1993; Wood et al., 1993). Collectively these pioneering studies provided significant impetus to the understanding of the effects of TCDD on human B cells. Recently, Allan and Sherr demonstrated activation of AHR in human lymphocytes and cell lines further paving the way for molecular studies (Allan and Sherr, 2005). In addition, the TCDD: AHR complex was found to bind to DREs in the human CD19 gene

thereby causing a decrease in CD19 expression upon TCDD treatment (Masten and Shiverick, 1995). These studies demonstrated the transcriptional role of AHR in directly impairing human B cell function by down regulating a mature B cell marker, CD19. Yet, a significant data gap existed in the field with regards to understanding 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 model, B cells were activated in a T cell-dependent manner using CD40 ligand and cytokines (Lu et al., 2009). TCDD treatment of human primary B cells increased the expression of AHR-responsive genes such as CYP1A1, AHRR and TIPARP as expected. Concurrent with previous observations, a suppression of the primary IgM response was observed in a TCDD-concentration dependent manner in TCDD-responsive human donors. Three out of twelve donors were refractory to the suppression of the IgM response (non-responders) possibly due to differences in AHR polymorphisms in human donors (Lu et al., 2010). One of striking observations of 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 strategy. It is likely that these differences are a result of an approximately 10-fold difference in the binding affinity of TCDD between mouse and human AHR. In addition it is known that genes involved in the immune response were differentially regulated in mice expressing either mouse or human AHR (Flaveny et al., 2010). This possibly explains the dichotomy observed between mouse and human responses to TCDD. It was also noted that TCDD did not alter the expression of plasmacytic differentiation genes such as Blimp-1 and Pax-5 in human cells as observed in mouse B cells. 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 (Lu et al., 2011). It was also eluded that TCDD could potentially abrogate a proximal signaling event in human and mouse cells but which could lead to varied downstream effects owing to phenotypic differences between mouse and humans. 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 toxic action of TCDD in these two species.

These studies by Lu et al, were pivotal in comprehensively characterizing the immunotoxicity of TCDD in human cells but the mechanism underlying the effects on human cells, specifically human B cell activation remains to be investigated.

Rationale

TCDD is a ubiquitous environmental contaminant and the most toxic congener of the family of compounds termed halogenated aromatic hydrocarbons. Exposure to TCDD is known to suppress the humoral immune response by specifically affecting the processes of B cell activation and differentiation. Decades of research have contributed towards understanding the immunotoxic effects of TCDD in mouse and human cells but several questions regarding the role of specific transcription factors or direct targets of AHR, and detailed mechanisms still remain unanswered. Hence, the overarching goal of my dissertation research was to elucidate the mechanism by which TCDD alters B cell

activation and differentiation. This dissertation research is comprised of three components each centering on individual genes directly involved in regulating B cell differentiation through B cell signaling networks. I am specifically testing the hypothesis that *TCDD impairs B cell differentiation through BTB and CNC homology protein 2 (Bach2) and through decreased B cell activation involving dysregulation of B cell lymphoma-6 (BCL-6) and Src homology phosphatase-1 (SHP-1) in human primary B cells*.

As mentioned earlier, ChIP-on-chip and gene expression microarray studies had previously identified 78 genes that were altered in expression and regulated by the AHR in the presence of TCDD. Of the 78 potential targets, an initial study focused on investigating the role of Bach2 in TCDD-mediated suppression of B cell differentiation. Another gene identified through the same analysis was SHP-1. The regulation of SHP-1 will be studied in context of disruption of B cell activation given the role of SHP-1 in early B cell signaling. The latter part of this study will focus on understanding the role of TCDD in impairing B cell activation through dysregulation of the transcription factor BCL-6. Understanding the mechanism of TCDD action in human B cells will help to develop a better risk assessment framework for dioxins and related compounds.

CHAPTER 2: MATERIALS AND METHODS

2.1. Chemicals and Reagents

TCDD in dimethyl sulfoxide (DMSO) (purity 99.1%) and 1-chlorodibenzo-*p*-dioxin (MCDD) in DMSO were purchased from AccuStandard Inc., (New Haven, CT). DMSO and LPS (*Salmonella typhosa*, Catalog No. L4391-1mg) were purchased from Sigma-Aldrich (St. Louis, MO). The previously characterized BCL-6 small molecule inhibitor 79-6 was purchased from EMD Millipore (Darmstadt, Germany) and used at a final concentration of 100μM *in vitro*. DMSO was used as a solvent for the inhibitor 79-6. The specificity and the activity of this inhibitor are described previously (Cerchietti et al., 2010). Sodium stibogluconate (SSG), also known as sodium antimony gluconate is a potent inhibitor of SHP-1 phosphatases (Pathak and Yi, 2001). SSG was purchased from EMD Millipore and used a final concentration of 10μg/ml *in vitro*.

2.2. Cell lines

The CH12.LX B-cell line, derived from the murine CH12 B-cell lymphoma, has been previously characterized by Bishop and Haughton (Bishop and Haughton, 1986) and was a generous gift from Dr. Geoffrey Haughton (University of North Carolina). CH12.LX cells were grown in Advanced Roswell Park Memorial Institute (RPMI) medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated bovine calf serum (Invitrogen, Carlsbad, CA), 13.5 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 50 μM β-mercaptoethanol. Cells were maintained at 37°C in a 5% CO_2 humidified incubator.

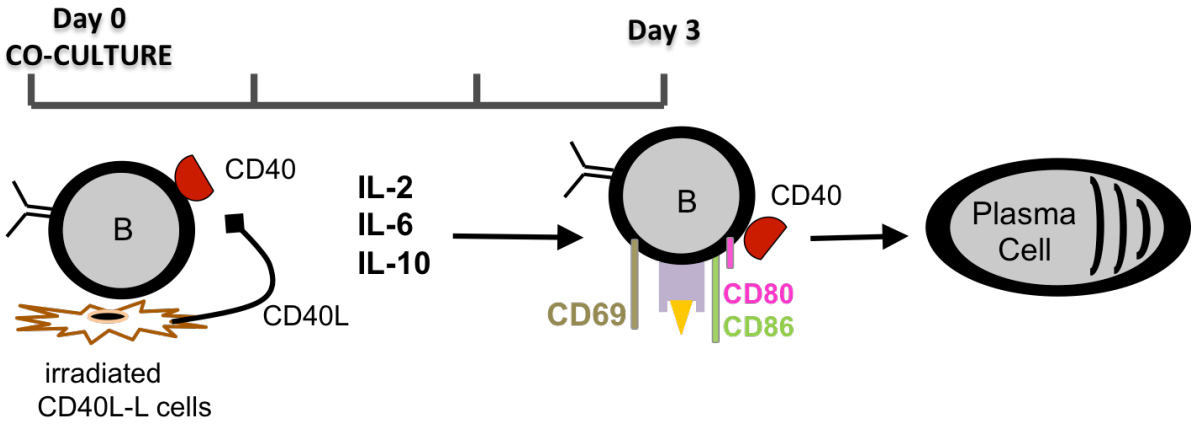


Figure.2.1. CD40:CD40 ligand model of human B cell activation

The figure illustrates the system wherein irradiated CD40 ligand-L cells are co-cultured with naïve B cells in presence of cytokines IL-2, IL-6 and IL-10. Three days post-activation cells are harvested and used for flow cytometry or for qPCR measure ments

CD40 ligand - L cells were obtained as a generous gift from Dr. David Sherr (Boston University). CD40 ligand-L cells are a mouse fibroblast cell line containing the stably transfected human CD40 ligand. The cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% bovine calf serum (ThermoScientific, Lafayette, CO), 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen), 50mM of 2-mercaptoethanol and 1X HT supplement (Invitrogen). CD40 ligand-L cells were thawed at least 4 days prior to use and irradiated with 3500 Gy of X-Rays using X-Rad 320 (Precision X-Ray Inc., North Branford, CT) a day prior to co-culture with B cells. CD40 ligand-L cells were seeded at a concentration of 1×10^4 cells/ml in 500μl of media per well in 48 well tissue culture plates (Invitrogen) for the culture period. Figure.2.1 illustrates the co-culture model used for human B cell experiments. The expression of CD40 ligand on the surface of these cells was monitored routinely to select high CD40 ligand-expressing cells. Human peripheral blood B cells were cultured in RPMI 1640 medium supplemented with 10% human AB serum (Valley Biomedical, VA), 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen), 50mM of 2-mercaptoethanol. In all cases cells, were cultured in 5% CO_2 incubator at 37^0C.

2.3. Flow cytometry

Antibodies used for flow cytometry are as follows: PE anti-mouse/human BCL-6 (Clone: IG191E/A8), PE/Cy5 anti-human CD80 (Clone:2D10), PE anti-human CD86 (Clone: IT2.2), PE/Cy7 anti-human CD69 (Clone: FN50) Biolegend (San Diego, CA), PE

anti-human/mouse BCL-6 (Clone: 603406) R&D Systems (Minneapolis, MN), PE anti-human BCL-6 (Clone : K112-91) BD Pharmingen (San Jose, CA), Alexa-488 p-44/42 (Y202/Y204) pERK1/2 mAb (Clone: D13.14.4E) Cell Signaling technology (Boston, MA) and FITC anti-human SHP-1 (Biorbyt, UK). For BCL-6 proliferation assays, Cell Trace Violet Proliferation kit from Invitrogen Molecular Probes (Eugene, OR) was used. For each staining, approximately 0.5 - 1 x 10^6 cells were harvested at the indicated time points and viable cells were identified by Fixable Live/Dead Near-IR dye (Invitrogen) following manufacturer's instructions prior to cell surface or intracellular staining. Surface Fc-Receptors were blocked using human AB serum before staining for intracellular or extracellular proteins. For surface staining of activation markers CD80, CD86 or CD69, cells were resuspended in FACS buffer (1X Phosphate buffered saline, 1% bovine serum albumin (BSA) Calbiochem (San Diego, CA) and 0.1% sodium azide Sigma (St. Louis, MO) pH: 7.6) in the presence of 20% human AB serum and the specific antibodies were added at the company recommended concentrations and incubated at 4^0C for 15- 30 min. Following incubation with antibody, the cells were washed twice with FACS buffer to remove excess antibody and then fixed by incubation in the BD Cytofix cell fixation buffer (BD Biosciences, CA) for 10 min, washed and stored at 4^0C until they were ready to be analyzed by flow cytometry . For intracellular protein antigen staining, cells that were previously fixed after surface staining were permeabilized with 1X BD PermWash buffer (BD Biosciences, CA) by washing twice and then incubating them for an additional 30 min at 4^0C. Antibodies specific to the intracellular antigens were then added to the cells and allowed to incubate for 30 min at

4^0C. No difference was observed in the trends of surface activation markers due to the effect of fixation followed by permeabilization as opposed to just fixation followed by Flow cytometry analysis. In all cases, cells were analyzed on BD FACSCanto II using FACSDiva software (BD Biosciences) and subsequently analyzed using FlowJo (version 8.8.7, Treestar Software Ashland, OR) or Kaluza (version 1.1 or 1.2, Beckman Coulter Inc., Brea, CA). Unless stated, cells were gated on singlets, live (as determined by Live/Dead dye) followed by gating on lymphocyte populations. Gates were drawn on the basis of unstimulated cells at day 0 (Resting B cells not stimulated with CD40 ligand or IL-2, IL-6 or IL-10) or unstained cells as appropriate.

2.4. Human Leukocyte packs and Isolation of human B cells

Buffy coats collected from anonymous donors were obtained from Gulf Coast Regional Laboratories (Houston, TX). All human leukocyte packs were tested for presence of HIV, HBV, HCV and HTLV before shipment. In brief, blood packs were diluted with HBSS and overlaid on Ficoll-Paque Plus density gradient (GE Healthcare, Piscataway, NJ) and centrifuged at 1800 rpm for 40 min with low brake. The peripheral blood mononuclear cells were isolated post-centrifugation, washed, counted and subjected to a magnetic column-based separation that enables isolation of untouched >95% pure $CD19^+CD27^-$ B cells. This negative selection was conducted using MACS Naïve human B cell isolation kits (Miltenyi Biotec, Auburn CA) following manufacturer's instructions. Purified B cells at a concentration of 1×10^6 cells/ml are then co-cultured with sub-lethally irradiated CD40 ligand-L cells (1×10^4 cells/ml) in a 48 well tissue

culture plate as mentioned earlier. Cells were cultured in presence of recombinant human cytokines IL-2 at 10U/ml, IL-6 at 100U/ml (Roche Applied Sciences, Indianapolis, IN) and IL-10 at 20ng/ml (BioVision Inc., Milpitas, CA) for a period of three or four days depending on the endpoints assayed.

2.5. Quantitative Reverse Transcription PCR (qRT-PCR)

RNA Isolation from mouse CH12.LX cells

Naïve or LPS (10 μg/ml)- activated CH12.LX cells ($2x10^4$ cells/ml in 10 ml of media per 100 mm^2 petri dish) were treated with either 10 nM TCDD and/or vehicle (0.02% DMSO) for 0, 2, 4, 8 or 24 h for time course experiments or 0.1, 1.0 or 10 nM TCDD and/or vehicle (0.02% DMSO) for concentration-response experiments. Total RNA was isolated using the SV40 Total RNA Isolation System (Promega Corporation, Madison, WI) and RNA concentrations were quantified using a Nanodrop ND-1000 spectrophotometer (Wilmington, DE). Double stranded cDNA was synthesized using 500 ng of total RNA using the Applied Biosystems high capacity cDNA reverse transcription kit (Foster City, CA). qRT-PCR was performed according to manufacturer's instructions using the Taqman Universal PCR Master Mix and Taqman gene expression assays for Bach2 (Mm00464379_m1) or Prdm1 (Mm00476128_m1). All qRT-PCR measurements were made on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The change in gene expression was calculated using the $\Delta\Delta C_t$ method using 18S ribosomal RNA (4319413E) as an internal control. For statistical analysis, unpaired two-tailed Student's t-tests were performed between treatments and their corresponding controls.

RNA Isolation from human peripheral blood B cells

RNA was isolated from human peripheral blood B cells using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA from each sample was quantified using Nanodrop ND-1000 Scientific spectrophotometer (Thermo Scientific, Wilmington, DE) and 500ng of RNA from each treatment sample was reverse-transcribed using Applied Biosystems High capacity cDNA reverse transcription kit as per the company's protocol. The cDNA was amplified using Applied Biosystems TaqMan Gene Expression Assays inventoried probe binding to human BCL-6 (Hs00277037_m1), Fbxo11 (Hs00251516_m1), Ptpn6 (Hs00169359_m1), AHR (Hs00169233_m1). All quantitative real-time PCR reactions were performed on an Applied Biosystems model ABI Prism 7900 Sequence Detection System. 18S ribosomal RNA (Applied Biosystems, Foster City, CA) was used as an internal control gene and the fold change in gene expression from the reference was calculated using the $\Delta\Delta C_t$ method as described in Livak and Schmittgen (Livak and Schmittgen, 2001).

2.6. Electrophoretic Mobility Shift Assays (EMSA)

EMSA for Blimp1

Nuclear proteins were isolated from naïve or LPS (10 µg/ml)-activated CH12.LX cells ($2x10^5$ cells/ml in 10 ml of media per 100 mm^2 petri dish) that were treated with 10 nM TCDD or vehicle (0.02% DMSO) for 0 and 4 h as previously described (Andrews and Faller, 1991). Cells were pelleted, washed in cold 1X PBS, resuspended in 400 µl cold buffer A (10 mM HEPES-KOH pH 7.9 at 4^0C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM PMSF), and allowed to swell on ice for 10 min. Samples were

vortex mixed for 10 sec, centrifuged for 10 sec, and the supernatants discarded. The pellets were resuspended in 100 µl of cold buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF) and incubated on ice for another 20 min for high salt extraction. Cellular debris was removed by centrifugation and the protein concentration of the supernatant was quantified using the BCA assay (Sigma-Aldrich, St. Louis, MO). The binding reaction was performed by adjusting the final NaCl concentration to 150 mM by the addition of Buffer C devoid of NaCl followed by incubation of 5 µg of nuclear protein with 0.5 µg of poly dI-dC (Roche, Indianapolis, IN) on ice for 10 min. Following incubation with dI-dC, the double stranded ^{32}P-labeled probes (45,000 cpm of promoter MARE: 5'-ATAGTGGTGCTGACTCAGCATCG-3' or 45,000 cpm of intron 5 MARE: 5'-ATCGAAAATGTGAGTCGGCATAATTAA- 3') were added to the reaction and incubated at room temperature for another 30 min. To assess the specificity of DNA binding activity, the nuclear extracts were incubated with 100-fold excess of unlabeled probe prior to addition of the radiolabeled probe. The resulting protein-DNA complexes were resolved on a 4% polyacrylamide gel in 0.5X TBE buffer (1X = 89 mM Tris, 89 mM borate, and 2 mM EDTA). The gel was then dried on 3 mm filter paper (Whattman, Hillsboro, OR) and autoradiographed. Autoradiograph bands were quantified by densitometry using the UN-SCAN IT software (Silk Scientific, Orem, UT). For the supershifts, the nuclear proteins were incubated with anti-Bach2 (L- 17) antibody sc-14704X or normal goat IgG (sc-2028) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) for 30 min at RT prior to the addition of radiolabeled probes.

EMSA for Bach2

Nuclear proteins for the DRE3 and Bach2 intron1 EMSA were prepared from resting CH12.LX cells treated with 10nM TCDD or 0.02% DMSO as vehicle for 1 h as previously described (Probst et al., 1993; Williams et al., 1996) with modifications. The cells were harvested by centrifugation at 300 x g for 5 min followed by one wash with 1X HBSS and incubated in HB buffer (10 mM HEPES (pH:7.5), 1mM $MgCl_2$) for 10 min. Nuclei were isolated by centrifugation at 1500 x g for 5 min and the pellet was washed twice with MDHK buffer (3 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES and 100 mM KCl) and resuspended in 100 ml of HEDK buffer (25 mM HEPES, 1 mM EDTA, 1 mM DTT, 400 mM KCl) and incubated on ice for 30 min for high-salt extraction. The samples were then centrifuged at 14,000 g for 2 min after an equal volume of HEDG buffer (25 mM HEPES, 1 mM EDTA, 1 mM DTT, 10% glycerol) was added to the supernatants and the proteins were quantified using the BCA assay (Sigma-Aldrich). For the binding reaction, 10 mg of nuclear extract was first incubated with 0.6 μg of poly dI-dC on ice for 20 min. Following incubation, ^{32}P-labeled double stranded probes (50,000 cpm of DRE3: 5'-GATCCGGAGTTGCGTGAGAAGAGC-3' (Denison and Yao, 1991) or 50,000 cpm of Bach2 intron1: 5'-TAATAACACAGCGTGAGCCCTT-3') were added for an additional 30 min at room temperature. The resulting protein-DNA complexes were resolved on a 4% polyacrylamide gel in 0.5X TAE buffer (1X = 6.7 mM Tris, 3.3 mM acetate, and 1 mM EDTA).

EMSA for BCL-6

Human primary B cells were harvested at specific time points and nuclear

proteins from TCDD-treated and VH-treated cells were isolated using standardized protocols as previously described (Andrews and Faller, 1991; De Abrew et al., 2011). 5μg of nuclear protein as estimated using the BCA protein determination assay (Sigma-Aldrich, St. Louis, MO) was used in the binding reaction containing 0.5μg poly dI/dC (Roche, Indianapolis, IN) with a final salt concentration of 180mM NaCl. Double stranded DNA probes specific for the enhancer element within the BCL-6 gene were synthesized and end-labeled by γ-^{32}P-ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) and column purified to remove unbound γ-^{32}P using Illustra Probe Quant columns (GE Healthcare, Piscataway, NJ). The sequence of the BCL-6 probe used is 5' AGGGTCA**TCTTAGAA**CATGAA 3' with the BCL-6 binding site highlighted in bold. The nuclear protein and poly dI/dC reaction and was incubated on ice for 10 min followed by addition of 45,000cpm of the BCL-6 radiolabeled probe and incubation for an additional 30 min at room temperature. The protein-DNA complexes were resolved on a 4% polyacrylamide gel in 0.5X TBE buffer (1X = 89mM Tris, 89mM borate and 2mM EDTA). Following electrophoresis, the gel was dried and exposed overnight for detection. To assess specific DNA binding activity, nuclear extracts were incubated with 100-fold excess of unlabeled probe before addition of the radiolabeled probe. The bands on the autoradiograph were quantified by densitometry using UN-SCAN IT software (Silk Scientific, Orem, UT). For the supershift analysis, nuclear protein and dI-dC reactions were incubated with BCL-6 antibody (C-19 X) SantaCruz Biotechnology Inc., (SantaCruz, CA) for 15 min at RT after addition of the radiolabeled probe. IgG from goat serum (Sigma-Aldrich, St. Louis, MO) was used as the control.

2.7. 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, Invitrogen) at 1 X 10^6 cells/ml for 20 min in dark at 37^0C. The labeled cells are then washed twice with complete RPMI and then the cell density is adjusted as desired prior to treatment of cells with TCDD (30nM) or VH (0.02% DMSO) and activation with CD40 ligand and cytokines IL-2, IL-6 and IL-10. The cells were harvested at Day 1, Day 2, Day 3 and Day 4 for intracellular staining.

2.8. AHR polymorphism study

Genomic DNA was isolated from human leukocytes using DNAeasy Blood and Tissue kit (Qiagen, Inc) with manufacturer's instructions. Three TCDD-responders and non-responders were submitted for AHR exon sequencing. Primers were designed for each exon of human AHR and sequencing was conducted by Functional Biosciences, Inc (Madison, WI). The AHR exon sequence from different donors was compared to the reference exon to identify existing and potentially new polymorphisms.

2.9. Statistical analysis

Statistical analysis to obtain Mean Fluorescence Intensity (MFI) of flow cytometry data was performed within the FlowJo software. Data obtained as percentage of gated cells by flow cytometry were log transformed before performing statistical analysis. Unconcatenated samples were used to calculate statistical significance using one-way

ANOVA followed by the Bonferroni's post-hoc test, Dunnet's post-hoc test. The Fisher's uncorrected least significant difference (LSD) test was used following a significant one-way ANOVA was also used wherever appropriate. Significant outliers were identified using Grubb's outlier test and eliminated from the analyses. For comparison between just the VH and TCDD groups, a Student's two-tailed t-test was employed.

CHAPTER 3: EXPERIMENTAL RESULTS

3.1. Regulation of Bach2 by AHR in TCDD-mediated suppression of B cell differentiation

Identification of Bach2 and confirmation of AHR binding within DRE of Bach2 by EMSA

Bach2 was identified as a potential candidate gene directly regulated by AHR in mouse B cells through a genome-wide study used to characterize AHR binding (De Abrew et al., 2010). In order to verify the ChIP-chip results, a ChIP-qPCR was performed on the same region using mouse B cells treated with TCDD. As expected, increased binding of AHR to the same genomic region was observed in presence of TCDD as compared to VH-control cells (De Abrew et al., 2011). To further confirm binding of AHR to the DRE within intron1, electrophoretic mobility shift assays were also performed using nuclear extracts isolated from TCDD-treated CH12.LX cells. As a control for AHR binding, DRE from CYP1A1 was also used. Figure 3.1.1 illustrates the presence of a TCDD-inducible binding complex at the DRE in Bach2 intron1 at a similar mobility compared to the control DRE probe. Another binding complex with lower mobility of unknown composition is also visible using both probes.

Time Course and Dose Response of Bach2 Expression following TCDD-treatment

The temporal changes in Bach2 expression were evaluated in both LPS-activated and non-activated B cells at 0, 2, 4, 8, and 24 h following TCDD treatment (Figures 3.1.2 A and B). In the absence of TCDD-treatment, LPS activation of B cells resulted in a modest increase in Bach2 mRNA levels at 2 h. The LPS-induced increase in Bach2 expression decreased over the following 24 h period. In the presence of LPS

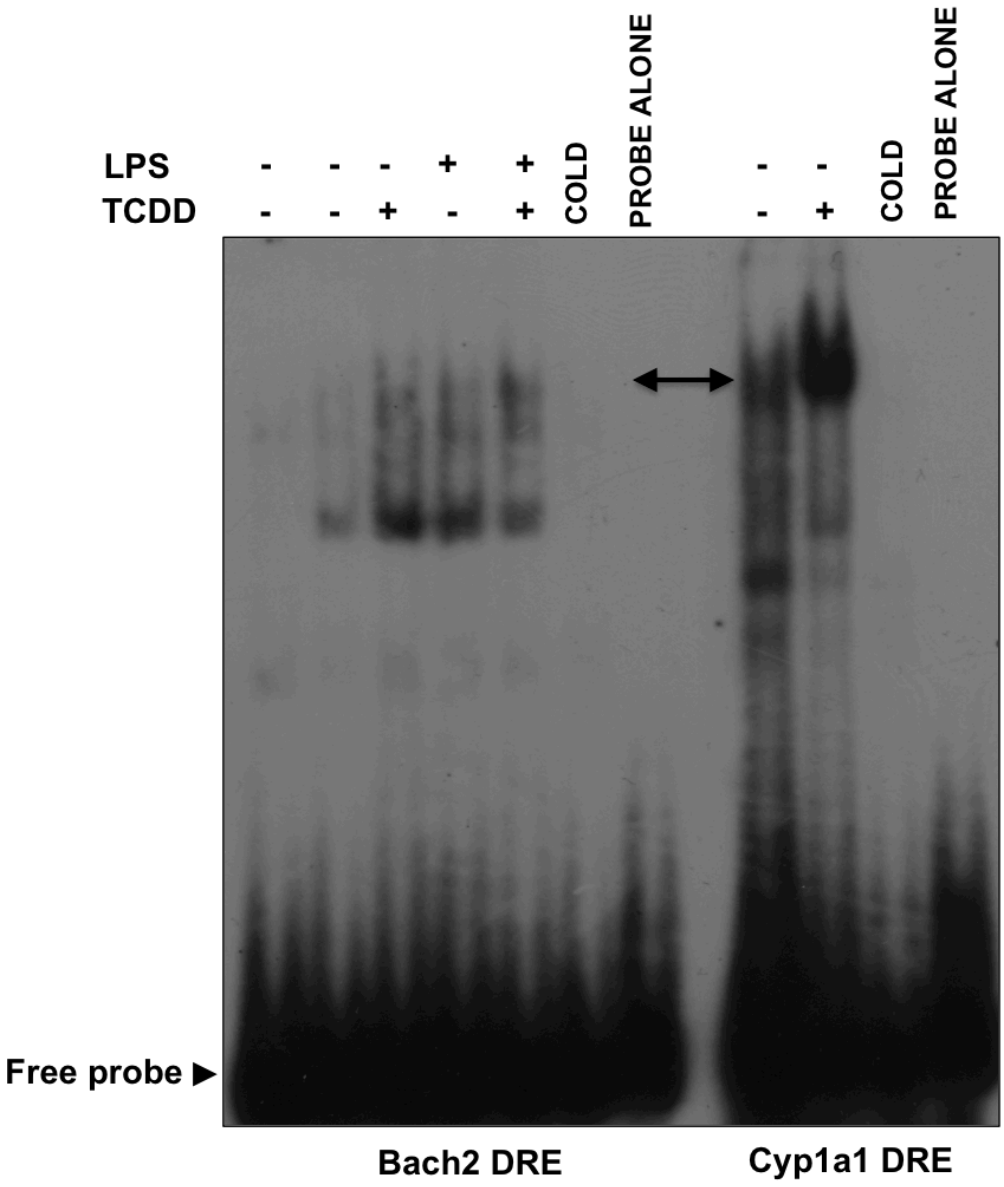


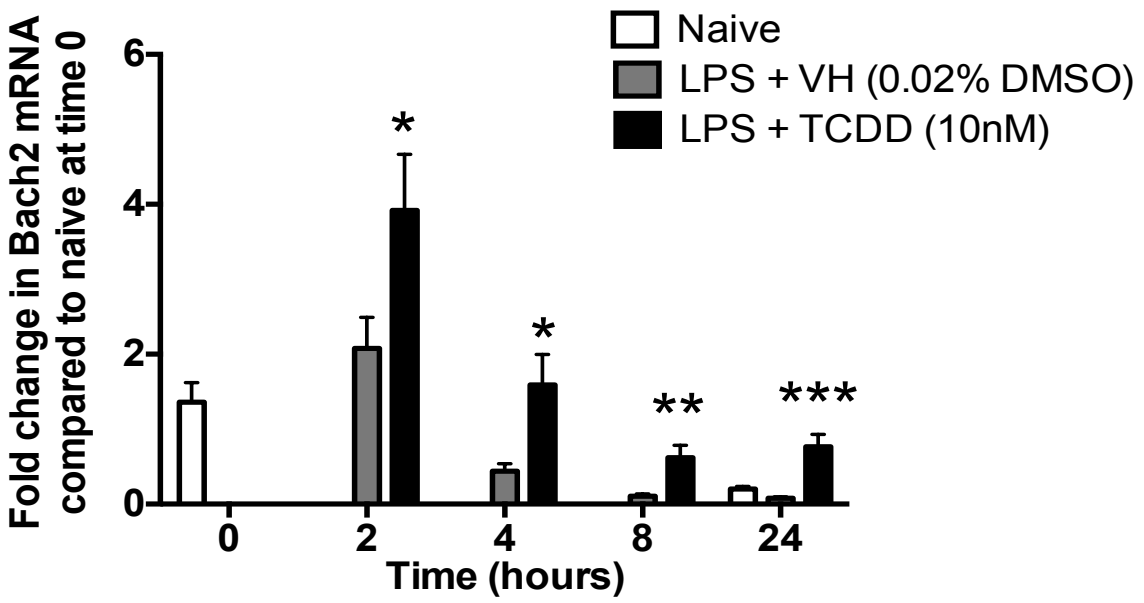
Figure 3.1.1: Confirmation of AHR binding to intron1 of Bach2 in presence of TCDD

B cells were activated with 10 µg/ml LPS and treated with 10 nM TCDD or 0.02% DMSO. Nuclear extracts were isolated 1h post-treatment and used for electrophoretic mobility shift assays. The DRE3 region from CYP1A1 was used as a control. Binding

Figure 3.1.1 (cont'd)

reactions were performed with ^{32}P-labeled Bach2 and DRE3 probes. The arrow in the center of the gel represents the relative location of the binding complexes.

A.



B.

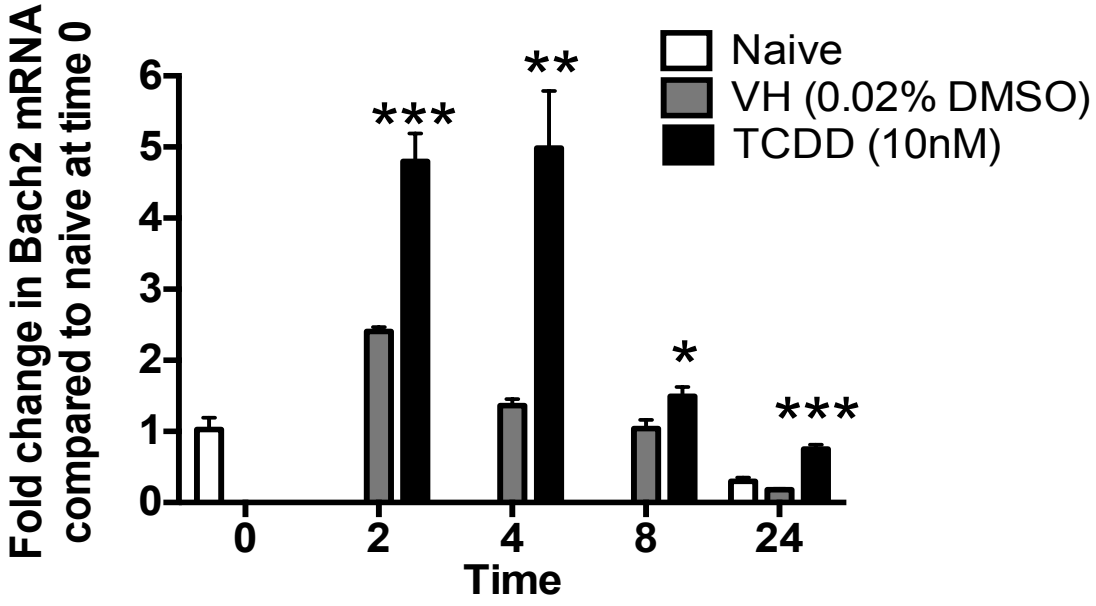


Figure 3.1.2: Time course of Bach2 mRNA levels following TCDD-treatment.

Figure 3.1.2 (cont'd)

A. B cells were activated with 10 μg/ml LPS and treated with 10 nM TCDD or 0.02% DMSO and analyzed for Bach2 mRNA using qRT-PCR at the indicated times. Naive cells were not activated with LPS nor treated with TCDD or DMSO and were analyzed at 0 and 24 h. B. Resting B cells were treated with 10 nM TCDD or 0.02% DMSO and analyzed for Bach2 mRNA using qRT-PCR at the indicated times. Naive cells were not treated with TCDD or DMSO and were analyzed at 0 and 24 h. The data in these graphs (A and B) represent mean ± S.E. of quadruplicate measurements at each time point of three experimental replicates. Comparisons were made between the TCDD-treated group and DMSO-treated group at each time point. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ using unpaired Student's t-test.

and TCDD, Bach2 mRNA showed a significant increase in expression at each time point. A similar temporal profile in both vehicle control cells and TCDD-treated cells was observed for Bach2 mRNA in non-activated B cells demonstrating that LPS activation is not required for the TCDD-induced expression.

Concentration-response changes were examined in LPS-activated B cells at the 4 h time point (Figure 3.1.3). Although no change in Bach2 mRNA was observed for the DMSO (vehicle) control compared to naive cells, Bach2 mRNA levels in the TCDD-treated samples exhibited a concentration-dependent increase in expression with a statistically significant increase at the 1.0 nM concentration.

Time Course Expression of Prdm1.

Given the transcriptional repression of Prdm1 by Bach2, differentiating B cells have shown reciprocal expression patterns for Bach2 and Prdm1 depending on the developmental stage of the cells (Ochiai et al., 2006). To assess whether this reciprocal expression pattern is observed following TCDD treatment, time course gene expression measurements of Prdm1 were also performed using qRT-PCR (Figure 3.1.4). Although Prdm1 expression showed a significant TCDD-induced increase in expression at the 2 h time point, a significant down-regulation was observed at the 24 h time point.

TCDD-inducible DNA Binding in Prdm1 Promoter and Intron 5 Maf Response Elements (MARE)

Bach2 has been shown to dimerize with MafK and bind to two separate Maf response elements (MARE) to repress transcription of Prdm 1. One MARE is within the

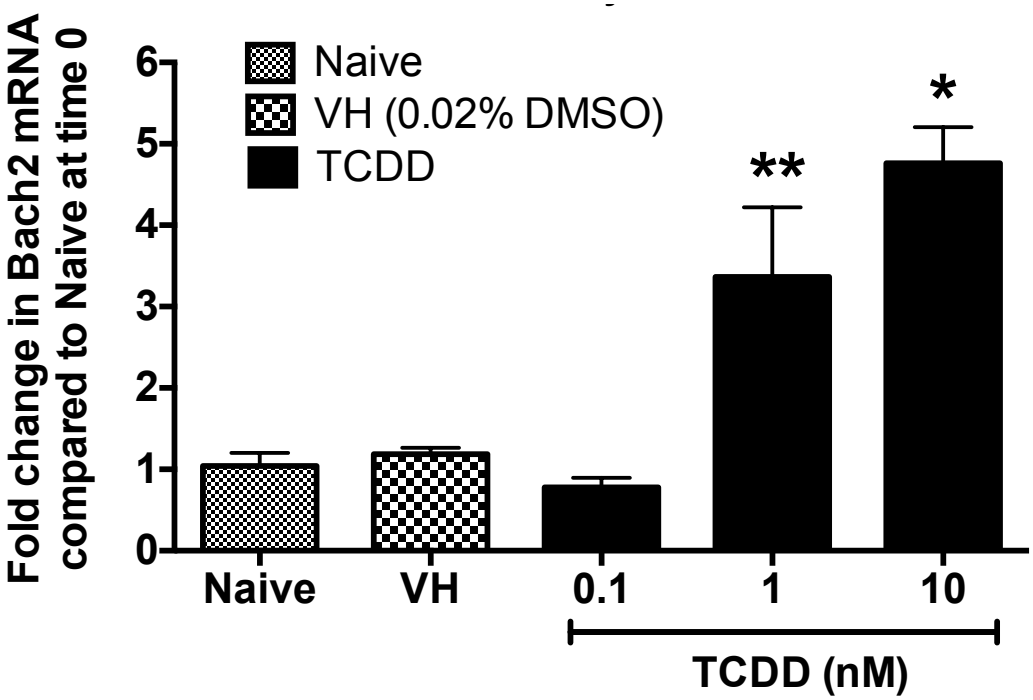


Figure 3.1.3: Concentration response changes in Bach2 mRNA levels

B cells were activated with LPS (10 μg/ml) and treated with 0.1, 1.0, 10 nM TCDD or 0.02% DMSO. Bach2 mRNA was analyzed at 2 h using qRT-PCR. The data in all graphs represent mean ± S.E. of quadruplicate measurements at each treatment group of two experimental replicates. Comparisons were made between the TCDD-treated group and the DMSO group *, $p < 0.05$; **, $p < 0.01$.

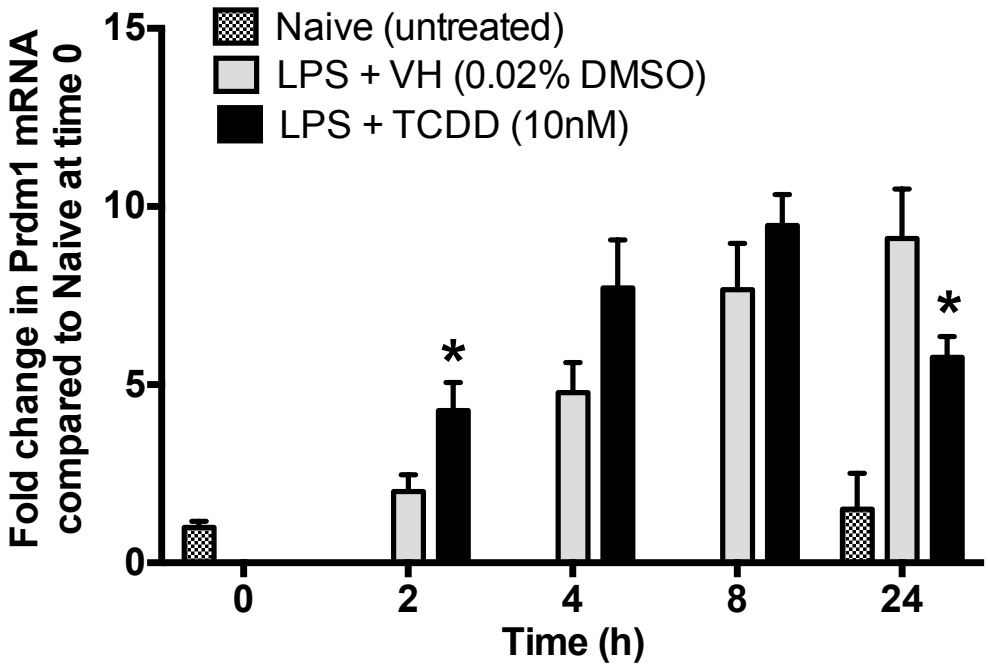


Figure 3.1.4: Time course changes in Prdm1 expression levels in LPS-activated B cells

B cells were activated with LPS (10 μg/ml) and treated with TCDD (10 nM) or DMSO (0.02%). Prdm1 mRNA was analyzed by qRT-PCR at the indicated time. The data represent mean ± S.E. of quadruplicate measurements at each time-point of three experimental repliates. Comparisons were made between the LPS + TCDD-treated group and LPS + DMSO-treated group at each time point. *, $p < 0.05$ using unpaired Student's t-test.

Prdm1 promoter (Ochiai et al., 2006) and the other is within intron 5 (Ochiai et al., 2008b). To evaluate whether TCDD-induced Bach2 expression resulted in increased DNA binding at the two MAREs, electrophoretic mobility shift assays (EMSA) were performed on nuclear extracts from activated and resting B cells treated with TCDD and in LPS-activated B cells with increasing concentrations of TCDD. In the promoter MARE, only a small increase in DNA binding was observed when cells were treated with both LPS and TCDD for 4 h as compared to either treatment alone (Figure 3.1.5 panel A). Maximal DNA binding in the promoter MARE was observed at 0.1 nM TCDD (Figure 3.1.5 panel B). Higher doses of TCDD decreased the amount of DNA binding in the promoter MARE. In the intron 5 MARE, a more robust increase in DNA binding was observed in cells treated with both LPS and TCDD for 4 h compared to either treatment alone (Figure 3.1.6 panel A). In addition, DNA binding in the intron 5 MARE showed a clear concentration-dependent response (Figure 3.1.6 panel B). No clear increase in DNA binding activity was observed for either MARE following TCDD-treatment at the 8 and 24 h time points (data not shown).

TCDD-inducible binding of Bach2 in the intron 5 MARE but not in promoter MARE

In order to identify the TCDD-inducible DNA-protein complexes at the Prdm1 MARE sites, an anti-Bach2 antibody was used for a supershift assay. The TCDD-inducible nuclear protein complex at the intron 5 MARE was partially reduced in presence of the specific anti-Bach2 antibody whereas, loss of binding activity was not observed using the control goat IgG (Figure 3.1.7 panel A). However, the TCDD-inducible DNA- protein complex at promoter MARE was not ablated using the same

A.

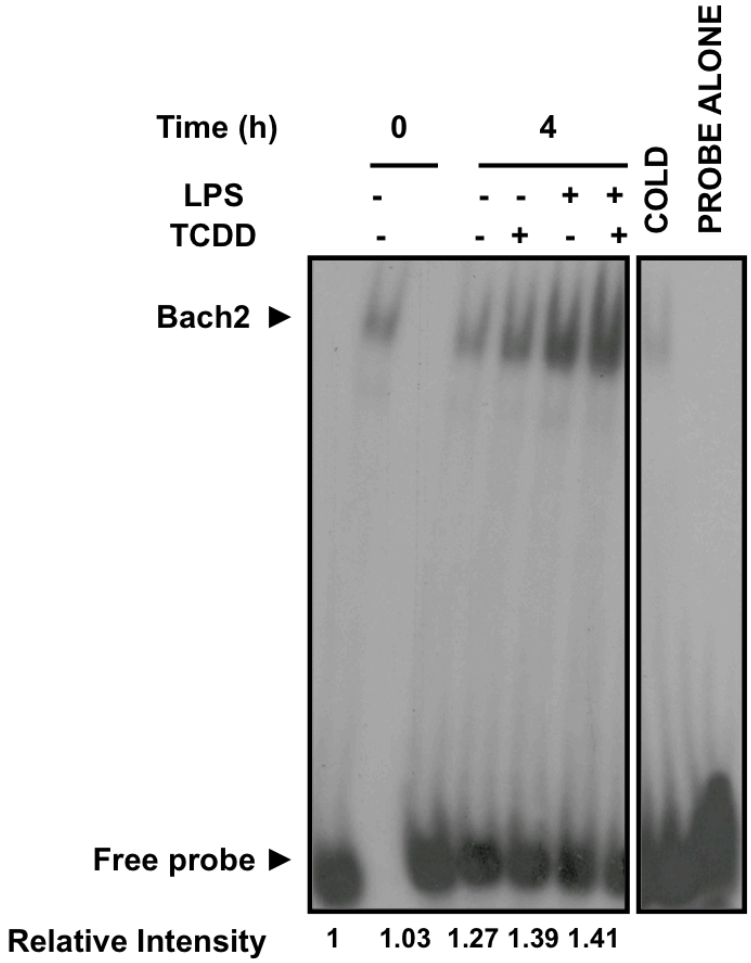


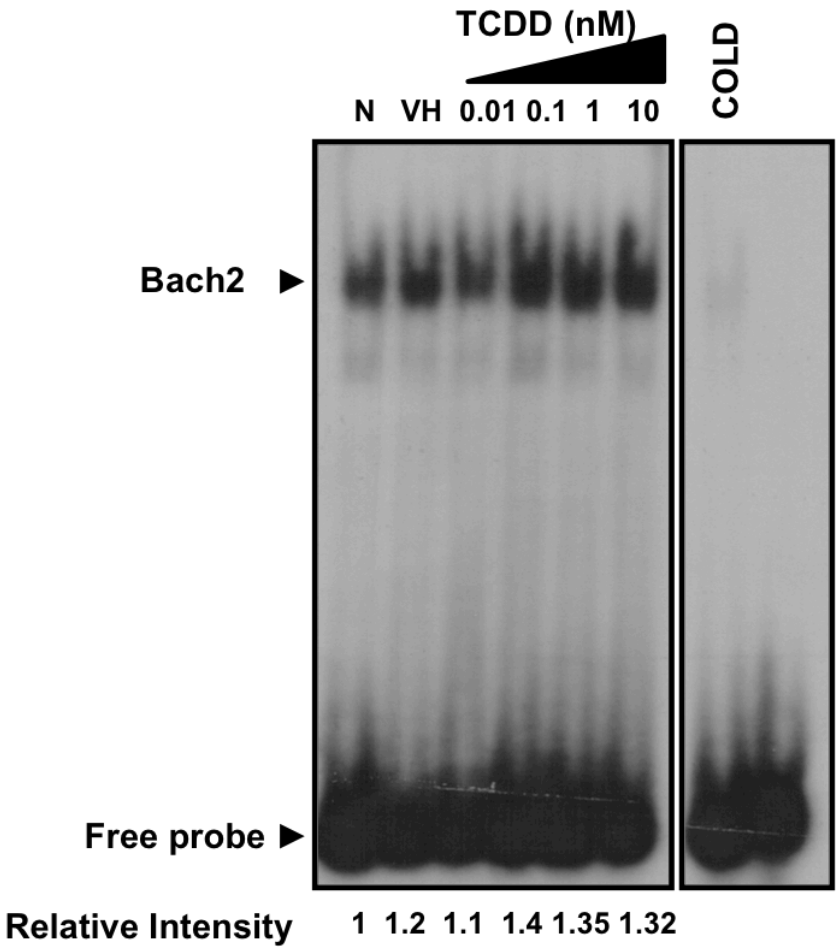
Figure 3.1.5: TCDD-inducible and concentration-dependent DNA binding at Prdm1 promoter MARE

A. Electrophoretic mobility shift assay performed on naive and LPS-activated (10 μg/ml) B cells treated with TCDD (10 nM) or DMSO (0.02%) for 0 or 4 h. The 0 h indicates background binding in untreated B cells. B. Electrophoretic mobility shift assay performed on LPS-activated (10 μg/ml) B cells treated with the indicated concentrations of TCDD or 0.02% DMSO for 4 h. In both assays, binding reactions were set up with ^{32}P-labeled promoter MARE probe as described in experimental methods. The arrow

Figure 3.1.5 (cont'd)

head indicates the TCDD-inducible DNA-protein complex. Results are representative of three separate experiments.

B.



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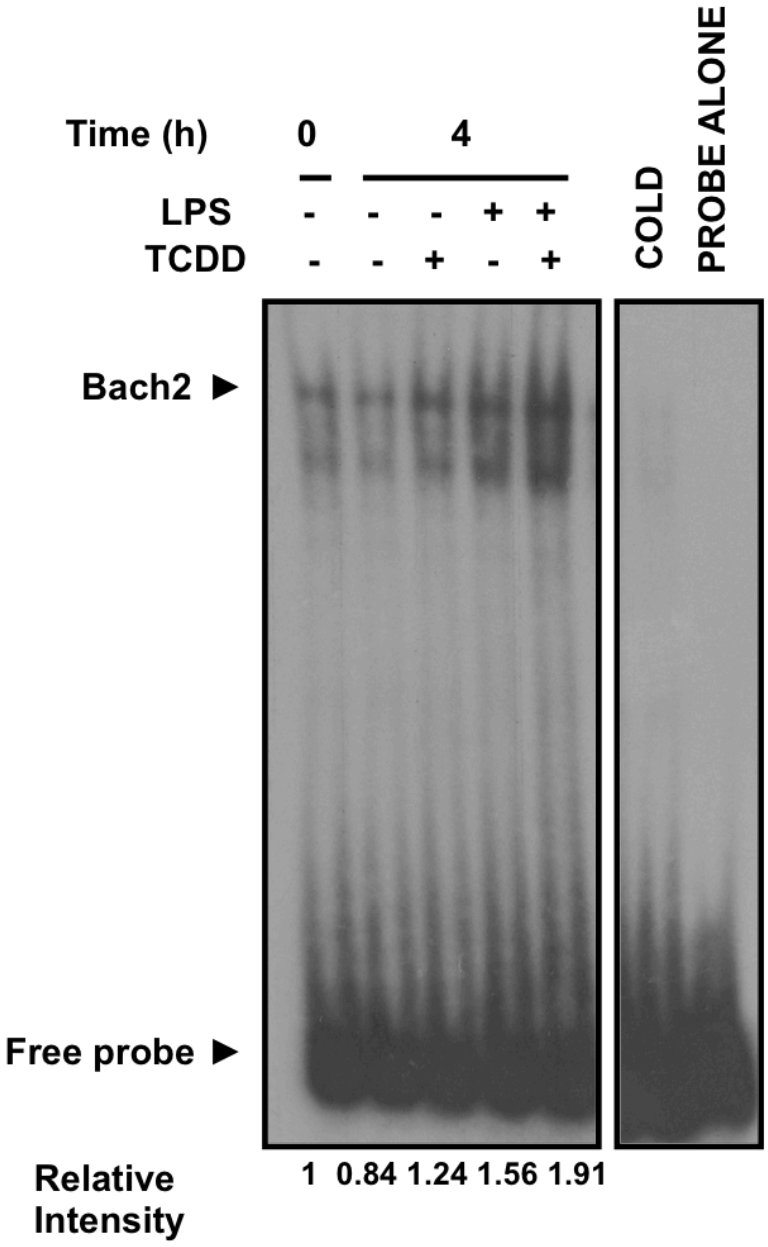


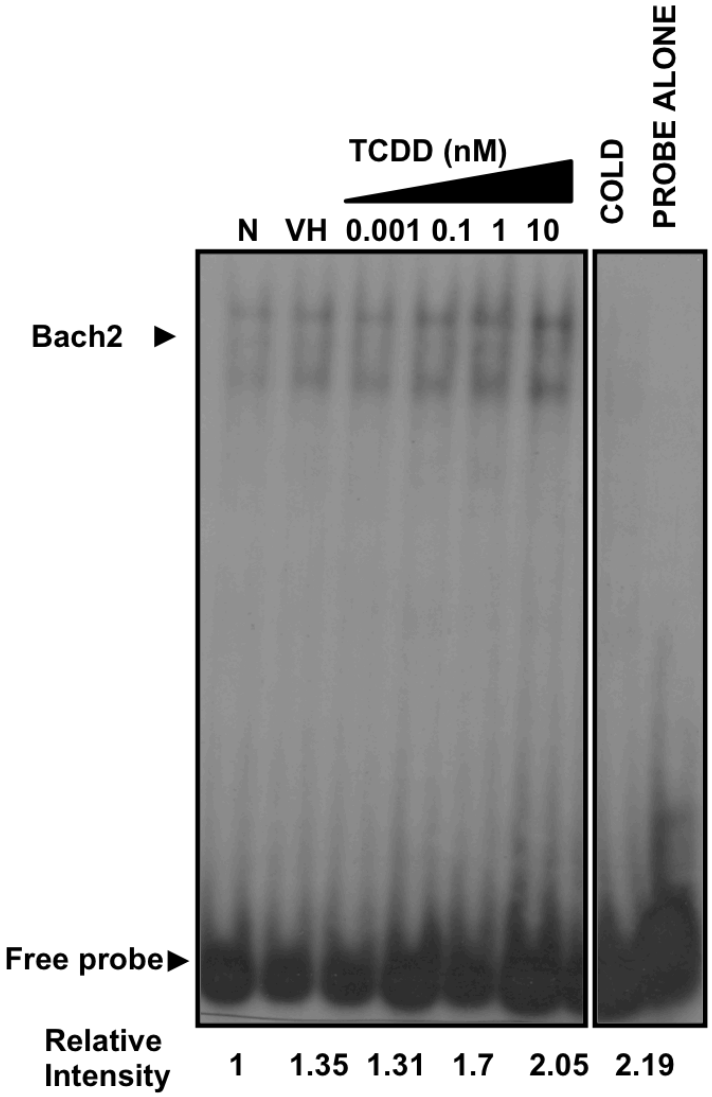
Figure 3.1.6: TCDD-inducible and concentration-dependent DNA binding at Prdm1 intron 5 MARE

A. Electrophoretic mobility shift assay performed on naive and LPS-activated (10 μg/ml) B cells treated with TCDD (10 nM) or DMSO (0.02%) for 0 or 4 h. The 0 h indicates background binding in untreated B cells. B. Electrophoretic mobility shift assay

Figure 3.1.6 (cont'd)

performed on LPS-activated (10 µg/ml) B cells treated with the indicated concentrations of TCDD or 0.02% DMSO for 4 h. In both assays, binding reactions were set up with ^{32}P-labeled intron 5 MARE probe as described in experimental methods. The arrow head indicates the TCDD-inducible DNA-protein complex. Results are representative of three separate experiments.

B.



A.

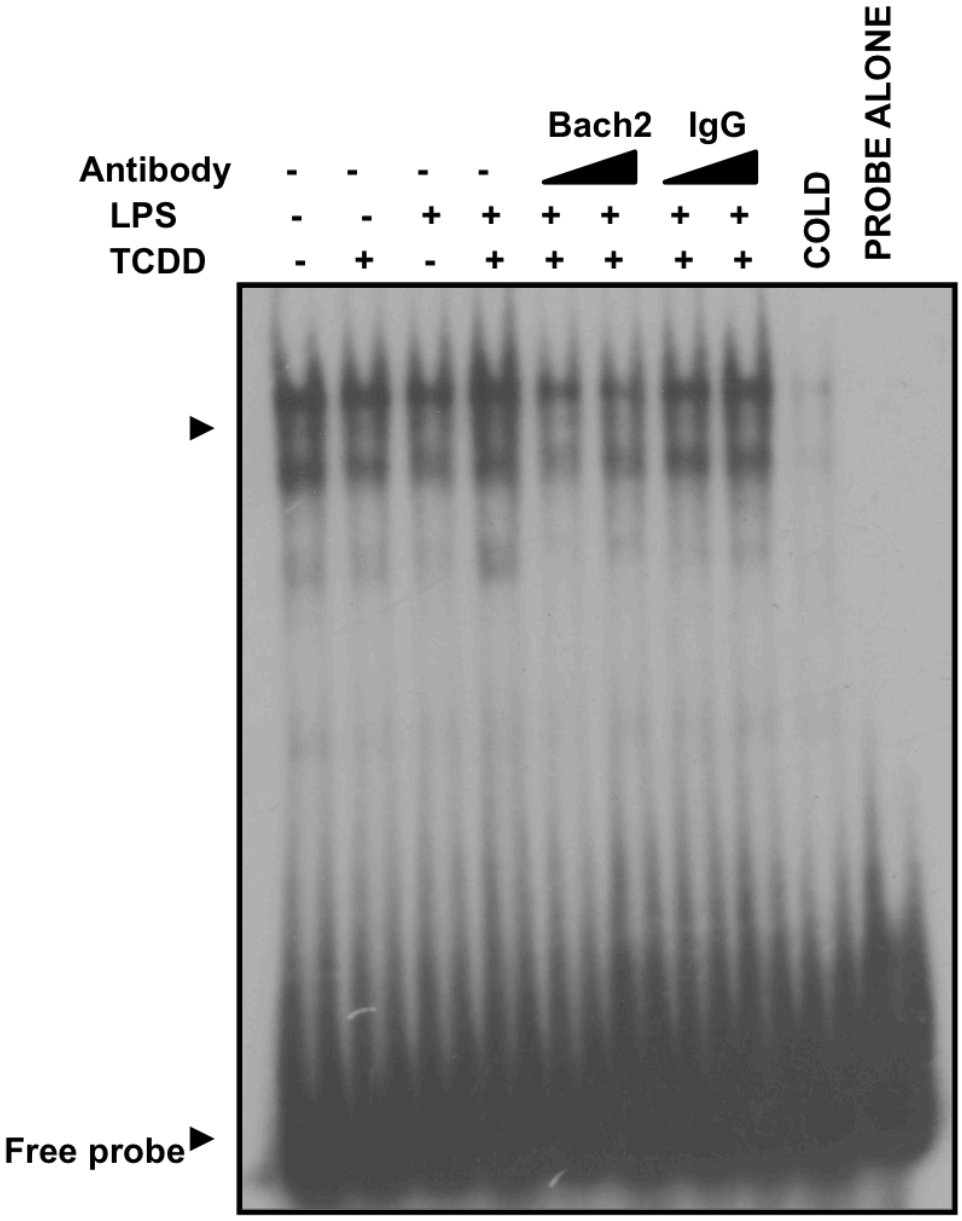


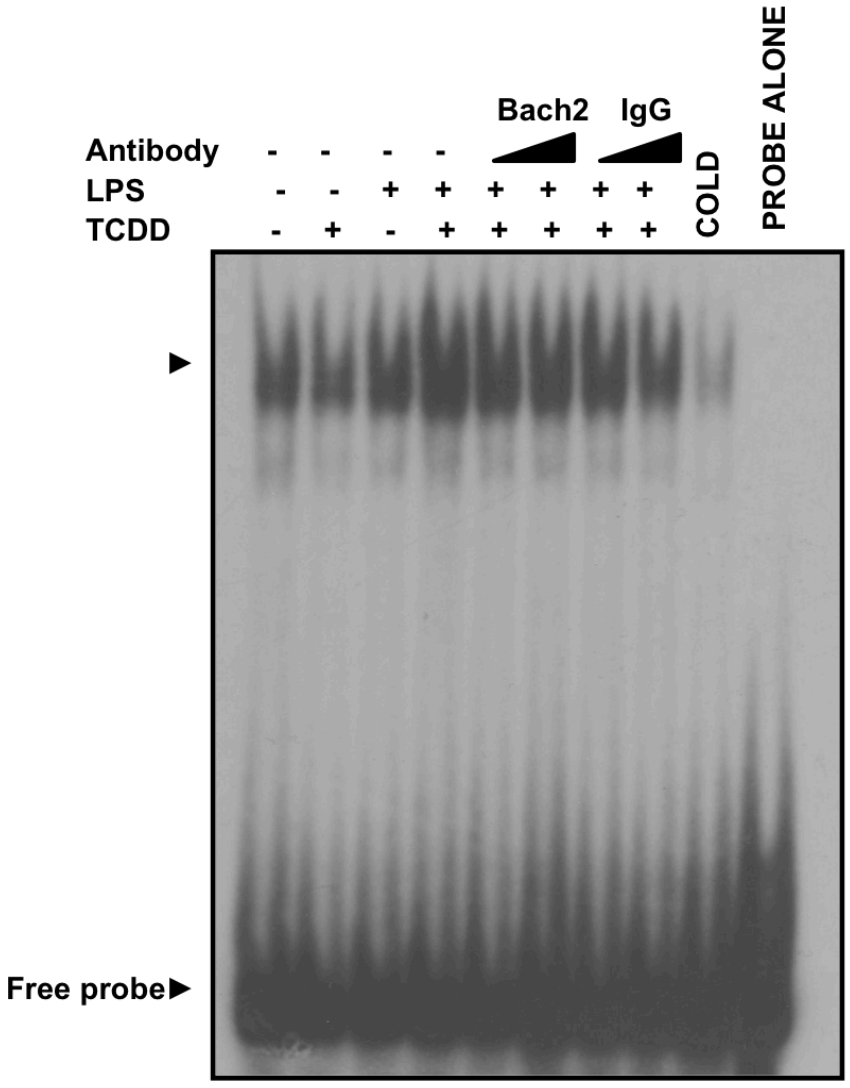
Figure 3.1.7: Supershift analysis of TCDD-inducible binding activity of Bach2 at Prdm1 intron 5 MARE and promoter MARE

Electrophoretic mobility shift assays were performed on resting and LPS-activated (10 μg/ml) B cells treated for 4 h with TCDD (10 nM) or vehicle (0.02% DMSO). Binding reactions were set up with A. ^{32}P-labeled intron5 MARE probe and B. ^{32}P-labeled

Figure 3.1.7 (cont'd)

promoter MARE probe. In both assays, anti-Bach2 antibody at 1 µg and 2 µg (lanes 5 and 6, respectively) and isotype control goat IgG at 1 µg and 2 µg (lanes 7 and 8, respectively) were used. The arrow head indicates the TCDD-inducible DNA-protein complex. Results are representative of two separate experiments.

B.



anti-Bach2 antibody (Figure 3.1.7 panel B lanes 5 & 6) as compared to the control goat IgG (Figure 3.1.7 panel B lanes 7 & 8). Taken together these results demonstrate TCDD-inducible DNA binding activity to both the *prdm1* promoter and the intron5 MARE in CH12.LX cells but specific binding of Bach2 only to the MARE element in intron5 of *prdm1.*

3.2. BCL-6 suppresses B cell activation markers in presence of TCDD in human primary B cells

TCDD treatment alters BCL-6 protein levels in primary human B cells

Sub-optimal activation of B cells leads to a decrease in their viability thereby affecting their proliferation and the primary IgM response (Jelinek and Lipsky, 1983). In a previous study it was seen that TCDD impaired human B cell activation as evidenced by a decrease in the expression of CD80 and CD86 (Lu et al., 2011). To understand the mechanism underlying altered TCDD-mediated impaired B cell activation, the current studies focused on BCL-6 as a putative candidate gene. Hence, BCL-6 protein levels were measured in human primary B cells. In naïve resting B cells the percentage of BCL-6^{hi} cells was low but increased upon activation with CD40 ligand plus cytokines on Day 2 and was further increased on Day 3. Correspondingly, TCDD treatment produced a 5% increase in the proportion of BCL-6^{hi} cells at Day 2 and a 20% increase in the proportion of BCL-6^{hi} cells on Day 3 (Figure 3.2.1 panel A). The mean fluorescence intensity (MFI), which is a measure of the total amount of BCL-6 protein expressed by individual B cells also increased with activation and was significantly higher when

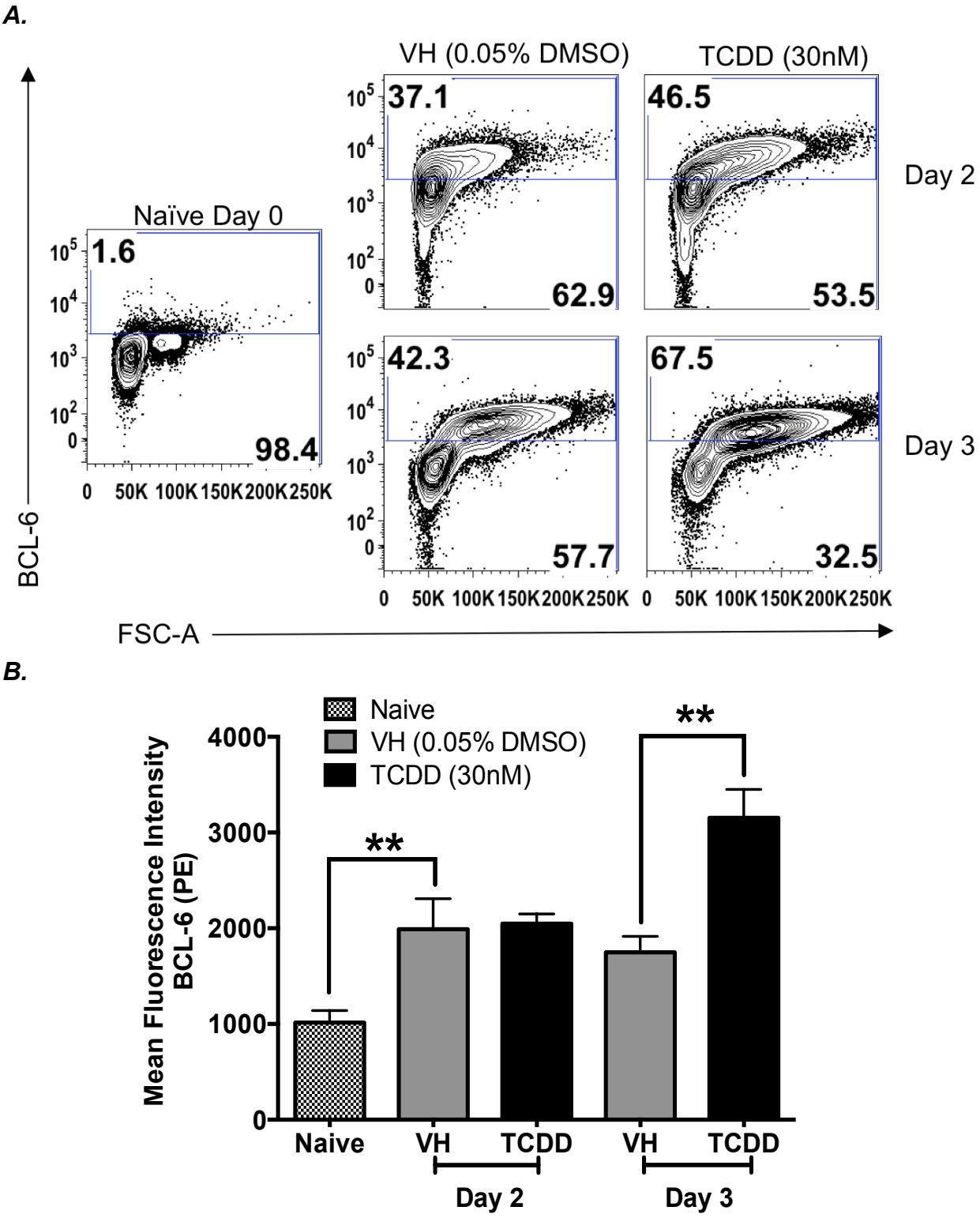


Figure 3.2.1: BCL-6 protein levels in TCDD-treated human primary B cells. A. Human primary B cells were activated with CD40 ligand and cytokines IL-2, IL-6, IL-10

Figure 3.2.1 (cont'd)

and treated with TCDD (30nM) or VH (0.05% DMSO). At the time points indicated above, cells were permeabilized and BCL-6 protein levels were measured by flow cytometry. Dead cells were excluded. The numbers in the left corner represent percentage of BCL-6^{hi} cells. B. Graph depicts mean fluorescence intensity measurements from cells treated with VH and TCDD from the same donor depicted in panel A. ** $p<0.01$ by one-way ANOVA followed by Uncorrected Fisher's LSD test. Data is representative of fifteen TCDD-sensitive human donors.

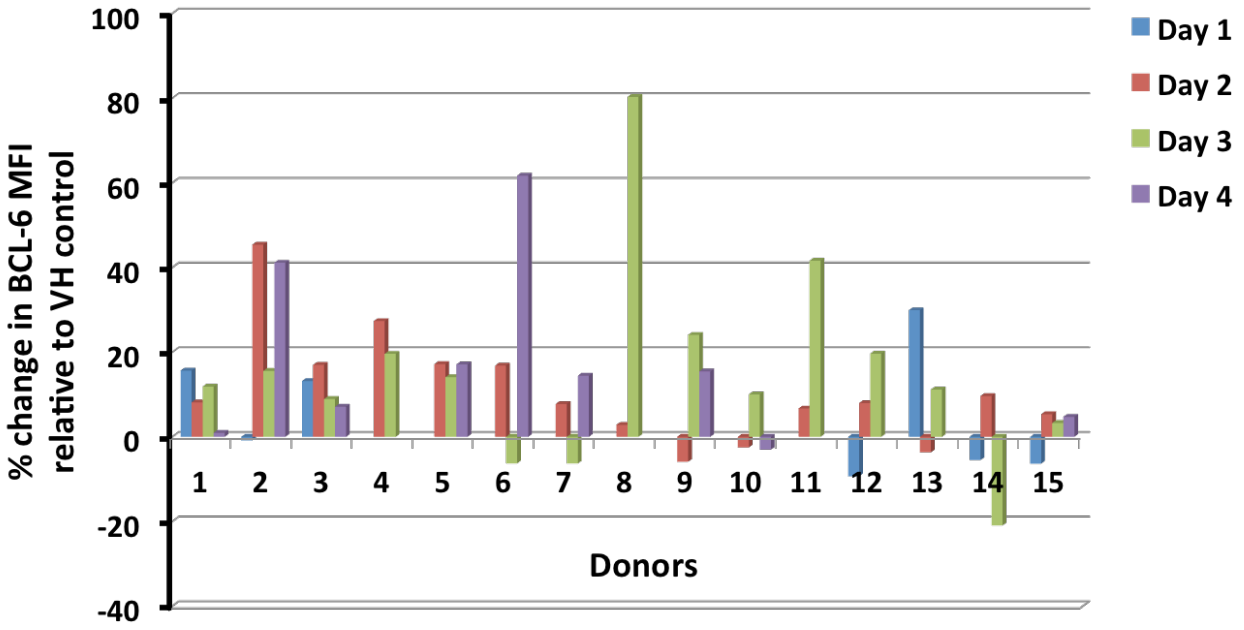


Figure 3.2.2: Relative change in BCL-6 protein levels across fifteen individual human donors treated with TCDD

BCL-6 protein levels were measured by flow cytometry in human primary B cells at the indicated time points and the change in BCL-6 MFI was plotted relative to the respective VH control for each donor. Human B cells in all donors were treated with TCDD (30nM) or VH (0.05% DMSO).

compared to the VH-treated control group on Day 3 (Figure 3.2.1 panel B). Figure 3.2.2 displays the change in BCL-6 MFI relative to the vehicle control in fifteen TCDD-sensitive human donors. The levels of BCL-6 are normalized to the vehicle control of each donor with each donor serving as their own internal control. The overall trend shows at least a 10% increase in BCL-6 levels across fifteen TCDD-sensitive human donors though the magnitude of change and the peak time in BCL-6 levels varied across donors. Due to limitations on the number of B cells that could be obtained from each human donor, all of the time points for each donor could not be assessed. These results show that TCDD-treatment induced a higher level of BCL-6 compared to VH-treated B cells.

The above studies were further extended to determine if BCL-6 protein levels increased in a concentration-responsive manner to TCDD treatment. Figure 3.2.3 panel A shows changes in BCL-6^{hi} proportion of cells in an individual human donor. The increase in proportion of BCL-6^{hi} cells is seen in a concentration-responsive manner at the higher concentrations of 10nM and 30nM but not at the lower concentrations. When the percentage of BCL-6^{hi} cells was measured across 6 TCDD-sensitive human donors, a TCDD-mediated concentration dependent response was not evident owing to differences among individual human donors in background BCL-6 expression (Figure 3.2.3 panel B). mRNA levels of BCL-6 in human primary B cells were also measured; however, no differences were observed between the VH-treated and TCDD-treated groups (Figure 3.2.4). These results are in accordance with previous reports suggesting that alterations in BCL-6 regulation occur at the protein level through changes in

recruitment of BCL-6 to different regulatory regions or protein modifications such as phosphorylation of BCL-6 (Niu et al., 1998b).

Inverse relationship between expression of BCL-6 and activation markers CD80 and CD69

Owing to the repressive action of BCL-6 on B cell activation, it has been observed in the B cell that the expression of BCL-6 and CD80 is mutually exclusive (Niu et al., 2003). To test if BCL-6 and CD80 regulate each other in presence of TCDD, primary B cells from human donors were activated with CD40 ligand plus cytokines and treated with TCDD. BCL-6 and CD80 levels were measured in the same population of cells by multi-parametric flow cytometry. As observed earlier, TCDD-treatment increased the percentage of BCL-6^{hi} cells on day 3 (Figure 3.2.5 panel A). The cells expressing higher levels of BCL-6 exhibited lower CD80 levels as is evident in flow cytometry contour images. Furthermore, figure 3.2.5 panel B shows the MFI of CD80 in BCL-6^{hi} cells in a representative TCDD-sensitive human donor. The expression of activation marker CD80 in the VH group increases upon activation at day 2 and is subsequently higher at day 3 in the BCL-6^{hi} cells. In contrast, cells treated with TCDD, showed a slight increase at day 2 and day 3 but were significantly lower as compared to the VH group at day 3. Conversely, the cells expressing lower BCL-6 levels (Figure 3.2.5 panel C) had significantly higher expression of CD80 in presence of TCDD at day 3. A significant decrease in CD80 MFI within the BCL-6^{hi} cells was also observed in five TCDD-sensitive human donors (Figure 3.2.7 panel A) and trend towards an increase in

A.

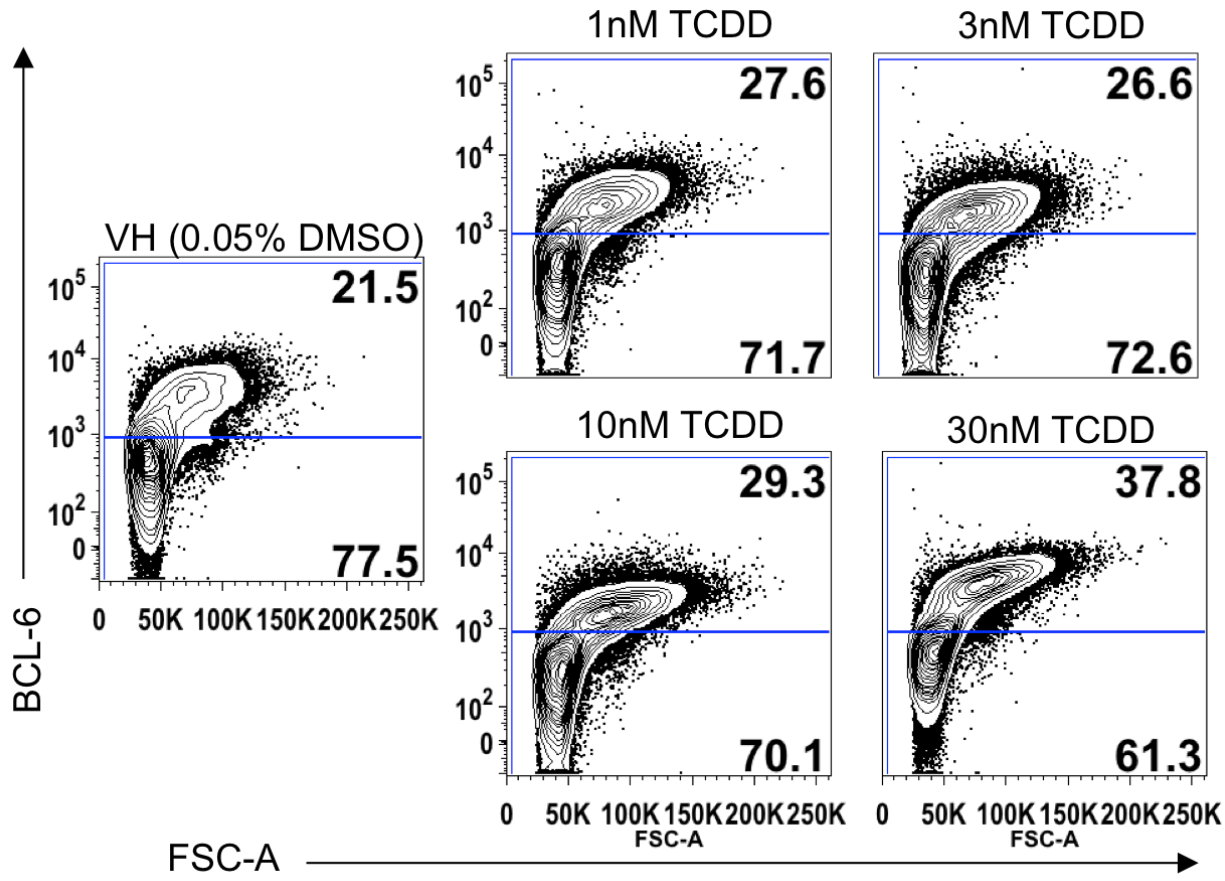


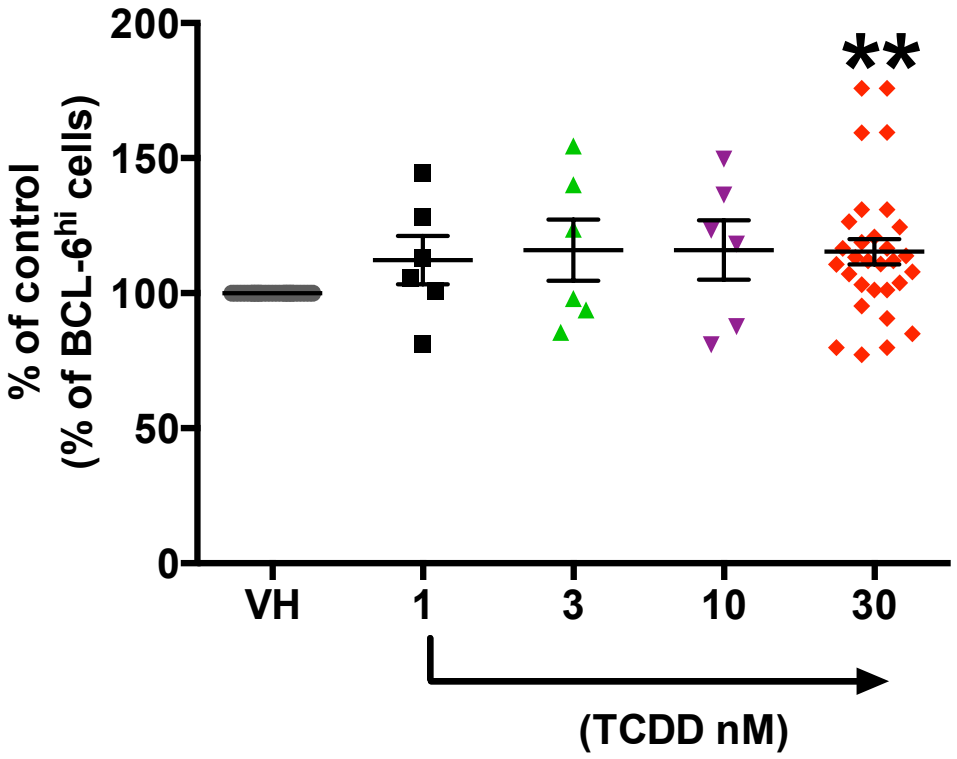
Figure 3.2.3: TCDD-concentration-dependent increase in BCL-6 protein levels in human primary B cells

A. B cells were harvested on day 3 after being activated with CD40 ligand and cytokines and treated with the indicated concentrations of TCDD or VH (0.05% DMSO). BCL-6 levels were measured by flow cytometry. Numbers in the top and bottom corners depict the percentage of BCL-6^{hi} and BCL-6^{lo} cells respectively. Data shown in panel A is representative of six TCDD-sensitive donors. B. Graph represents percent of BCL-6^{hi} cells at each concentration of TCDD. N=6 for TCDD concentrations 1, 3, 10nM and N=

Figure 3.2.3 (cont'd)

31 for VH and 30nM TCDD groups. Dead cells excluded. ** p<0.01 using Kruskal Wallis test followed by Bonferroni's post-hoc test

B.



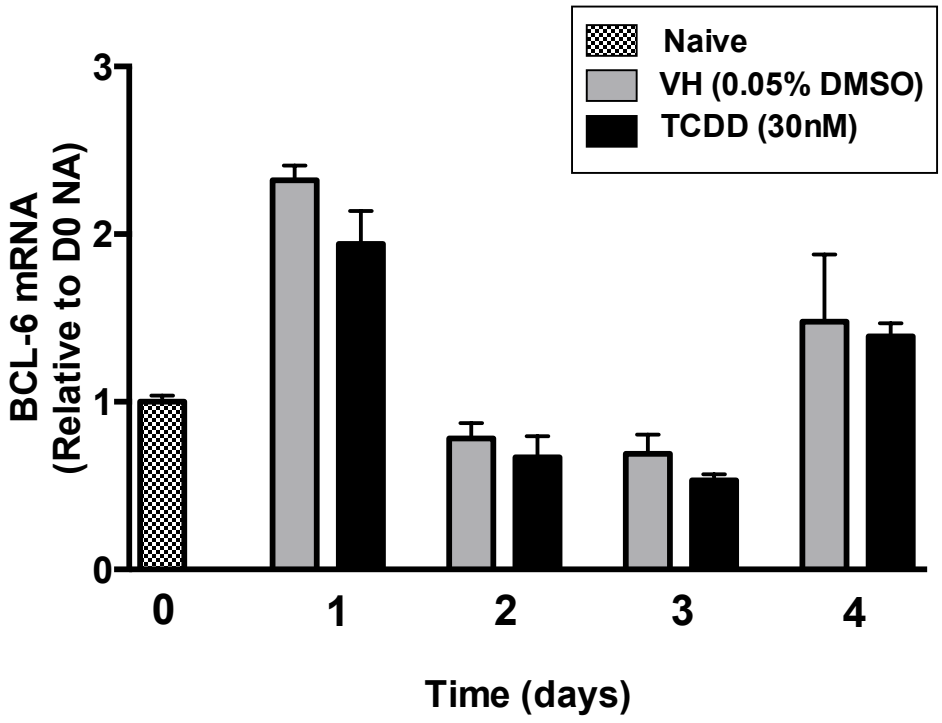


Figure 3.2.4: BCL-6 mRNA levels in primary human B cells unchanged upon treatment with TCDD.

Human primary B cells were treated with TCDD (30nM) or DMSO (0.05%) as the vehicle control for the indicated time. RNA was isolated and quantitative RT-PCR was performed. The data represent mean ± SE of triplicate measurements at each time point. Data shown in the figure is representative of five individual human donors.

CD80 MFI was observed within BCL-6^{lo} cells (Figure 3.2.7 panel B). These results indicate that BCL-6 and CD80 exhibit an inverse expression profile suggesting that BCL-6 could potentially play a role in regulating CD80 in presence of TCDD. The activation marker CD69 was repressed in microarray experiments performed on cell lines deficient in BCL-6 but transfected with BCL-6 overexpressing constructs (Shaffer et al., 2000). Similar to the observations made in figure 3.2.5, TCDD-treatment increased BCL-6 levels with the BCL-6^{hi} cells exhibiting lower CD69 levels (Figure 3.2.6 panel A). CD69 expression in BCL-6^{hi} cells decreased significantly in the presence of TCDD on day 3 (Figure 3.2.6 panel B) and in contrast, was higher in BCL-6^{lo} cells (Figure 3.2.6 panel C). The expression of CD69 on the surface of B cells increased as expected over time with activation (Figure 3.2.6 panels A and B). CD69 MFI measured in five TCDD-sensitive human donors was significantly lower in BCL-6^{hi} cells and the same trend was reversed in the case of BCL-6^{lo} cells (Figure 3.2.7 panels C and D). Overall, these experiments demonstrate an inverse relationship between the expression of BCL-6 and CD80, and BCL-6 and CD69 in presence of TCDD in human peripheral blood B cells.

Increased TCDD-inducible DNA binding ability of BCL-6 at the CD80 enhancer region

To determine if altered BCL-6 expression was sufficient to regulate CD80 in presence of TCDD, we focused on the CD80 promoter to identify existing BCL-6 binding sites. The expression of human CD80 is regulated by an enhancer element located 3kb

A.

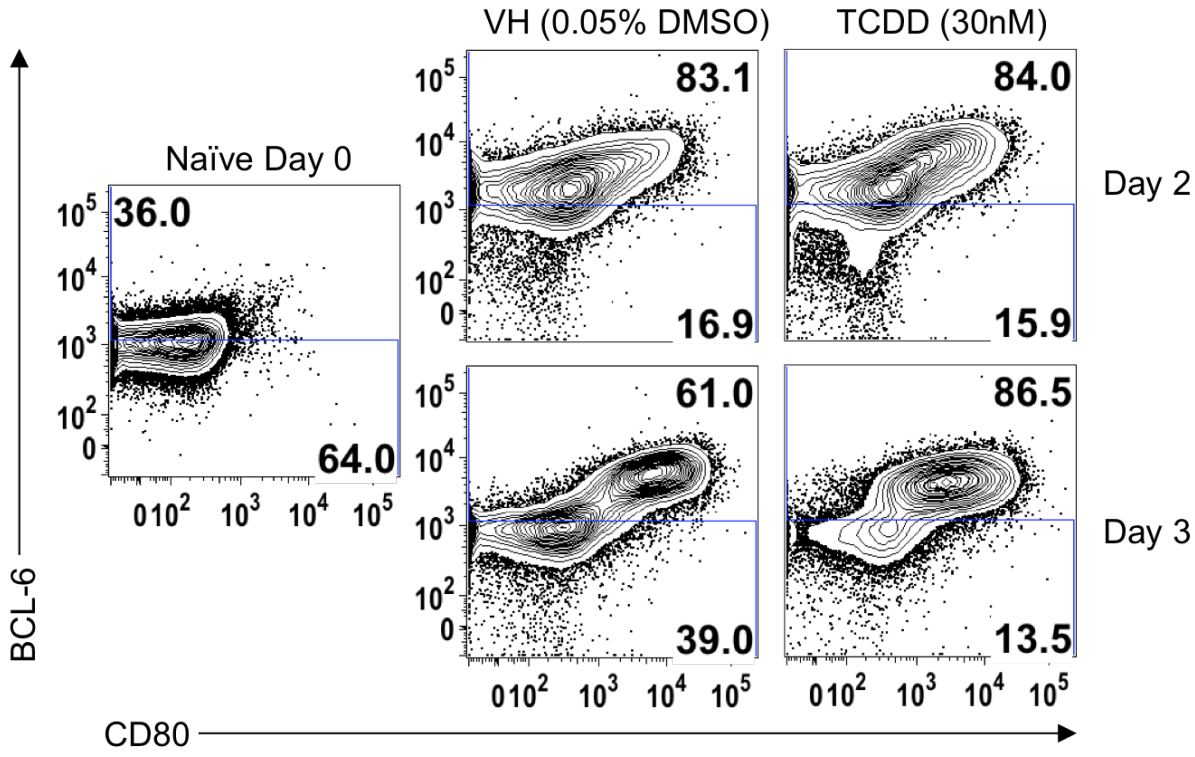
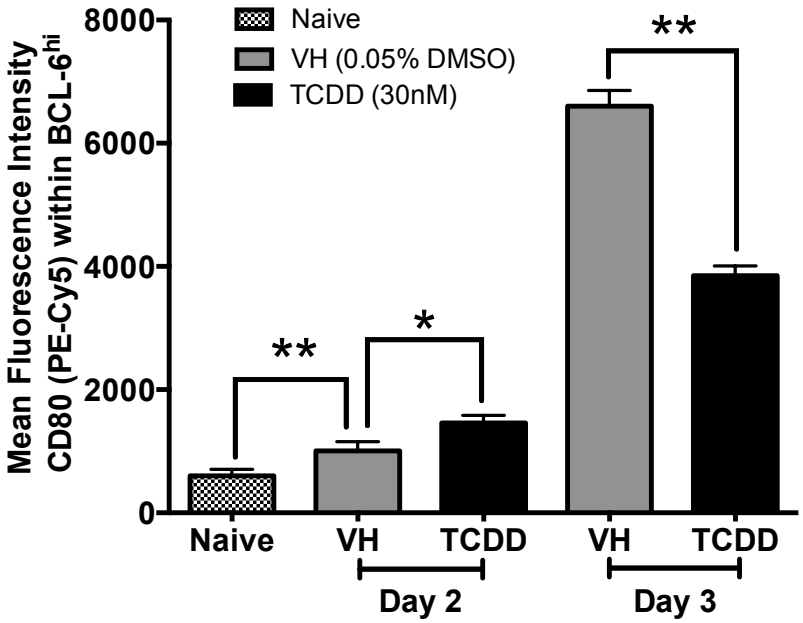


Figure 3.2.5: Decreased expression of CD80 corresponds to higher BCL-6 expression in TCDD-treated human primary B cells

A. BCL-6 and CD80 protein levels were assessed by multi-parametric flow cytometry in human primary B cells activated with CD40 ligand plus cytokines and treated with TCDD (30nM) or VH (0.05% DMSO) at the indicated time points. Data is representative of five TCDD-sensitive human donors. B. Graph depicts mean fluorescence intensity of CD80 in BCL-6^{hi} cells at the time points indicated C. Graph depicts mean fluorescence intensity of CD80 in BCL-6^{lo} cells at the time points indicated. Dead cells excluded. *p<0.05, **p<0.01, ****p<0.0001 using one-way ANOVA followed by Fisher's Uncorrected LSD test.

Figure 3.2.5 (cont'd)

B.



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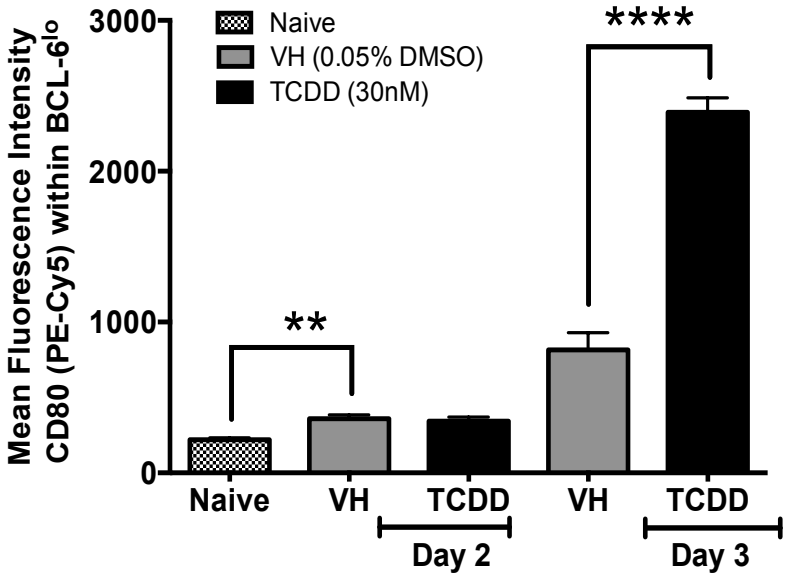

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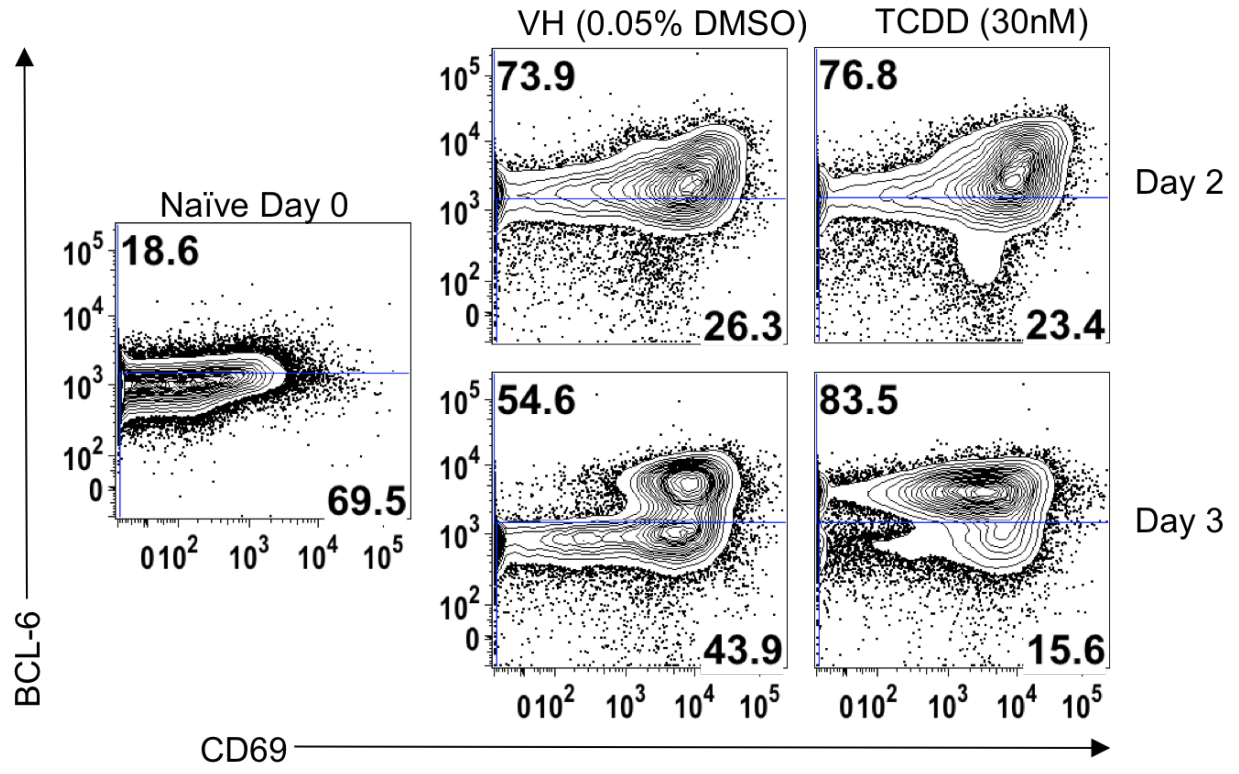
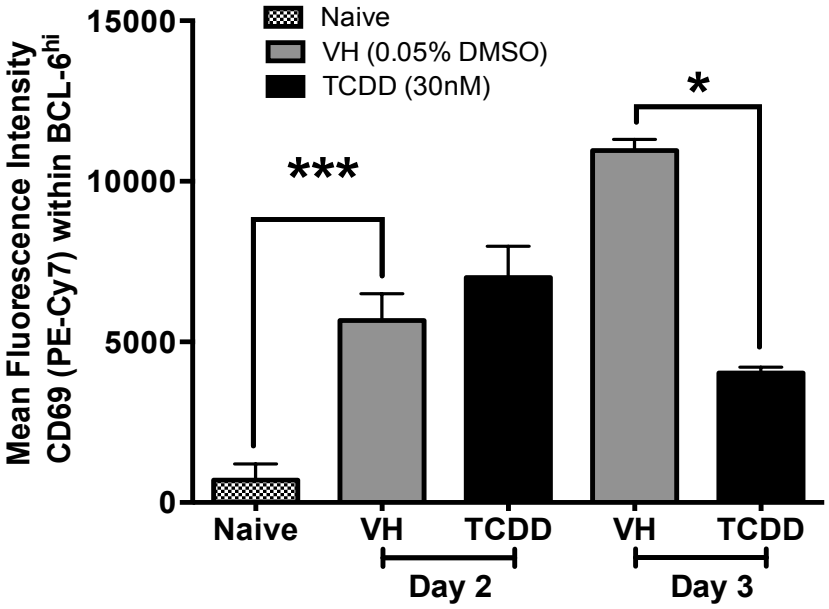


Figure 3.2.6: Decreased expression of CD69 corresponds to higher BCL-6 expression in TCDD-treated human primary B cells

A. BCL-6 and CD69 protein levels were assessed by multi-parametric flow cytometry in human primary B cells activated with CD40 ligand plus cytokines and treated with TCDD at the indicated time points. B. Graph depicts mean fluorescence intensity of CD69 in BCL-6^{hi} cells at the time points indicated C. Graph depicts mean fluorescence intensity of CD69 in BCL-6^{lo} cells at the time points indicated. Dead cells excluded. *p<0.05, ***p<0.001 using one-way ANOVA followed by Uncorrected Fisher's LSD test. Data is representative of five TCDD-sensitive human donors.

Figure 3.2.6 (cont'd)

B.



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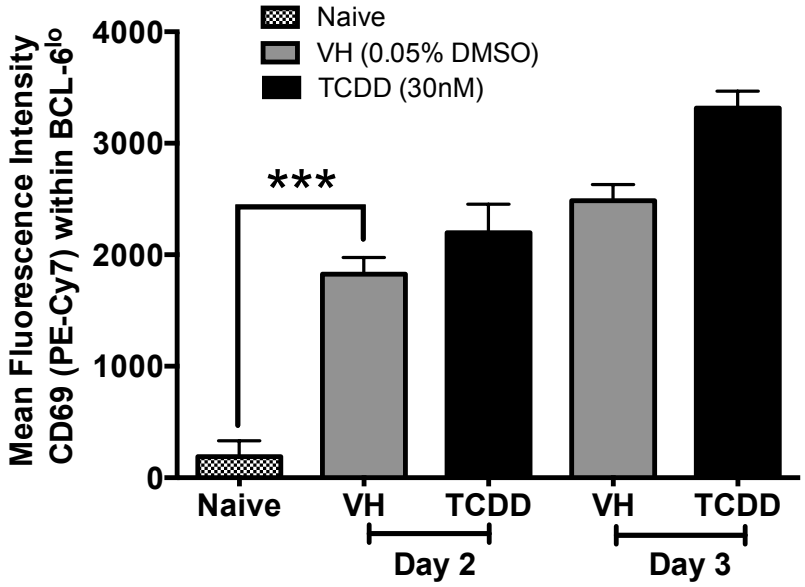

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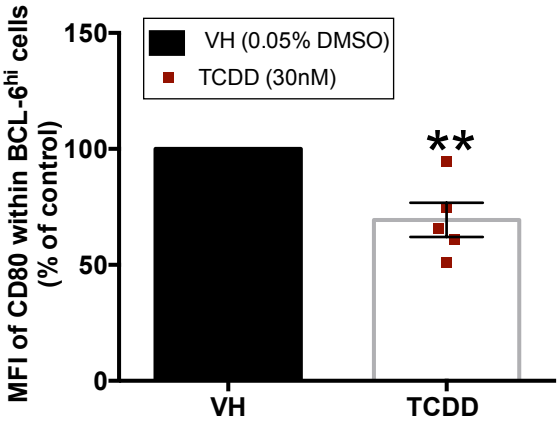

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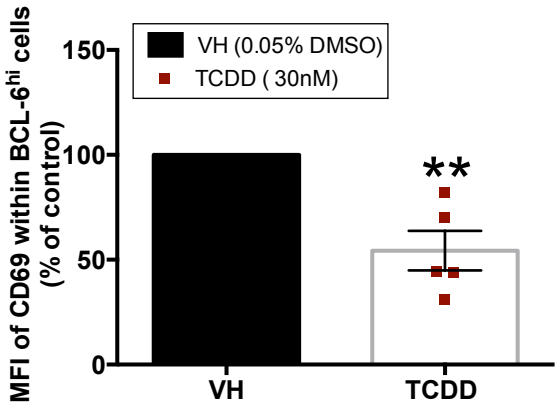

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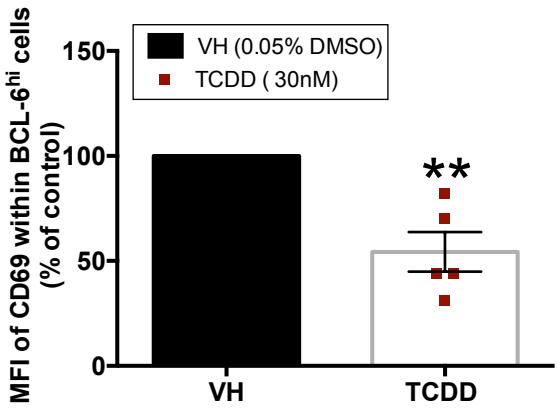
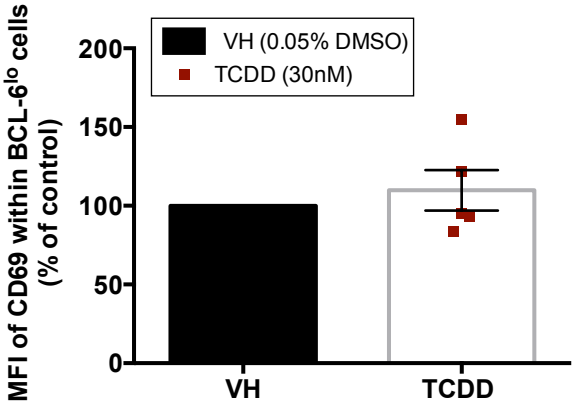


Figure 3.2.7: Changes in MFI of CD80 and CD69 in BCL-6^{hi} and BCL-6^{lo} gated human B cells in five TCDD-sensitive human donors

Figure 3.2.7 (cont'd)

A. MFI of CD80 within BCL-6^{hi} cells as measured by flow cytometry. B. MFI of CD69 within BCL-6^{hi} cells. C. MFI of CD80 within BCL-6^{lo} cells, D. MFI of CD69 within BCL-6^{lo} cells. Dead cells excluded. **p<0.01 using one-way ANOVA followed by Uncorrected Fisher's LSD test. Data in each panel is normalized to the VH-treated group and depicted as percent of control. Each small square represents data from one donor.

D.



upstream of the transcriptional start site (Zhao et al., 1996). BCL-6 is known to bind within the enhancer region of CD80 at a site slightly different from the consensus binding site (Niu et al., 2003). Hence, electrophoretic mobility shift assays were performed using a radiolabeled CD80 enhancer probe harboring the BCL-6 binding site. Nuclear proteins were isolated from human peripheral blood B cells treated with TCDD or VH at various time points. Naïve human B cells showed higher binding of BCL-6 on the CD80 enhancer region that was relieved upon activation of B cells on day 1 (Figure 3.2.8 lane 1). An increase in TCDD-induced DNA binding activity was observed at day 2 and day 3 with maximal binding seen at day 2 (Figure 3.2.8 lanes 4-7). To assess BCL-6 binding specificity an anti-BCL-6 antibody was employed. Though a supershift of the DNA-binding complexes in Figure 3.2.8 was not evident, a decrease in binding intensity was observed in the presence of an anti-BCL-6 antibody. The TCDD-inducible complex was competed off upon addition of the antibody but not in presence of the non-specific rabbit IgG (Figure 3.2.8 lanes 8 and 9, 10 and 11). The kinetics of DNA binding activity of BCL-6 demonstrated in the gel-shift assays is consistent with the pattern of decrease in expression of activation marker CD80 in several TCDD-sensitive human donors at Day 3.

BCL-6 inhibitor reverses suppression of B cell activation markers

Most of the repressive effects of BCL-6 are mediated by the BTB domain, which plays an important role in binding to the corepressors recruited by BCL-6 (Ahmad et al., 2003; Ghetu et al., 2008). To further delineate the role of BCL-6 in suppression of B cell activation, a small molecule inhibitor of BCL-6, 79-6, was added to cells *in vitro* in the

presence of TCDD. This small molecule inhibitor of BCL-6 was designed to specifically bind the BTB domain thereby disrupting the activity of BCL-6 by blocking its interaction with the corepressors (Cerchietti et al., 2010). To this end, human primary B cells were activated with CD40 ligand plus cytokines IL-2, IL-6 and IL-10 and treated *in vitro* with the inhibitor, 79-6, in the absence or presence of TCDD. As illustrated in Figure 3.2.9 panel A, the treatment of cells with the BCL-6 inhibitor reversed the suppression by TCDD as the repressive action of BCL-6 on B cell activation markers was blocked. As compared to cells treated with inhibitor, cells treated with the vehicle of inhibitor still showed moderate suppression of activation marker expression. The control cells treated with VH and TCDD alone, showed the typical pattern of suppression of activation marker expression as evident from the MFI and the pattern of the histograms. The effects of BCL-6 inhibitor on multiple TCDD-sensitive human donors were also assessed. Reversal of suppressed activation marker expression was marked in case of CD80 (Figure 3.2.9 panel B) and was seen to a lesser extent for activation markers CD86 and CD69 owing to a VH effect (Figure 3.2.9 panels C and D). Notably, suppression of activation marker expression by TCDD also occurs independent of the mode of activation of B cells. B cells could also be robustly activated via ligation of surface CD40 receptor and with a combination of cytokines such as IL-2 and IL-21. This CD40 ligand plus IL-2 and IL-21 response was significantly suppressed by TCDD treatment (data not shown). Addition of 100μM of 79-6, in presence of TCDD caused reversal of suppressed activation marker expression. However, treatment of activated human B cells with VH in absence of inhibitor also led to suppression of activation marker expression due to higher vehicle concentrations (Figure 3.2.10).

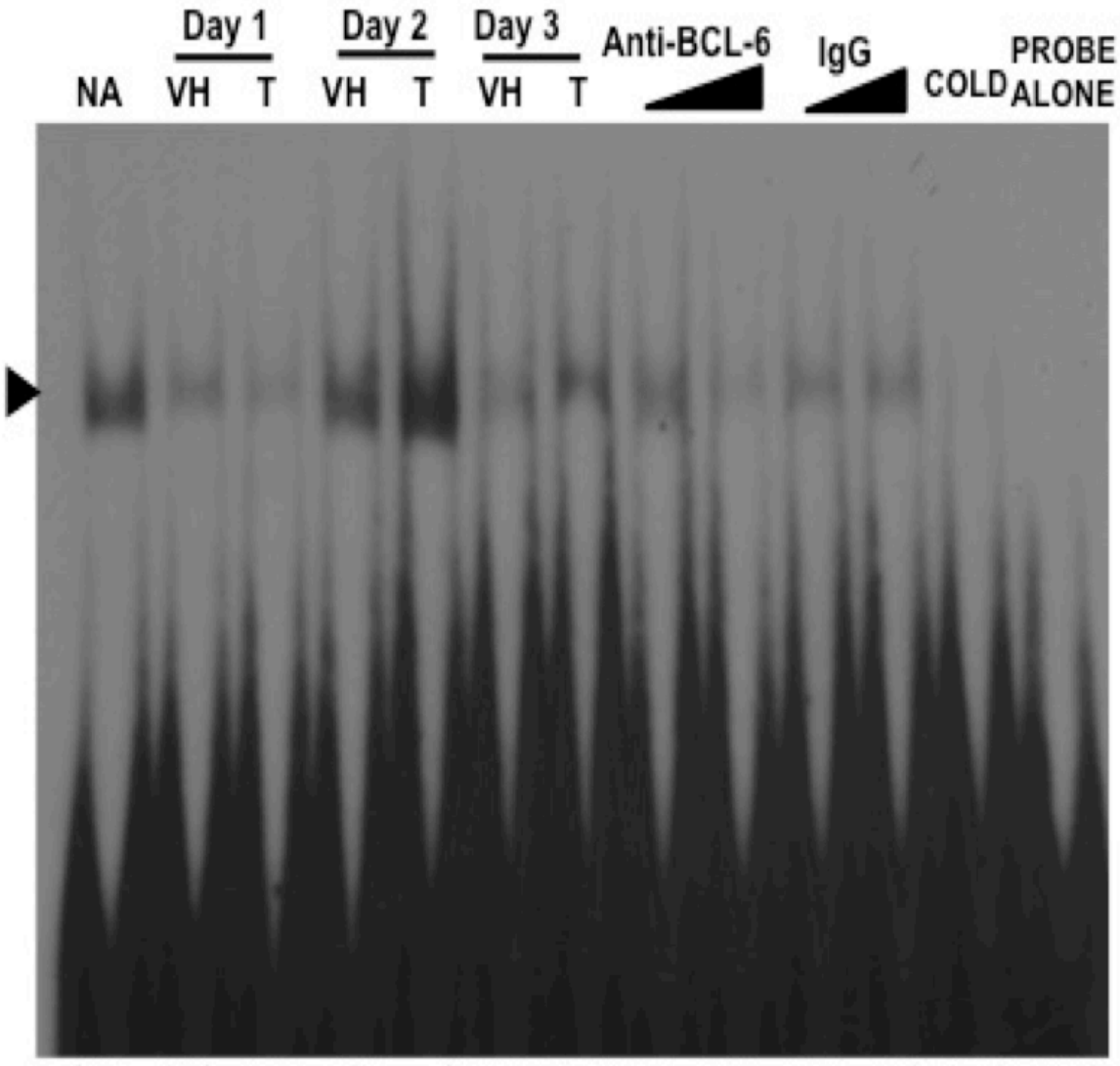


Figure 3.2.8: TCDD-mediated increase in DNA-binding activity of BCL-6 at the CD80 enhancer region.

Electrophoretic mobility shift assay performed on nuclear extracts isolated from human primary B cells treated with TCDD (30nM) or VH (0.05% DMSO) for the indicated days. Binding reactions were set up with ^{32}P-labeled CD80 enhancer region probe. BCL-6 antibody at 0.5μg and 1μg (lanes 7 and 8 respectively) was used for supershift assays. An isotype control rabbit IgG was used at the same concentration as

Figure 3.2.8 (cont'd)

the specific antibody (lanes 9 and 10). The arrowhead indicates TCDD-inducible BCL-6 binding to the CD80 enhancer region. Data shown is representative of five TCDD-sensitive human donors.

A.

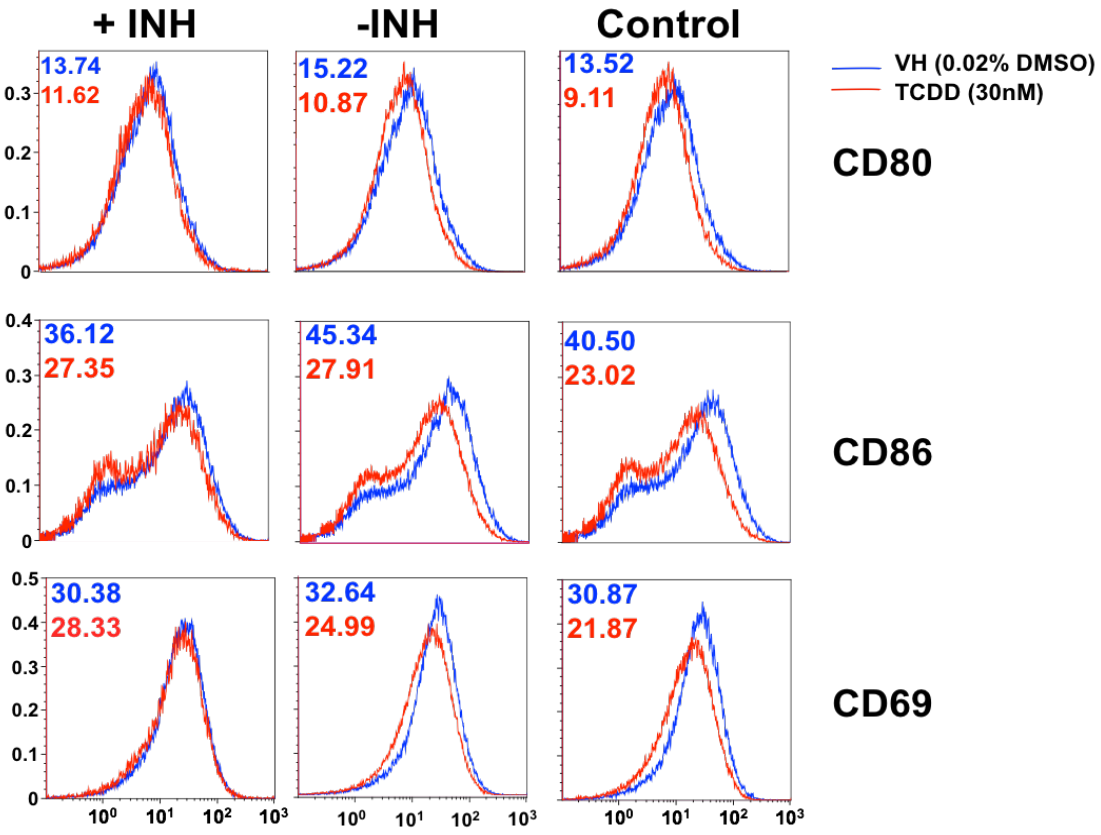


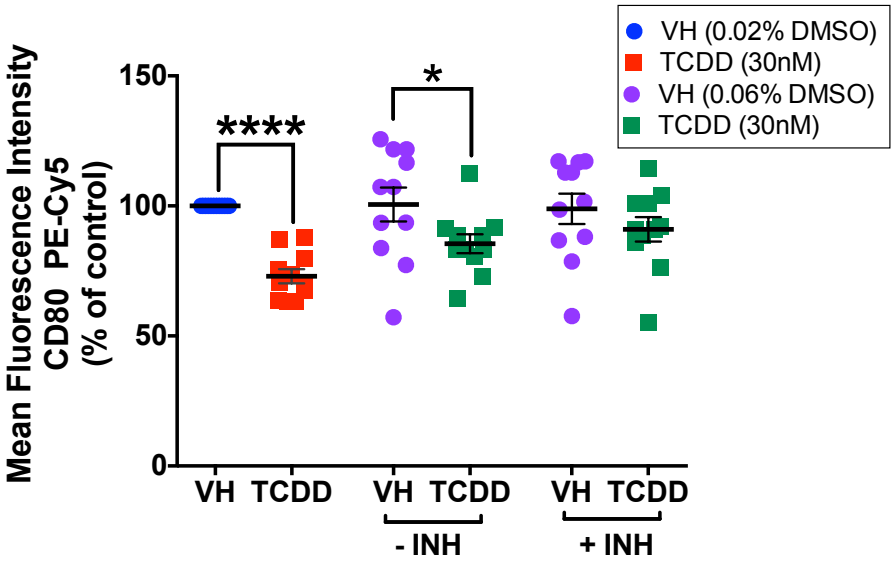
Figure 3.2.9: BCL-6 inhibitor reverses suppressed activation marker expression in human B cells activated with CD40 ligand and cytokines IL-2, IL-6 and IL-10

A. B cells were isolated on Day 3 post-activation and treatment with TCDD or VH and BCL-6 inhibitor. Cells were permeabilized and stained for activation markers. Numbers in the left corner depict the MFI for the indicated activation marker. Data shown in the figure is representative of seven TCDD-sensitive human donors. B-D. Graphs depict the MFI of CD80 (top), CD86 (middle) and CD69 (bottom) in presence and absence of inhibitor along with treatment with TCDD or VH. Data in each graph is normalized to the VH control. Graphs represent percent of control with each dot representing one donor.

Figure 3.2.9 (cont'd)

Data from 9 donors is shown in panel B whereas data from 12 donors is shown in panels C and D.

B.



C.

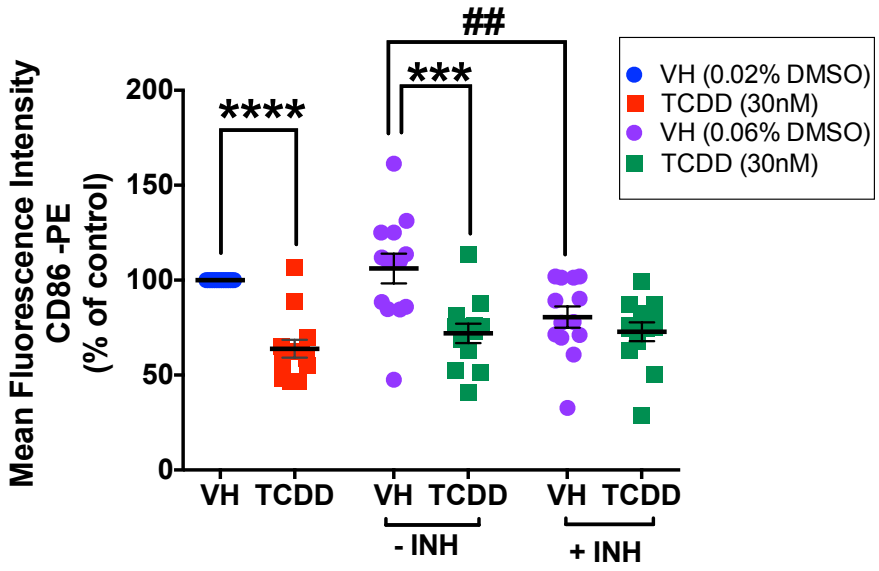
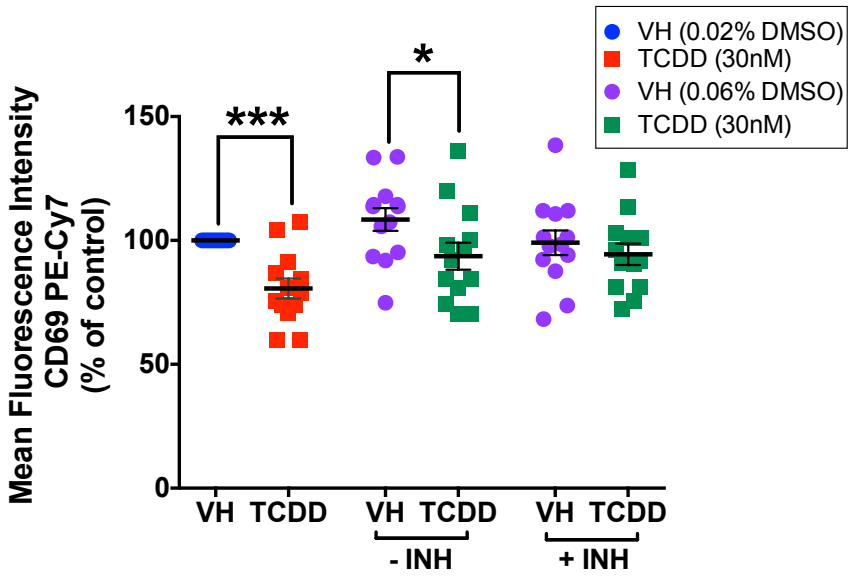
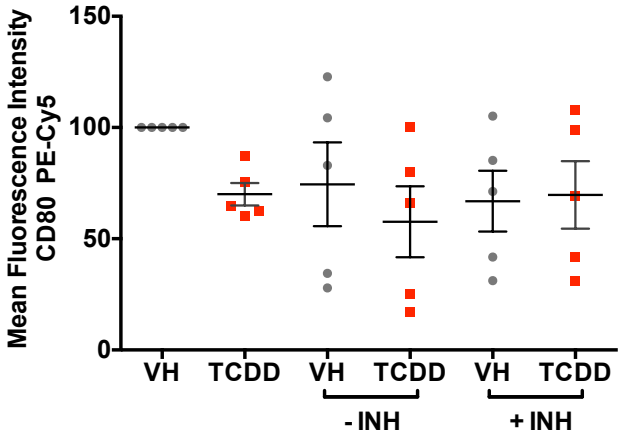


Figure 3.2.9 (cont'd)

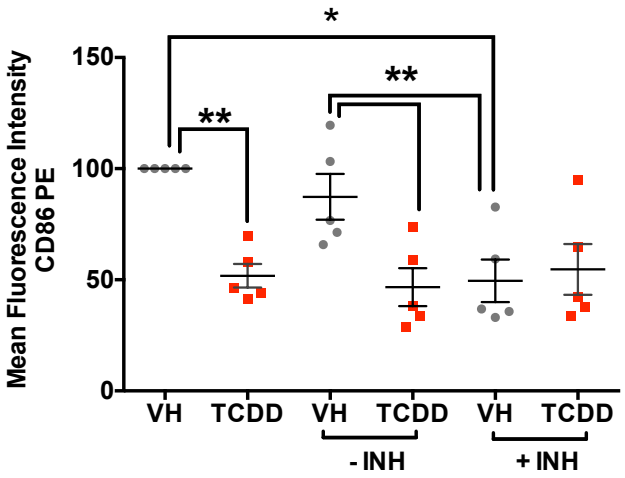
D.



A.



B.



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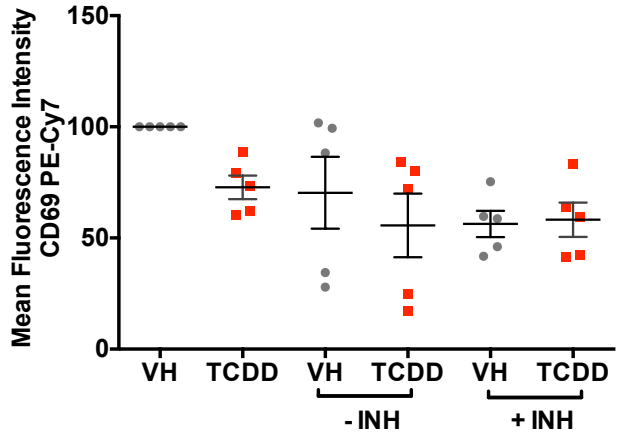


Figure 3.2.10: Effect of BCL-6 inhibitor on human B cells treated activated with CD40L and cytokines IL-2 and IL-21

Figure 3.2.10 (cont'd)

B cells were isolated on Day 3 post-activation and treatment with TCDD or VH and BCL-6 inhibitor. Cells were permeabilized and stained for activation markers. Graphs depict the MFI of CD80, CD86 and CD69 in presence and absence of inhibitor along with treatment with TCDD or VH. Data in each graph is normalized to the VH control. Graphs represent percent of control with each dot representing one donor. Data from 5 TCDD-sensitive donors.

BCL-6 expression in highly proliferating human B cells

BCL-6 helps the B cells to tolerate rapid proliferation (Reljic et al., 2000; Shvarts et al., 2002) and high genomic instability by repressing the genes such at ATR, ATM and p53 in the germinal center (Phan and Dalla-Favera, 2004). We expected that cells expressing high levels of BCL-6 would have greater proliferation owing to the function of BCL-6 in B cells and given its role as an oncogene in lymphomas. Figure 3.2.11 shows the proliferation profile of human B cells activated with CD40 ligand plus cytokines. As expected, B cells underwent two to four rounds of proliferation post-activation. Cells expressing higher levels of BCL-6 comprised the higher proliferating cells as highlighted by the gate in Figure 3.2.11 thereby providing a proof-of-principle for the expression of BCL-6 in proliferating B cells. However, TCDD- treated cells, also showed a similar proliferation profile with no significant differences in the percentage or the number of cells proliferating at each day as compared to the vehicle control (data not shown).

Impaired downregulation of BCL-6 involves AHR

It is well known that most biological and toxic effects of TCDD are mediated through the AHR (Okey, 2007a; Rowlands and Gustafsson, 1997). The involvement of the AHR in mediating TCDD toxicity has been shown extensively through the use of AHR null mouse models and cell lines (Fernandez-Salguero et al., 1995; Mimura et al., 1997; Schmidt et al., 1996). To determine if alteration of BCL-6 levels in presence of TCDD involves AHR, we used MCDD, a congener of the dibenzo-*p*-dioxins that has minimal binding affinity for the AHR and an inability to induce CYP1A1 and EROD activity. CD40 ligand and cytokine-activated human B cells were treated with VH, TCDD

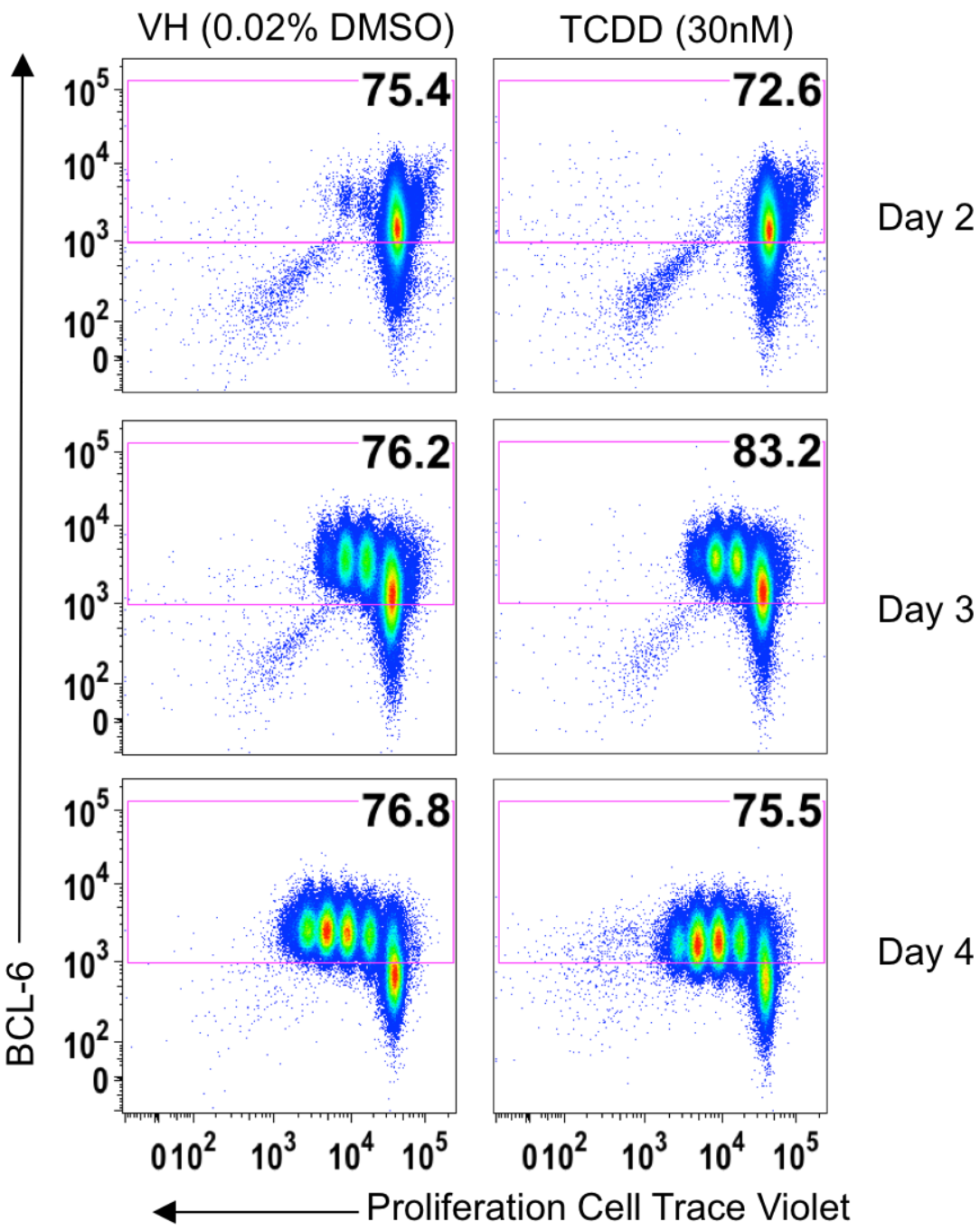


Figure 3.2.11: BCL-6^{hi} cells show increased proliferation.

Human primary B cells were stained with Cell trace violet on day 0 following which cells were treated with TCDD or VH and activated via CD40 ligand and cytokines. At the

Figure 3.2.11 (cont'd)

indicated time points, cells were permeabilized and stained for intracellular BCL-6 levels. The gate and the numbers in the corner indicate BCL-6^{hi} cells. Dead cells excluded. Data shown is representative of the proliferation of three TCDD-sensitive human donors.

or MCDD for 3 days. TCDD-treatment increased the proportion of BCL-6^{hi} cells compared to the VH control cells at both time points as seen previously (Figure 3.2.12 panel A). Concurrently, cells treated with MCDD showed a significantly lower proportion of BCL-6^{hi} cells as compared to the VH-treated or TCDD-treated cells (Figure 3.2.12 panel A and B). This suggests that the increase in BCL-6 levels is specific to the TCDD-treated group and thereby by extension also suggests a role for the AHR pathway in suppression of activation. Figure 3.2.12 panel C shows the percentage of BCL-6^{hi} cells as a fraction of the control from five TCDD-sensitive donors. Treatment of cells with MCDD failed to show an increase in BCL-6 levels as expected and was not different from the VH-treated cells. To further determine if suppression of activation markers in presence of TCDD involves the AHR pathway, human primary B cells were treated cells with MCDD or with VH or TCDD for comparison. The magnitude of suppression of activation marker expression by TCDD was the greatest whereas cells treated with MCDD or VH showed no suppression of activation markers CD80, CD86 or CD69 (Fig. 3.2.13). Due to donor variability in background response and sensitivity to TCDD, we observed that in some donors the suppression of activation markers was in the rank order of TCDD > MCDD > VH (Figure 3.2.13 panel B).

Effect of TCDD on pERK levels in CD40 ligand plus cytokine-activated B cells

B cell receptor signaling activates the MAP kinase pathway increasing pERK levels. Phosphorylation of BCL-6 by pERK causes BCL-6 degradation via the ubiquitin proteasome pathway (Niu et al., 1998b). This is one way by which BCL-6 protein levels are controlled in B cells. Previous studies assessing the effects of TCDD on MAPK

signaling have suggested that TCDD suppressed CD40 ligand-induced immediate pERK activation at 30 min (Lu 2011). We hypothesized that changes in pERK levels in presence of TCDD during the course of B cell activation could potentially affect BCL-6 levels. To this end, pERK levels were measured by intracellular flow cytometry in CD40 ligand plus cytokine activated human B cells in presence of TCDD. At day 1, TCDD-treatment led to a significant decrease in pERK levels (Figure 3.2.14) but the levels recovered on day 2 and were significantly higher in comparison with the VH-treated cells. On day 3 no change in pERK levels was observed. These results demonstrate modulation of pERK levels by TCDD.

Effect of TCDD on F-box protein (Fbxo11) mRNA levels

Another mechanism controlling stability of BCL-6 is through association with FBXO11, an orphan F-box protein. FBXO11 targets BCL-6 for degradation via the SKP1-CUL1-F-box protein (SCF) ubiquitin ligase pathway. Expression of FBXO11 is known to reduce BCL-6 levels due to increase proteolysis (Duan et al., 2012). So, we investigated changes in Fbxo11 mRNA levels in presence of TCDD. Fbxo11 was also identified as a direct AHR target through ChIP-on-chip experiments and this suggested presence of putative DREs in Fbxo11 regulatory regions. Fbxo11 mRNA levels decreased with B cell activation and were maintained throughout the time course (Figure 3.2.15). Contrary to our expectations, a significant increase in Fbxo11 mRNA levels was observed on day 2 following TCDD-treatment and the levels decreased back on day 3.

A.

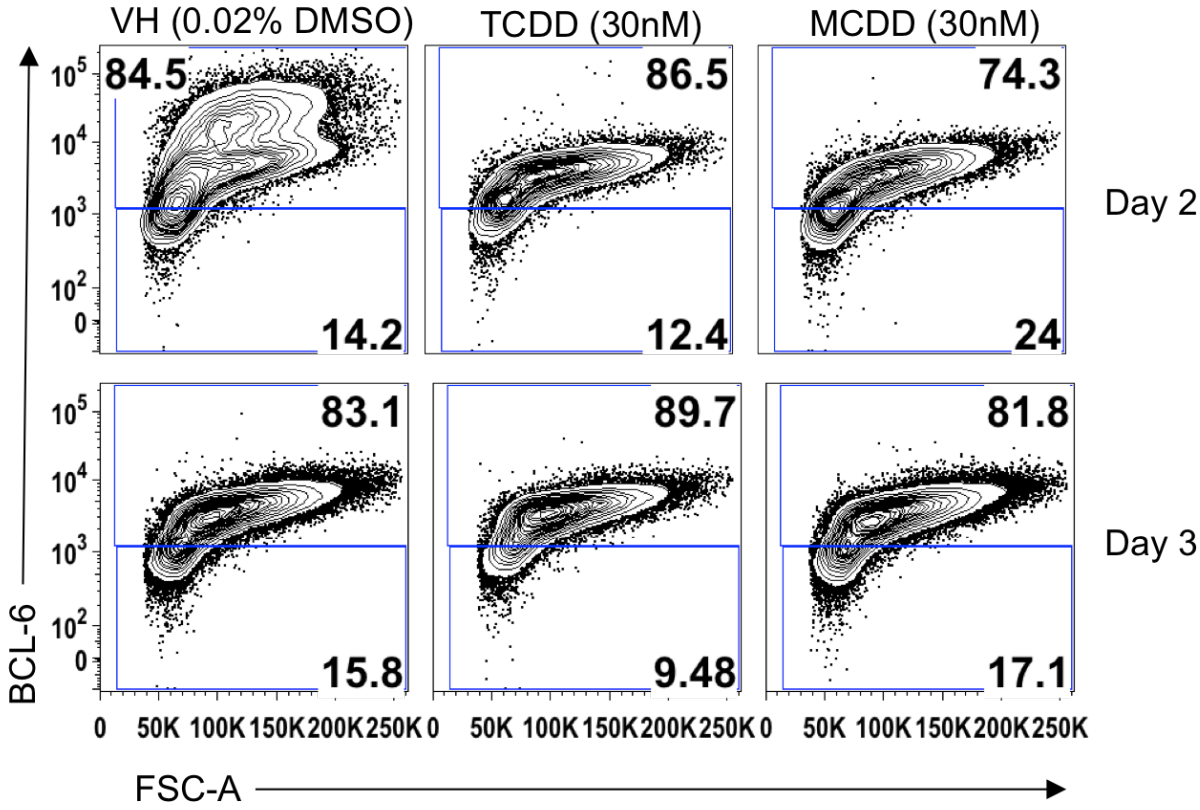
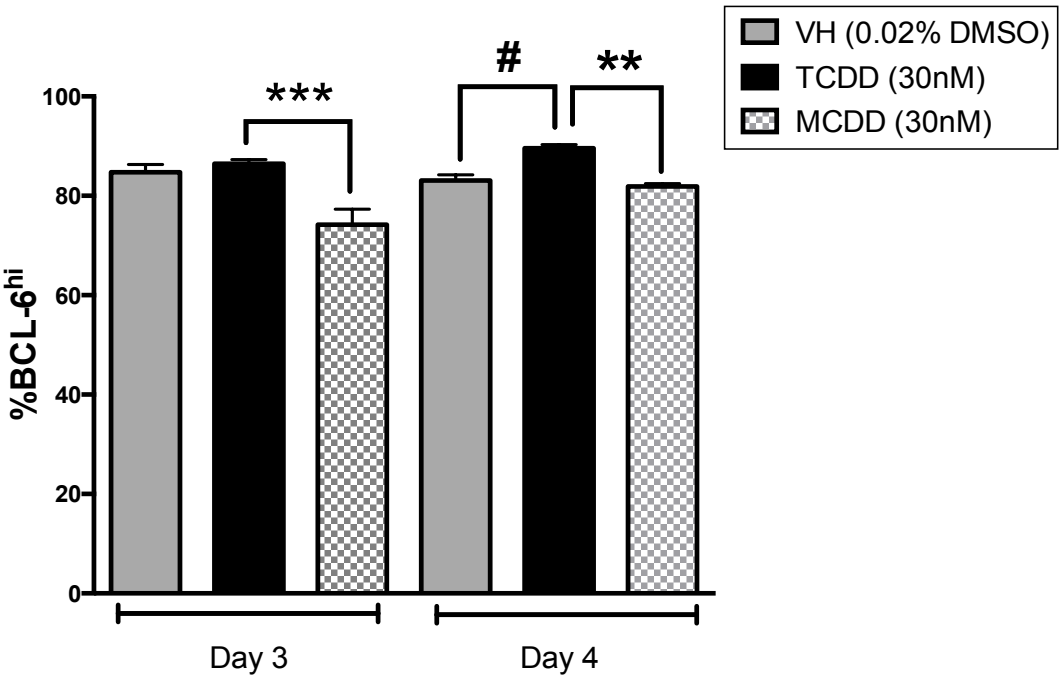


Figure 3.2.12: Impaired BCL-6 regulation involves AHR.

A. BCL-6 protein levels were measured by flow cytometry on Day 2 and Day 3 post-activation and TCDD- treatment. The gates and the numbers in the corner depict the % BCL-6^{hi} and % BCL-6^{lo} cells. Data representative of five TCDD-sensitive human donors. B. Graph shows % BCL-6^{hi} cells in VH, TCDD and MCDD-treated groups on Day 3 and Day 4 from the same donor as panel A. C. Graph depicts the percent of BCL-6^{hi} cells as a percent of control. Data is normalized to the VH group of each donor. Each square represents one donor. # $p<0.05$, ** $p<0.01$, ***$p<0.001$ by One-way ANOVA followed by Fisher's Uncorrected LSD test.

Figure 3.2.12 (cont'd)

B.



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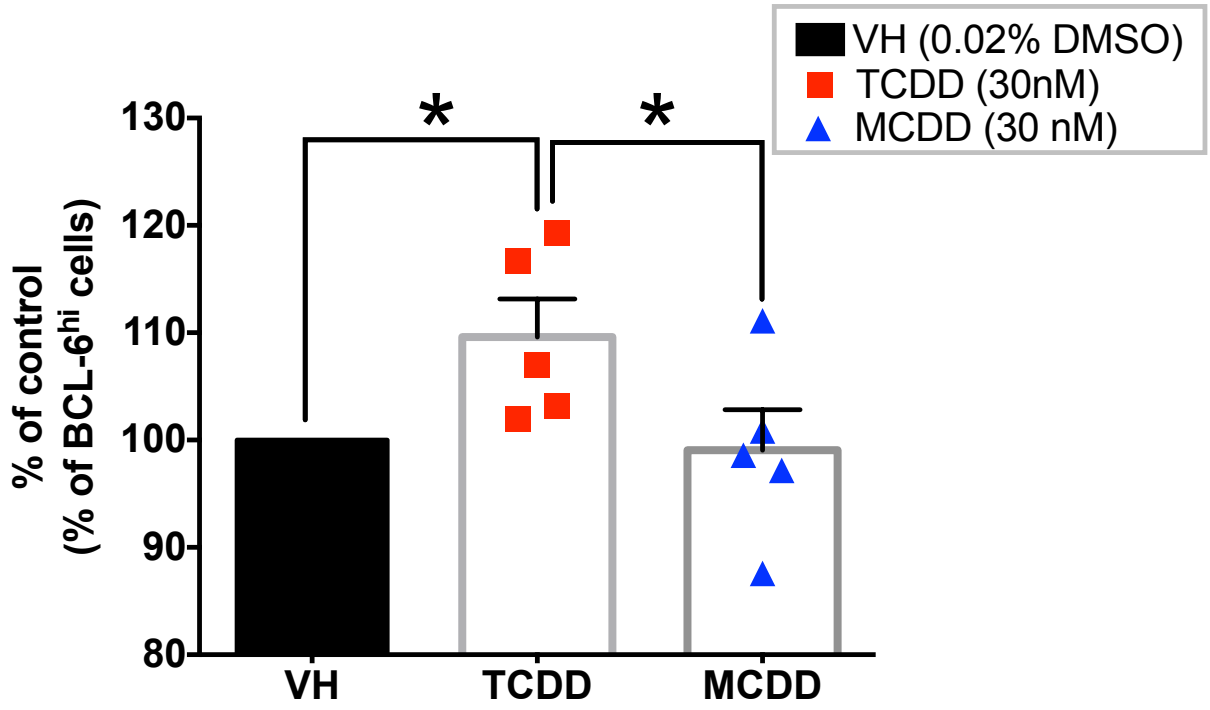
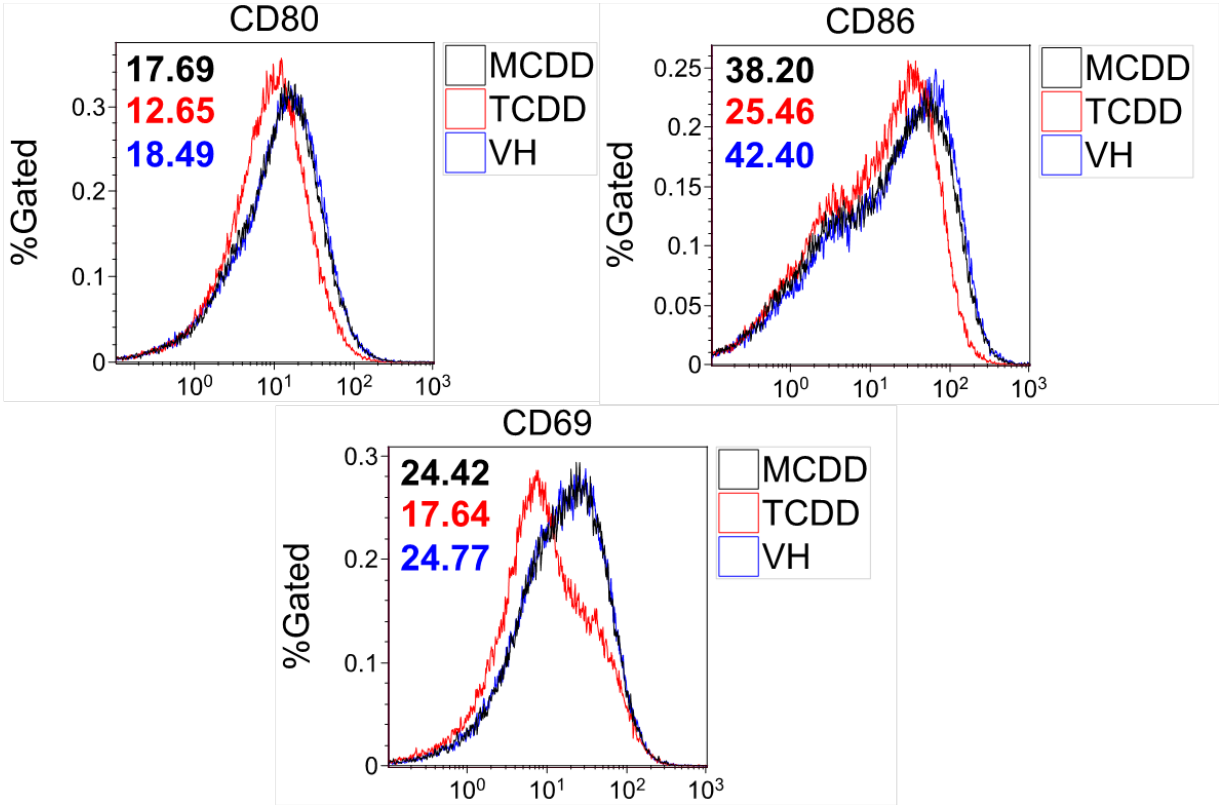


Study of the polymorphisms in AHR of TCDD-responders and non-responders

The human AHR gene harbors few polymorphisms with previously characterized polymorphisms in the 5'UTR (-459 G>A), codon 44 (132T>C) in exon 2, codon 517 (1549C>T), codon 554 (1661G>A) and codon 570 (1708G>A) in exon 10 (Harper et al., 2002). Among these, polymorphisms in the transactivation domain especially codon 554 were found to be important to induce CYP1A1 activity in lymphocytes (Smart and Daly, 2000). Understanding the relationship between polymorphisms in AHR and the phenotypic effects determining responsiveness to TCDD can provide an insight into the molecular basis of TCDD toxic effects. We used suppression of activation markers as a phenotypic marker to determine responsiveness or non-responsiveness to TCDD. Donors with greater than 50% suppression of B cell activation markers CD80, CD86 and CD69 were selected and the AHR gene was sequenced. As seen in Figure 3.2.16, one of the three non-responders harbored the 1661G>A polymorphism in exon 10. To our surprise, one of the three responders also harbored the 1661G>A and the 1708G>A polymorphism. The three non-responders also showed a common polymorphism C>A in exon 1 which has not been reported previously.

A.



B.

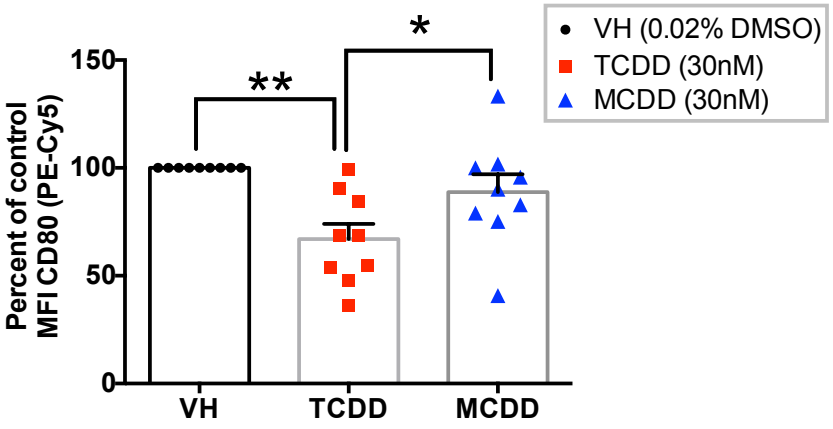


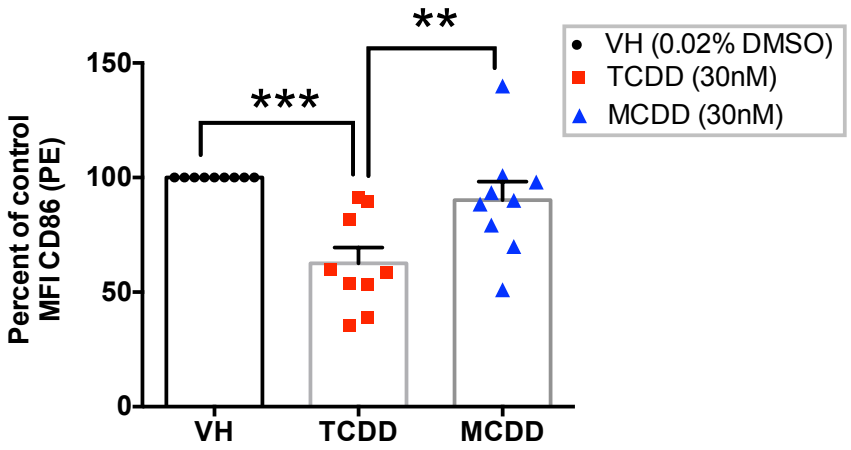
Fig.3.2.13: Suppression of activation markers by TCDD involves AHR.

A. CD40 ligand and cytokine activated- B cells were treated with VH (0.02% DMSO) or TCDD (30nM) or MCDD (30nM). The numbers to the left depict the MFI of each of the

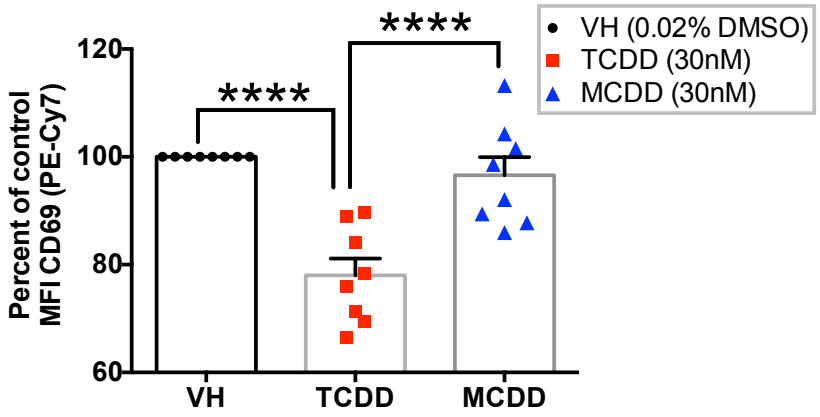
Figure 3.2.13 (cont'd)

groups. Data representative of nine TCDD-sensitive human donors. B-D. Graphs represent the MFI of CD80, CD86 and CD69 as a percent of VH control. Data is normalized to the VH group of each donor. Each dot represents an individual human donor. * p< 0.05, ** p< 0.01, **** p< 0.0001 by one-way ANOVA followed by Fisher's Uncorrected LSD test.

C.



D.



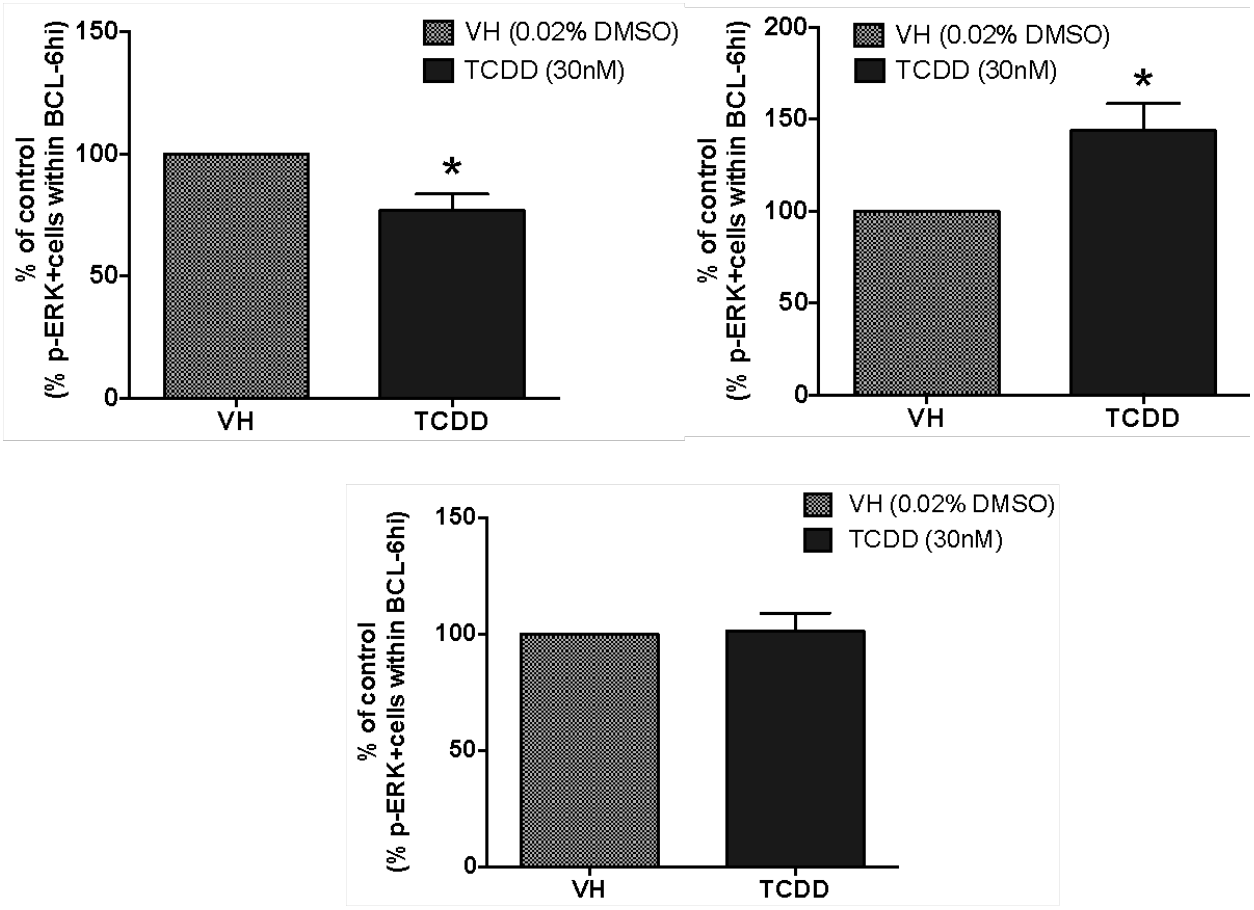


Figure 3.2.14: Effect of TCDD on pERK levels in human B cells

Naïve human B cells were activated with CD40 ligand plus cytokines and treated with VH or TCDD for three days. pERK levels were measured by intracellular flow cytometry on day1 (left panel), day 2 (right panel) and day 3 (bottom-center panel). Graph depicts the percentage of p-ERK+ cells as a percent of control. Data shown in the graph is pooled from three donors for day 1 and four donors for day 2 and day 3 with values normalized to the VH control of each donor. * $p < 0.05$ by unpaired Student's t-test.

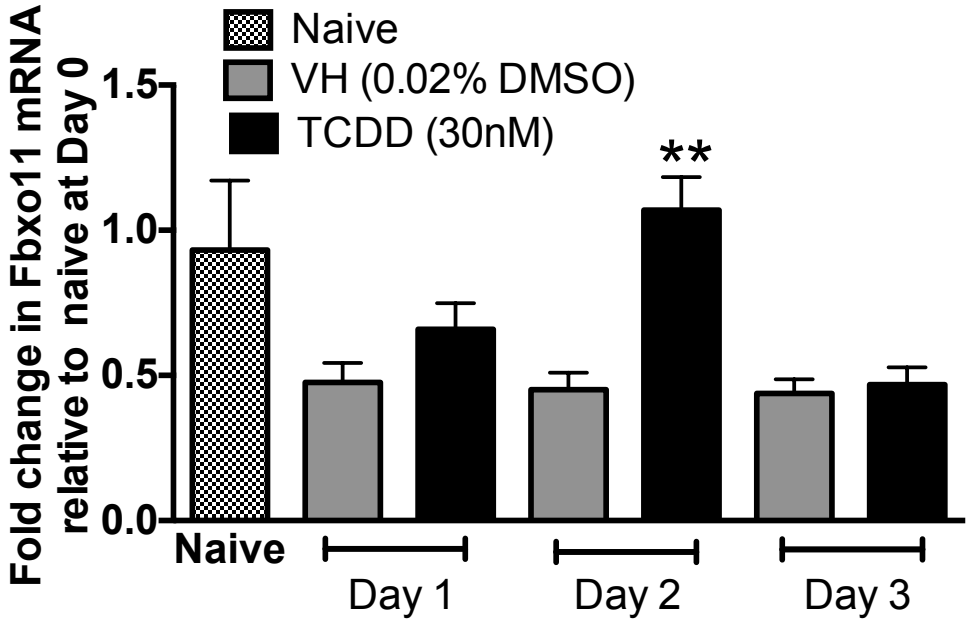


Figure 3.2.15: Effect of TCDD on Fbxo11 mRNA levels in activated human B cells

Human primary B cells were activated with CD40 ligand plus cytokines and treated with TCDD or DMSO for the indicated time. RNA was isolated and qRT-PCR was performed. The data in the graph represents mean ± SE of triplicate measurements at each time point. Data is representative of two individual human donors. Comparisons were made between the TCDD and VH-treated groups at each time point. **p< 0.01 by one-way ANOVA.

#Exon Name	Donor #/Primer #	Pos On Gene	Pos On Ref Exon	Pos On Genome	Ref Base	Sample Base	Responders/ NonResponders	Polymorphism
1	**8/8_1_-f**	**759**	**759**	17339034	C	A	N	New?
1	**15/15_1_-f**	**759**	**759**	17339034	C	A	N	New?
1	**13/13_1_-f**	**759**	**759**	17339034	C	A	N	New?
2	**15/ 15_2_F1**	**11794**	**510**	**17350069**	**A**	**G**	**N**	New?
2	**8/8_2_F1**	**11794**	**510**	**17350069**	**A**	**G**	**N**	New?
10	**8/8_10_.2F**	**40835**	**501**	**17379110**	**G**	**A**	**N**	**1661G>A**
10	**9/9_10_.2R**	**40835**	**501**	**17379110**	**G**	**A**	**R**	**1661G>A**
10	***9/9_10_.2F***	***40835***	***501***	***17379110***	***G***	***A***	***R***	**1661G>A**
10	***9/9_10_.2R***	***40882***	***548***	***17379157***	***G***	***A***	***R***	**1708 G>A**
10	***9/9_10_.1.1F***	***40882***	***548***	***17379157***	***G***	***A***	***R***	**1709 G>A**

Table 3.1: Known and putatively novel polymorphisms in AHR in human donors

Table displays location of known polymorphisms and putatively novel polymorphisms in three TCDD-non-responsive human donors (8, 13, 15) and one TCDD-responsive human donor (9). PosOnGene- refers to position of AHR gene, PosONRefExon-refers to position on reference AHR exon, PosOnGenome-refers to position on genome, RefBase- refers to reference base in the sequenced AHR exon, N-refers to TCDD-non-responder and R- TCDD-Responder based on suppression of activation markers in presence of TCDD. Cells highlighted in yellow refer to C>A polymorphism in human donors 8,15 and 13. Cells highlighted in orange refer to A>G polymorphism in human donor 8 and 15.

3.3. Direct regulation of SHP-1 by TCDD in human B cells

Increase in SHP-1 mRNA levels in presence of TCDD

SHP-1 was identified as a potential target of AHR through genome-wide ChIP-on-chip studies in mouse B cells (De Abrew et al., 2010). SHP-1 expression was also significantly elevated in presence of TCDD as compared to control cells in gene expression microarrays performed in conjunction with the ChIP-on-chip studies. To confirm these findings and to determine if SHP-1 expression is altered by TCDD in human B cells, qRT-PCR was performed. Figure 3.3.1 panel A, shows up-regulation of SHP-1 mRNA levels upon B cell activation at day 2 and a maintenance of the mRNA levels at the later time points. However, TCDD-treatment further elevated SHP-1 mRNA levels on days 2, 3 and 4 in activated primary human B cells with significant effects observed on day 2 and day 3 as compared to the vehicle control cells.

TCDD-mediated concentration response changes in SHP-1 mRNA levels were measured on Day 3 in CD40 ligand plus cytokine-activated human primary B cells (Figure 3.3.1 panel B). No change was observed in SHP-1 mRNA levels in the vehicle-treated cells as compared to naïve cells on day 0. Lower concentrations of TCDD did not result in an increase in SHP-1 mRNA levels but treatment of cells with 30nM TCDD showed a significant elevation in SHP-1 mRNA levels as compared to the vehicle-treated cells.

Time course and concentration response of SHP-1 protein levels in presence of TCDD

In order to assess if changes in SHP-1 mRNA levels result in changes in SHP-1 protein levels, the percentage of SHP-1^{hi} cells were measured by intracellular flow

A.

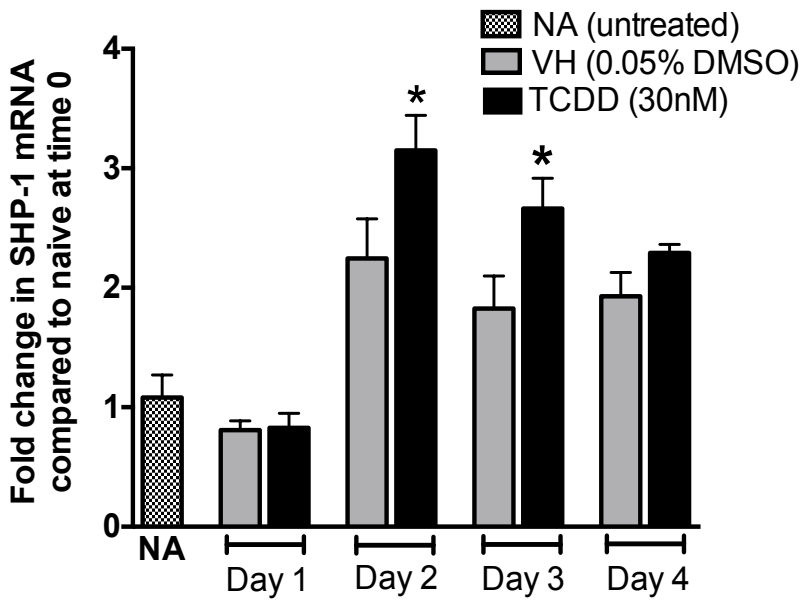
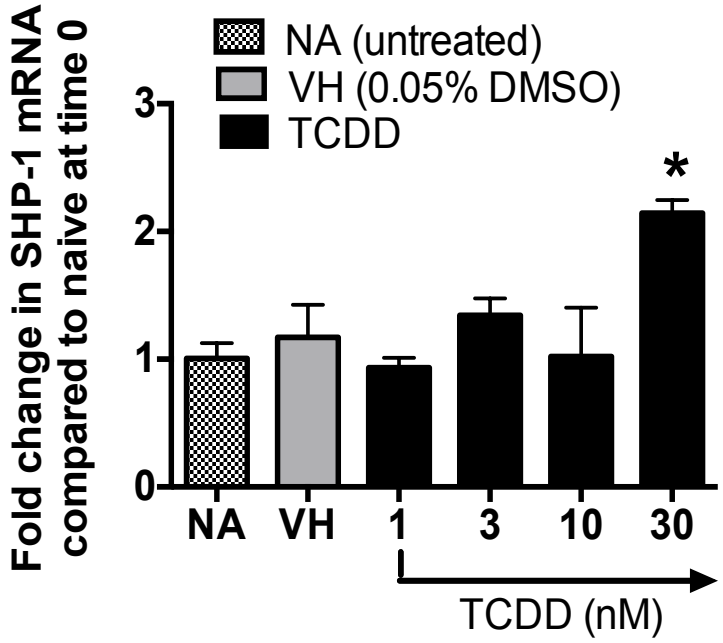


Figure 3.3.1: Time course and concentration-dependent induction of SHP-1 mRNA levels following TCDD-treatment in human B cells

A. Human B cells were activated were CD40 ligand plus cytokines and treated with the indicated concentration of TCDD or VH for four days. SHP-1 mRNA levels were measured by qRT-PCR. Naïve cells were activated with CD40 ligand and cytokines and harvested on day 0. B. Human B cells were activated with CD40 ligand and cytokines and treated with 1, 3, 10 and 30nM TCDD or 0.05% DMSO. SHP-1 mRNA levels were analyzed by qRT-PCR on day 3. The data in the graphs represents mean ± SE of triplicate measurements at each time point. Data in panel A is pooled from three TCDD-sensitive human donors. For statistical analysis comparisons were made between the TCDD and VH-treated group at each time point. * $p < 0.05$ by Student's t-test.

Figure 3.3.1 (cont'd)

B.



cytometry at Days 2, 3 and 4 following B cell activation and TCDD-treatment. The percentage of SHP-1^{hi} cells increased with activation of B cells but TCDD-treatment further elevated the percentage of SHP-1^{hi} cells as seen by the profile of cells in Figure 3.3.2 panel A. The increase in SHP-1^{hi} population of cells and the MFI of SHP-1 was significant at day 3 in presence of TCDD as compared to the VH-treated cells (Figure 3.3.2 panels B and C). This increase in SHP-1 protein levels parallels the increase in mRNA levels observed at similar time points. The change in SHP-1 MFI relative to the VH control cells was also measured in seven TCDD-sensitive human donors. Though the magnitude of change and relative time of increase in SHP-1 levels varied across donors (data not shown), the overall trend was observed across human donors (Figure 3.3.2 panel D and E). Furthermore, concentration-response changes of SHP-1 protein levels following TCDD-treatment were examined on day 3. Figure 3.3.3 panel A shows a TCDD-mediated concentration-dependent increase in the percentage of SHP-1^{hi} cells in one TCDD-sensitive human donor. In another study, SHP-1 protein levels were assessed in five TCDD-sensitive human donors in presence of increasing concentrations of TCDD. A similar trend showing an increase in SHP-1^{hi} population of cells was evident with a peak increase at 10nM TCDD concentration (Figure 3.3.3 panel B).

Effect of CD40 ligand and differential cytokine stimulus on SHP-1 protein levels

As mentioned earlier, SHP-1 was identified as a direct target of the TCDD: AHR complex. SHP-1 exerts its phosphatase activity upon B cell activation via ligation of

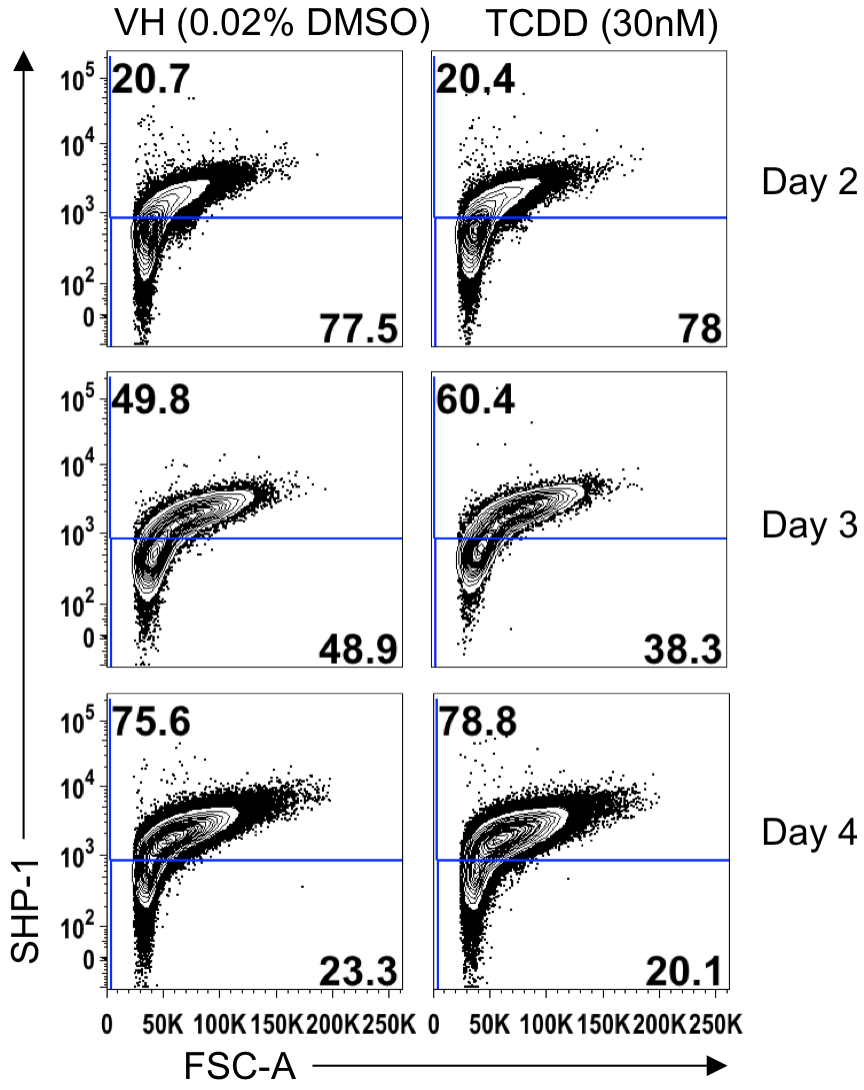


Figure 3.3.2: Increase in SHP-1 protein levels in presence of TCDD

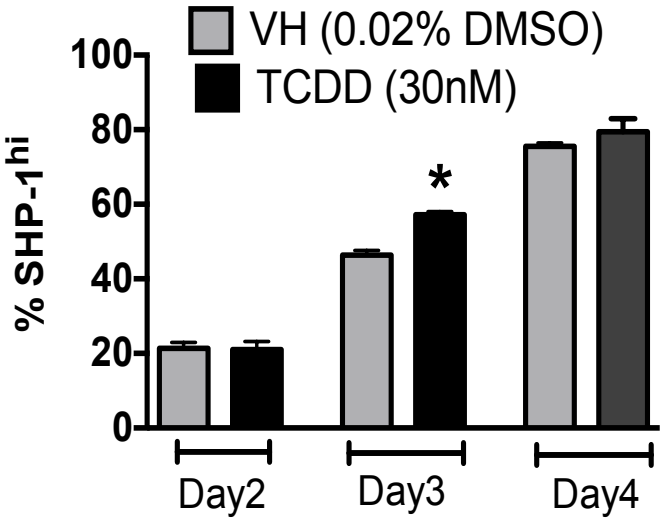
A. Human primary B cells were activated with CD40L plus cytokines and treated with TCDD or VH. Cells were harvested at the indicated time points and permeabilized. SHP-1 levels were measured by flow cytometry. The gates mark the SHP-1^{hi} and SHP-1^{lo} populations of cells and the numbers within each gate depict the % SHP-1 cells. Data shown in the figure is representative of fifteen TCDD-sensitive human donors. B. Changes in %SHP-1^{hi} cells at different time points in the same donor. C. Graph depicts change in SHP-1 MFI in the same donor. Dead cells were excluded from the analysis.

Figure 3.3.2 (cont'd)

*p<0.05 using one-way ANOVA followed by Fisher's Uncorrected LSD test. D. %SHP-1^{hi} cells in 13 TCDD-sensitive donors. E. MFI SHP-1 across 10 TCDD-sensitive donors

* p< 0.05 using unpaired Student's t-test.

B.



C.

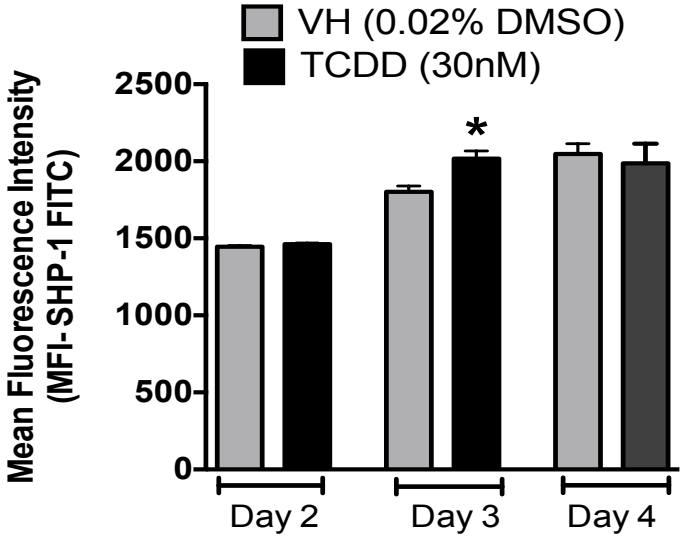
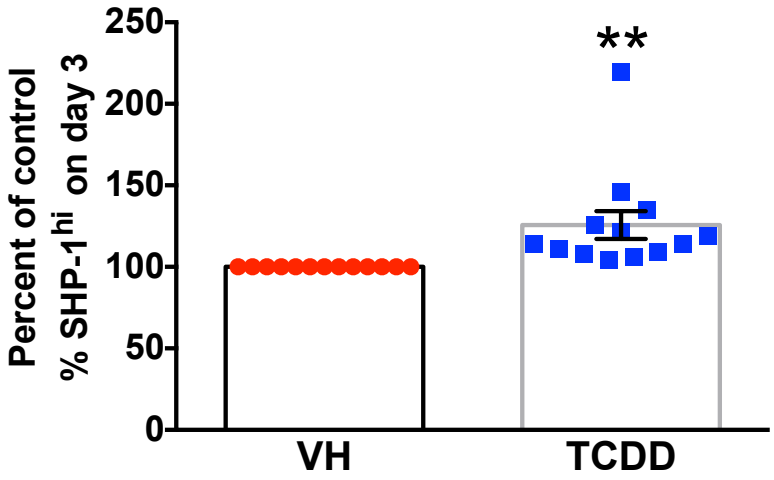
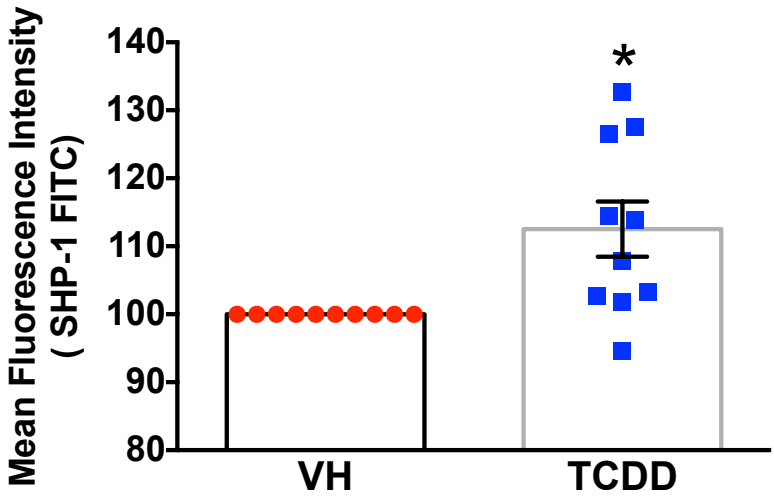


Figure 3.3.2 (cont'd)

D.



E.



A.

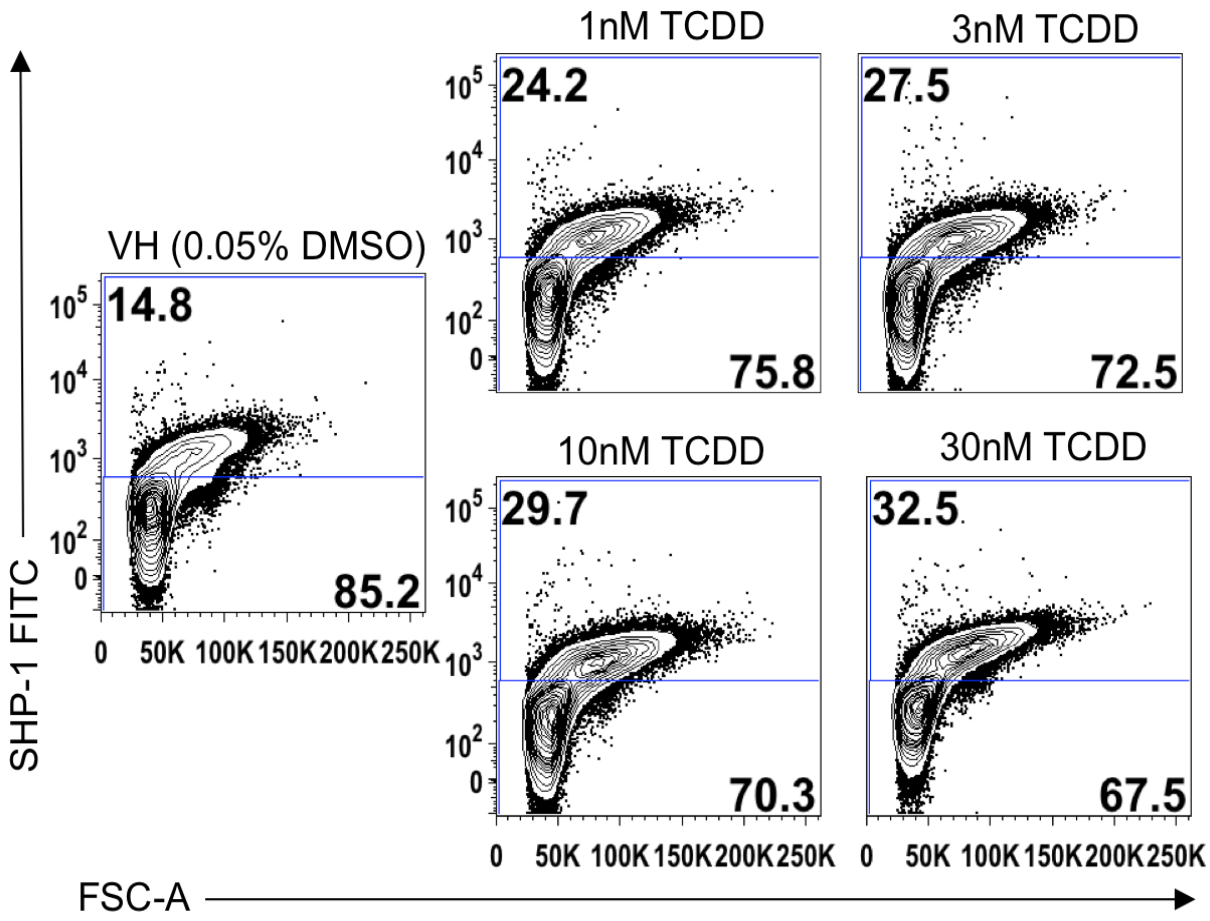


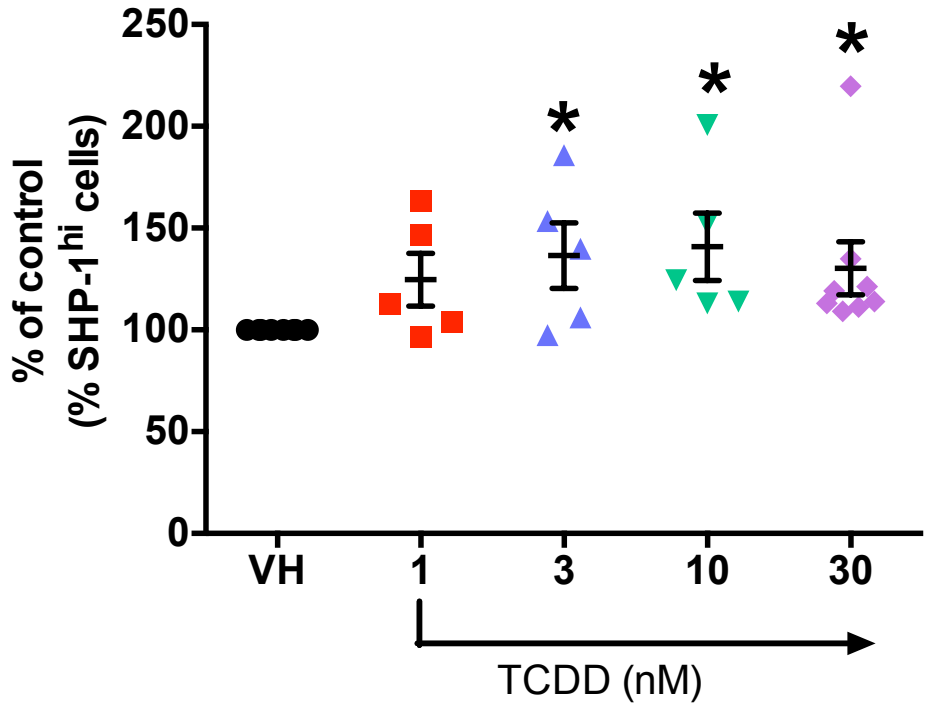
Figure 3.3.3: TCDD-concentration dependent increase in SHP-1 protein levels

A. Primary human B cells were treated with VH or TCDD at the indicated concentrations for a period of three days. Cells were harvested and then permeabilized for staining by flow cytometry. SHP-1 protein levels were measured on day 3. The gates depict the SHP-1^{hi} and SHP-1^{lo} cell populations with the numbers within the gates indicating the percentage of SHP-1 cells. Data shown in the figure is representative of five TCDD-sensitive human donors. B. Graph showing % SHP-1^{hi} cells at each concentration of TCDD. N=5 for TCDD concentrations of 1, 3, 10nM and N=8 for TCDD concentration of

Figure 3.3.3 (cont'd)

30nM. * p< 0.05 by one-way ANOVA followed by Fisher's uncorrected LSD post-hoc test.

B.



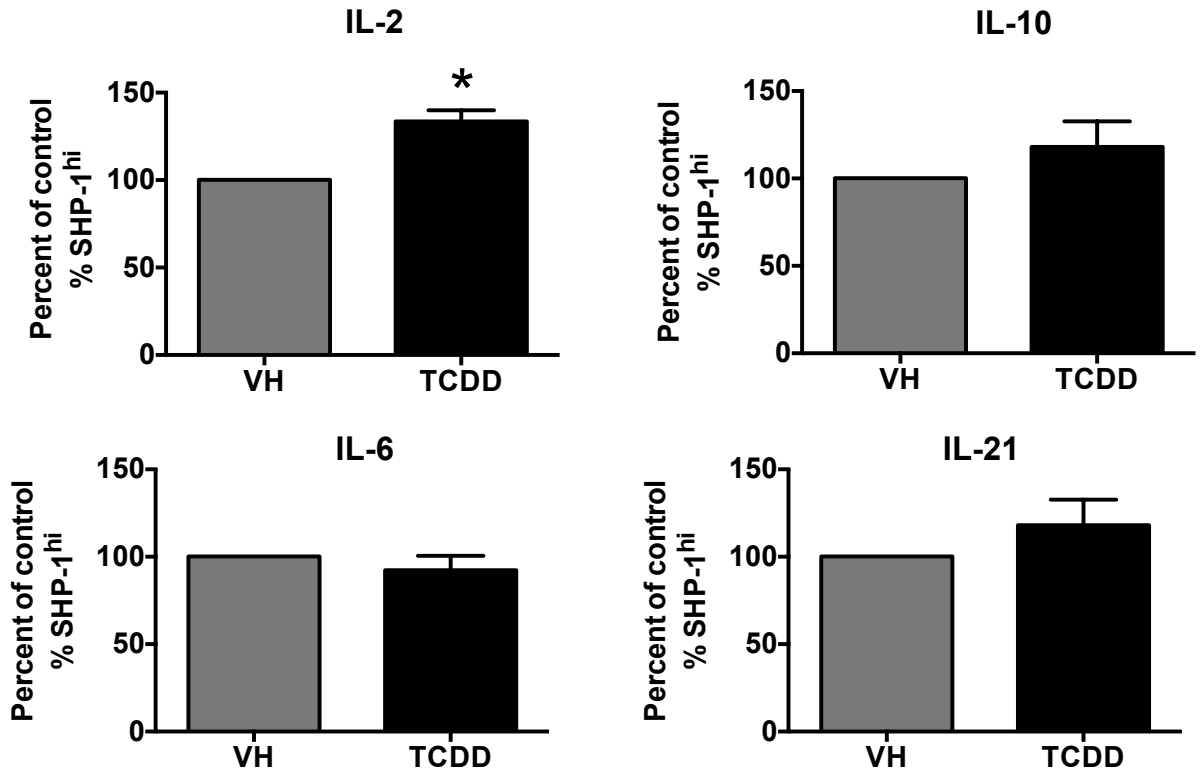


Figure 3.3.4: Effect of different cytokines on induction of SHP-1 protein levels in presence of TCDD

Human primary B cells were activated with CD40 ligand and individual cytokines as indicated above for a period of three days. Cells were then harvested, permeabilized and SHP-1 protein levels were measured. Graphs depict the percent of SHP-1^{hi} cells as a percent of VH control. For each graph, SHP-1 levels from three TCDD-sensitive human donors were pooled. * $p < 0.05$ by one-way ANOVA followed by Fisher's Uncorrected LSD post-hoc test.

surface CD40 or via B cell receptor signaling. To know if cytokine signaling through specific JAK-STAT pathways leads to increase in SHP-1 levels, human B cells were activated for a period of three days with CD40 ligand and individual cytokines IL-2, IL-6, IL-10 or IL-21. As a control, cells were also activated with CD40 ligand and a combination of IL-2, IL-6 and IL-10. SHP-1 protein levels were increased by TCDD-treatment in CD40 ligand and IL-2, 6, 10 activated B cells as seen before (data not shown). In the same donors, SHP-1 protein levels were significantly induced by TCDD-treatment only in presence of IL-2 (Figure 3.3.4). Activation with CD40 ligand and IL-10 or IL-21 alone, showed a slight trend towards an increase in SHP-1 levels.

Time of TCDD addition identifies a critical window of sensitivity leading to altered SHP-1 protein expression

qRT-PCR and flow cytometric analysis have suggested that an increase in SHP-1 expression occurs on days 2 or 3 following TCDD-treatment in activated primary human B cells. As SHP-1 is involved in proximal B cell signaling, we hypothesized that the induction of SHP-1 levels in presence of TCDD would be an early signaling event triggered by TCDD-AHR pathway. To this end, TCDD was added to primary human B cells either 30 min prior to or at the time of B cell activation or at days 1, 2 or 3 post-B cell activation. Figure 3.3.5 panel A and D show that the percentage of SHP-1^{hi} cells is higher in presence of TCDD especially when TCDD is added 30 mins before activation or at the time of activation and remain elevated post-activation on day 1. Significant effects of TCDD on SHP-1 protein levels are seen mostly when TCDD is added at the time of B cell activation or one day later (Figure 3.3.5 panels B-D).

Effect of sodium stibogluconate on B cell activation markers in the presence of TCDD

TCDD-treatment of human primary B cells decreased B cell activation as seen by an overall reduction in the expression of activation markers CD80, CD86 and CD69. SHP-1 is known as a negative regulator of B cell activation (Neel, 1997; Zhang et al., 2000). Hence, to determine the functional significance of elevated SHP-1 mRNA and protein levels in presence of TCDD, the effect of decreased SHP-1 on B cell activation markers was examined. For this purpose, sodium stibogluconate, a potent inhibitor of Src-homology phosphatases, known to inhibit 99% of SHP-1 activity was employed (Pathak and Yi, 2001). We hypothesized that suppression of SHP-1 activity would prevent its phosphatase activity thereby activating upstream of B cell signaling networks leading to increase B cell activation. To our surprise, impaired SHP-1 activity in human B cells treated with TCDD failed to reverse the suppression of activation markers as shown in figure 3.3.6.

Increase in BCL-6^{hi} SHP-1^{hi} double positive cells in the presence of TCDD

Network analysis performed on the 78 genes obtained as direct targets of AHR through the ChIP-chip and gene expression microarray studies showed associations with three key transcription factors BCL-6, Pax-5 and BLIMP-1 involved in B cell differentiation. Of particular relevance to this study is the association of SHP-1 (direct AHR target gene) with BCL-6 involved in B cell activation. Through an earlier study, we have demonstrated the role of BCL-6 in suppression of B cell activation in presence of TCDD. Putative associations between BCL-6 and SHP-1 highlight the functional role of SHP-1 in B cell activation. As both BCL-6 and SHP-1 are known to inhibit B cell activation. We measured the protein levels of BCL-6 and SHP-1 in the same population

A.

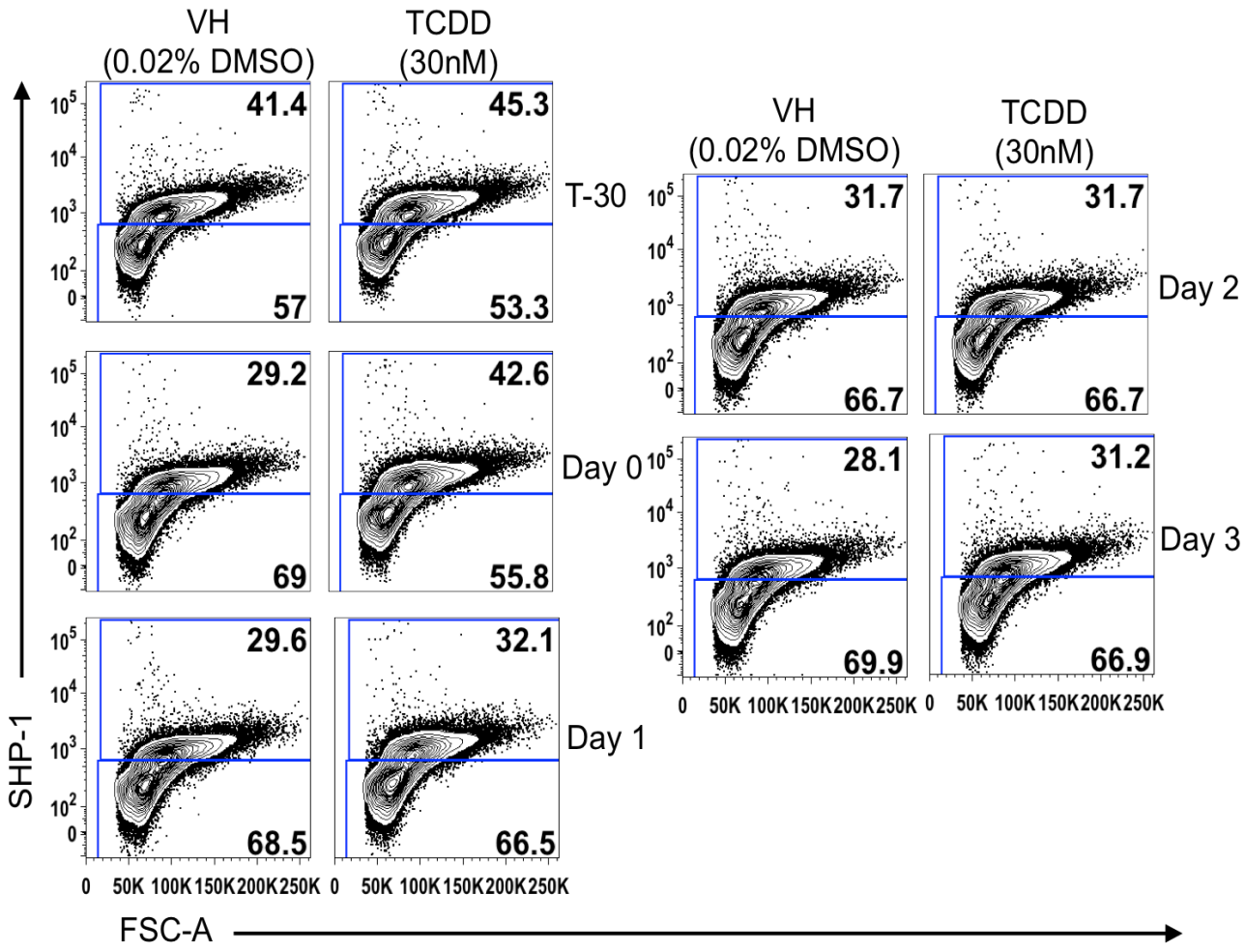


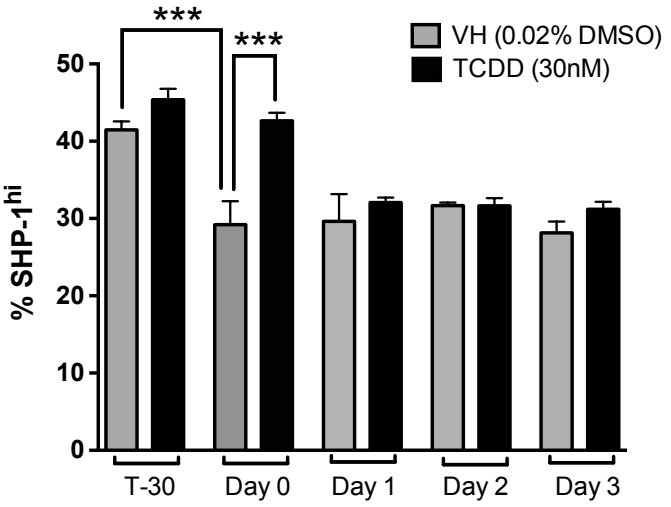
Figure 3.3.5: Increase in SHP-1 protein levels is dependent on time of TCDD addition

A. Human primary B cells were activated with CD40 ligand plus cytokines. TCDD was added to the cells either 30 min before activation or at the time of activation or at the indicated time points. Cells were harvested on day 4 and permeabilized in order to measure intracellular SHP-1 protein levels by flow cytometry. Graphs depict the % SHP-1^{hi} and SHP-1^{lo} cells as indicated by the gates and the frequency numbers in the corner.

Figure 3.3.5 (cont'd)

B. Percent of SHP-1^{hi} cells in the same TCDD-sensitive human donor C. MFI SHP-1 in the same donor. * p< 0.05, ***p< 0.001 by one-way ANOVA followed by Fisher's Uncorrected LSD test. D. %SHP-1^{hi} cells as a percent of VH control. Each dot represents data from one individual TCDD-sensitive human donor.

B.



C.

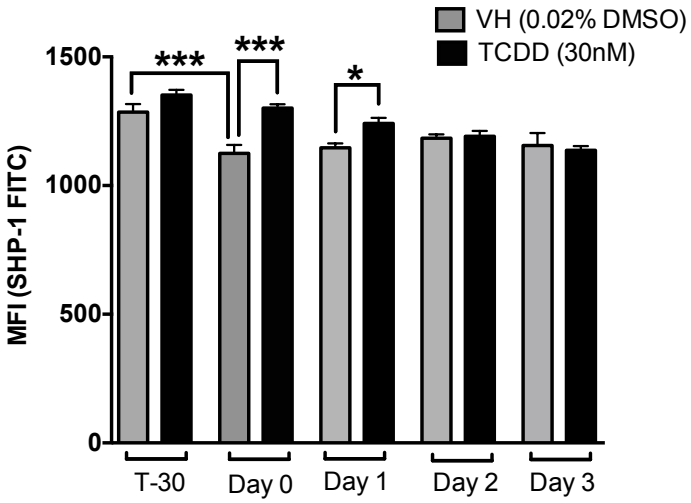
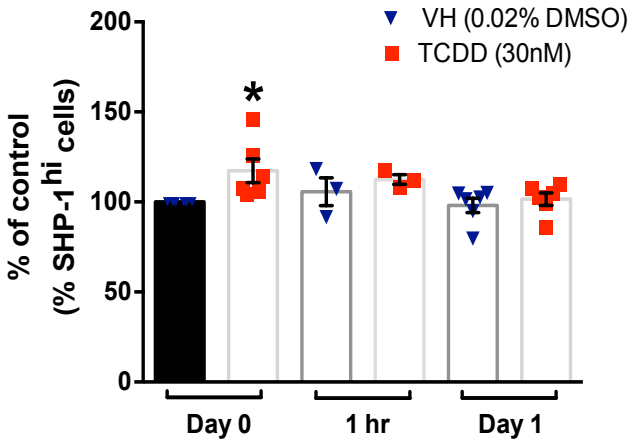
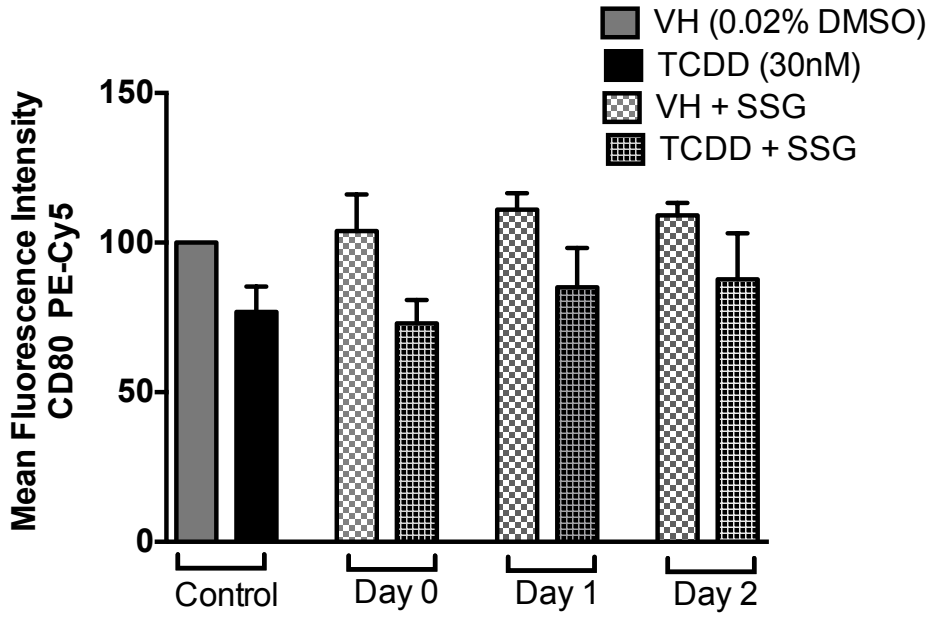


Figure 3.3.5 (cont'd)

D.



A.



B.

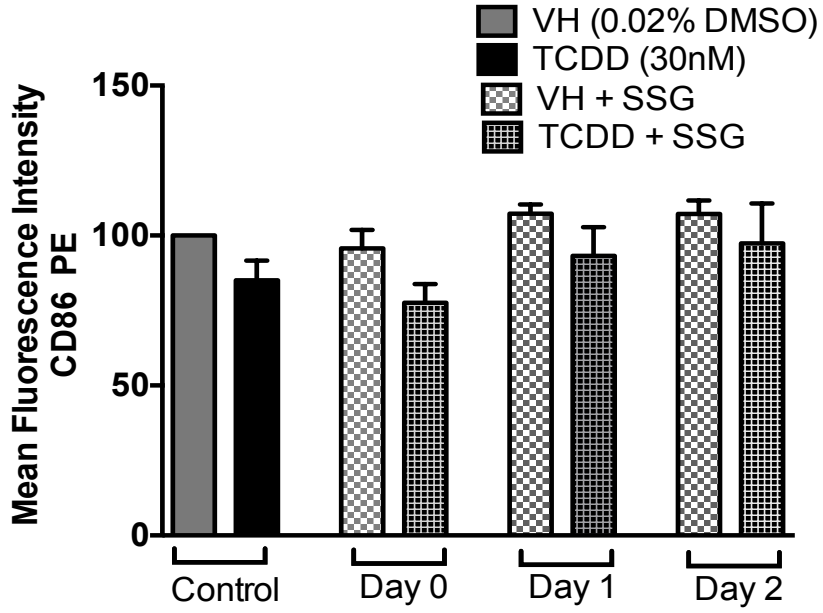


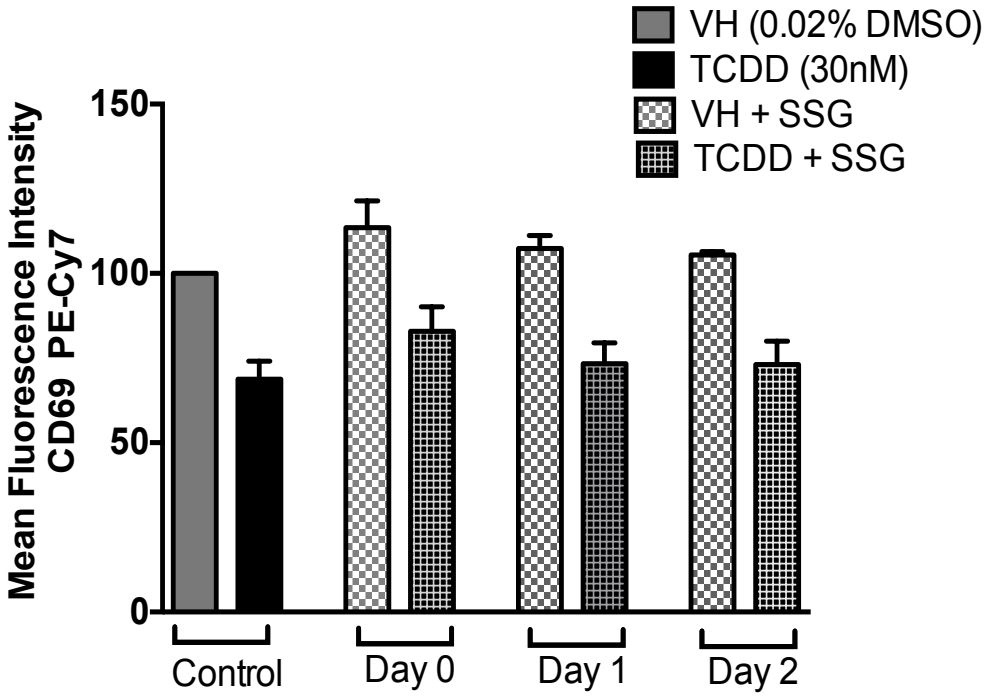
Figure 3.3.6: Effect of sodium stibogluconate on CD80, CD86 and CD69 levels in presence of TCDD

Human B cells were treated activated with CD40 ligand plus cytokines and treated with TCDD or VH. At the indicated time points, Sodium stibogluconate (10μg/ml) was added

Figure 3.3.6 (cont'd)

to the treated cells. Cells were harvested at day 3, permeabilized and stained for surface activation markers. Control cells did not receive any SHP-1 inhibitor. Graphs represent MFI of CD80, CD86 and CD69 from three TCDD-sensitive human donors.

C.



A.

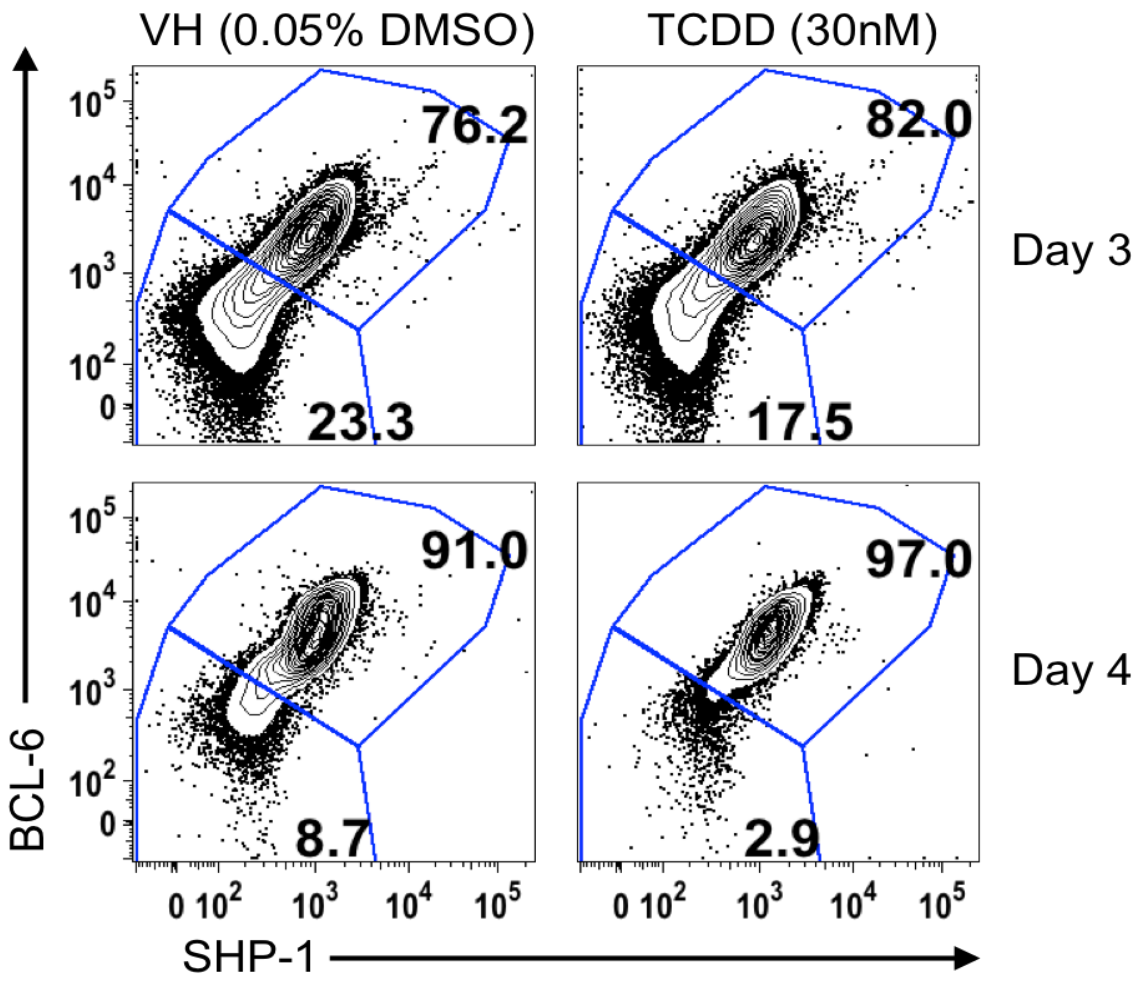


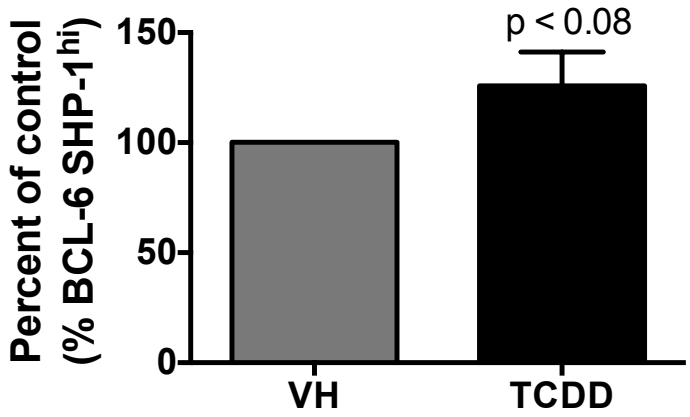
Figure 3.3.7: Increase in BCL-6^{hi} SHP-1^{hi} double positive cells in presence of TCDD

A. Human B cells were harvested at Day 2 and Day 3 following activation with CD40 ligand plus cytokines and treatment with TCDD or VH. The gates indicate the BCL-6^{hi} SHP-1^{hi} and BCL-6^{lo} SHP-1^{lo} populations of cells with numbers indicating the percent of double positive cells. B. Increase in %BCL-6^{hi} SHP-1^{hi} cells on day 3 shown as percent of VH control. Data pooled from five TCDD-sensitive human donors. C. Effect of increasing concentrations of TCDD on BCL-6hi SHP-1hi cells. Each dot represents data

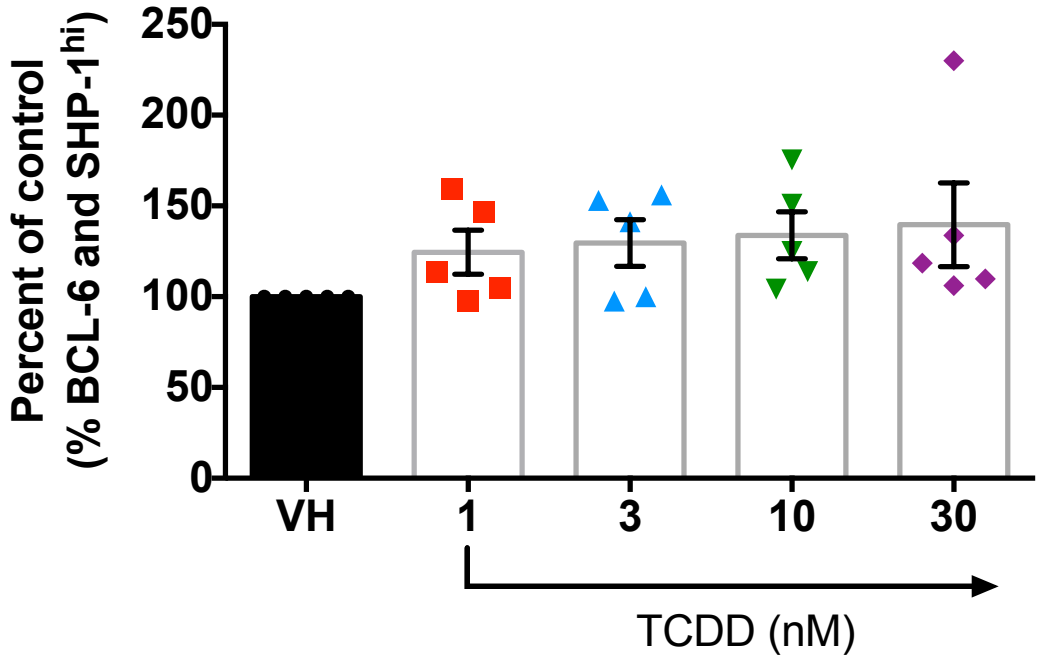
Figure 3.3.7 (cont'd)

from one individual TCDD-sensitive human donor.

B.



C.



of cells using multiparametric flow cytometry. Figure 3.3.7 panel A shows the increase in the percentage of BCL-6 SHP-1^{hi} cells in presence of TCDD on days 3 and 4. This increase in the BCL-6^{+} SHP-1^{+} population is seen in five TCDD-sensitive human donors and is slightly altered by the increasing concentrations of TCDD (Figure 3.3.7 panel B and C).

Putative cross talk between SHP-1 and BCL-6

Given the role of SHP-1 as phosphatase, we hypothesized that SHP-1 could dephosphorylate BCL-6 in B cells thereby increasing stability of BCL-6 causing an increase in BCL-6 levels. Moreover, it has been shown that SHP-1 can translocate to the nucleus and retain its phosphatase activity(Yang et al., 2002). To this end, the SHP-1 inhibitor was added to human peripheral blood B cells activated with CD40 ligand plus cytokines. As shown in Figure 3.3.8 panel A, an increase in BCL-6 levels was observed in naïve human B cells in absence of SHP-1 inhibitor for 2-3 days. In contrast, naïve cells cultured in presence of SHP-1 inhibitor fail to show a change in BCL-6 levels. The increase in the percentage and MFI of BCL-6 expressing cells is evident through the graphs in Figures 3.3.8 panels B and C.

A.

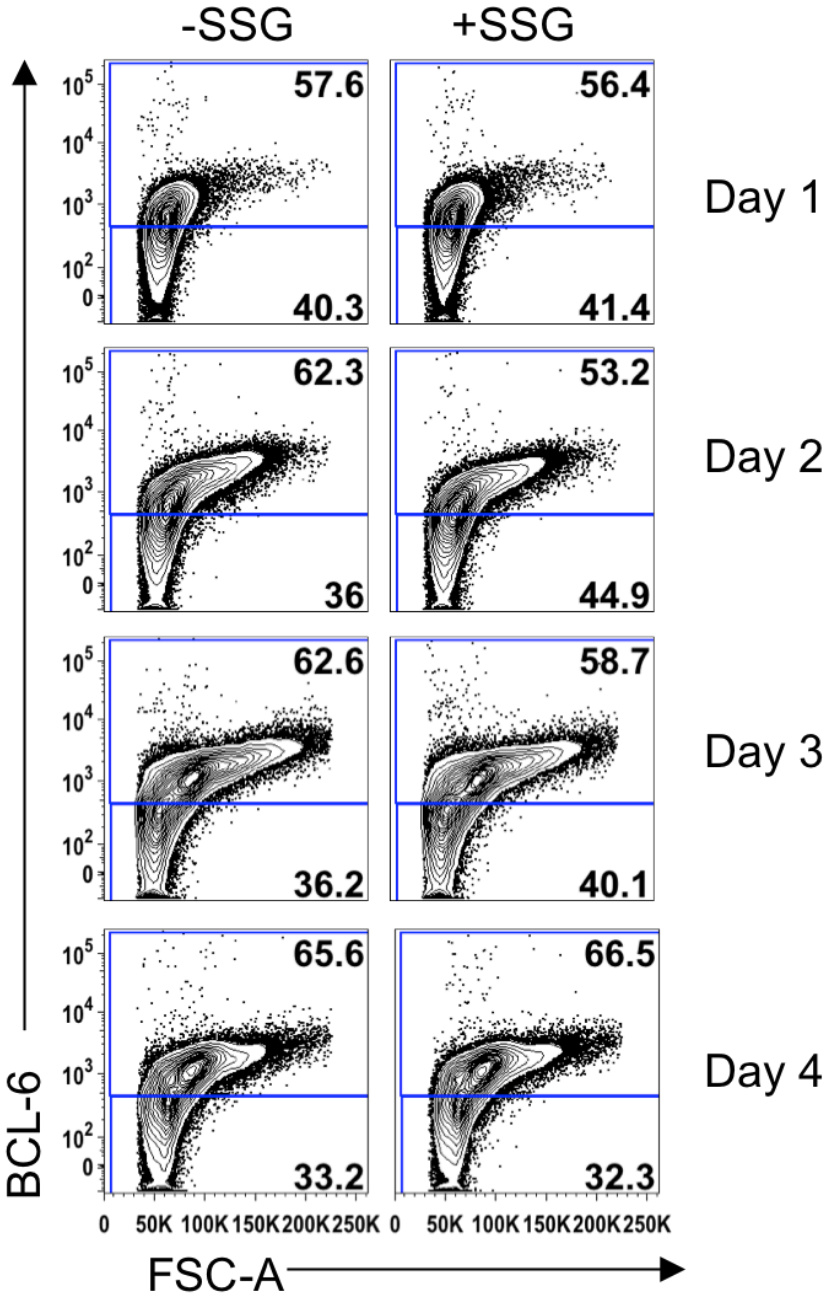


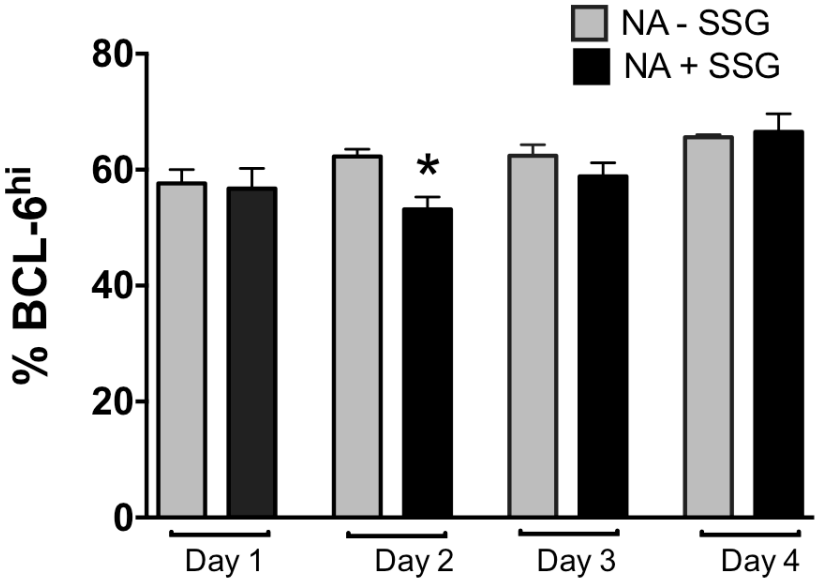
Figure 3.3.8: Changes in BCL-6 protein levels in presence of SHP-1 inhibitor

A. Naïve human B cells were activated with CD40 ligand plus cytokines and 10μg/ml of SHP-1 inhibitor SSG. Cells were harvested at the indicated time points and stained for BCL-6. Data in the flow cytometry plots is representative of two TCDD-sensitive human

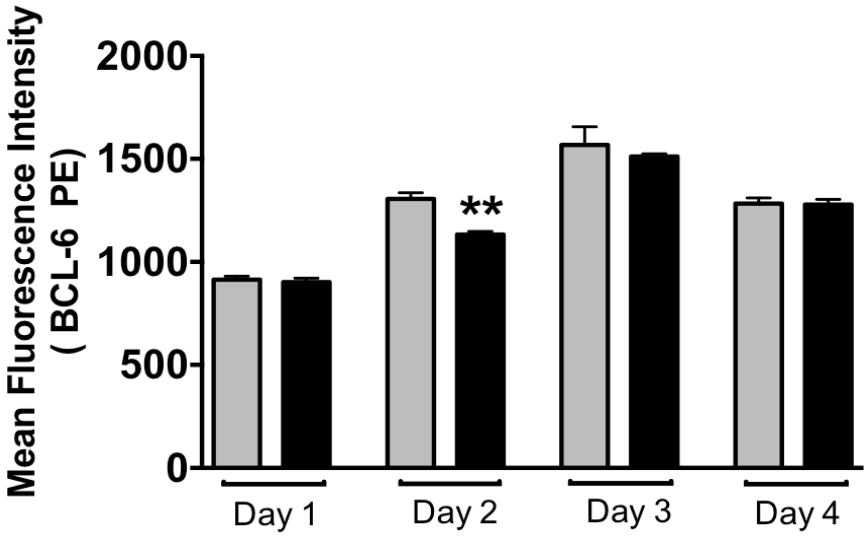
Figure 3.3.8 (cont'd)

donors. B. Changes in percentage of BCL-6hi cells in the same donor in presence and absence of SHP-1 inhibitor. C. Changes in MFI of BCL-6 in the same donor.

B.



C.



CHAPTER 4: DISCUSSION

This dissertation research aims to investigate the roles of Bach2, BCL-6 and SHP-1 in TCDD-mediated suppression of B cell function. For this purpose, the effect of TCDD on alteration of Bach2 mRNA levels and on Bach2 function was evaluated. This aspect of Bach2 regulation is discussed in section 4.1. BCL-6 and SHP-1 were identified as putative candidate genes affecting human B cell activation in presence of TCDD. The roles of BCL-6 and SHP-1 in suppression of B cell activation are discussed in sections 4.2 and 4.3, respectively.

4.1. Role of Bach2 in TCDD-mediated suppression of mouse B cell differentiation

TCDD, an AHR agonist is known to disrupt the process of B cell differentiation by altering key transcription factors such as Blimp-1, Pax-5, AP-1 and BCL-6 finally affecting IgM production (Schneider et al., 2008; Schneider et al., 2009; Sulentic et al., 1998; Sulentic et al., 2004b). In order to identify novel targets of the TCDD: AHR complex affecting plasmacytic differentiation in mouse B cells, an integrated genomic analysis involving ChIP-on-chip study and gene expression microarray analysis was performed (De Abrew et al., 2010). This study identified Bach2 as a direct target of AHR. AHR binding was observed at a DRE within intron1 of the Bach2 gene leading to an increase in Bach2 expression (De Abrew et al., 2010). Bach2 is a B cell specific transcriptional repressor that disrupts the plasmacytic differentiation in B cells to promote antibody class switching (Muto et al., 2010). Hence the aim of this study was to functionally determine the role of Bach2 in suppression of B cell differentiation in mouse B cells.

The binding of AHR to a consensus DRE “GCGTG” within the intron1 of Bach2 was confirmed by performing a ChIP-qPCR (De Abrew et al., 2011) and by performing EMSAs using radiolabeled DRE probe one hour post-TCDD treatment. The increase in Bach2 mRNA in presence of TCDD was verified in LPS-activated and resting B cells at several time points ranging from 2-24h. Time course studies showed at least a two-fold increase in Bach2 mRNA levels at each time point in presence of TCDD. TCDD-concentration dependent changes in Bach2 mRNA were observed 2h post-TCDD treatment in a concentration-responsive manner with significant effects at low concentrations of 1nM.

Among the various transcription factors known to regulate B cell differentiation, Blimp-1 is the master regulator that drives plasmacytic differentiation. Blimp-1 was recently identified to be a crucial target of Bach2 (Ochiai et al., 2006; Ochiai et al., 2008b). Blimp-1 and Bach2 represent two transcription factors associated with distinct stages of B cell differentiation; Blimp-1 expressed at high levels in plasma cells and Bach2 expressed at high levels in naïve B cells with low or no expression in plasma cells (Ochiai et al., 2008b). Owing to the reciprocal gene expression profiles of Bach2 and Blimp-1, time course measurements of Blimp-1 mRNA were performed to examine whether this reciprocal relationship existed in TCDD-treated B cells. Blimp-1 mRNA levels increased with B cell activation and were significantly higher at 2h in presence of TCDD but showed a significant suppression at 24h time point. This decrease in Blimp-1 mRNA at 24h correlates with previous observations (Schneider et al., 2009) and is reflective of the time required for translation, folding and subsequently transcriptional repression by Bach2. Blimp-1 expression is up regulated by the transcription factor AP-

1 in activated B cells and this occurs by binding of AP-1 to TPA-responsive elements (TRE) in the Blimp-1 promoter (Ohkubo et al., 2005). Additional mechanisms involving AHR-mediated effects on the transcription factor AP-1 may also play a role in regulating Blimp-1 in presence of TCDD as suggested by experiments showing impaired AP-1 binding to the three TRE sites in presence of TCDD (Schneider et al., 2009). Collectively, these results suggest that the increase in Bach2 expression by the TCDD-AHR complex contributes to the decrease in Blimp-1 expression partly through suppression by Bach2 and AP-1 in TCDD-treated B cells.

The mechanism of transcriptional repression by Bach2 involves heterodimerization of Bach2 with small Maf proteins such as Mafk, MafG and MafF followed by binding to the Maf recognition elements (MAREs) (Igarashi et al., 1994; Motohashi et al., 1997; Muto et al., 1998). Repression of Blimp-1 by Bach2 occurs through two separate MAREs, one in the promoter (Ochiai et al., 2006) and another within the intron 5 of Blimp-1 gene (Ochiai et al., 2008a). EMSAs performed to examine the effect of TCDD on binding of Bach2 to these MAREs showed a modest increase in DNA binding activity at the promoter MARE and a stronger increase at the intron 5 MARE. The increase in DNA binding activity of Bach2 at the intron 5 MARE occurred in a TCDD-concentration dependent manner. EMSA-supershifts performed to identify specific binding of Bach2 to the MAREs in the Blimp-1 gene revealed specific binding of Bach2 only at the intron 5 MARE in presence of TCDD as the binding intensity decreased upon addition of an anti-Bach2 antibody but not in the presence of a non-specific isotype control antibody. A supershifted band of decreased mobility was not observed due to an interference of the anti-Bach2 antibody with the DNA binding of

Bach2 at that site. To our surprise, specific binding of Bach2 was not detected at the promoter MARE, though a report published by Ochiai *et. al.,* had suggested binding of Bach2 at that site (Ochiai et al., 2006). This result suggests differential regulation of Bach2 at the two MARE sites within the Blimp-1 gene in presence of TCDD in CH12.LX cells. It also reflects on the differential role of Bach2 in controlling Blimp-1 in activated B cells in presence of TCDD.

Ablating Bach2 expression using RNA interference in CH12.LX cells demonstrated functional validation of the role of Bach2 in suppression of B cell differentiation (De Abrew et al., 2011). Transient transfection with Bach2 siRNA showed 40% reversal of IgM suppression (De Abrew et al., 2011) suggesting that transcriptional regulation of Bach2 by AHR is one of the mechanisms by which TCDD suppresses mouse B cell differentiation.

The role of Bach2 in suppressing B cell differentiation is consistent with previous observations. The B cell differentiation network is comprised of three cardinal transcriptional factors Blimp-1, Pax-5 and Bcl-6. The interplay between these transcription factors is altered by TCDD in activated B cells. Previous studies show that TCDD-mediated suppression of B cell differentiation, as evidenced by decreased IgM secretion, occurs through decreased expression of IgH, κ light chain, and J chain (Yoo et al., 2004). The decrease in IgH is caused by binding of TCDD:AHR to the DRE in 3'α enhancer regions (Sulentic et al., 2004b). A subsequent study showed that downregulation of Pax-5 was impaired by TCDD. The upstream regulator of Pax-5, Blimp-1 mRNA was inhibited by TCDD so was the binding of Blimp-1 within the Pax-5 promoter (Schneider et al., 2008; Schneider et al., 2009). The ChIP-on-chip study

identified other proteins such as Irf8, Myc and Xbp-1 as potential direct AHR targets (De Abrew et al., 2010). Bach2 was recently shown to cooperatively bind to BCL-6 and regulate gene expression in GC B cells through transcriptional and biochemical mechanisms controlling plasmacytic differentiation (Huang et al., 2013a). In addition, Bach2 also negatively regulates the Ig heavy chain 3'α enhancer region thereby directly controlling B cell differentiation (Muto et al., 1998). This aspect of Bach2-mediated repression of IgH 3'α enhancer remains to be verified in presence of TCDD. Overall, these studies involving identification of Bach2 and elucidation of the role of Bach2 in suppression of B cell differentiation in presence of TCDD have provided greater mechanistic insight into the understanding of the transcriptional network altered in B cells by AHR agonists.

4.2. Role of BCL-6 in TCDD-mediated suppression of B cell activation in human primary B cells

Numerous signaling pathways, feedback loops and transcriptional circuits regulate immune responses in mammalian systems. Disruption of any of the signals either transcriptional, cell signaling or both can lead to severe consequences finally culminating in compromised immune system function. Use of environmental contaminants possessing high affinity for AHR, as probes to understand the role of AHR in B cell function have proven effective in determining the importance of AHR from a physiological standpoint. Several recent reports have eluded that AHR is involved in influencing the kinetics, magnitude and/or direction of immune responses (Stevens et al., 2009). TCDD, one such environmental contaminant is known to adversely affect

primary humoral immune responses altering the ability of B cells to produce antibody. Using a primary human B cell differentiation model wherein B cells were activated with CD40 ligand and cytokines IL-2, IL-6 and IL-10, TCDD treatment showed attenuation of the B cell activation phenotype with an overall decrease in the number of active and viable cells that form antibody secreting cells (Lu et al., 2011; Lu et al., 2010). For B cell differentiation to ensue, activation of B cells is an indispensible precursor event as the threshold of activation dictates the outcome of immune responses. BCL-6 was a prime candidate for our investigation given the role of BCL-6 in regulating B cell activation, proliferation, cell cycle and plasmacytic differentiation.

TCDD-treatment of activated human B cells increased BCL-6 protein levels. In our model, this TCDD-mediated increase in the percent and in the MFI of BCL-6 was seen in several TCDD-sensitive human donors. Though differences were observed in the magnitude and kinetics of BCL-6 levels between human donors, the overall trends were constant irrespective of the donor. The alteration in BCL-6 protein levels was also seen at different concentrations of TCDD with a significant effect at the highest concentration of TCDD. However, no changes were observed at the mRNA level upon TCDD treatment. This result is not surprising considering the high expression level of BCL-6 in naïve and activated B cells in our model. Moreover, BCL-6 expression is controlled at the protein level in numerous different ways. BCL-6 regulation is complex and involves several post-transcriptional processes. BCL-6 expression is controlled by acetylation (Bereshchenko et al., 2002), repression via the transcription factors Blimp-1 and Mad1 (Kusam and Dent, 2007), by STAT-5 (Walker et al., 2007), and also by negative autoregulation through CtBP (Mendez et al., 2008).. Recently, BCL-6 was

found to interact with FBXO11 (Duan et al., 2012). FBXO11 is an F-box protein that directly controls degradation of BCL-6 via the SCF-cullin proteolysis pathway. As FBXO11 levels and BCL-6 levels were inversely correlated, we measured mRNA levels of FBXO11 in presence of TCDD. FBXO11 was also identified in the genome-wide ChIP-on-chip analysis performed in mouse CH12.LX cells. In human B cells, Fbxo11 mRNA levels were elevated upon treatment with TCDD contrary to our expectations, thus suggesting that Fbxo11 did not play a role in maintenance of BCL-6 levels. Another mechanism for downregulation of BCL-6 levels involves phosphorylation of BCL-6 by ERK, which triggers BCL-6 degradation via the ubiquitin proteasome pathway (Niu et al., 1998b). In a previous study, TCDD-treatment significantly attenuated the percentage of p-ERK^{+} p-p38^{+} p-AKT^{+} cells in human B cells 30 min post-activation with recombinant CD40L (Lu et al., 2011) and decreased pERK levels on day 1 and day 2 post-activation with recombinant CD40L (Lu et al., unpublished results). In my studies, pERK levels significantly decreased only on day 1 post-activation with CD40 ligand plus cytokines but the pERK levels recovered back on day 2 in presence of TCDD. This suggests that a disruption of early B cell signaling may be partially responsible for the maintenance of elevated BCL-6 protein levels in human B cells. Though we have not completely ruled out the possibility that TCDD would lead to a decreased downregulation of BCL-6 by impairing its ubiquitination via the SCF-cullin or ubiquitin pathway, further studies need to be conducted to address this issue.

Multi-parametric analyses measuring BCL-6 and CD80 or BCL-6 and CD69 levels in the same population of cells suggested an inverse relationship between BCL-6 expression and the amount of CD80 or CD69 expressed by human B cells. This finding

is consistent with the observations made in Burkitt's lymphoma cell lines demonstrating mutually exclusive expression patterns of CD80 and BCL-6 (Niu et al., 2003) thus suggesting that BCL-6 may be playing a role in downregulating CD80 expression *in vivo.* cDNA microarrays have been useful in identifying novel targets of BCL-6 repression. CD69 was one of the genes repressed by BCL-6 in BJAB and SUDHL5 cell lines and was also known to possess high affinity BCL-6 binding sites within the promoter region (Shaffer et al., 2000). Furthermore genetic deletion of BCL-6 in mice caused increased CD69 expression (Shaffer et al., 2000) and CD80 expression (Niu et al., 2003) therefore supporting our finding that BCL-6^{hi} cells showed lower CD80 and CD69 expression in presence of TCDD.

The activation marker CD80 is directly regulated by BCL-6 (Niu et al., 2003). We found that BCL-6 binds to a previously identified consensus BCL-6 binding site within the CD80 enhancer in presence of TCDD *in vitro*. The CD80 enhancer region also harbors an NFκB binding site downstream of the BCL-6 binding site. Expression of CD80, in human B cells in the presence of TCDD may also be regulated partly through the action of NFκB and BCL-6. Previously, it has been observed that TCDD-treatment of human primary B cells leads to a decrease in phospho-p65 levels and phospho-c-jun (Lu et al., unpublished results). In addition, several studies have established a possible complex interaction between AHR and NFκB signaling pathways, which may contribute to this effect (Tian et al., 2002; Vogel and Matsumura, 2009). Hence, it is tempting to speculate that the effect of TCDD on CD80 expression could be due to a combination of the repressive action of BCL-6 and due to decreased NFκB levels in presence of TCDD.

BCL-6 is expressed at high levels in rapidly proliferating cells of the germinal center (Ranuncolo et al., 2007). In our model system, we too observed that cells expressing higher BCL-6 levels displayed greater proliferation with no effect of TCDD on B cell proliferation similar to reports by others (Holsapple et al., 1986b; Luster et al., 1988). BCL-6 has a broad range of target genes that it suppresses including genes involved in DNA damage and cell cycle. The survival and proliferation of DLBCL or germinal center cells is governed by the lateral groove of BCL-6 BTB domain (Huang et al., 2013b). The BTB domain has autonomous repressor activity along with an ability to recruit corepressors aiding BCL-6 in its repressive activity (Ahmad et al., 2003; Ghetu et al., 2008). This domain of BCL-6 is being targeted for development of drugs to kill lymphoma cells. Treatment of human primary B cells with TCDD and the BCL-6 inhibitor reversed the suppressed expression of BCL-6 target genes CD80, CD86 and CD69. The magnitude of reversal of suppressed activation marker expression was greater for CD80 as compared to CD86 and CD69. This suggests that the effect of BCL-6 repression may be strongest on CD80. BCL-6 is just one of the transcription factors controlling expression of CD86 and CD69 thus suggesting that other transcription factors could play a greater role in regulating expression of these activation markers. Differences are also observed in the magnitude of response of different donors to the BCL-6 inhibitor. One reason for this could be the differences in basal and activation-induced expression levels of activation markers and BCL-6 in different human donors. In addition, the levels of different corepressors and their ability to be recruited or sequestered from BCL-6 could be different across individuals. Collectively, the data

support the conclusion that suppression of activation marker function is, in part, due to elevated expression of BCL-6 and BCL-6-mediated repression.

The AHR is known to contribute at multiple levels to the suppression of immune system function. Activation of mouse and human B cells leads to an increase in AHR levels (Allan and Sherr, 2005; Crawford et al., 1997; Sulentic et al., 1998). The increase in BCL-6 levels and the suppression of B cell activation marker expression was dependent on AHR as demonstrated by using the high and low-affinity congeners of the dibenzo-*p*-dioxins. Several studies have also suggested an AHR-dependent but a DRE-independent mechanism of action of TCDD in different cell types (Puga et al., 2000; Tappenden et al., 2011). Taken together the results suggest that the TCDD-AHR complex is involved in altering BCL-6 levels, the mechanism of which remains unanswered. Through this study, we have tried to bring forth a different perspective for the role of AHR in human B cells, in regulating B cell activation and possibly in B cell malignancies owing to the oncogenic potential of BCL-6. Our study provides interesting correlations to the observations made in the epidemiological studies performed in areas of dioxin exposure. This study also presents a framework of investigations into the molecular mechanisms underlying suppression of B cell activation in human cells in the presence of the environmental contaminant, TCDD. A complete understanding of the effects of impaired B cell activation within the context of immune responses will involve a detailed study of the effects of TCDD on B cell-mediated T cell activation, which remain to be investigated.

4.3. Role of SHP-1 in TCDD-mediated suppression of B cell activation in human primary B cells

The use of the CD40 ligand-dependent activation model has been pivotal in providing an understanding of the mechanism underlying suppression of B cell effector function by TCDD in human cells. TCDD-treatment decreased B cell differentiation specifically by suppressing of the primary IgM response (Lu et al., 2009). A recent study further extended the mechanistic investigation of TCDD immunotoxic effects and reported that TCDD attenuated expression of surface B cell activation markers CD80, CD86 and CD69 (Lu et al., 2011). Activation of B cells is critical prequel to the process of differentiation of B cells into antibody secreting plasma cells (Jelinek and Lipsky, 1983; Schmidlin et al., 2009). Hence, the overall aim of this study was to elucidate the mechanism by which AHR agonists perturb B cell activation. In a previous study, SHP-1 was identified as a potential transcriptional target of AHR in a mouse B cell line (De Abrew et al., 2010). SHP-1 negatively regulates BCR signaling by controlling the threshold for B cell activation (Pao et al., 2007b). The goal of the present study was to characterize the role of SHP-1 in TCDD-mediated impaired human B cell activation.

The increase in SHP-1 expression in presence of TCDD was confirmed by performing mRNA time course measurements in activated human B cells using qRT-PCR. These studies showed that the expression of SHP-1 was induced by TCDD at days 2 and 3 post-B cell activation. Concentration-response studies demonstrated a TCDD-mediated concentration-dependent increase in SHP-1 mRNA levels at day 3. Consistent with the changes in mRNA levels, SHP-1 protein levels too, were elevated upon TCDD-treatment at days 3 and 4 in CD40 ligand plus cytokine-activated human B

cells. The increase in the percentage of SHP-1^{hi} cells as well as change in MFI of SHP-1 was seen in several TCDD-sensitive human donors. The changes in protein levels of SHP-1 were also observed in a TCDD concentration-dependent manner. Collectively, these results confirm the findings of the ChIP-on-chip and gene expression microarray study.

One of the mechanisms by which SHP-1 expression is altered in presence of TCDD is via direct binding of AHR as shown by the ChIP-on-chip studies. Increase in SHP-1 expression in lymphoid cells is regulated by differential promoter requirement and by activation with PMA/Io through NFκB (Tsui et al., 2002). To further characterize the mechanism by which an increase in SHP-1 is observed in human B cells in presence of TCDD, individual cytokines were used to activate human B cells in presence of CD40 ligand-L cells. A combination of CD40 ligand and IL-2 showed a similar increase in the percentage of SHP-1^{hi} cells in presence of TCDD when compared to treatment with individual cytokines IL-6, IL-10 or IL-21. These findings suggest that signaling downstream of IL-2R pathway is critical for the increase in SHP-1 expression. IL-2 is responsible for promoting B cell proliferation as compared to IL-6, IL-10 or IL-21, which are important for B cell differentiation (Ding et al., 2013; Ettinger et al., 2005). Signaling downstream of IL-2R predominantly involves Jak3 mediated-phosphorylation of STAT5, PI3K-mediated activation of NFκB and PLCγ plus Ca^{++}-mediated activation of MEK and ERK pathways finally leading to changes in gene expression. In contrast, the IL-6, IL-10 and IL-21 pathways regulate gene expression via Ras or p38-mediated activation of ERK and Jak1 or Jak3-mediated activation of STAT3.

The differential downstream signaling intermediates may govern changes in SHP-1 expression. Overall, these results suggest that the increase in SHP-1 protein levels in presence of TCDD is mediated by a signaling intermediate downstream of the IL-2 signaling pathway.

Addition of TCDD to cultured human B cells at specific time points prior to activation helped identify the critical window of sensitivity of human B cells to TCDD. A significant increase in SHP-1 protein levels as measured by the MFI and percentage of SHP-1^{hi} cells was evident only when TCDD was added 30 min prior to or at the time of B cell activation. Addition of TCDD at day 1 post-activation also resulted in a small increase in SHP-1^{hi} cells with no effect seen at the later time points. Collectively, these results suggest that an increase in SHP-1 levels occurs due to a change in early signaling events occurring at the time of B cell activation in presence of TCDD. The time-of-addition study results are consistent with previous observations wherein TCDD-mediated suppression of B cell activation in human cells occurred only when TCDD was added at the time of activation or 24h post- activation of B cells (Lu, H unpublished observations). Moreover, this observation also corroborates with earlier studies in which, the window of sensitivity of TCDD in the sRBC-elicited IgM response was determined. Greatest suppression of anti-sRBC IgM AFC response was observed when TCDD was added to splenic cultures either 60 min prior to or at the time of antigen sensitization or day 1 post-antigen sensitization. The effect diminished when TCDD was added at days 2 - 5 post-antigen sensitization (Tucker et al., 1986). In addition, inhibition of LPS-induced IgM AFC response was produced when TCDD was added before 3h post- B cell activation (Holsapple et al., 1986a). Suppression of early kinase

phosphorylation by TCDD, especially kinases associated with the CD40 receptor and cytokine signaling pathways may provide an explanation for the window of sensitivity surrounding the effects observed in human cells. It is well known that kinases in B cells respond very rapidly to activation stimuli and transmit the signal downstream to affect B cell effector functions before returning to normal levels (Irish et al., 2006). It can be speculated that the initial signaling pathway induced via ligation of CD40 and binding of cytokines may have reached peak activity within hours of activation and then decreased back to basal levels, the critical window of sensitivity may have passed thereby rendering the cell refractory to the toxic effects of TCDD at the later time points.

Further, to functionally validate the role of SHP-1 in suppressing B cell activation in presence of TCDD, SHP-1 phosphatase function was impaired using sodium stibogluconate, a specific inhibitor of SHP-1 activity (Pathak and Yi, 2001). Addition of the SHP-1 inhibitor failed to reverse suppression of B cell activation markers such as CD80, CD86 or CD69 in presence of TCDD. SHP-1 dephosphorylates numerous kinases in the B cell especially LYN, SYK which are associated with the B cell receptor. SHP-1 also inhibits B cell activation by impairing p-STAT3 (Han et al., 2006), and the PI3K-AKT signaling pathway. This suggests that suppression of B cell activation markers may be occurring independent of SHP-1 or another phosphatase may be complementing the role of SHP-1 in presence of the inhibitor. Also, as complete ablation of SHP-1 activity is not achievable using an inhibitor other strategies involving knockdown of SHP-1 activity using RNA interference may have to be employed in future to observe phenotypic effects.

To identify which genes among the 78 genes identified by the ChIP-on-chip and gene expression microarrays were involved in B cell differentiation, putative signaling networks were constructed to connect the 78 genes to Pax5, Blimp-1 and BCL-6 (De Abrew et al., 2010). Through the network analysis, SHP-1 was seen to negatively regulate STAT1, STAT5 and IRF8, which in turn were regulating BCL-6 expression. Hence given the possibility of SHP-1-mediated regulation of BCL-6, SHP-1 and BCL-6 levels were measured concomitantly in human B cells treated with TCDD. An increase in the percentage of SHP-1^{+} BCL-6^{+} cells was observed in presence of TCDD in human B cells from five TCDD-sensitive human donors. The percentage of BCL-6^{hi} SHP^{hi} cells were elevated at the lowest concentration of TCDD but showed only a slight dependence on TCDD concentrations beyond 1nM.

Taken together, these results suggest that SHP-1 expression is induced by TCDD. Elevated SHP-1 levels may have a significant impact on B cell function through the alteration of upstream kinases. TCDD treatment was shown to decrease the percentage of $pERK^{+}p38^{+}pAKT^{+}$ cells upon immediate early activation of B cells (Lu et al., 2011). TCDD time-of-addition studies also suggested that changes in SHP-1 expression occur if TCDD is present 30 min before or at the time of B cell activation. Hence it is possible that decrease in levels of B cell kinases may be occurring through elevated SHP-1 levels in human B cells. Moreover, SHP-1 has been reported to inhibit Akt, pSTAT3 and pSTAT5 (Han et al., 2006; Mittal et al., 2011). Akt is important for cell survival (Andjelic et al., 2000; Calo et al., 2003). Hence impaired B cell activation phenotype could be a result of impaired activation of B cells implicated as a result of

decreased Akt and decreased survival in presence of elevated SHP-1 levels. SHP-1 and BCL-6 multiparametric flow cytometry revealed that SHP-1^{hi} cells also showed elevated BCL-6 levels thus suggesting potential cross-talk between SHP-1 and BCL-6 in presence of TCDD. We further explored if BCL-6 levels were altered in presence of SHP-1 inhibitor in naïve B cells and interestingly observed a decrease in BCL-6 as opposed to no change or stable BCL-6 levels in absence of SHP-1 inhibitor. This is suggestive of role of SHP-1 in dephosphorylating BCL-6 thereby stabilizing BCL-6 levels in absence of inhibitor. These studies show for the first time a relationship between SHP-1 and BCL-6 levels in human primary B cells. In the earlier part of this thesis we have also shown BCL-6-mediated suppression of B cell activation with direct effects of BCL-6 on activation marker CD80 in presence of TCDD (manuscript in preparation). Collectively, these studies demonstrate for the first time that SHP-1, may, in part be responsible for TCDD-induced effects on human B cells.

CHAPTER 5: FINAL CONCLUDING REMARKS

Environmental contaminants such as TCDD have been a long-standing concern to human health. The results from this dissertation study provide a novel insight into the mechanistic basis underlying TCDD-mediated effects on B cell activation and differentiation. The first part of this thesis elaborates on the role of Bach2, a novel gene identified through a genome-wide study focusing on direct targets of AHR in mouse B cells. Specifically, the study shows that binding of AHR in presence of TCDD to an intronic site within the Bach2 gene increases Bach2 expression. Bach2 regulates Blimp-1 by binding to the intron 5 site within the Blimp-1 promoter thereby affecting B cell differentiation in presence of TCDD. The role of Bach2 is consistent with the dynamics of other transcription factors that are regulated by TCDD-AHR complex in B cells. The interplay between the TCDD: AHR complex and B cell differentiation network is illustrated in Figure 5.1. A 'bi-stable' switch controls the decision of activated B cells to differentiate into antibody secreting plasma cells (Delogu et al., 2006; Mora-Lopez et al., 2007). TCDD reduces the number of IgM-secreting cells in an 'all-or-none' manner rather than decreasing the total amount of antibody produced by a plasma cell (Zhang et al., 2013). With the identification of Bach2, we could add an additional transcription factor to the existing network of three main transcription factors including Prdm1, BCL-6 and Pax-5. Cell fate decisions often rely on functional association between transcription factors. Several recent reports have eluded to the possibility that Bach2 associates with BCL-6 in repressing Blimp-1 in mouse B cells (Ochiai et al., 2008a). Moreover, BCL-6 and Bach2 cooperatively orchestrate gene expression in GC B cells through distinct transcriptional and biochemical mechanisms (Huang et al., 2013a). Deregulated Bach2

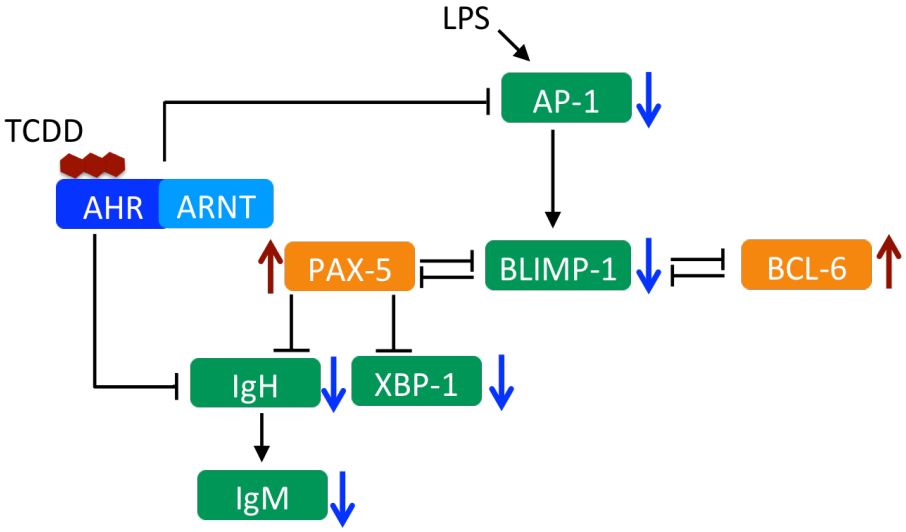


Figure 5.1: Illustration of the B cell differentiation gene regulatory network disrupted by TCDD

The TCDD-AHR complex directly induces Bach2, which represses Blimp-1. Downregulation of Pax-5 is blocked by TCDD thereby leading to decreased Blimp-1 possibly through decreased AP-1 levels and increased BCL-6 levels. Changes in Bach2, Bcl-6 and Pax-5 lead to a decrease in IgM.

expression has been associated with lymphoid malignancies, especially in the presence of mutations at the Bach2 locus that are observed in approximately 30% pre-B acute lymphoblastic leukemia cases (Merup et al., 1998; Sasaki et al., 2000). These observations provide strength to our identification of the involvement of Bach2 in TCDD-mediated suppression of B cell differentiation given the epidemiological evidence suggesting increased incidence of lymphomas in areas of dioxin exposure. In our study, the mRNA levels of Bach2 were unaltered in human primary B cells in the presence of TCDD in contrast to the observations made in the mouse B cells. These findings though surprising are not completely unexpected owing to differences in the regulation of transcription factors between mouse and human cells. Genes with homologous sequences are known to be differentially expressed suggesting that genes are used in different functional contexts even when the sequence is conserved (Hoffmann, 2005). Another recent study measuring gene expression changes during human B cell development showed upregulation of Bach2 in a Pax-5 dependent manner at the multipotent progenitor to the pro-B cell transition stage. In human cells, Bach2 is implicated in the pre-BCR checkpoint-signaling network unlike its role in mature mouse B cells (Swaminathan et al., 2013). This aspect of Bach2 is yet unexplored in the process of B cell development from TCDD-treated human hematopoietic stem cells to pro-B cells and is an avenue for future research. Whether the interactions between Bach2 and BCL-6 exist in presence of TCDD in the process of early B cell development is also an impending research question.

Animal models have been used extensively to characterize the toxic responses of the immune system to TCDD, which have more recently been replaced or

supplemented with human studies. The increasing emphasis on human risk assessment and the identification of hazards to human health has provided an impetus to research using human materials. It was also recognized that investigations exclusively using mouse models might not be predictive of toxic responses in human cells (Selgrade, 1999). Despite significant homology between mouse and human cells, several differences exist in the composition of leukocyte sub-populations, phenotypic markers and other regulatory factors, which could contribute to differences in toxic responses or pathways that toxicants regulate.

Human are highly outbred species and hence greater inter-individual variability is expected among toxic responses. This extreme variability was taken into account while designing experiments and a higher N was included depending on the availability of cells. Despite the inherent variability, consistent and significant effects of TCDD were observed in the mechanistic studies conducted using human cells. There is increasing evidence for associations between suppression of immune function and increased incidence of infectious and neoplastic disease in humans (Biagini, 1998). Studies on human cells have become easier with the development of sophisticated approaches to study immune system function and increased sensitivity of detection of the subtle consequences of TCDD toxicity.

This dissertation research utilized the CD40 ligand and cytokine-dependent activation of human B cells to study the effect of TCDD on B cell activation and differentiation. Freshly isolated naïve human peripheral blood B cells were used as they represent resting B cells that have never seen antigen before and are relevant to the *in*

vivo human physiology as compared to transformed or lymphoma-derived cell lines. These cells also closely recapitulate the phenotype of mouse splenic B cells.

With the advent of modern flow cytometers, high dimensional flow cytometric analysis using up to 7 colors has enabled mechanistic investigations in human cells. Single cell analysis offers identification and characterization of subpopulations of immune cells, which are not apparent in bulk lysis biochemical techniques thereby generating an edge over classical approaches (Chattopadhyay et al., 2014). In this dissertation, flow cytometric approaches were used for analysis of transcription factors, phospho-kinases and cell surface markers within single cells at the same time.

As a continuation of the studies by *Lu et. al.,* the second part of this dissertation aimed to dissect the molecular mechanism underlying impaired B cell activation by TCDD in human primary B cells. We used a systematic candidate gene approach to understand the molecular basis of impaired human B cell activation. BCL-6 was chosen as a putative candidate gene not only due to its role as a key repressor of B cell differentiation but also a repressor of B cell activation and cell cycle genes (Shaffer et al., 2000). My research demonstrated that BCL-6 protein levels increased with TCDD treatment in activated primary B cells also explaining the possible mechanistic basis to the observed epidemiological outcomes of TCDD exposure in the environment. The magnitude and the timing of increase in BCL-6 levels though variable still showed a consistent trend among TCDD-responsive donors. The increase in protein levels of BCL-6 was also seen in a TCDD-mediated concentration dependent manner. The increase in BCL-6 levels correlated with a simultaneous decrease in expression of B cell activation markers CD80 and CD69. Especially, in the case of activation marker CD80,

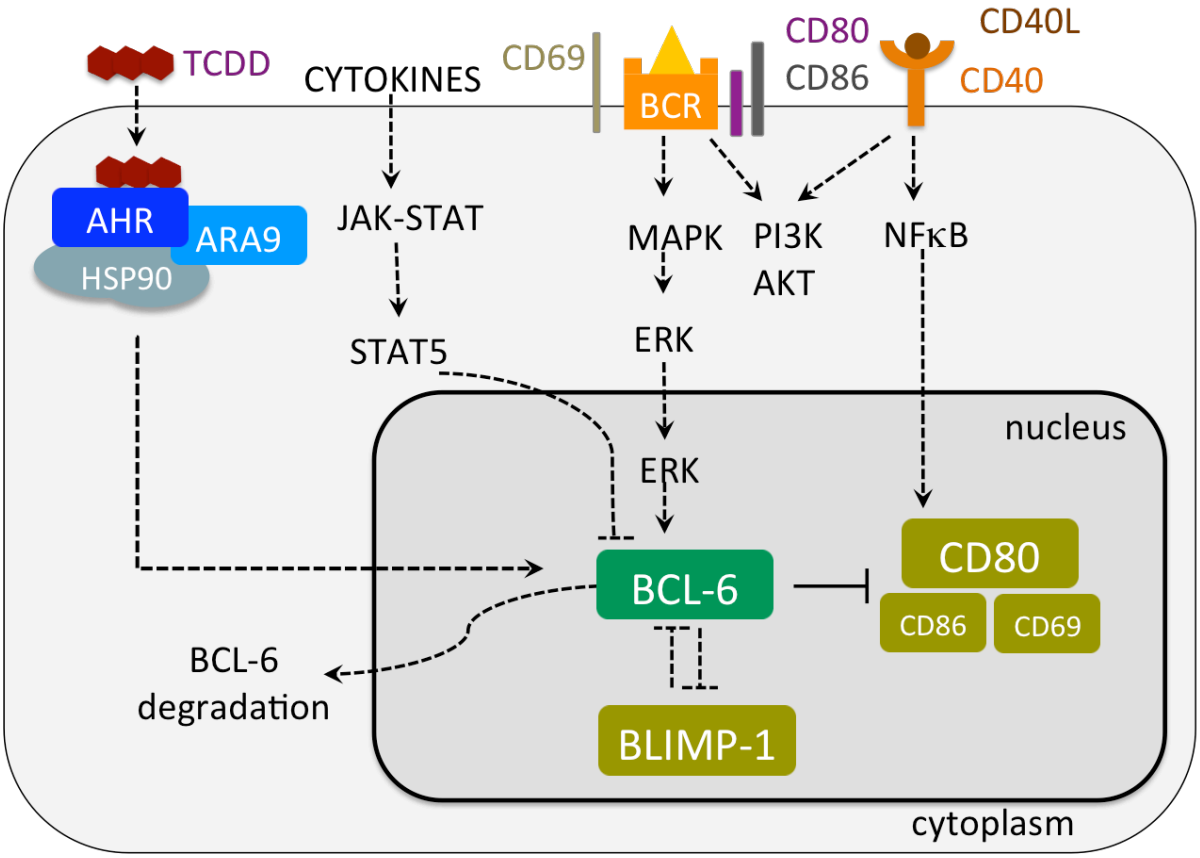


Figure 5.2: Schematic illustration of the series of events occurring upon B cell activation in presence of TCDD with a focus on BCL-6

The figure above illustrates signaling pathways activated upon ligation of BCR with antigen and upon stimulation of the B cell with CD40L and cytokines. These signaling pathways result in B cell activation exemplified by an increase in CD80, CD86 and CD69 levels. Sustained ERK activation upon B cell signaling controls BCL-6 degradation along with STAT5. In presence of TCDD, BCL-6 levels are increased which in turn decreases expression of CD80 and CD69 and Blimp-1 thereby suppressing B cell activation and differentiation.

BCL-6 binding within the enhancer region of CD80 was suggestive of BCL-6-mediated suppression of CD80 in presence of TCDD. Further justification for the role of BCL-6 in suppression of B cell activation was obtained upon using a BCL-6 small molecule inhibitor 79-6, which, when added to TCDD-treated B cells led to a reversal of suppressed activation marker levels. Moreover, changes in BCL-6 levels and suppression of activation markers in presence of TCDD are AHR-dependent as revealed by the structure-activity relationship experiments. It remains to be determined whether the transcriptional activity of AHR is required for this effect or if alternative mechanisms involving associations of AHR with other binding proteins play a role in this process. Figure 5.2 illustrates the proposed model of BCL-6 regulation in human B cells in presence of TCDD. Another interesting finding from this study was the correlation of suppressed activation marker phenotype with TCDD responsiveness. This was addressed by analyzing the AHR exon sequences to identify either existing or novel AHR polymorphisms. To our surprise, one of the TCDD-responders showing a greater than 40% suppression of activation markers harbored two polymorphisms (1661 G >A) and (1708 G>A) previously reported to be responsible for non-responsiveness to TCDD toxic effects especially up-regulation of CYP1A1. A TCDD non-responsive donor showed the previously characterized polymorphism (1661 G> A) but interestingly, two other non-responsive donors did not. However, the three non-responsive donors chosen for this analysis showed a previously unreported polymorphism. This study is inconclusive owing to the very small sample size. Also the biological outcome of these polymorphisms with regards to immune function is yet unknown. Other factors including a lack of background information about these donors, polymorphisms in genes other

than AHR, could be associated with non-responsiveness to TCDD. An ongoing project in the Kaminski lab will be exploring the basis of this effect by transfecting SKW6.4, a human B cell lacking AHR with polymorphic AHR harboring the 1661 G>A polymorphism. Such a model system will enable systematic understanding of the role of AHR polymorphisms in governing immunotoxic effects of TCDD in human B cells. Questions regarding regulation of BCL-6 by AHR were addressed by monitoring levels of pERK and Fbxo11 although the involvement of other pathways such as AP-1, NFκB and IRF-8 in regulating BCL-6 levels in the presence of TCDD still need to be explored.

The final part of this dissertation research focused on elucidating the role of SHP-1 in TCDD-mediated impaired human B cell activation. SHP-1 was identified through the genome-wide analysis of AHR binding in mouse B cells. SHP-1 is a negative regulator of B cell activation and inhibits signaling downstream of the BCR, by inhibiting kinases associated with the BCR and CD40 receptor (Zhang et al., 2000). In the current study, an increase in SHP-1 mRNA and protein levels was observed in presence of TCDD and in a TCDD-concentration dependent manner. However, the increase in SHP-1 protein levels was contingent on the time of TCDD addition. This provides an insight into the temporal window wherein disruption of early signaling events by TCDD could potentially regulate SHP-1 levels. One of the ways by which SHP-1 expression is altered in presence of TCDD is via binding of AHR to the putative DREs in the promoter region of SHP-1 as seen in the ChIP-on-chip studies. In addition, early kinase signaling downstream of the IL-2R signaling along with TCDD-AHR signaling drives the expression of SHP-1. The exact nature of the early signaling

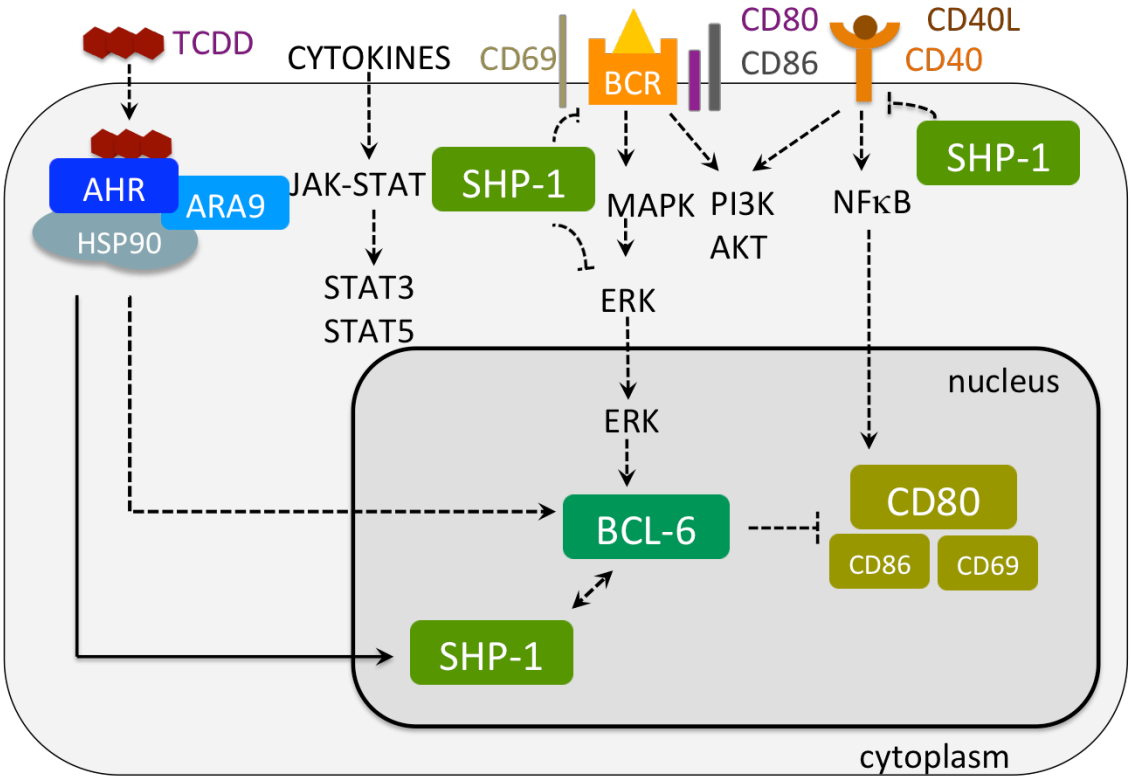


Figure 5.3: Schematic illustration of the interaction between BCL-6 and SHP-1 in presence of TCDD in activated human B cells

The figure above illustrates signaling pathways activated upon ligation of BCR with antigen and upon stimulation of the B cell with CD40L and cytokines. These signaling pathways result in B cell activation exemplified by an increase in CD80, CD86 and CD69 levels. The TCDD:AHR complex results in an increase of SHP-1. SHP-1 potentially regulates BCL-6 and thereby affecting B cell activation.

molecules is yet unknown but future studies focusing on the JAK-STAT, MAPK, PI3K-AKT pathways can help identify the signaling intermediate important for activation of SHP-1. An interesting observation from these studies suggests that TCDD-treatment leads to an increase in both SHP-1 and BCL-6 protein levels in the same population of cells. Both SHP-1 and BCL-6 normally repress B cell activation and are dysregulated in presence of TCDD. This is suggestive of crosstalk between SHP-1 and BCL-6. It is tempting to speculate that SHP-1 being a phosphatase that retains its phosphorylation activity upon translocation to nucleus may prevent phosphorylation of BCL-6 thereby preventing BCL-6 degradation in presence of TCDD. Another mechanism by which SHP-1 could regulate BCL-6 levels is through amplification of double-negative feedback loop involving B cell signaling and transcription factors BCL-6 and Blimp-1 (Reth and Brummer, 2004). SHP-1 is known to inhibit Lyn, Syk and Btk kinases downstream of BCR, thereby keeping BCR activation at a minimum. This would lead to a decrease in ERK activation ultimately preventing BCL-6 phosphorylation and degradation. BCL-6 would then suppress B cell activation and differentiation through repression of its target genes such as CD80, CD69 and Blimp-1. Figure 5.3 illustrates the current model of SHP-1 regulation in presence of TCDD in human B cells. Taken together, these studies suggest that BCL-6 and SHP-1 may, in part, explain the impaired B cell activation phenotype seen in presence of TCDD. Future studies involving an RNA-seq analysis in TCDD-responsive human B cells may help to identify novel regulators of B cell activation. In addition, large-scale phospho-kinase studies focusing on early B cell signaling may help in the identification of kinases altered by TCDD. Studying the effect of inhibition of critical B cell signaling pathways in presence of TCDD can help to identify

targets of SHP-1 in the B cells thereby broadening our understanding of the mechanisms involved in impaired B cell activation.

In summary, this dissertation research has addressed the basic question- How does TCDD affect B cell activation and differentiation? The mechanistic findings presented in this thesis have highlighted the role of a novel gene Bach2, which, has now led to an updated picture of the B cell transcriptional network affected by TCDD in B cells. BCL-6 and SHP-1 were identified as two candidate genes altered in presence of TCDD with BCL-6, specifically affecting expression of B cell activation markers. These studies provide the mechanistic underpinnings for the epidemiological findings associated with exposure to dioxins in the environment. To date, this is one of the first studies to provide the molecular mechanism through which ligand-bound AHR could contribute to B-lineage cancers by dysregulation of BCL-6 and demonstrate putative cross talk between BCL-6 and SHP-1. Bach2, BCL-6 and SHP-1 provide an understanding of a part of the enigma behind TCDD: AHR- mediated immune toxic effects. There is still the potential for identification of novel regulators altered by AHR, activated by either endogenous or exogenous ligands ultimately affecting B cell function.

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