ARYL HYDROCARBON RECEPTOR ACTIVATION BY 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN IMPAIRS HUMAN B LYMPHOPOIESIS

By

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ABSTRACT

ARYL HYDROCARBON RECEPTOR ACTIVATION BY 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN IMPAIRS HUMAN B LYMPHOPOIESIS

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Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates biological responses to endogenous and environmental chemical cues. Increasing evidence shows that the AHR plays physiological roles in regulating development, homeostasis and function of a variety of cell lineages in the immune system; however, the role of the AHR in human B lymphopoiesis remains to be elucidated. The objective of this study was to investigate the effects of persistent AHR activation by environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on human B lymphopoiesis. *In vitro* human B cell development model systems were established by culturing human cord blood-derived $CD34^+$ hematopoietic stem and progenitor cells (HSPC). Using these *in vitro* models, we found that TCDD significantly suppressed the total number of cells derived from HSPCs in a concentration-dependent manner. Cell death analysis demonstrated that the decrease in cell number was not due to apoptotic or necrotic cell death. TCDD also markedly decreased CD34 expression on HSPCs. Moreover, the generation of lineage committed B cells from HSPCs was significantly suppressed by TCDD treatment, indicating the impairment of human B lymphopoiesis. Structure-activity relationship assays and studies using an AHR antagonist demonstrated that AHR mediated the effects of TCDD on human B cell development. Gene expression

analysis revealed a significant decrease in the mRNA levels of early B cell factor 1 (EBF1) and paired box 5 (PAX5), two critical transcription factors directing B cell lineage commitment. In addition, binding of the ligand-activated AHR to the putative dioxin response elements in the *EBF1* promoter was demonstrated by electrophoretic mobility shift assays and chromatin immunoprecipitation analysis suggesting transcriptional regulation of EBF1 by AHR. Taken together this study, for the first time, demonstrates that AHR activation by TCDD impairs human B cell development, and suggests that transcriptional alterations of EBF1 by the AHR are involved in the underlying mechanism.

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KEY TO ABBREVIATIONS

2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
Ah	aromatic hydrocarbon
AHR	aryl hydrocarbon receptor
AHRR	aryl hydrocarbon receptor repressor
ARNT	AHR nuclear translocator
BCL-6	B cell lymphoma-6
BCR	B cell receptor
Blimp1	B lymphocyte-induced maturation protein 1
DAG	diacylglycerol
ChIP	chromatin immunoprecipitation
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
cy	cytoplasmic
DLC	dioxin-like compound
DMBA	7,12-dimethylbenz[a]anthracene
DRE	dioxin response element
EBF1	early B cell factor 1
EMSA	electrophoretic mobility shift assays
HAH	halogenated aromatic hydrocarbon
HMSC	human marrow stromal cell

HSC	hematopoietic stem cell
HSPC	hematopoietic stem/progenitor cell
HxCDD	1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin
Ig	immunoglobulin
IL7R	interleukin-7 receptor
IL7Rα	interleukin-7 receptor α chain
IP_3	inositol trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
LPS	lipopolysaccharide
LSK	Lin^- $Sca\text{-}1^+$ $cKit^+$
MAPK	mitogen-activated protein kinase
MCDD	1-chlorodibenzo-*p*-dioxin
MMS	matrix similarity scores
MPP	multipotent progenitors
PAX5	paired box 5
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PIP_2	phosphatidylinositol bisphosphate
PLC-γ	phospholipase C-γ
SHP-1	Src homology phosphatase 1
sIgM	cell surface IgM
SPADE	spanning-tree progression analysis of density-normalized events

sRBC	sheep red blood cell
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TLR	Toll-like receptor
TriCDD	2,3,7-trichlorodibenzo-*p*-dioxin
VH	vehicle

CHAPTER 1: LITERATURE REVIEW

1.1 Purpose of this research

Dioxins and dioxin-like compounds (DLC) are ubiquitous, persistent, and lipophilic environmental contaminants that bioaccumulate in the food chain. Within the family of dioxin and DLCs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent compound that exhibits a wide range of toxic effects including immunotoxicity. Epidemiological studies have identified the association between TCDD exposure and B cells disorders, most notably compromised humoral immunity and B cell related cancers such as non-Hodgkin's lymphoma. *In vitro* studies using primary human B cells have demonstrated that TCDD impairs the function of mature B cells; however, the effects of TCDD on the developmental process of human B cells still represent a significant data gap in the field. This dissertation research aims to bridge the gap by charactering the effects of TCDD on human B cell development and providing molecular insight into the underlying mechanism.

1.2 Humoral immune response and B cell development

Introduction to immune system

The immune system is an integrated network of lymphoid tissues, cells and humoral factors, which plays an essential role in host defense against infectious pathogens and diseases (Parkin and Cohen 2001). To effectively protect individual, the immune system must be able to fulfill the following major tasks: 1) pathogen recognition that distinguishes between self and non-self; 2) immune effector response to eliminate infectious pathogens; 3) immune regulation that keeps immune responses under control; and 4) immunological memory for protection against subsequent exposure. Based on the differences in the speed of immune responses, the specificity of pathogen recognition, and the ability of establishing immunological memory, the immune system can be divided into two parts: the innate and adaptive. The innate immune system provides immediate and non-specific defense against pathogens. The extent of innate immune responses remain the same regardless of how many times the pathogen is encountered. In contrast, the adaptive immune responses take days to develop, carry out exquisitely specific recognition of pathogens, and improve on repeated exposure to a given pathogen.

The innate immune system consists of physical and chemical barriers, phagocytic and inflammatory cells, and molecular components, which provide the first line of host defense. The epithelia that line the internal and external surfaces of the body represent a physical barrier against pathogen entry. The epithelia also produce a wide variety of antimicrobial enzymes and peptides to prevent the invading

microorganisms. The pathogens that breach the epithelial barrier will then encounter the cellular and molecular components of the innate immune system. The cellular component of the innate immune system comprises monocytes, macrophages, granulocytes, dendritic cells and natural killer cells, which are involved in directly killing of microorganisms by phagocytosis, releasing cytokines and chemokines that induce inflammation, and eliminating infected cells. In addition, the molecular components of the innate immune system, including acute-phase proteins and complement, play a role in facilitating the phagocytosis and pathogen lysis. The innate immune system distinguish between non-self and self by utilizing pattern recognition receptors that recognize repetitive molecular structures on many microorganisms known as pathogen-associated molecular patters. The binding of pathogens by these pattern recognition receptors not only mediates phagocytic activity of innate immune cells, but also promote the capability of antigen presentation, which is essential for initiation of the adaptive immune responses.

The adaptive immune responses are highly specific, which can recognize and efficiently eliminate vast variety of pathogens. This specificity results from the enormously diverse repertoire of antigen receptors possessed by B and T lymphocytes. Antigen recognition by B and T lymphocytes occurs in lymph nodes, to which the pathogen antigens are carried by antigen presenting cells, primarily dendritic cells, from sites of infections. Upon antigen recognition, T cells become activated and differentiate into effector cells. Two major types of T effector cells are CD8 cytotoxic T cells and CD4 helper T cells. Cytotoxic T cells function to kill viral

infected cells or tumor cells, known as cell-mediated immune responses. On the other hand, CD4 effector T cells comprise several subsets with a variety of different functions, including macrophage activation, promoting inflammation, immunological regulation and facilitating B cell activation. Naive B cells interact with antigens and helper T cells leading to cell activation and differentiation into antibody secreting cells. The antibodies produce by B cells mediate the humoral immune responses that eliminate microorganism in extracellular fluids.

One of the most important characteristic of adaptive immune system is to provide protective immunity against recurrent infections, i.e., immunological memory. In the primary immune response, naive lymphocytes encounter a pathogen for the first time and become activated and differentiate into effector T cells and antibody-secreting B cells. During this process, long-lived memory B and T cells are also generated that ensure rapid induction of immune responses against the same pathogen on subsequent encounters, therefore providing long-lasting protection.

Humoral immune response

Humoral immune responses are mediated by antibodies that eliminate extracellular microorganisms and prevent the spread of intracellular infections. The role of antibody includes: 1) binding to toxins, virus and bacteria to prevent them from entering cells; 2) coating pathogens to promote phagocytosis; and 3) activating complement system to lyse or opsonize microorganisms. The production of antibodies in response to antigens involves B cell activation and differentiation into antibody secreting plasma cells.

B cell activation requires signal delivered through its antigen receptor, B cell receptor (BCR). The immunoglobulin (Ig) on the B cell surface serves as a BCR for antigen recognition. Each Ig molecule consists of two identical heavy chains and light chains, each of which is comprised of a variable region (V region) and constant region (C region). The V region is extensively variable and responsible for antigen binding. The enormously diverse BCRs are generated through a genetic mechanism called gene rearrangement at an early stage of cell development (Delves and Roitt 2000). Specifically, the V region of a Ig heavy or light chain is encoded by discrete gene segments, including V (variable), D (diverse) and J (joining). Each of the gene segments is present in multiple diverse copies in the genome. Taking the human Ig heavy chain gene loci for example, there are 46 V segments, 23 D segments and 6 J segments. Gene recombination brings together 1 copy of V, D and J to form a functional gene encoding variable region of the Ig heavy chain. The random selection of gene segment copies during the rearrangement results in a large number of

possible combinations that accounts for the diversity of the receptor repertoire. This gene rearrangement process is mediated by lymphoid specific recombinase RAG-1 and RAG-2. Additional diversity of the Ig repertoire is introduced at the joints between the different gene segments as nucleotides are removed by exonuclease meanwhile random nucleotides are added by terminal deoxynucleotidyl transferase during recombination processes. The greatly diverse repertoire of BCR enables specific recognition of a vast variety of antigens.

The BCR complex is composed of cell surface Ig and two additional signaling molecules: Igα and Igβ. Antigen binding and BCR clustering activate the receptor associated Src-family kinases (Blk, Fyn, or Lyn) (Cambier et al. 1994). These kinases phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) in the Igα and Igβ, which then bind and activate protein kinase Syk. The activated Syk in turn phosphorylates phospholipase C-γ (PLC-γ), which cleaves the membrane phospholipid phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3), and initiates downstream signaling pathways, including calcium release, MAP kinase cascade, and PKC activation (Kurosaki 2000). Ultimately, there signaling pathways culminate in the activation of transcription factors, such as NFκB, NFAT and AP-1 to regulate genes involved in cell proliferation and differentiation.

In addition to signaling from BCR, a second signal is required for B cell activation by either T cell independent or T cell dependent antigens. For T cell independent antigens, a second signal can be generated by activating Toll-like

receptors (TLR) on B cells. For example, lipopolysaccharide (LPS) activates TLR-4, single stranded RNA from virus activates TLR-7, and bacteria DNA with unmethylated CpG can activate TLR-9. For T cell dependent antigens, the second signal required for B cell activation is delivered by a helper T cell that recognized the processed antigen presented by B cell in the form of a peptide:MHC II complex. The interaction between CD40 ligand on the T cell and CD40 on the B cell provides the second signal, which activates NFκB, MAP kinase and PLC-γ pathways and leads to B cell activation (Elgueta et al. 2009). In addition, the helper T cells also produces B cell stimulatory cytokines IL-4, IL-5 and IL-6, which drive the proliferation and differentiation of B cells.

Activated B cells undergo somatic hypermutation by introducing mutations in immunoglobulin V regions in a effort of generating high-affinity mutants. This process is known as affinity maturation. The mutations that improve the binding affinity of BCR to antigens increase the chance of B cells to interact with helper T cells, leading to B cell proliferation and differentiation into high affinity antibody secreting plasma cell or memory B cell (Li et al. 2004). In addition to somatic hypermutation, activated B cells also undergo class switching. In this process, different isotypes of the exon encoding the C region of Ig heavy chain will be associated with V region exon by gene recombination. There are five major classes of immunoglobulins: IgM, IgD, IgG, IgE and IgA, which have distinct biological functions and distributions.

The terminal differentiation of B cell to antibody secreting plasma cells associates with marked changes in the morphology and gene expression profiles.

Plasma cells have a prominent perinuclear Golgi apparatus and an extensive endoplasmic reticulum that reflect its commitment to the production and secretion of large amount of antibodies. In addition, the expression of MHC II and CD19 is diminished while the expression of CD38 and Syndecan-1 is increased on the surface of plasma cells. The regulatory circuit underlying B cell differentiation involves two key transcription factors: B lymphocyte-induced maturation protein 1 (Blimp1) and paired box 5 (Pax5), which form a mutually repressive feedback loop (Zhang et al. 2010). Pax5 is expressed from pro-B cell to mature B cell stage and plays a critical role in maintaining B cell identity (Cobaleda et al. 2007b). Pax5 functions to suppress B cell differentiation and represses the expression of genes that encode components of antibodies (e.g., *IgH, Igκ*, and *J chain*) and Xbp-1, an essential regulator for antibody secretion. Pax5 also negatively regulates Blimp1. Blimp1 is a critical regulator required for B cell differentiation and antibody secretion (Kallies et al. 2007; Tellier et al. 2016). The elevated expression of Blimp1 in activated B cells downregulates Pax5 and leads to B cell differentiation into antibody secreting plasma cells.

Hematopoiesis and B cell development

All leukocytes, the cells that carry out innate and adaptive immune responses, are derived from hematopoietic stem cells (HSC) that reside in the bone marrow. HSCs are pluripotent stem cells that are capable of self renew as well as differentiation into all types of blood cells. The generation of blood cells from HSCs is known as hematopoiesis. The roadmap of hematopoiesis resembles a tree structure, in which HSCs first generate progenitor cells with limited lineage potential, then further differentiate into cells that are irreversibly committed to each cell lineage (Figure 1.1). HSCs first derive multipotent progenitors (MPP), which have lost self-renewal capacity but maintain the potential of multilineage differentiation. The following cell fate decision of MPPs gives rise to either common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). Further development of CLPs generates B cells, T cells, natural killer cells and dendritic cells, whereas CMPs are capable to derive dendritic cells, macrophages, granulocytes, platelets and red blood cells.

The development of B cells from HSC involves stepwise cell fate choices and lineage restrictions. This process is governed by a lineage-specific transcription factor network (Figure 1.2). The lymphoid priming in HSC-derived multipotent progenitors is governed by transcription factor IKZF1 (IKAROS), SPI1 (PU.1) and TCF3 (E2A) (Dias et al. 2008; Scott et al. 1997; Yoshida et al. 2006). The interplay between these transcription factors leads to the loss of myeloid lineage potential and gives rise to CLPs. CLPs are capable of developing into T, B, nature killer and

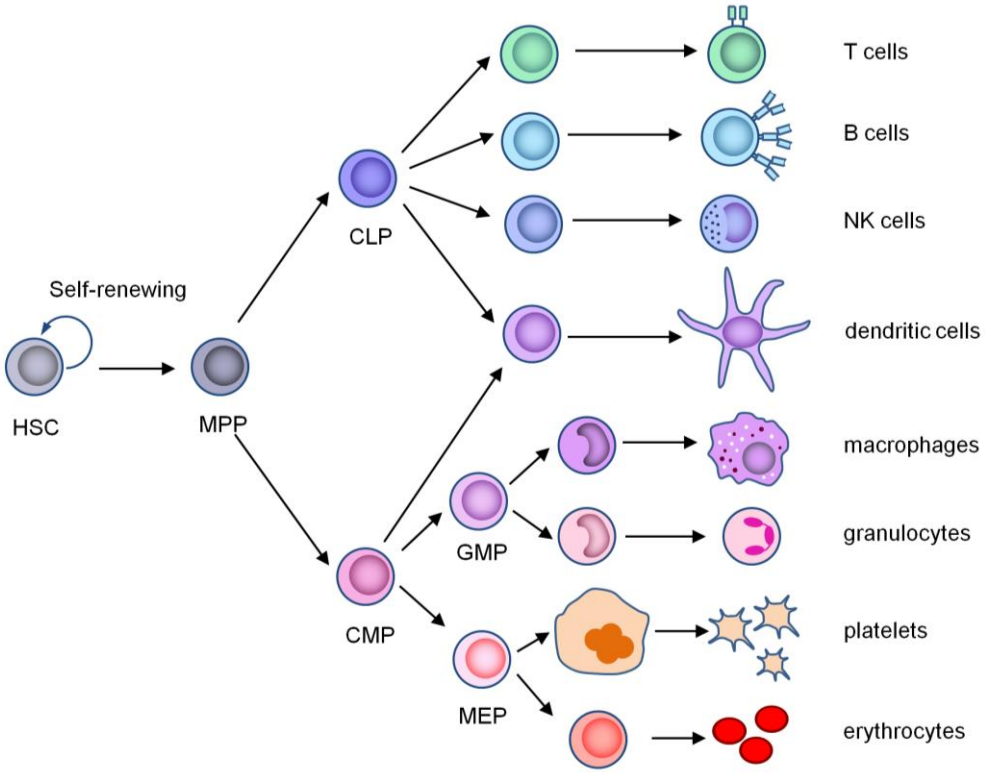


Figure 1.1: Schematic representation of hematopoiesis

All cellular components of the blood, including the cells of the immune system, are derived from pluripotent hematopoietic stem cells (HSC). This developmental process is known as hematopoiesis. MPP, multipotent progenitors; CLP, common lymphoid progenitor; CMP, common lymphoid progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/erythrocyte progenitor.

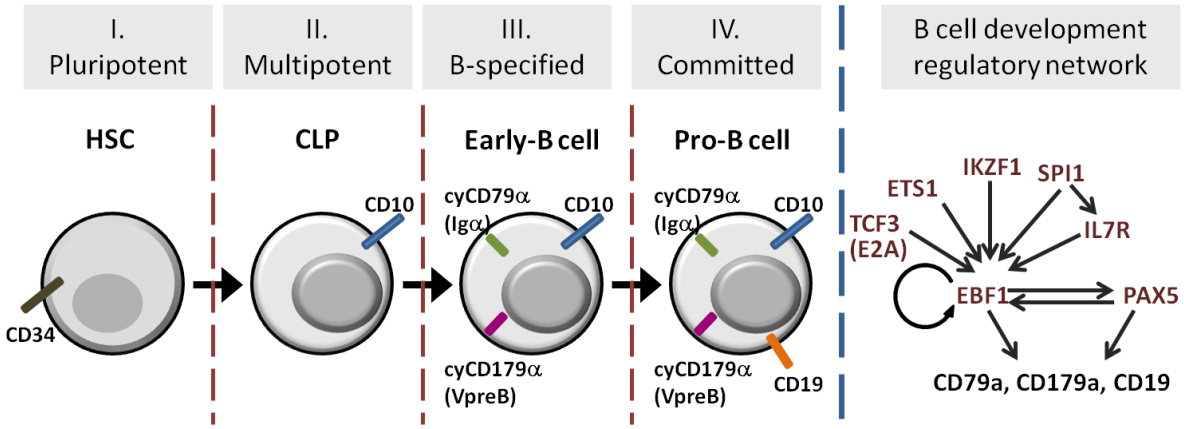


Figure 1.2: The stages and regulatory network in B cell development

The different stages in B cell development is illustrated in the figure above. The cell surface and intracellular proteins that can serve as cell markers demarcating different stages include: CD34, CD10, cytoplasmic (cy) CD79α, cyCD179α and CD19. The network directing B cell development involves EBF1-PAX5 axis and regulators of EBF1. Arrows indicate positive regulation.

lymphoid dendritic cell lineages (Galy et al. 1995). CLPs have been identified by the expression of CD34,CD10 and CD45RA, but not T, B or nature killer cell lineage surface markers.

The next step in B lymphopoiesis is the generation of early-B cells from CLPs. Early-B cells are characterized by the initiation of DJ_H rearrangements and expression of genes that are functionally important for B cells, including VpreB (CD179α) and Igα (CD79α). The expression of B cell specific genes indicates B cell lineage specification. One of the required transcription factors that governs the specification of the B cell developmental program is early B cell factor 1 (EBF1). EBF1 is present at low levels in CLPs and is under the regulation of SPI1, ETS1 and TCF3 (Roessler et al. 2007). EBF1 is a master regulator for B cell development. It activates many B cell specific genes that are required for B lymphopoiesis, including *Cd79a, Cd79b and Vpreb1*(Lin and Grosschedl 1995). In addition, EBF1 induces the expression of RAG1 and RAG2, which are responsible for gene rearrangement of the Ig heavy chain locus. Evidence supporting a critical role of EBF1 in B cell development comes from the observation that *Ebf1* knockout mice lack B cells and exhibit a complete block in B cell development at the CLP-like stage (Lin and Grosschedl 1995), whereas enforced expression of EBF1 can activate the B cell program in the absence of other upstream regulators (Medina et al. 2004; Reynaud et al. 2008). EBF1 also activates the expression of PAX5, which reciprocally upregulates EBF1 through a positive feedback loop (Roessler et al. 2007; Zandi et al. 2008). The positive feedback loop between EBF1 and PAX5 forms a bistable

memory module, which is a characteristic regulatory motif that governs cell fate decisions during cellular development (Xiong and Ferrell Jr 2003; Zhang et al. 2013). PAX5 plays a critical role in B cell development as well as maintaining B cell identity. The elevated expression of EBF1 and PAX5 eliminates alternative cell fates by suppressing non-B cell genes, activates many B cell specific genes that confer B cell identity, and ultimately leads to B cell lineage commitment (Nechanitzky et al. 2013a; Nutt et al. 1999; Schebesta et al. 2007).

Lineage committed B cells are identified as pro-B cells, which express cell surface protein CD19. CD19 is a hallmark of B cells, which plays a role regulating B cell receptor signaling (Del Nagro et al. 2005). One critical event that occurs during the pro-B cell stage is VDJ rearrangement at the Ig heavy chain loci. The successfully rearranged heavy chain couples with surrogate light chain to form a pre-B cell receptor, which demarcates the generation of pre-B cells.

The functional pre-B cell receptor signals the cell to develop to the stage of light chain gene rearrangement. The successful rearrangement of heavy and light chain loci allows expression of IgM on cell surface (sIgM). sIgM associates with accessory molecule Igα (CD79α) and Igβ (CD79β) to form a functional B cell receptor complex, which characterizes the generation of immature B cells. At this stage, cells are tested for their activity to self antigens. Immature B cells bearing receptors that strongly respond to self antigen will be eliminated, and only those that have no strong response to self antigen are allowed to mature.

Mature B cells leave the bone marrow and enter lymph nodes via peripheral blood. Circulating human mature B cells have an average half-life of 18 days (Macallan et al. 2005). As a result, the homeostasis of the peripheral B cell population requires lifelong replenishment through B lymphopoiesis.

The role of EBF1 in B lymphopoiesis

Many studies have demonstrated EBF1 as a primary determining factor of B cell lineage specification during B lymphopoiesis. Mice lacking EBF1 exhibit a complete block in B cell development at the CLP-like stage (Lin and Grosschedl 1995). In addition, *Ebf1* knockout mice failed to express genes encoding key proteins required for B lymphopoiesis (*Cd79a, Cd79b, Vpreb1, and Rag1*) and did not show gene rearrangement at Ig loci. In contrast, ectopic expression of *Ebf1* can rescue B lymphopoiesis from developmentally arrested multipotent progenitors due to deletion of PU.1 or Ikaros (Medina et al. 2004; Reynaud et al. 2008). Likewise, the expression of EBF1 overcomes the block in B lymphopoiesis imposed by the absence of E2A (Seet et al. 2004). Moreover, enforced expression of EBF1 in HSCs skews development favoring B cell lineage commitment (Zhang et al. 2003), further supporting a critical role for EBF1 in the regulatory circuitry of B lymphopoiesis.

In addition to directly regulating B cell specification, EBF1 also contribute to B cell lineage commitment by: 1) inducing expression of PAX5, which in turn exerts B cell commitment; and 2) directly suppressing the expression of key regulators that determine alternative cell fates. In the early stage of B cell development, EBF1

activates the PAX5 promoter by inducing chromatin remodeling and active histone modification (Decker et al. 2009). In addition, EBF1 suppressed the expression of transcriptional regulators of myeloid, innate lymphoid and T cell fates, including PU.1, C/EBPα, Id-2, GATA-3 and TCF-1 (Nechanitzky et al. 2013b; Pongubala et al. 2008; Thal et al. 2009). The direct role of EBF1 in B cell lineage commitment is also evidenced by the finding that *Ebf1*-deficient *Pax5*-expressing lineage committed pro-B cells can dedifferentiate and generate T cells (Nechanitzky et al. 2013b). Thus, EBF1 plays a critical role in both B cell specification and lineage commitment.

The role of PAX5 in B lymphopoiesis

The development of B cells from HSCs involves successive rounds of lineage restrictions, in which cells gradually lose the multilineage potential and become committed to the B cell lineage. B cell lineage commitment is regulated by PAX5 (Nutt et al. 1999). The expression of PAX5 is restricted to the B cell lineage from pro-B to the mature B cell stage (Fuxa and Busslinger 2007). PAX5 is required in both initiating and maintaining B cell lineage commitment. Loss of PAX5 leads to arrest of B cell development at the early pro-B cell stage and failure to differentiate to mature B cells (Nutt et al. 1997). In addition, *Pax5*$^{-/-}$ pro-B cells exhibit capability of developing into a broad spectrum of hematopoietic cell lineages (Nutt et al. 1999; Rolink et al. 1999). More interestingly, conditional loss of Pax5 in mature B cells results in B cell dedifferentiates into uncommitted progenitors and generates functional T cells (Rolink et al. 1999).

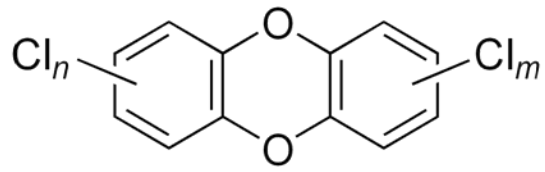
PAX5 regulates B cell lineage commitment by its dual role of repressing non-B-lineage genes and activating B-lineage-specific genes (Nutt et al. 1999). Genome-wide transcriptional profiling has identified 110 genes that are suppressed by PAX5, including genes that are functionally important in myeloid cells and T cells (Delogu et al. 2006; Pridans et al. 2008). Conversely, PAX5 activates 170 genes in lineage committed pro-B cells, which are involved in B cell receptor signaling, adhesion, migration and immune function (Pridans et al. 2008; Schebesta et al. 2007). Taken together, aforementioned studies highlight the crucial role played by PAX5 in initiating and maintaining B lineage commitment.

1.3 Dioxin and AHR pathway

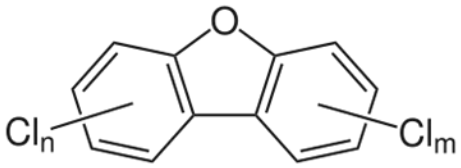
The production and general toxicity of dioxin

Dioxins and dioxin-like compounds (DLC) are ubiquitous environmental contaminants, which comprise structurally related halogenated aromatic hydrocarbons, including polychlorinated dibenzo-*p*-dioxins (PCDD), polychlorinated dibenzofurans (PCDF) and polychlorinated biphenyls (PCB) (Figure 1.3). Among the family of dioxins and DLCs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is identified as the most toxic compound and a prototypical dioxin (Poland and Knutson 1982; Whitlock 1990). Dioxins can be generated in naturally occurring thermal reactions, such as forest fires and volcanic eruptions. However, human activities are primarily responsible for the generation of dioxins in the past two centuries (White and Birnbaum 2009). Historically, dioxins were mainly generated as a side product during herbicide manufacture, including 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Poland and Knutson 1982). Other thermal processes can also lead to dioxin production, such as metal-processing, bleaching of paper pulp with free chlorine and incineration of municipal solid waste.

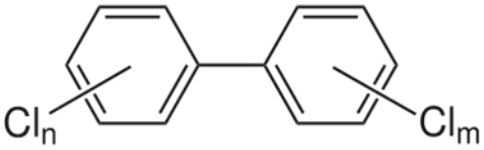
Owning to the chemical stability and lipophilicity, dioxins are persistent in environment and tend to bioaccumulate in food chain (Poland and Knutson 1982). Hence, human are exposed to dioxin primarily through consumption of high fat-containing foods, which account for over 90 percent of the body burden of dioxin in people (Travis and Hattemer-Frey 1987; Whitlock 1990). Public concern of dioxin has been evoked by the toxic effects of dioxin observed in incidents of environmental



Polychlorinated dibenzo-*p*-dioxins (PCDDs)
75 possible congeners



Polychlorinated dibenzofurans (PCDFs)
135 possible congeners



Polychlorinated biphenyls (PCBs)
209 possible congeners

Figure 1.3: The molecular structure of dioxins and dioxin-like compounds

The molecular structures of dioxins and dioxin-like compounds are illustrated in the figure above. Depending on the number and position of chlorination, there are hundreds of possible congeners in the family.

and occupational exposure as well as laboratory animal studies (White and Birnbaum 2009). Exposure to TCDD and other DLCs can produce a broad spectrum of species- and tissue-specific toxic and biological effects. Epidemiological studies based on the incidences of human exposure to dioxin have identified a variety of adverse health effects, including diabetes (Michalek and Pavuk 2008), increased thyroid stimulating hormone in neonates (Baccarelli et al. 2008), uterine fibroids (Eskenazi et al. 2007), decreased sperm count and motility (Mocarelli et al. 2008), decreased plasma immunoglobulin G levels (Baccarelli et al. 2002), developmental dental defects (Alaluusua et al. 2004), and carcinogenesis (Crump et al. 2003). Animal studies also reveal a similar spectrum of toxic effects by dioxin exposure, including wasting syndrome, hepatotoxicity, dermal toxicity, endocrine disruption, reproductive and developmental toxicity, immunotoxicity and tumor promotion (Denison et al. 2011; Peterson et al. 1993; Poland and Knutson 1982).

Aryl hydrocarbon receptor signaling pathway

Numerous studies have demonstrated that most, if not all, of the toxic and biological effects produced by TCDD and DLCs are mediated by aryl hydrocarbon receptor (AHR) (Burbach et al. 1992; Denison et al. 2011; Harper et al. 1991; Poland and Knutson 1982; Whitlock 1990). One of the most convincing lines of evidence comes from studies that demonstrate resistance to TCDD toxicity in AHR-null mice (Gonzalez and Fernandez-Salguero 1998; Mimura et al. 1997). AHR was first identified as the receptor for TCDD in hepatic cytosol by Poland, Glover and Kende (Poland et al. 1976). AHR is a ligand-activated transcription factor which belongs to Per-ARNT-Sim (PAS) protein superfamily. As other PAS proteins, AHR acts as a sensor of endogenous or exogenous chemicals and trigger signaling cascades to evoke cellular responses, including the induction of drug-metabolizing cytochrome P450-encoding genes. The classic mechanism of AHR-dependent gene regulation is shown in Figure 1.4. The AHR interacts with a homodimer of heat shock proteins (hsp90), Ah receptor-associated protein-9 (ARA9) and other chaperone proteins in the absence of ligand binding in the cytosol (Bell and Poland 2000; Carver et al. 1998; Hankinson 1995; Perdew 1988). By binding with its ligand, the AHR undergoes a conformation change and exposes the N-terminal nuclear localization sequence, which facilitate the translocation of the ligand bound AHR complex from cytosol into nucleus. Once in the nucleus, AHR dissociates with hsp90 and other chaperone proteins and interacts with a structurally related nuclear protein called AHR nuclear translocator (ARNT). Then, the ligand:AHR:ARNT complex transforms into a

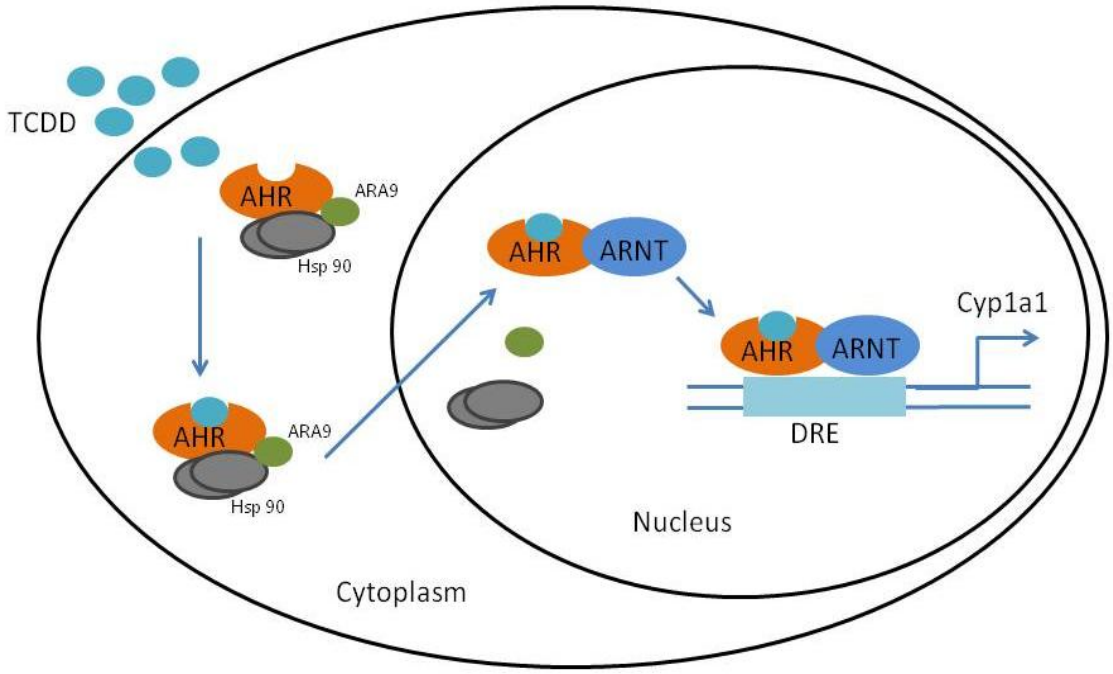


Figure 1.4: Schematic representation of the classic AHR pathway

This figure illustrates the classic TCDD-AHR pathway. TCDD diffuses across cell membrane and enters cytoplasm, where it binds to AHR that is associated with hsp90, AHA9 and other chaperon proteins. TCDD binding induces conformational changes in AHR, resulting in translocation of TCDD:AHR into nucleus. TCDD:AHR complex then binds to ARNT, recognizes the cognate DNA sequence (DRE) in the regulatory region of target genes, and regulates gene expression. AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element.

high affinity DNA binding form, binding to dioxin-responsive elements (DREs) in the regulatory regions of target genes to exert transcriptional regulation (Denison and Nagy 2003; Poland and Knutson 1982; Whitlock 1990). AHR has also been reported to interact with a variety of coactivators and corepressors (Nguyen et al. 1999). The termination of AHR-mediated transcriptional regulation involves dissociation of ligand:AHR:ARNT complex from DRE, nuclear export of AHR into cytosol, and ubiquitin-mediated AHR degradation (Pollenz 2002). The AHR transcriptional activity can also be attenuated by upregulation of the aryl hydrocarbon receptor repressor (AHRR), which can compete with AHR for dimerizing with ARNT and binding to DREs (Mimura et al. 1999).

Given the ability of AHR to act as a ligand-dependent transcription factor, it is hypothesized that many of the toxic and biological effects of AHR ligands result from alterations in gene expression. In addition, the overt toxic effects produced by metabolically stable AHR ligands (i.e., TCDD and DLCs) but not metabolically labile AHR ligands (i.e., polycyclic aromatic hydrocarbons) suggests that the persistent activation of AHR is responsible for toxicity (Denison et al. 2011).

In addition to the classic AHR mechanism that involves ligand:AHR:ARNT: DRE complex dependent gene regulation, alternative non-classic mechanisms have also been observed. AHR:ARNT has been found to bind to alternative DREs or inhibitory DREs to repress transcription (Safe et al. 1998). AHR can also dimerize with other transcription factors (i.e., RelB), in place of ARNT, to regulates gene expression (Vogel et al. 2007). Independent to DNA binding, AHR may interact with

other transcription factors and lead to their sequestration (RelA) or degradation (ER) (Ohtake et al. 2003; Sheppard et al. 1998). Additionally, the AHR competes with other transcription factors (i.e., HIF-1α) or receptors (i.e., ERβ) for ANRT binding thereby disrupts their function (Ruegg et al. 2008). Moreover, the AHR can directly interacts with various intracellular signaling pathways, including protein kinases, mitogen-activated protein kinases (MAPKs), c-Src kinases, NF-κB signaling, and many others (de Oliveira and Smolenski 2009; Haarmann-Stemmann et al. 2009; Matsumura 2009). In addition, some AHR ligands are believed to stimulate calcium influx and calcium-dependent signaling in an AHR-independent manner (Tijet et al. 2006).

The physiological role of AHR

Although early studies were focused on the role of AHR in response to xenobiotics, including adaptive responses of inducing xenobiotic metabolizing enzymes and toxic effects triggered by persistent ligands (dioxin and DLCs), recent studies have also demonstrated that the AHR plays an important role in normal physiology. From an evolutionary perspective, the amino acid sequence of AHR is highly conserved across vertebrate species (Hahn 2002). In addition, AHR orthologs found in invertebrate species lack ligand binding but possess physiological functions (Duncan et al. 1998; Qin and Powell-Coffman 2004). Moreover, the aberrant phenotypes observed in *Ahr*-null mice, such as defects in ovarian and vascular development, provide compelling evidence for the physiological role of AHR and the existence of endogenous AHR ligands (Benedict et al. 2000; Schmidt et al. 1996). The search for endogenous AHR ligands has identified a growing list of compounds, including indigoids, equilenin, tryptophan metabolites, arachidonic acid metabolites and heme metabolites (Nguyen and Bradfield 2008). It is believed that endogenous AHR activation is controlled via an autoregulatory feedback pathway such that endogenous ligands activate AHR, which in turn upregulate the expression of cytochrome P450 enzymes that degrade endogenous ligands (Chiaro et al. 2007).

Studies utilizing endogenous and exogenous AHR ligands, AHR antagonists, and *Ahr* knockout animal models have suggested physiological roles for the AHR in regulating various biological processes, including cell cycle regulation (Andrysik et al. 2007; Ge and Elferink 1998), apoptosis (Marlowe et al. 2008), tumor progression

(Opitz et al. 2011), neuronal development (Qin and Powell-Coffman 2004), vascular development (Schmidt et al. 1996), and ovarian development (Benedict et al. 2000).

In the immune system, the AHR has been demonstrated to be involved in inflammation (Beamer and Shepherd 2013; Qiu and Zhou 2013), autoimmune responses (Nguyen et al. 2013) and allergy (Schulz et al. 2013) by regulating the development, homeostasis and function of immune cell populations (Hanieh 2014). Examples include a role by the AHR in the activation and proliferation of HSCs (Boitano et al. 2010; Singh et al. 2011), differentiation of Th17 cells and regulatory T cells (Quintana et al. 2008; Veldhoen et al. 2008), maintenance of innate lymphoid cells (Qiu et al. 2012), immunogenicity of dendritic cells (Nguyen et al. 2010), and function of mature B cells (Lu et al. 2010; Sulentic and Kaminski 2011).

1.4 The effects of TCDD on B cells

The alteration of immune responses by TCDD in animal models

The immune system has been recognized as one of the most sensitive target organs for TCDD since 1970s. In animal studies, alterations in immune functions by TCDD has been consistently observed across many species at low doses that do not produce obvious signs of toxicity (Holsapple et al. 1991; Kerkvliet 2002; Sulentic and Kaminski 2011). The immunomodulatory effects of TCDD have been reported in innate, cell-mediated, and humoral immunity.

Several studies have shown that TCDD alters the innate immune responses in mice, including dendritic cell function and neutrophil recruitment. Dendritic cells exposed to TCDD showed increased expression of MHC class II, adhesion molecules, and costimulatory molecules, thereby possessing enhanced T-cell stimulating capacity (Lee et al. 2007; Vorderstrasse and Kerkvliet 2001). These effects appear to be dependent on AHR as *Ahr* null mice do not show the similar effects (Vorderstrasse and Kerkvliet 2001). Interestingly, TCDD treatment decreased the number of dendritic cells and increased Fas-mediated apoptosis (Ruby et al. 2005; Vorderstrasse and Kerkvliet 2001). TCDD treatment also led to excessive neutrophil recruitment that contributes to decreased survival and more severe bronchopneumonia in mice infected with influenza A virus (Luebke et al. 2002; Teske et al. 2005).

It has been established for long time that TCDD exposure produces suppressive effects on cell-mediated immunity, in which thymus-derived T cells play

a critical role in defense against infected cells and tumor cells. One of the first indications of immunotoxicity produced by TCDD exposure is the prominent involution of the thymus in various animal species (Gupta et al. 1973; Harris et al. 1973; Holsapple et al. 1991; Vos et al. 1973). Later, studies found that TCDD exposure suppressed the differentiation of thymic epithelial cells and reduced maturation of T lymphocytes, which might contribute to the involution of the thymus (Greenlee et al. 1985; Kerkvliet and Brauner 1990). In keeping with the reduced cellularity in thymus, *in vivo* administration of TCDD also suppressed the activity of cytotoxic T lymphocyte in an acute graft-versus-host response. This suppression was dependent on the expression of AHR in both CD4 and CD8 T cells (Kerkvliet et al. 2002). In addition, Funatake *et al.* have shown that TCDD treatment decreased the number of CD4 T cells on day 4 of the immune response elicited by antigenic challenge. Meanwhile, the expression of several genes associated with cell survival/death in purified CD4 T cells were altered by TCDD (Funatake et al. 2004). Concordantly, TCDD exposure significantly decreased the numbers of CD4 and CD8 superantigen-responsive T cells and increased Fas-dependent cell apoptosis (Camacho et al. 2002). In addition, AHR activation by TCDD interfered Th17 cell differentiation and promoted the differentiation of regulatory T cells (Funatake et al. 2005; Quintana et al. 2008).

Numerous studies have consistently demonstrated the suppression of humoral immunity by TCDD, which is mediated through alterations in the function of B cells. Since 1970's, researchers have been reporting the suppression of antibody

responses by *in vivo* or *in vitro* TCDD exposures in animal models (Hinsdill et al. 1980; Holsapple et al. 1986; Thomas and Hinsdill 1979; Vecchi et al. 1980a; Vecchi et al. 1980b; Vecchi et al. 1983; Vos et al. 1973). For example, i*n vivo* studies have shown that TCDD exposure profoundly suppressed primary and secondary antibody responses (Hinsdill et al. 1980; Thomas and Hinsdill 1979). Likewise, *in vitro* TCDD treatment to isolated spleen cells also produced significantly reduced antibody responses (Holsapple et al. 1986). In the effort of identifying the subsets of leukocyte interfered by TCDD that were responsible for the suppression of humoral immunity, studies were conducted using variety of antigens, including T cell dependent antigens (e.g., sheep red blood cells (sRBC)), T cell independent antigens (e.g., dinitrophenyl-ficoll or trinitrophenyl-LPS), or polyclonal B-cell activators (e.g., LPS or anti-Ig). *In vivo* TCDD exposure produced comparable suppression of antibody responses to both T-independent and T-dependent antigens (administered *in vivo* or directly to splenic culture) in mice, suggesting the primary target of the TCDD-induced suppression of IgM antibody production is the B lymphocyte (Dooley and Holsapple 1988). To further identify the cellular target of TCDD in humoral immunity, Dooley and Holsapple separated spleen cells into B cell, T cell and macrophage populations, then reconstituted the *in vitro* cell culture in various combinations. They showed that only those experimental groups reconstituted with B cells from TCDD-treated mice exhibited suppression of the IgM response, demonstrating that the B cell was the primary target of TCDD in suppressed humoral immunity (Dooley and Holsapple 1988). In addition, Morris et al. using purified mouse B cells also showed the decreased primary antibody responses by TCDD treatment (Morris et al. 1993).

Concordantly, TCDD treatment suppressed the LPS-induced IgM response in murine B cell line, CH12.LX, further confirming the direct effects of TCDD on B cells (Sulentic et al. 2000).

The involvement of AHR in TCDD-elicited suppression of antibody responses has been demonstrated by multiple independent experimental observations. First lines of supporting evidence come from studies using congenic mouse strains that possess distinctive AHR alleles with either low (AHR^d) or high (AHR^b) TCDD binding affinity. The strong suppression of antibody production by a low dose of TCDD (1.2 μg/kg) was observed in a mouse stain expressing AHR^b but not AHR^d allele, suggesting AHR involvement (Vecchi et al. 1983). Additionally, structure-activity-relationship assays using dioxin congeners showed a correlation between the suppression of antibody responses by congeners and the ability of congeners to bind to AHR, suggesting that immune suppression was mediated by the AHR (Tucker et al. 1986). Additional support for the AHR involvement was generated from studies using murine B cell lines, the CH12.LX and the BCL-1, which differ in their expression of the AHR. TCDD treatment led to marked suppression of LPS induced IgM production from CH12.LX cells, whereas no suppression was observed in the AHR-deficient BCL-1 cells (Sulentic et al. 1998). Moreover, later studies using *Ahr* null mice and rats demonstrated that TCDD suppressed sRBC- or LPS-induced antibody responses in wild type controls but not in *Ahr* null animals, providing definitive evidence that AHR mediates TCDD-elicited suppression of the IgM responses (Phadnis-Moghe et al. 2016a; Vorderstrasse et al. 2001).

Humoral immune responses to antigens require B cell activation and differentiation into antibody secreting plasma cells. To elucidate the molecular mechanism by which TCDD suppresses antibody responses, many studies were devoted to exploring the effects of TCDD on B cell activation and differentiation. Some of the earliest studies have shown that *in vitro* TCDD treatment increased basal protein kinase activity in naive murine B cells (Kramer et al. 1987) and enhanced phosphorylation of kinases in activated murine B cells (Snyder et al. 1993). Likewise, purified mouse splenic B cells treated with TCDD showed increased phosphorylation of membrane associated proteins, which correlated with the suppression of B cell antibody production (Clark et al. 1991). On the other hand, TCDD treatment decreased the phosphorylation of ERK, AKT, and JNK in mouse B cells activated using Toll-like receptor ligands (North et al. 2010). Overall, TCDD-mediated changes in various aspects of cell signaling suggest that TCDD perturbs early B-cell activation events.

In addition to altered B cell activation, the impairment of B cell differentiation into antibody secreting plasma cells by TCDD were also investigated. Many studies have shown that along with reduced antibody production, the number of antibody-secreting cells was also decreased by TCDD treatment in mice, suggesting an impairment of B cell differentiation (Crawford et al. 2003; Dooley and Holsapple 1988; Tucker et al. 1986). The regulatory circuit underlying B cell differentiation involves two key transcription factor: Blimp1 and Pax5, which reciprocally repress each other. Pax5 plays a critical role in B cell development as well as maintaining B cell identity

(Cobaleda et al. 2007b). Hence, Pax5 acts as a negative regulator of B cell differentiation and is downregulated in plasma cells. Pax5 can repress the expression of genes that encode components of antibodies (e.g., *IgH, Igκ*, and *J chain*) and Xbp-1, an essential regulator for antibody secretion. As predicted, TCDD treatment promoted the retention of Pax5 mRNA and protein levels. Concordantly, *IgH, Igκ*, *J chain* and *Xbp-1* genes were suppressed by TCDD (Yoo et al. 2004). Blimp-1 represses Pax5, which in turn downregulates Blimp-1, forming a mutually repressive feedback loop (Zhang et al. 2010). Therefore, Blimp-1 functions as a positive regulator of B cell differentiation. As expected, TCDD treatment decreased the expression of Blimp-1 as well as the binding of AP-1 in the promoter region of Blimp-1 (Schneider et al. 2009). Consistently, TCDD treatment in mouse splenocytes produced significant suppression of Igμ, Igκ, J chain, Xbp-1, and Blimp-1, along with a decrease in percentage of plasma cells (North et al. 2009), suggesting the disruption of B cell differentiation by TCDD. Taken together, these studies suggest alterations in B cell activation and differentiation by TCDD contribute to the suppression of humoral antibody responses.

The effects of TCDD on human B cells

Although impairment of B cell function by TCDD has been consistently observed in various animal species, the effects of TCDD on human B cells are not extensively characterized. Evidence from epidemiological studies suggest an association between exposure to TCDD and human B cell disorders, most notably decreased humoral immune competence and B cell-derived cancers (Baccarelli et al. 2002; Becher et al. 1996; Floret et al. 2003; Kogevinas et al. 1997; Kramarova et al. 1998; Viel et al. 2008). The suppression of humoral immune responses were first identified in a study of a cohort of Dutch children (ten Tusscher et al. 2003). In this study, the increased perinatal dioxin exposure was correlated with reduced vaccine titers and an increased incidence of chicken pox and otitis media. In another study 20 years after an industrial accident of TCDD contamination in Seveso, Italy, investigators identified a significant association between decreased plasma immunoglobulin G (IgG) levels and increasing TCDD plasma concentration in the population from highly contaminated zone and surrounding noncontaminated area (Baccarelli et al. 2002). Another notable B cell disorder associated with TCDD exposure is B cell-related cancers (Becher et al. 1996; Kogevinas et al. 1997; Kramarova et al. 1998). The Institute of Medicine (US) Committee to Review the Health Effects in Vietnam Veterans of Exposure to Herbicides concluded a positive association between non-Hodgkin's lymphoma and exposure to the defoliant, 2,4-D, 2,4,5-T and its contaminant TCDD (Institute of Medicine (US) Committee 1994). Several epidemiological studies also investigated the relative risk of non-Hodgkin's

lymphoma incidence among the population living close to solid waste incinerators, which generate dioxin-like compounds by combustion. These studies found individuals living in the area of high dioxin exposure had a significantly higher incidence of non-Hodgkin's lymphoma than individuals in the low dioxin exposure area (Floret et al. 2003; Viel et al. 2008).

In addition to epidemiological studies, the direct effects of TCDD on human mature B cell function has also been studied *in vitro*. The pioneering studies were conducted by Wood and co-workers who has demonstrated that TCDD suppressed LPS-induced IgM antibody production in human tonsillar lymphocytes (Wood et al. 1993). In addition, superantigen induced IgM responses was also reduced by TCDD in human primary lymphocytes (Wood and Holsapple 1993). More recently, studies in the Kaminski's lab were conducted using naive B cells isolated from human peripheral blood to evaluate the effects of TCDD (Lu et al. 2011; Lu et al. 2009; Lu et al. 2010). In these studies, B cells were activated in a T cell-dependent manner using CD40 ligand and cytokines (Lu et al. 2009). Consistent with previous studies, the primary IgM responses were significantly suppressed by TCDD in a concentration-dependent manner (Lu et al. 2010). In addition, TCDD treatment also led to reduced B cell activation, which is indicated by decreased expression of CD80, CD86 and CD69 on B cells (Lu et al. 2011). Subsequent studies focusing on the underlying mechanisms revealed the involvement of B cell lymphoma-6 (BCL-6) and Src homology phosphatase 1 (SHP-1). BCL-6 is a multifunctional transcriptional repressor known to regulate critical events of B cell activation. TCDD-induced

expression of BCL6 and concomitant increase in BCL6 binding to an enhancer region of CD80 suggested a mechanism underlying impaired B cell activation (Phadnis-Moghe et al. 2015). In addition, TCDD treatment also increased SHP-1 expression in activated human B cells. This increase in SHP-1 expression might result from direct transcriptional regulation by AHR because TCDD-activated AHR was able to bind to DREs in the promoter region of SHP-1 (Phadnis-Moghe et al. 2016b). Inhibition of SHP-1 activity decreased BCL-6 levels, suggesting possible regulation of BCL-6 by SHP-1 (Phadnis-Moghe et al. 2016b). Taken together, these studies suggested that alterations in SHP-1 and BCL-6 was involved in TCDD-mediated impairment of human B cell activation.

Collectively, the aforementioned studies have demonstrated that TCDD affects already established mature B cells. Given that human mature B cells have a short life span (i.e., 18 days on average) (Macallan et al. 2005), the peripheral B cell population requires lifelong continuous B cell development from HSCs. However, it is presently unclear whether TCDD also affects human B cell developmental process from HSCs.

The effects on TCDD on B cell development

B cell development from HSCs occurs in the bone marrow and requires a sophisticated microenvironment, which makes it difficult to study the perturbation of this process by chemicals. Indeed, there are few studies, primarily in mice, investigating the effects of TCDD on B cell development. The first observations that implicate the perturbations of B cell development by TCDD come from studies identifying alterations in the function, survival and proliferation of HSCs by TCDD. For example, treatment of adult C57BL/6J mice with TCDD increased the number of Lin^- $Sca\text{-}1^+$ $cKit^+$ (LSK) HSCs, which might result from an arrest in HSC development (Murante and Gasiewicz 2000). Likewise, Sakai *et al.* investigated the effects of TCDD on $CD34^-$ LSK HSCs, the HSC subpopulation that possesses the ability of long-term multilineage reconstitution. They found administration of TCDD to C57BL/6J mice increased the number of $CD34^-$ LSK HSCs; however, these TCDD-treated cells lost long-term reconstitution activity. This defect was not observed in *Ahr* knockout mice, suggesting AHR involvement (Sakai et al. 2003). Moreover, TCDD treatment also led to reduced migration of HSCs to bone marrow *in vivo*, and to chemokine CXCL12 *in vitro* (Casado et al. 2011). TCDD also altered circadian rhythms, quiescence, and expression of clock genes in murine hematopoietic precursor populations (Garrett and Gasiewicz 2006). Taken together, these studies show that TCDD interferes with the function, proliferation and development of HSCs in mice.

The effects of TCDD on B cell development have also been demonstrated using mouse models. *In vivo* studies showed that TCDD treatment decreased pro/pre-B cells and immature B cells in mice (Thurmond and Gasiewicz 2000; Thurmond et al. 2000). This effect appears to be mediated by AHR because the number of pro/pre-B cells was not affected by TCDD in *Ahr* null mice (Thurmond et al. 2000). Subsequent studies revealed that TCDD skewed the differentiation of HSC by increasing the number of myeloid progenitors and decreasing lymphoid progenitors, which give rise to B cells (Singh et al. 2009). Moreover, HSCs from *Ahr* null mice showed elevated proliferation activity as compared to HSCs from heterozygote controls (Singh et al. 2011). Also, competitive repopulation assays demonstrated that HSCs from *Ahr* null mice had increased capacity to generate splenic $B220^+$ B cells than HSCs from wild type mice (Singh et al. 2011). In addition, perinatal exposure to TCDD decreased the capacity of HSCs to generate both B and T lymphocytes in mice (Ahrenhoerster et al. 2014). Overall, these studies demonstrate the impairment of B cell development by TCDD in mice.

The mechanism by which TCDD alters B cell development has been suggested to involve a number of proteins that are functionally important for B cell development, including NF-κB, PAX5, and CD19 (Andersson et al. 2003; Thurmond and Gasiewicz 2000; Thurmond et al. 2000). Rel/NF-κB activity is critical for normal B cell development and survival (Gugasyan et al. 2000). Hence, the interaction between the AHR and Rel/NF-κB signaling pathways, as demonstrated by Vogel et al., might contribute to the effects of TCDD on B cell development (Vogel and

Matsumura 2009; Vogel et al. 2007). As discussed in chapter 1.2, PAX5 plays a critical role in regulating B cell lineage commitment during B cell development by activating many B cell specific genes, including CD19 (Cobaleda et al. 2007b). CD19 is critically involved in signaling through the pre-B cell receptor and B cell receptor, thereby modulating B-cell fate decisions at multiple stages of development (Del Nagro et al. 2005). In keeping with the disruptions of B cell development by TCDD, Masten and Shiverick has demonstrated that TCDD treatment led to decreased mRNA level of CD19. Interestingly, they have identified the binding of TCDD activated AHR to a DRE located in a PAX5 binding site in the CD19 promoter, suggesting that the AHR could interfere with PAX5-regulated CD19 gene transcription by competition for a common DNA binding site (Masten and Shiverick 1995).

The aforementioned studies demonstrating the effects of TCDD on B cell development and illustrating the underlying mechanisms are conducted primarily in a mouse model. Although the mouse has historically served as the primary model for mechanistic studies in immunotoxicology, there exist significant differences in the development, activation and response of the mouse and human immune system (Carninci 2014; Mestas and Hughes 2004; Seok et al. 2013). To date, the effects of TCDD on human B cell development have not been investigated. Interestingly, it has been reported that treatment of human HSC with an AHR antagonist produced retention of the stem cell marker, CD34, and markedly promoted the expansion of human HSCs (Boitano et al. 2010). This study suggests a role of the AHR in

regulating human HSC development; however, the effects of AHR activation by persistent environmental ligand (i.e., TCDD) on the developmental process of human HSC to lineage committed B cell remains to be investigated.

1.5 Rationale of this research

TCDD is a ubiquitous environmental contaminant that exhibits a wide range of toxic effects including immunotoxicity. Epidemiological studies have identified the association between TCDD exposure and B cells disorders, most notably compromised humoral immunity and B cell related cancers such as non-Hodgkin's lymphoma. Although studies have demonstrated that TCDD impairs the function of already established human mature B cells (Lu et al. 2011; Lu et al. 2010; Wood and Holsapple 1993), it is presently unclear whether the associations between TCDD exposure and B cell anomalies are also attributable to alterations in human B cell lymphopoiesis by TCDD. Since circulating human B cells have an average half-life of 18 days (Macallan et al. 2005), the homeostasis of the peripheral B cell population requires lifelong replenishment through B lymphopoiesis. As a result, the mature B cell repertoire is susceptible to disruptions of lymphopoiesis.

The receptor that mediates most of the effects of TCDD, AHR, has been recently demonstrated to play a role in regulating human HSC development (Boitano et al. 2010). Hence, the goal of my dissertation study was to test the overarching hypothesis that *AHR activation by TCDD alters the sequential progression required for development of HSC to lineage committed B cell*.

The first part of the dissertation will focus on characterizing the effects of TCDD on human B lymphopoiesis using three *in vitro* models of B cell development starting from human cord blood derived $CD34^+$ hematopoietic stem/progenitor cells. The second part of the dissertation aims to elucidate the molecular mechanisms by

which AHR activation by TCDD impairs human B cell lymphopoiesis. Finally, the future directions of the current study is also described in Chapter 4.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1-chlorodibenzo-*p*-dioxin (MCDD), 2,3,7-trichlorodibenzo-*p*-dioxin (TriCDD), and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HxCDD) were purchased from AccuStandard (New Haven, CT). DMSO and AHR antagonist CH223191 were purchased from Sigma Aldrich (St. Louis, MO).

2.2 Cells

Primary human marrow stromal cells (HMSC) were obtained from Cell Applications (San Diego, CA). Fresh human $CD34^+$ hematopoietic stem/progenitor cells (HSPC) isolated from cord blood of mixed donors were purchased from All Cells (Emeryville, CA).

2.3 Human $CD34^+$ HSPC cultures

Three different *in vitro* culture systems were utilized for human B lymphopoiesis in this study. The first culture system was a co-culture system previously described by Parrish *et al.* (Parrish et al. 2009) in which HMSCs were used as feeder cells to support lymphopoiesis of HSCs. HMSCs were cultured in marrow stromal cell growth medium (Cell Applications, Inc) for less than 8 rounds of cell division. Then, 24 hr prior to co-culture, HMSCs were sub-lethally irradiated

(2000 rad) and seeded ($1x10^4$cells/well) in 96-well tissue culture plate. Fresh human $CD34^+$ HSPCs ($1x10^4$cells/well) were co-cultured with irradiated HMSCs in complete RPMI media (RPMI-1640 medium (Life Technologies) supplemented with 5% human AB serum (serum from human blood type AB donors) (Valley Biomedical), 100 U/ml of penicillin (Life Technologies), 100 µg/ml of streptomycin (Life Technologies), and 50 µM 2-mercaptoethanol). In addition, the cultures were supplemented with IL-3 (1ng/ml) (week 1 only), Flt3 ligand (1ng/ml), IL-7 (5ng/ml) and stem cell factor (25ng/ml) (Miltenyi Biotec). Half of the media was replaced weekly with fresh media containing supplements as described above. At indicated time points, the HSPC derived non-adherent cells were harvested by gentle resuspension without disrupting the monolayer of HMSCs.

The second culture system was stromal cell-free as described previously (Ichii et al. 2010). Briefly, fresh cord blood $CD34^+$ HSPCs ($1x10^4$cells/well) were cultured in complete RPMI media supplemented with cytokines as described in co-culture system. In addition, conditioned media, which was supernatant of one week HMSC culture, was filtered and added into stromal cell-free culture (20% v/v) to support B lymphopoiesis (Ichii et al. 2010). Half of the media was replaced weekly with fresh media containing supplements as described above.

The third culture system was feeder-free modified based on a previous study (Kraus et al. 2014). Specifically, $CD34^+$ HSPCs ($1x10^4$cells/well in 96-well tissue culture plates) were cultured in complete RPMI media with the addition of IL-6 (25 ng/ml; Sigma Aldrich), Flt3 ligand (25 ng/ml; Miltenyi Biotec), and stem cell factor

(SCF; 25ng/ml; Miltenyi Biotec). On day 7, half of the media was replaced with fresh media containing IL7 (20 ng/ml; Miltenyi Biotec), Flt3 ligand (25 ng/ml) and SCF (25 ng/ml). After day 14, cytokine-free media was used to replace half of the media weekly.

In all cases, cells were treated with TCDD (0.01, 0.1, 1, 10 or 30 nM) or vehicle (VH, 0.02% DMSO) only on day 0 prior to addition of cytokines. In studies using AHR antagonist CH223191, cells were treated with antagonist 30 min prior to TCDD treatment. After day 0, no additional VH, TCDD or CH223191 was added when the media was replenished.

2.4 Isolation of human naive B cells

Leukocyte packs were obtained from Gulf Coast Regional Laboratories (Houston, TX), diluted with HBSS (pH 7.4, Invitrogen), overlaid on Ficoll-Paque Plus density gradient (GE Healthcare, Piscataway, NJ), and centrifuged at 1300g for 25 min with low acceleration and brake rate. The peripheral blood mononuclear cells were isolated from the buffy coat post-centrifugation, washed, counted and subjected to a magnetic column-based separation that enriched $CD19^{+}CD27^{-}$ naive human B cells (more than 95% purity). This negative selection was conducted using the MACS Naive human B cell isolation kits (Miltenyi Biotech, Auburn, CA) following manufacturer's instructions.

2.5 Flow cytometric analysis

Antibodies used for flow cytometry included Alexa Fluor 488 anti-human CD34 (clone: 581), Pacific Blue anti-human CD45 (clone: HI30), APC anti-human CD127 (IL7Rα) (clone: A019D5), BV421 anti-human CD10 (clone: HI10a), APC anti-human CD79α (clone: HM47), and PE/Cy7 anti-human CD19 (clone: HIB19) from Biolegend (San Diego, CA), PE anti-human CD127 (IL7Rα) (clone: hIL-7R-M21) from BD Bioscience (San Jose, CA). At the indicated time points, cells were harvested and washed using 1X Hank's Balanced Salt Solution (HBSS, pH 7.4, Invitrogen). Viable cells were identified using Live/Dead Fixable Aqua Dead Cell Stain (Invitrogen) prior to cell surface and intracellular staining. Cell surface Fc receptors were blocked by incubating cells with human AB serum (Valley Biomedical). For cell surface staining, cells were incubated with antibodies in FACS buffer (1X HBSS containing 1% BSA and 0.1% sodium azide, pH 7.4–7.6) for 30 min and then fixed using Cytofix fixation buffer (BD Biosciences) for 10 min. For intracellular staining, fixed cells were permeabilized by incubating in Perm/Wash Buffer (BD Biosciences) for 20 min and incubated with antibodies (anti-CD79a) for 30 min. To assess cell death, cells were harvested and stained with PE Annexin V and 7-aminoactinomycin D (7-AAD) using Apoptosis Detection Kit (BD Pharmingen) per manufacturer's instructions. In all cases, flow cytometric analyses were performed on a FACS Canto II cell analyzer (BD Biosciences) and data were analyzed using FlowJo or Kaluza software. For cell marker expression analysis, the gating strategy was to first gate on singlets and

viable cells, and then gated on lymphocytes. For cell death analysis, the level of Annexin V and 7-AAD was analyzed based on singlets gate.

2.6 PrimeFlow RNA assay

PrimeFlow RNA assay, a flow cytometry based RNA detection technology, was conducted following manufacturer's instructions (eBioscience, San Diego, CA). Specifically, 1 x 10^6 cells were harvested, permeabilized followed by intracellular staining using PE anti-human CD79a (clone: HM47). Cells were then incubated with *EBF1* mRNA specific target probes (VA1-19733; Affymetrix, Santa Clara, CA) for 2 hours. After incubation, cells were washed and incubated with pre-Amplification and Amplification probes for 3 h, followed by incubation with fluorophore conjugated label probes for 1 h. Cells were resuspended in FACS buffer (1X HBSS containing 1% BSA and 0.1% sodium azide, pH 7.4–7.6) and analyzed by flow cytometry.

2.7 Spanning-tree progression analysis of density-normalized events (SPADE)

The SPADE (Qiu et al. 2011) algorithm was used to visualize the dynamics of B lymphopoiesis and its alteration by TCDD. SPADE enables extraction of cellular hierarchy and heterogeneity from multi-parametric single-cell cytometry data in an unsupervised manner. The algorithm contains four computational modules: (i) density-dependent down-sampling of the cytometry data; (ii) agglomerative clustering of the down-sampled data into clusters of cells; (iii) construction of a minimum

spanning tree connecting the clusters into a hierarchy; and (iv) mapping of each cell in the original data to the most similar cluster in the tree (Qiu et al. 2011). Specifically, we used the SPADE 3.0 application (http://pengqiu.gatech.edu/software/SPADE/, accessed Jan. 17, 2017) based on MATLAB (R2014a, The MathWorks, Inc., Natick, MA). Algorithmic parameters were set to default values with number of desired clusters set as 100.

2.8 Real-time quantitative PCR

Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA) and was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The expression level of target gene was assessed by TaqMan Gene Expression Assays: CYP1B1 (Hs02382916_s1), AHR (Hs00907314_m1), CYP1A1 (Hs01054797_g1), EBF1 (Hs03045361_m1), PAX5 (Hs00277134_m1), ETS1 (Hs00428287_m1), TCF3 (Hs00413032_m1) and SPI1 (Hs02786711_m1). Real-time qPCR was performed on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The relative steady-state mRNA level for target gene was calculated by normalizing to the 18S ribosomal RNA and fold changes were calculated by $\Delta\Delta$CT method (Livak and Schmittgen 2001).

2.9 Western blot analysis

Cells were harvested and lysed using lysis buffer that consisted of 50 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 100mM NaCl, 1mM DTT, 1 mM Na3VO4, 25 mM NaF, 0.2% (v/v) Igepal, 5% (v/v) glycerol and protease inhibitor (Roche, Indianapolis, IN). Total protein samples were prepared and the protein concentrations were determined using the BCA assay (Sigma, St Louis, MO). Proteins were separated on 4–20% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA), transferred to nitrocellulose membranes, and probed with primary antibodies: anti-EBF1 rabbit mAb (Abcam EPR4183) or anti-Actin mouse mAb (Sigma A5441), and secondary antibodies: anti-rabbit IgG (Sigma A1949) or anti-mouse IgG (Sigma A3673). The blots were incubated with ECL Western blotting substrate (Pierce, Rockford, IL) and exposed to X-ray films.

2.10 Electrophoretic mobility shift assays (EMSA) and EMSA-Western analysis for AHR

Nuclear Protein Preparation

Nuclear protein was isolated from HEPG2 cells as previously described (Denison and Nagy 2003) with a few modifications. Briefly, HEPG2 cells were treated with vehicle (0.01% DMSO) or TCDD (30 nM in DMSO) for 2h at 37℃. Cells were washed twice with 10 mM HEPES (pH 7.5), incubated at 37℃ for 15 min, then harvested in MDH buffer (2 mM $MgCl_2$, 1 mM DTT, 2mM HEPES, pH 7.5, with

protease inhibitor (Roche, Indianapolis, IN)) and homogenized using a Dounce homogenizer. The homogenates were centrifuged at 1000g for 5 min, washed twice with MDHK buffer (2 mM $MgCl_2$, 1 mM DTT, 2mM HEPES, pH 7.5, and 100 mM KCl, with protease inhibitor) and centrifuged. The crude nuclear pellets were resuspended in HEDGK4 buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 400 mM KCl, 200 μM phenylmethylsulfonyl fluoride, with protease inhibitor) (50 μl per plate of confluent cell) and incubated on ice for 30 min for high-salt extraction followed by centrifugation at 14,000 x g, 4℃ for 15 min. The supernatants were ultracentrifuged at 99,000 x g, 4℃ for 1 h. The protein concentration in supernatants was determined using the BCA assay (Sigma, St Louis, MO).

DRE Oligonucleotides

The consensus DRE has been previously described (Sun et al. 2004). The oligonucleotide sequence for consensus DRE is: GGCTTGCGTGCGA. The oligonucleotide sequences for three putative DREs in the promoter region of human *EBF1* gene are DRE4 (-6371): CACCTTTGCGTGCTGCG, DRE6 (-5918): TGCCCTGGCGTGACCAT and DRE7 (-5789): TAGAGCTCACGCAAGCT. Complementary pairs of DRE oligomers were synthesized and HPLC purified (Integrated DNA Technologies), followed by annealing and end labeling using T4 polynucleotide kinase (New England BioLabs) and γ-^{32}P ATP (PerkinElmer).

EMSA and EMSA-Western

Nuclear extracts (10 µg of protein) were incubated with double stranded poly (dI-dC) (0.5 µg) (Sigma) for 30 min at room temperature. The ^{32}P-labeled DRE oligomer (240,000 - 480,000 cpm) (for EMSA) or unlabeled DRE oligomer (10 pmol) (for EMSA-Western) was added and incubated for another 30 min at room temperature. The final buffer condition in the binding reaction was: 25 mM Hepes (pH7.5), 1mM EDTA, 1mM DTT, 10% glycerol, 100 mM KCl. Protein:DNA complexes were resolved on a 4% non-denaturing PAGE gel in TGE buffer (25 mM Tris, 380 mM glycine, 2 mM EDTA). The radiolabeled portion of the EMSA gel was dried on 3-mm filter paper, and autoradiographed. The non-radiolabeled portion of the EMSA gel was incubated in soaking buffer (375 mM Tris-HCl, pH 7.5, 1% SDS) for 2 h at room temperature, transferred to nitrocellulose blotting membrane (GE Healthcare Life Sciences) overnight using transfer buffer (30 mM Tris, 240 mM glycine, 20% methanol). The protein:DNA complexes on the blot were blocked in TBST buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat milk for 1 h at room temperature. The anti-human AHR purified (Clone: FF3399, eBioscience) primary antibody was then added at 1:1000 dilution and incubated for 2 h at room temperature. The blot was washed using TBST buffer and incubated with the anti-mouse IgG-HRP (Sigma A3673) antibody in TBST buffer with 5% nonfat milk for 1 h. The blot was incubated with ECL Western blotting substrate (Pierce, Rockford, IL) and exposed to X-ray films.

Competition EMSA

Nuclear extracts (5 µg of protein) were incubated with double stranded poly (dI-dC) (0.5 µg) (Sigma) for 15 min at room temperature. The competitor DNA, which is an un-labeled consensus DRE oligonucleotide, was added at 10, 25 or 50 fold excess, relative to the labeled DRE oligonucleotides. After incubation for 15 min, the ^{32}P-labeled DRE oligomers (DRE4 or DRE7, 0.12 pmol, 480,000 cpm) were added incubated for an additional 15min. The final buffer condition in the binding reaction was: 25 mM Hepes (pH7.5), 1mM EDTA, 1mM DTT, 10% glycerol, 100 mM KCl. Protein:DNA complexes were resolved on a 4% non-denaturing PAGE gel in TGE buffer (25 mM Tris, 380 mM glycine, 2 mM EDTA). The competition EMSA gel was dried on 3-mm filter paper, and visualized by autoradiograph.

2.11 Electrophoretic mobility shift assays (EMSA) and EMSA-Western analysis for PAX5

Nuclear Protein Preparation

Nuclear protein was isolated from JM1 cells and Jurkat cells. JM1 cell is a human pre-B lymphoblast cell line that expresses high levels of PAX5 mRNA hence serves as a good source of PAX5 protein. In contrast, Jurkat cell is a T lymphocyte cell line that does not express PAX5 and is used as a negative control for PAX5 EMSA analyses. Cells were washed twice with 10 mM HEPES (pH 7.5), incubated at 37℃ for 15 min, then harvested in MDH buffer (2 mM $MgCl_2$, 1 mM DTT, 2mM

HEPES, pH 7.5, with protease inhibitor (Roche, Indianapolis, IN)) and homogenized using a Dounce homogenizer. The homogenates were centrifuged at 1000g for 5 min, washed twice with MDHK buffer (2 mM $MgCl_2$, 1 mM DTT, 2mM HEPES, pH 7.5, and 100 mM KCl, with protease inhibitor) and centrifuged. The crude nuclear pellets were resuspended in HEDGK4 buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 400 mM KCl, 200 μM phenylmethylsulfonyl fluoride, with protease inhibitor) (50 μl per plate of confluent cell) and incubated on ice for 30 min for high-salt extraction followed by centrifugation at 14,000 x g, 4℃ for 15 min. The supernatants were ultracentrifuged at 99,000 x g, 4℃ for 1 h. The protein concentration in supernatants was determined using the BCA assay (Sigma, St Louis, MO).

Oligonucleotides of PAX5 binding sites

The consensus PAX5 binding site is: CGA GGG CAG CCA AGC GTG AC. The oligonucleotide sequences for two putative PAX5 binding sites in the promoter region of human *EBF1* gene are -5791: CGA GCT CAC GCA AGC TTG CC, and -6793: CAA GGG CAG GCG TGC AGA CC. Complementary pairs of DRE oligomers were synthesized and HPLC purified (Integrated DNA Technologies), followed by annealing and end labeling using T4 polynucleotide kinase (New England BioLabs) and γ-^{32}P ATP (PerkinElmer).

EMSA and EMSA-Western

Nuclear extracts (4 µg of protein) were incubated with double stranded poly (dI-dC) (1 µg) (Sigma) for 30 min at room temperature. The ^{32}P-labeled DRE oligomer (480,000 cpm) (for EMSA) or unlabeled DRE oligomer (10 pmol) (for EMSA-Western) was added and incubated for another 30 min at room temperature. The final buffer condition in the binding reaction was: 25 mM Hepes (pH7.5), 1mM EDTA, 1mM DTT, 10% glycerol, 100 mM KCl. Protein:DNA complexes were resolved on a 4% non-denaturing PAGE gel in TGE buffer (25 mM Tris, 380 mM glycine, 2 mM EDTA). The radiolabeled portion of the EMSA gel was dried on 3-mm filter paper, and autoradiographed. The non-radiolabeled portion of the EMSA gel was incubated in soaking buffer (375 mM Tris-HCl, pH 7.5, 1% SDS) for 2 h at room temperature, transferred to nitrocellulose blotting membrane (GE Healthcare Life Sciences) overnight using transfer buffer (30 mM Tris, 240 mM glycine, 20% methanol). The protein:DNA complexes on the blot were blocked in TBST buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat milk for 1 h at room temperature. The anti-PAX5 (clone 1H9, EMD Millipore) primary antibody was then added at 1:1000 dilution (1 µg/ml) and incubated for 2 h at room temperature. The blot was washed using TBST buffer and incubated with the HRP goat anti-rat IgG antibody (Biolegend) at 1:1000 dilution in TBST buffer with 5% nonfat milk for 1 h. The blot was incubated with ECL Western blotting substrate (Pierce, Rockford, IL) and exposed to X-ray films.

2.12 Chromatin immunoprecipitation (ChIP) and ChIP-qPCR analysis

Human $CD34^+$ HSPCs were cultured as described above for 28 days. On day 28 cells were treated with vehicle (VH, 0.02% DMSO) or TCDD (1 nM). Three hours post treatment cells were harvested for ChIP assays using ChIP-IT High Sensitivity kit following manufacturer's instructions (Active Motif). Specifically, cells were fixed at room temperature for 15 min followed by quenching for 5 min. The cells were washed twice with ice-cold PBS and lysed on ice. The cross-linked chromatin was sheared by sonication. Chromatin immunoprecipitation was conducted by incubating sheared chromatin (18 µg) with either anti-AHR antibody (4 µg, BML-SA210, Enzo Life Sciences) or negative control antibody (4 µg, Active Motif) (mock ChIP reaction) overnight. The AHR-containing chromatin complexes bound to anti-AHR antibody were isolation by incubating with protein G agarose beads followed by filtration. The isolated chromatin complexes were incubated with proteinase K at 55℃ for 30 min and at 80℃ for 2 h to reverse cross-links. Thereafter, the ChIP enriched genomic DNA was purified using DNA purification column (Active Motif).

ChIP enriched DNA was analyzed using quantitative PCR (qPCR). Primers were designed to amplify regions in *EBF1* promoter that contains putative AHR binding sites (DREs). The sequence of primers were: DRE4 (-6371): ACT TCC TTC GAG GGA CAA TTT (Forward), ATC ATA CAC ATC TCG CAT CCC (Reverse); DRE6 (-5918): CTT GCG GAT GTG CTT TAA TGG (Forward), CTG TAT TCT CCC GAC TCA GAA TG (Reverse); and DRE7 (-5789): CCA CAT TTA CTA TGT GAC CTC CT (Forward), ATG GGC ATC AGG AAC ATC C (Reverse). In addition, positive

control and negative control PCR primer pairs were included in analysis. The positive control primer set amplifies an AHR binding region in CYP1A1 promoter. The sequence of the primer is: CTG ACC TCT GCC CCC TAG A (Forward), GGG TGG CTA GTG CTT TGA TT (Reverse). The negative control primer set amplifies a region in a gene desert on human chromosome 12 (Human Negative Control Primer Set 1, Active Motif). A standard curve was produced using Input DNA isolated from sheared chromatin. For each primer set, SYBR Green based qPCR were conducted using ChIP DNA samples along with the dilution series of Input DNA standards. The data was expressed as a percent of input.

2.13 JM1 cell transduction

HEK293T cells were plated at 1.2×10^6 cells/well in 6-well plates and transfected with 6 μg of the *pTRIPZ-AHR-GFP* constructs using lentiviral packing mix (Open Biosystems, Huntsville, AL) according to the manufacturer's instructions. After a 16-hour incubation, HEK293T cell were overlaid with 2×10^6 JM1 cells/well and co-cultured for an additional 24 hours. After 24 hours of co-culture, JM1 cells were separated from HEK293T cells and cultured using IMDM media containing 0.5 μg/mL puromycin. Monoclonal population of transduced JM1 cells was established using cloning by limiting dilution. The expression of GFP in transduced cells was induced by doxycycline treatment (25 μg/ml) and measured by flow cytometry.

2.14 Statistics

Statistical analyses were performed using GraphPad Prism 5.00 (Graphpad Software, San Diego, CA). Data were graphed as mean ± SEM. Statistical comparisons were performed using t-test, one-way ANOVA with Bonferroni or Dunnett's multiple comparison posttest, or two way ANOVA with Bonferroni posttest depending on the experimental design. Data presented as fold-change were transformed using logarithmic transformation prior to statistical analysis.

CHAPTER 3: RESULTS

3.1 Characterization of TCDD-mediated alterations in human B lymphopoiesis

3.1.1 Using co-culture system to characterize the effects of TCDD on human B lymphopoiesis

An in vitro co-culture system supporting human B lymphopoiesis

An *in vitro* culture system was established to investigate the effects of TCDD on human B lymphopoiesis. Specifically, fresh human cord blood $CD34^+$ HSPCs were co-cultured with primary human marrow stromal cells (HMSC) and supplemented with cytokines. During the 3-5 week culture period, cell surface markers demarcating discrete stages of B cell development were measured to monitor the progression of B lymphopoiesis. The expression of interleukin-7 receptor α chain (IL7Rα), which identified lymphoid lineage restriction (Nutt and Kee 2007), increased from 0.3% on day 0 to 29.1% on day 20 (Figure 3.1), indicating the generation of common lymphoid progenitors. By day 24, 26.1% of cells expressed CD19, a hallmark of B cell lineage commitment and demonstrating that this *in vitro* co-culture system supports human B cell development from HSPCs (Fig. 3.1 B).

TCDD decreased the total number of HSPC-derived cells

To explore the effects of TCDD on early stages of human B cell development, HSPCs were treated with vehicle (VH, 0.02% DMSO) or TCDD (1, 10 or 30 nM) on day 0 and co-cultured with HMSCs for 5 weeks. The effects of TCDD on B cell

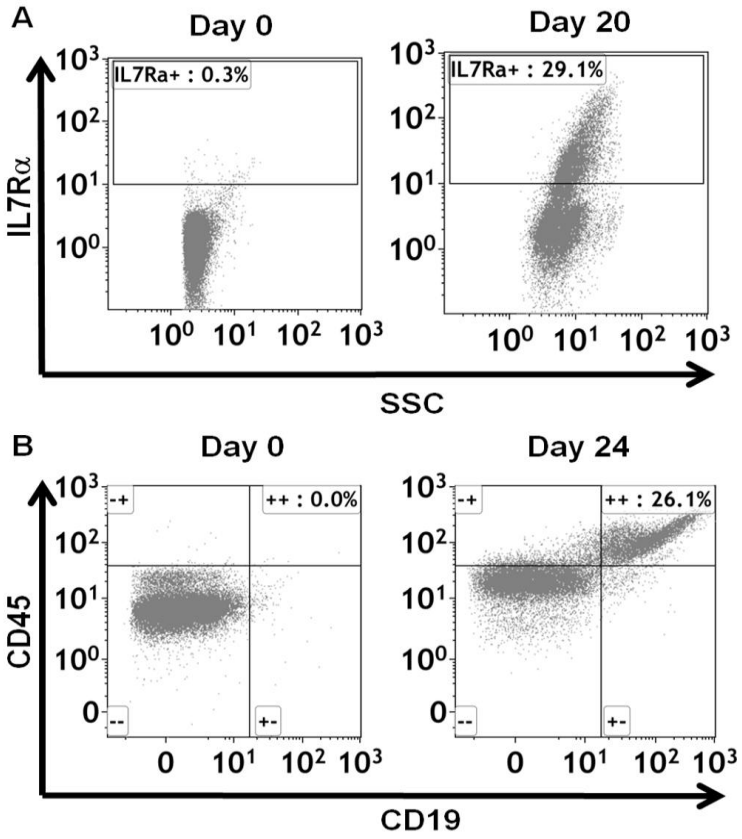


Figure 3.1: Establishment of an *in vitro* co-culture model system of human B cell development

Human HSPCs were co-cultured with HMSCs in RPMI media supplemented with cytokines. The HSPC-derived cells were harvested by gentle resuspension from co-cultures. The expression of cell surface IL7Rα (A) and CD19 (B) were analysed by flow cytometry. Data are representative of two independent experiments with similar results.

development were first assessed by enumerating the total number of cells derived from HSPCs. The cell growth curves show that TCDD treatment significantly decreased the total number of cells at each time point (Figure 3.2). To determine whether the decrease in cell number was due to TCDD-induced cell death, Annexin V and 7-AAD staining assay was performed. Consistently, a significant decrease in total cell number was observed with TCDD treatment on days 14, 18 and 21 (Figure 3.3 A). While the number of cells undergoing apoptosis (Annexin V^+ 7-$AAD^{+/-}$) or necrosis (Annexin V^- 7-AAD^+) was unchanged by TCDD treatment, a significant decrease in the number of live cells (Annexin V^- 7-AAD^-) was observed in the presence of TCDD (Figure 3.3 B). Collectively these findings suggest that the decrease in the total number of cells in the presence of TCDD is not due to necrotic or apoptotic cell death.

TCDD diminished CD34 expression on HSPCs

To further investigate impairment of B cell development by TCDD, the expression of CD34 on HSPCs was examined. CD34 has been used widely as a stem cell marker for HSPCs as it is absent from most mature hematopoietic lineages. In addition, the expression of CD34 by HSPCs has been identified as an indicator of stem cell activation status. Quiescent HSPCs, largely in G_0 phase of cell cycle, are $CD34^-$ whereas cytokine-activated HSPCs in G_0/G_1 phase are $CD34^+$ (Roberts and Metcalf 1995; Tajima et al. 2000). Using the HSPC-stromal cell co-culture, TCDD

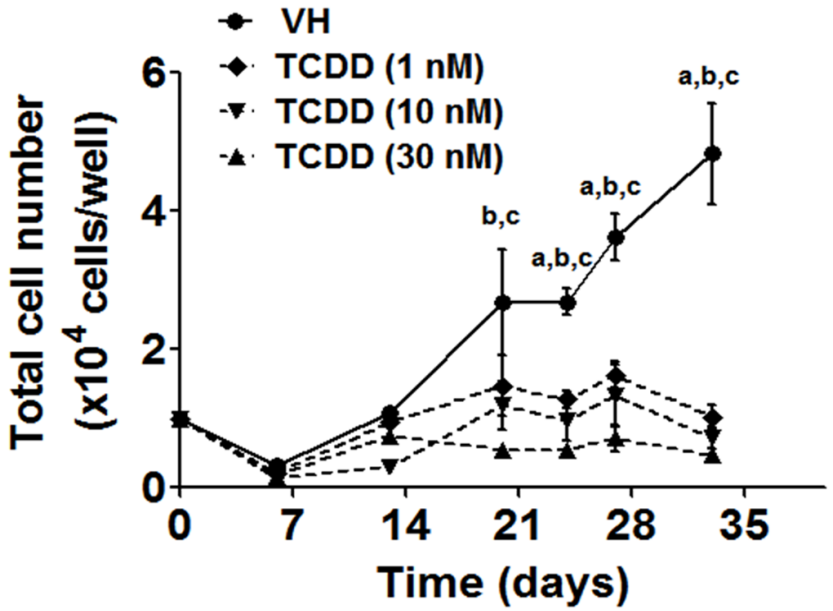


Figure 3.2: TCDD decreased the total cell number in co-culture

Human HSPCs (10^4 cells/well) were treated with vehicle (VH) (0.02% DMSO) or TCDD (1, 10 or 30 nM) on day 0 and co-cultured with HMSCs. The HSPC-derived cells were harvested by gentle resuspension from co-culture and enumerated using a hemocytometer. Data are presented as the mean ± SE of triplicate measurements. Statistic analysis was performed using two way ANOVA with Bonferroni posttest. The symbols a, b and c designate the significant differeces ($p<0.05$) between VH group and the three TCDD treatment groups respectively. Data are representative of two independent experiments.

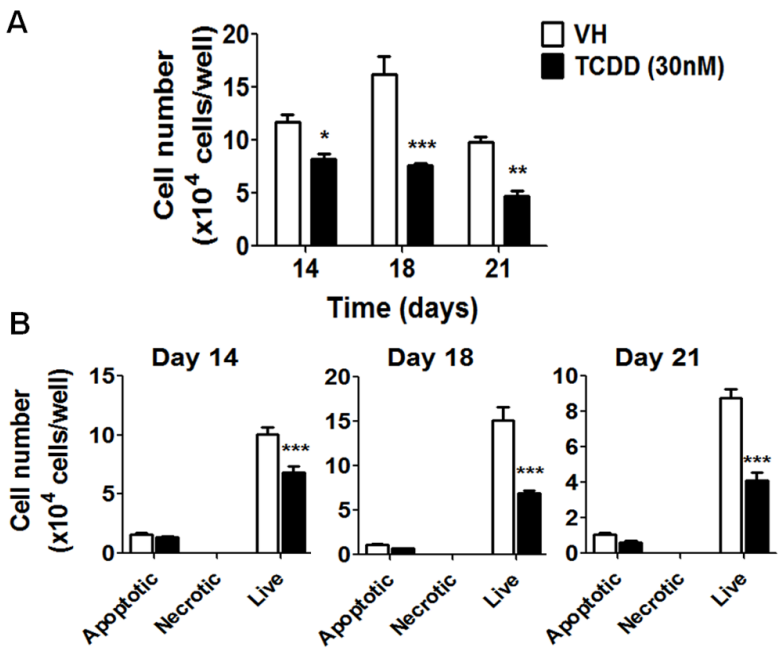


Figure 3.3: TCDD does not affect cell viability

Human HSPCs were treated with vehicle (VH) (0.02% DMSO) or TCDD (30 nM) on day 0 and co-cultured with HMSCs. **A)** The HSPC-derived cells were harvested by gentle resuspension from co-culture and enumerated using a hemocytometer. **B)** Cell death analysis was performed by Annexin V and 7-AAD staining. The numbers of live (Annexin V^- 7-AAD^-) , apoptotic (Annexin V^+ 7-$AAD^{+/-}$) and necrotic (Annexin V^- 7-AAD^+) HSPCs from VH or TCDD treated groups were enumerated on day 14, 18 and 21. Data are presented as mean ± SE of triplicate measurements. * $p < 0.05$, **$p<0.01$, ***$p < 0.001$, compared to the VH by two way ANOVA with Bonferroni posttest. Data are representative of four independent experiments .

significantly decreased the expression of CD34 on HSPCs (Figure 3.4) as evidenced by both the percentage of $CD34^+$ cells (Figure 3.4 A) as well as by the average level of CD34 surface expression on individual $CD34^+$ cells (represented by mean fluorescence intensity - MFI) (Figure 3.4 B). In light of the aforementioned association between CD34 expression and HSPC activation, decreased CD34 on HSPCs in the presence of TCDD suggests either suppression of HSPC activation or that HSPCs are being driven more rapidly toward a differentiated state. The former is more likely since TCDD treatment led to a reduction in the total cell number in culture (Figure 3.2).

Structure-activity-relationship studies to assess involvement of AHR in impairment of early B lymphopoiesis by TCDD

It is widely established that most of the effects produced by TCDD are mediated by the AHR (Hankinson 1995; Schmidt and Bradfield 1996). To explore the involvement of the AHR in TCDD-mediated impairment of B cell development, structure-activity-relationship studies were conducted using four polychlorinated dibenzo-p-dioxin (PCDD) congeners: 1-chlorodibenzo-p-dioxin (MCDD), 2,3,7-trichlorodibenzo-p-dioxin (TriCDD), 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin (HxCDD) and TCDD. First we measured the induction of *CYP1B1* expression by PCDD congeners, a AHR-mediated response. The rank order for *CYP1B1* induction was: MCDD < TriCDD < HxCDD < TCDD (Figure 3.5), which correlated with AHR binding affinity for the respective congeners (Poland et al. 1976; Sulentic et al. 2000).

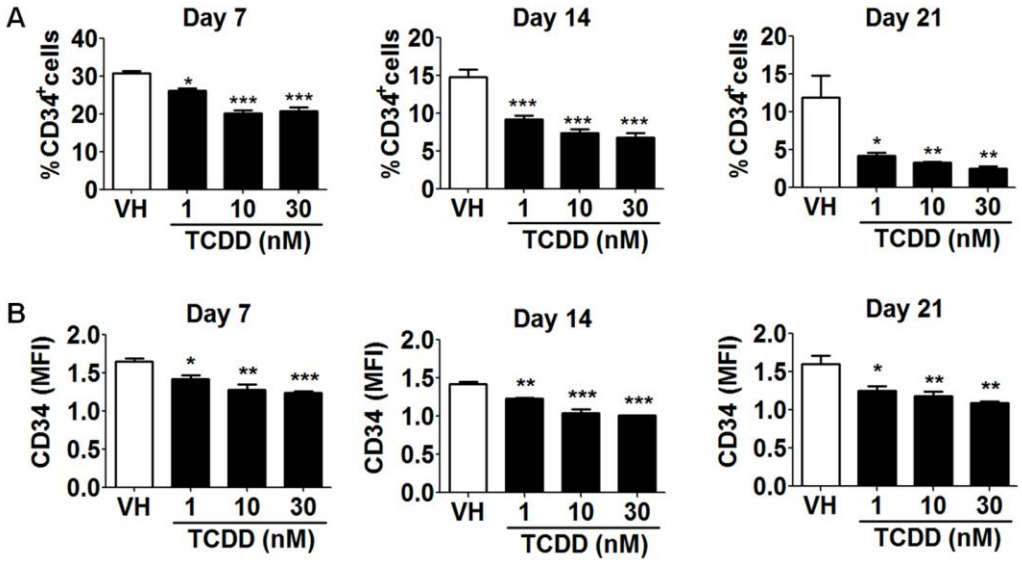


Figure 3.4: TCDD reduced the percentage of CD34$^+$ cells and the average expression level of CD34 on CD34$^+$ cells

HSPCs (CD34$^+$) were treated with vehicle (VH) (0.02% DMSO) or TCDD (1, 10 or 30 nM) on day 0 and co-cultured with HMSCs. The expression of CD34 on HSPC-derived cells was measured on day 7, 14 and 21 using flow cytometry. **A)** The percentage of CD34$^+$ cells in VH or TCDD treatment groups. **B)** The average expression level of CD34 on CD34$^+$ HSPCs is represented by MFI. Data are mean ± SE of triplicate measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to VH by one way ANOVA with Dunnett's multiple comparison test. Data are representative of three independent experiments.

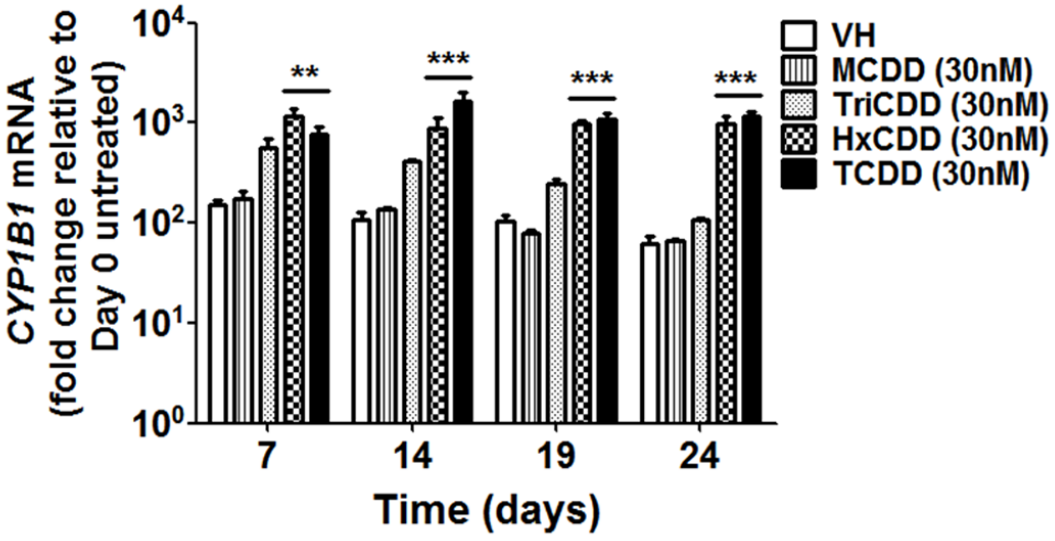


Figure 3.5: Effect of selected PCDD congeners on *CYP1B1* induction

HSPCs were treated with vehicle (VH) (0.02% DMSO) or PCDD congeners (30 nM) on day 0 and co-cultured with HMSCs. The mRNA level of *CYP1B1* in HSPC-derived cells was measured by RT-qPCR. The fold change was calculated relative to day 0 untreated cells. Data are presented as mean ± SE of triplicate measurements. **p<0.01, ***p <0.001, compared to the VH by two way ANOVA with Bonferroni posttest.

Consistently, the magnitude of reduction in the total cell number (Figure 3.6), down-regulation of CD34 expression, and reduction in the percentage of $CD34^+$ cells (Figure 3.7) all correlated with the congener's AHR binding affinity, suggesting AHR involvement.

TCDD diminished B cell lineage commitment

To investigate the effects of TCDD on human B cell lineage commitment, we assessed the capacity of HSPCs to give rise to pro-B cells in the absence and presence of TCDD (1, 10 and 30 nM). The generation of pro-B cells from HSPCs was identified based on the expression of CD19, a hallmark of B cell lineage commitment, using flow cytometry. During the culture period, $CD19^+$ cells started emerging after the third week and rapidly increased in number thereafter. In contrast, the generation of $CD19^+$ cells was significantly reduced in the presence of TCDD (Figure 3.8), suggesting impairment of B cell lineage commitment.

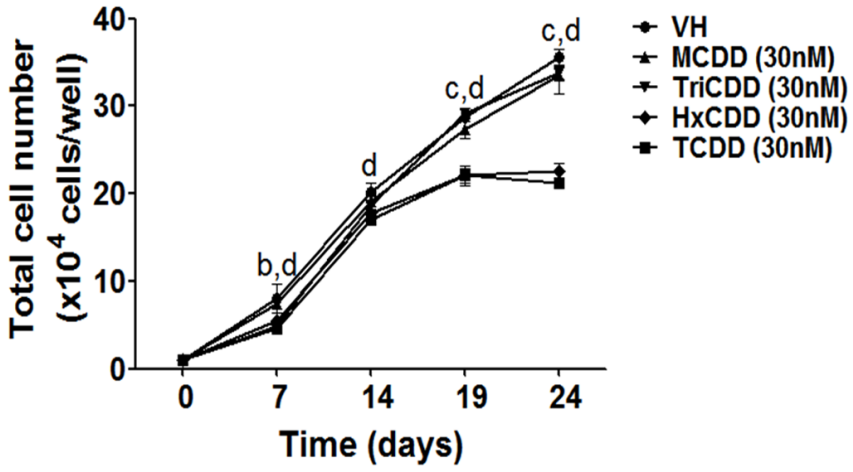


Figure 3.6: Effect of selected PCDD congeners on cell number

HSPCs (10^4 cells/well) were treated with vehicle (VH) (0.02% DMSO) or PCDD congeners (30 nM) on day 0 and co-cultured with HMSCs. Cells were harvested by gentle resuspension from co-culture and enumerated using a hemocytometer. Data are presented as mean ± SE of triplicate measurements. The symbols a, b, c and d designate significant differeces ($p<0.05$) between the VH group and four PCDD congeners treatment groups (MCDD, TriCDD, HxCDD and TCDD) respectively. Statistical analysis was performed using a two way ANOVA with Bonferroni posttest. Data are representative of two independent experiments.

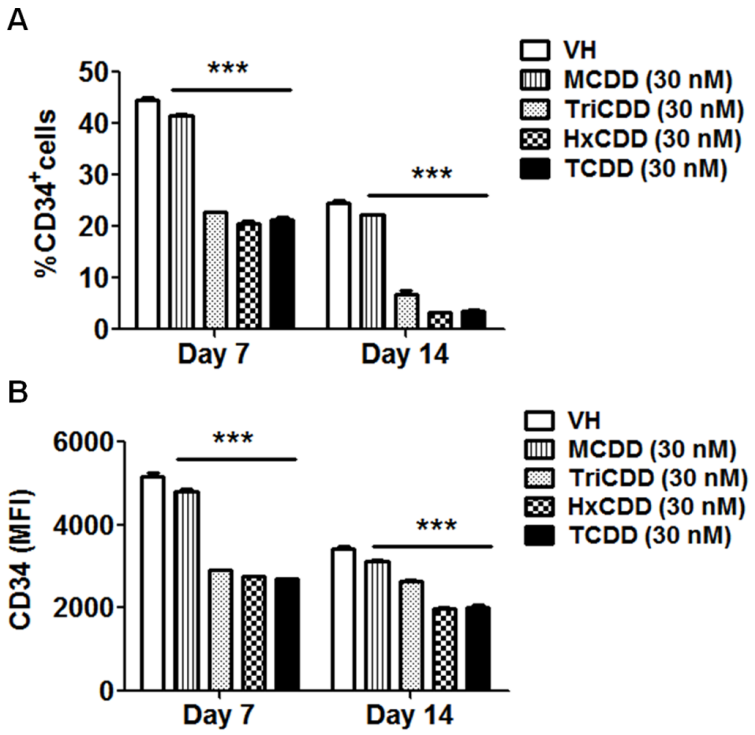


Figure 3.7: Effect of selected PCDD congeners on CD34 expression

HSPCs ($CD34^+$) were treated with vehicle (VH) (0.02% DMSO) or PCDD congeners (30 nM) on day 0 and co-cultured with HMSCs. The expression of CD34 on cells was measured on day 7 and 14 by flow cytometry. **A)** The percentage of $CD34^+$ HSPCs. **B)** The average expression level of CD34 on $CD34^+$ HSPCs, represented by MFI. Data are presented as mean ± SE of triplicate measurements. ***p <0.001, compared to VH by two way ANOVA with Bonferroni posttest. Data are representative of two independent experiments.

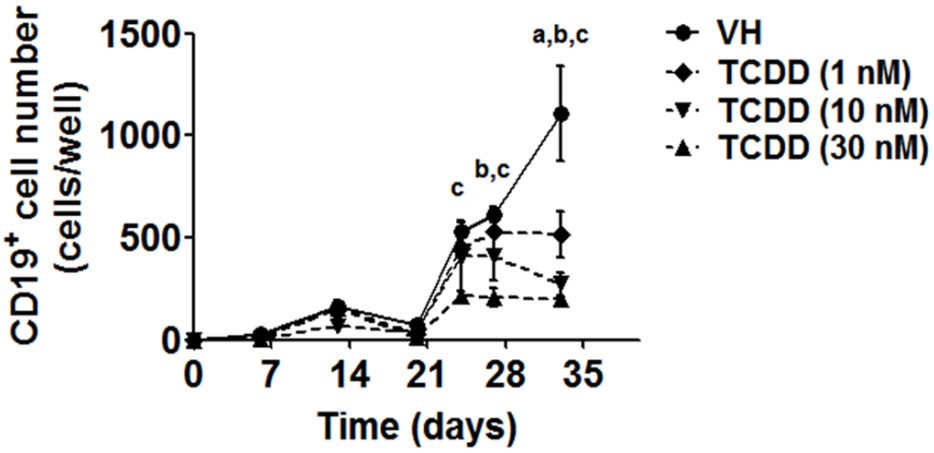


Figure 3.8: TCDD decreased the number of lineage committed B cells in co-culture

HSPCs (10^4 cells/well) were treated with vehicle (VH) (0.02% DMSO) or TCDD (1, 10 or 30 nM) on day 0 and were co-cultured with HMSCs. The $CD19^+$ lineage committed B cells were quantified by flow cytometry. Data are presented as mean ± SE of triplicate measurements. The symbols a, b and c designate significant differeces ($p<0.05$) between VH group and three TCDD treatment groups respectively. Statistic analysis was performed using a two way ANOVA with Bonferroni posttest. Data are representative of two independent experiments.

3.1.2 Using a stromal cell free culture system to characterize the effects of TCDD on human B lymphopoiesis

A previous study demonstrated that the AHR agonist, 7,12-Dimethylbenz[a]anthracene (DMBA), altered stromal cell cytokine production (Jensen et al. 2003). In addition, DMBA metabolites generated by stromal cells triggered bone marrow B cell apoptosis (Teague et al. 2010). Although TCDD is not readily metabolized and does not require metabolism to suppress B cell function, studies with DMBA raised the question whether TCDD-elicited impairment of early human B cell development was dependent on stromal cells. To investigate the direct effects of TCDD on HSPCs in the absence of stromal cells, we adopted and modified a previously described stromal cell-free culture system (Ichii et al. 2010). As observed in HSPC/HMSC co-cultures, the total number of cells in the stromal cell-free cultures was significantly reduced by TCDD treatment (Figure 3.9). In addition, TCDD treatment also decreased the percentage of $CD34^+$ cells as well as the magnitude of CD34 expression (represented by MFI) on $CD34^+$ cells in stromal cell-free cultures (Figure 3.10). Moreover, the number of $CD19^+$ lineage committed B cells was also diminished by TCDD treatment (Figure 3.11). Comparing between HSPC/HMSC co-cultures and stromal cell-free cultures, similar effects of TCDD on B cell development were observed, including the decrease in total cell number, decline in CD34 expression and reduction in lineage committed B cell production. This similarity, in spite of the difference in magnitude, suggests a direct effect by TCDD on HSPCs, which is sufficient to disrupt early B cell development.

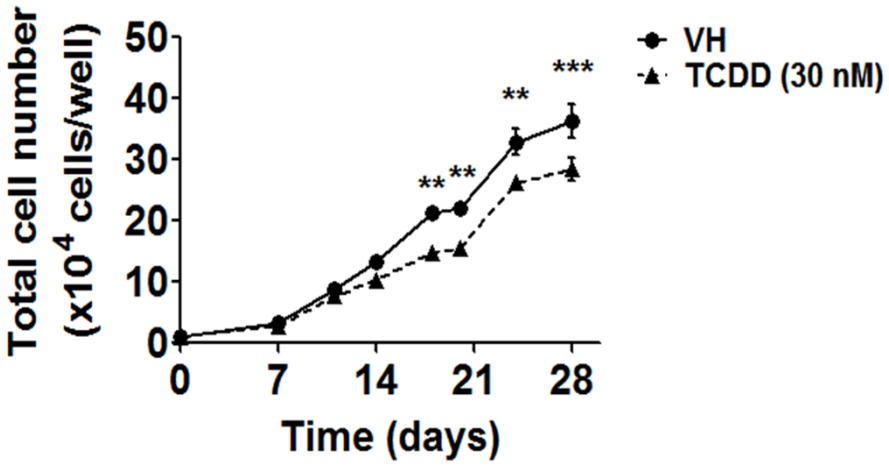


Figure 3.9: TCDD decreased the total cell number in stromal cell-free cultures

HSPCs (10^4 cells/well) were treated with vehicle (VH) (0.02% DMSO) or TCDD (30 nM) on day 0 and cultured in stromal cell-free cultures (RPMI media supplemented with cytokines and 20% v/v of conditioned media). Cells were harvested and enumerated using a hemocytometer. Data are presented as mean ± SE of triplicate measurements. **p <0.01, ***p <0.001, compared to the VH by a two way ANOVA with Bonferroni posttest. Data are representative of three independent experiments .

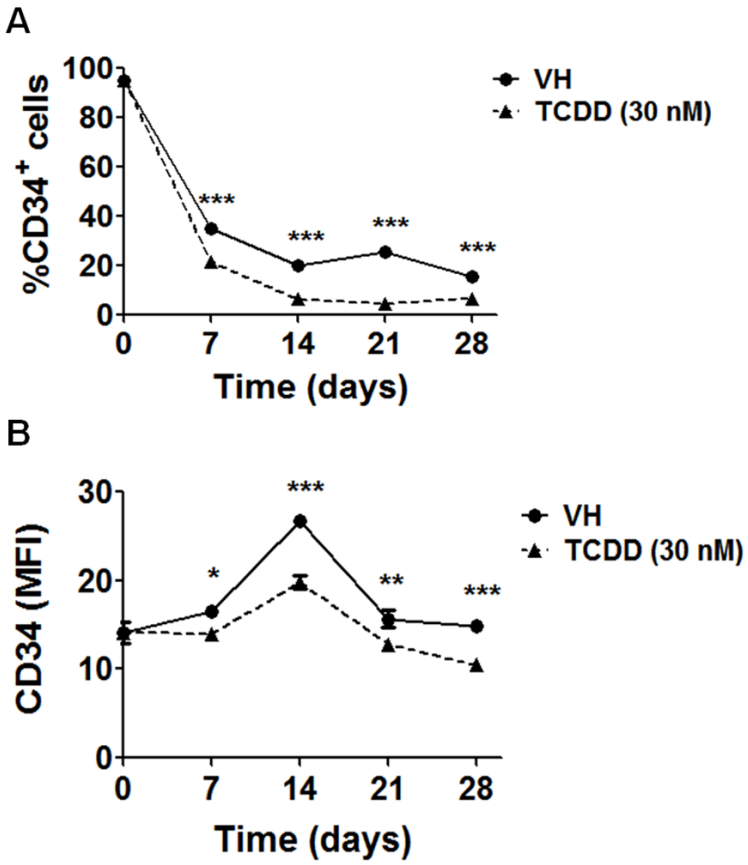


Figure 3.10: TCDD reduced CD34 expression in stromal cell-free cultures

HSPCs (10^4 cells/well) were treated with vehicle (VH) (0.02% DMSO) or TCDD (30 nM) on day 0 and cultured in stromal cell-free cultures (RPMI media with supplemented with cytokines and 20% v/v of conditioned media). The expression of CD34 on cells was measured by flow cytometry. **A)** The percentage of $CD34^+$ HSPCs. **B)** The average expression level of CD34 on $CD34^+$ HSPCs, represented by MFI. Data are presented as mean ± SE of triplicate measurements. * p <0.05, **p <0.01, ***p <0.001, compared to the VH by a two way ANOVA with Bonferroni posttest. Data are representative of three independent experiments.

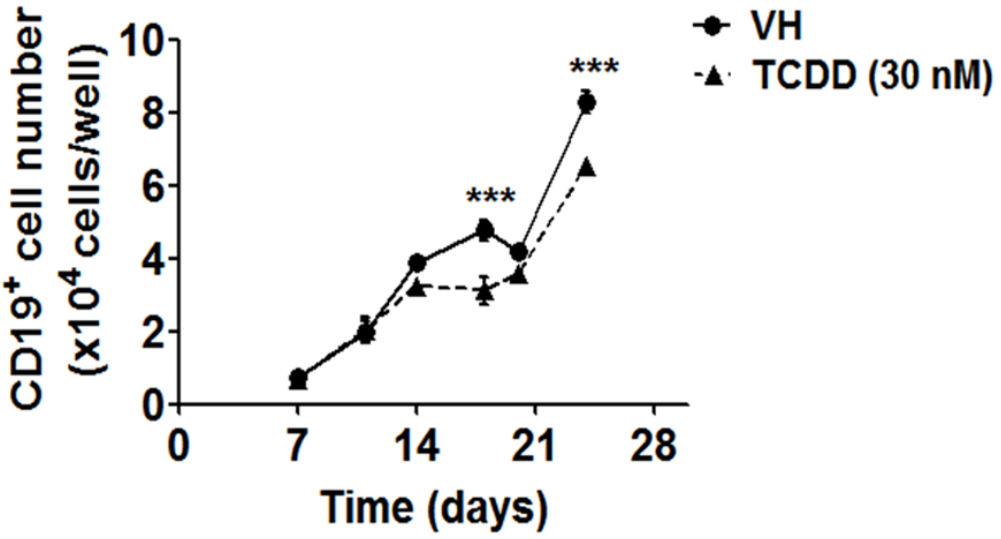


Figure 3.11: TCDD decreased the total number of lineage committed B cells in stromal cell-free culture

HSPCs (10^4 cells/well) were treated with vehicle (VH) (0.02% DMSO) or TCDD (30 nM) on day 0 and cultured in stromal cell-free cultures (RPMI media supplemented with cytokines and 20% v/v of conditioned media). The $CD19^+$ lineage committed B cells were identified by flow cytometry. Data were presented as mean ± SE of triplicate measurements. ***p <0.001, compared to the VH by two way ANOVA with Bonferroni posttest.

3.1.3 Using feeder cell free culture system to characterize the effects of TCDD on human B lymphopoiesis

In prior study, we employed co-culture system and stromal cell free culture system to investigate the effects of TCDD on human B lymphopoiesis. Both of the culture systems contain stromal cells and/or growth factors secreted by stromal cells. Since the influence of stromal cells and their secretory factors are poorly defined, here we employed a feeder-free culture system to explore the direct effects of AHR activation on human B lymphopoiesis and the underlying mechanism.

An in vitro feeder cell free system facilitates early-B and pro-B cell development from human CD34$^+$ hematopoietic stem/progenitor cells (HSPC)

The *in vitro* human B lymphopoiesis model was established using human cord blood-derived CD34$^+$ HSPCs based on a previous study (Kraus et al. 2014). To monitor the developmental progression for B lymphopoiesis, the expression of cell markers (CD34, CD10, CD79α and CD19) demarcating discrete B cell developmental stages was quantified by flow cytometry (LeBien 2000) (Figure 3.12). CD34$^+$ HSPCs (94.7% in purity) were cultured in media supplemented with specific growth factors and cytokines on day 0. After two weeks (day 14), the emergence of CD10$^+$ cells (approximately 2%) was observed, which contained common lymphoid progenitors (CLP). On day 21, a substantial population of early-B cells (CD10$^+$ CD79α$^+$ CD19$^-$) (LeBien 2000; Rose and Nahrwold 1976) was derived from CLPs (approximately 60% of CD10$^+$ cells) . Ultimately, pro-B cells (CD10$^+$ CD79α$^+$ CD19$^+$) emerged on

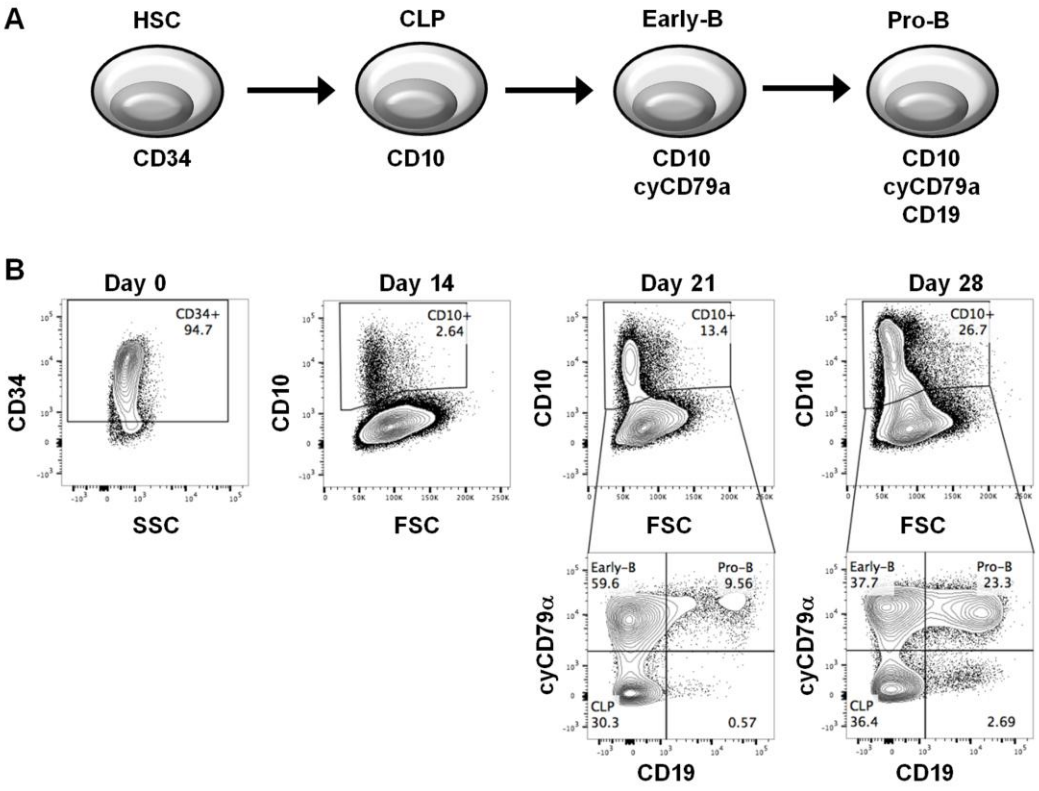


Figure 3.12: The developmental process of human CD34$^+$ HSPC to lineage committed B cells in feeder free culture

A) A schematic representation of different stages in B cell development. **B)** Cord blood-derived human CD34$^+$ HSPCs were cultured for up to 28 days. Cells were harvested at indicated time points for flow cytometric analysis of cell markers characterizing developmental stages, including CD34, CD10, cytoplasmic CD79α (cyCD79α) and CD19. Data are representative of four independent experiments.

day 28 (approximately 23% of $CD10^+$ cells), demonstrating the establishment of an *in vitro* human B lymphopoiesis model for assessing the consequence of AHR activation on B cell development.

TCDD suppressed the generation of early-B and pro-B cells

To assess the effects of AHR activation on human B lymphopoiesis, human HSPCs were treated with vehicle (0.2% DMSO) or TCDD (0.01, 0.1, 1 and 10 nM) on day 0. First, the effects of TCDD on the total cell number were assessed. Consistent with previous observations in co-culture and stromal cell free culture, the number of cells derived from HSPCs in the feeder free culture was also decreased by TCDD treatment in a concentration dependent manner (Figure 3.13). Likewise, TCDD treatment also led to a decline in the percentage of $CD34^+$ cells (Figure 3.14). Then, the effects of TCDD on B cell developmental process were investigated. In the vehicle (VH) treated samples, the $CD10^+$ cell population, which includes CLP, early-B and pro-B cells, increased with time (Figure 3.15 A). The step-wise emergence of CLP, early-B and pro-B cells from HSPC cultures demonstrated the progression of B lymphopoiesis. With TCDD treatment, the $CD10^+$ cell population was decreased in a concentration dependent manner, which is mainly attributable to the decrease in early-B cells and pro-B cells (Figure 3.15 A). Indeed, the percentage of early-B and pro-B cells was significantly decreased by TCDD at concentrations as low as 0.1 nM, whereas no significant change was observed in CLP population (Figure 3.15 B).

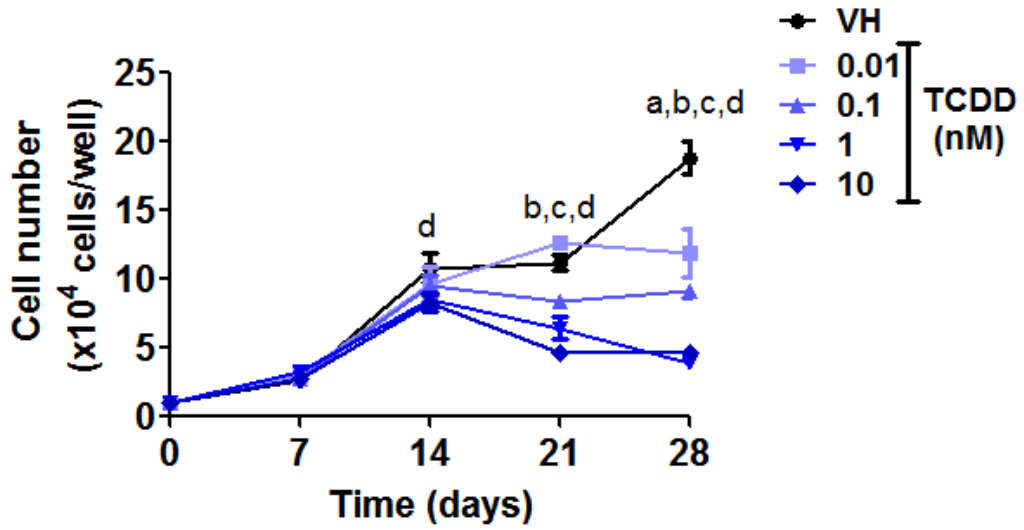


Figure 3.13: TCDD decreased the total cell number in feeder free culture

Cord blood-derived human $CD34^+$ HSPCs (10^4 cells/well) were treated with vehicle (VH, 0.02% DMSO) or TCDD (0.01, 0.1, 1 or 10 nM) on day 0 and cultured for up to 28 days. Cells were harvested at indicated time points and enumerated using a hemocytometer. Data are presented as the mean ± SE of triplicate measurements. Statistic analysis was performed using two way ANOVA with Bonferroni posttest. The symbols a, b, c and d designate the significant differeces ($p<0.05$) between VH group and the four TCDD (0.01, 0.1, 1 or 10 nM) treatment groups respectively. Data are representative of two independent experiments.

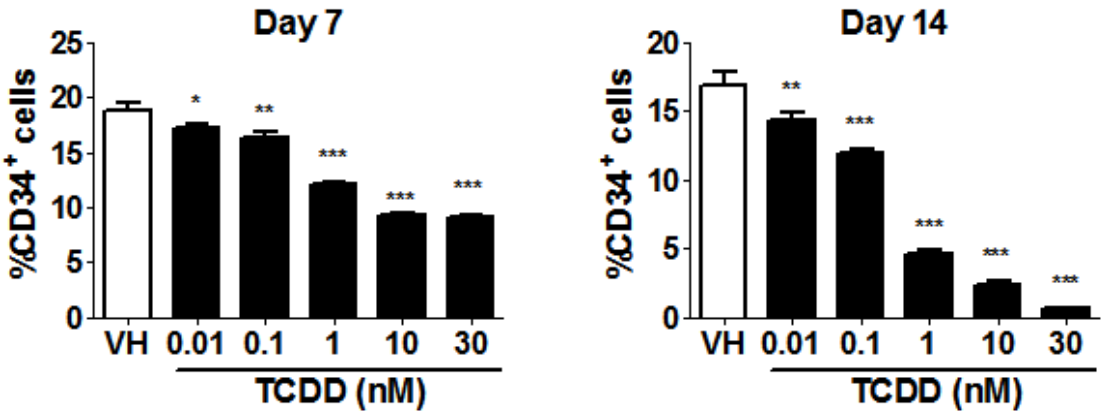


Figure 3.14: TCDD reduced the percentage of CD34$^+$ cells in feeder free culture

Cord blood-derived human CD34$^+$ HSPCs (10^4 cells/well) were treated with vehicle (VH, 0.02% DMSO) or TCDD (0.01, 0.1, 1 or 10 nM) on day 0. The expression of CD34 on HSPC-derived cells was measured on day 7 and 14 using flow cytometry. Data are mean ± SE of triplicate measurements. * p <0.05, **p <0.01, ***p <0.001, compared to VH by one way ANOVA with Dunnett's multiple comparison test. Data are representative of three independent experiments.

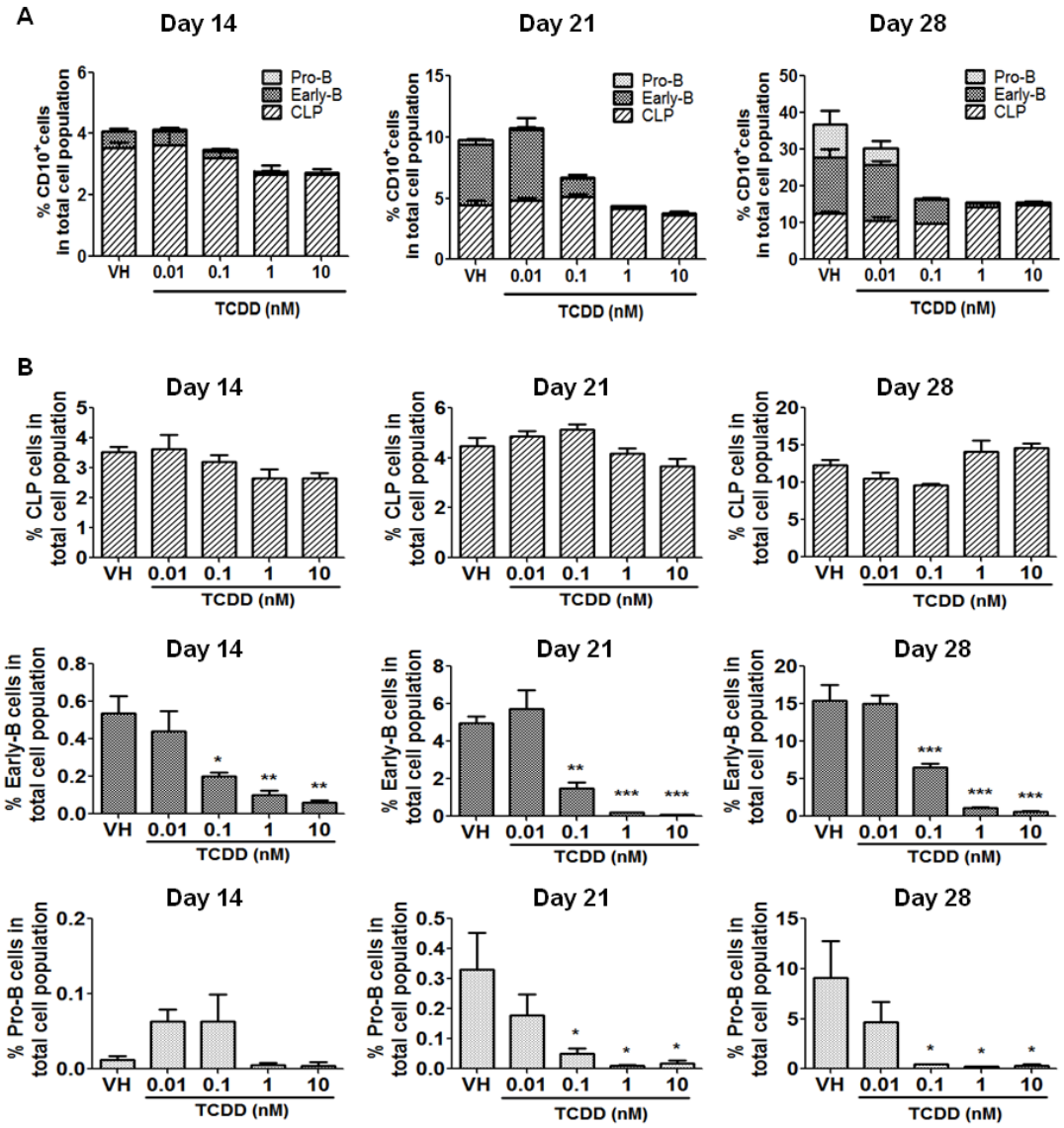


Figure 3.15: TCDD treatment suppressed the generation of early-B and pro-B cells in feeder free culture

Cord blood-derived human $CD34^+$ HSPCs were treated with vehicle (VH, 0.02% DMSO) or TCDD (0.01, 0.1, 1 and 10 nM) on day 0 and cultured for up to 28 days. Cells were harvested at indicated time points for measurement of cell markers by flow cytometry. **A)** The temporal and concentration response effects of TCDD

Figure 3.15 (cont'd)

treatment on the $CD10^+$ cell population, which includes common lymphoid progenitors (CLP), early-B and pro-B cells. **B)** The percentage of CLPs ($CD10^+$ $CD79\alpha^-$ $CD19^-$), early-B cells ($CD10^+$ $CD79\alpha^+$ $CD19^-$) and pro-B cells ($CD10^+$ $CD79\alpha^+$ $CD19^+$). Data are presented as mean ± SE of triplicate measurements. * p <0.05, **p <0.01, ***p <0.001, compared to VH by one way ANOVA with Dunnett's multiple comparison test. Data are representative of three independent experiments with similar results.

In addition, the SPADE computational tool was employed for extraction of cellular hierarchy from high-dimensional cytometry data (Qiu et al. 2011) to visualize the dynamics of B lymphopoiesis and its disruption by TCDD (Figure 3.16 A). Cells were clustered into nodes based on the expression of cell markers CD34, CD10, CD79α and CD19. Then the nodes of cell clusters were colored according to the expression intensity of CD79α, a marker for early-B and pro-B cells. In vehicle (VH) treatment, HSPCs developed along a definite trajectory of differentiation and gradually generated early-B and pro-B cells indicated by increasing expression of CD79α. With TCDD treatment, the progression of B lymphopoiesis was delayed or even arrested in a concentration-dependent manner (Figure 3.16 A). Quantification of the percentage of $CD79\alpha^{+}$ cells further confirmed suppression of B lymphopoiesis by TCDD (Figure 3.16 B).

AHR-mediated suppression of B lymphopoiesis by TCDD

To investigate AHR involvement in TCDD-elicited suppression of human B lymphopoiesis, the expression and functionality of AHR in hematopoietic stem and progenitor cells (HSPC) was determined. HSPCs expressed high levels of AHR mRNA, notably higher than naive peripheral blood B cells (Figure 3.17 A). The level of AHR mRNA in HSPCs increased modestly during the 28-day culture period, with TCDD treatment showing minimal effects (Figure 3.17 B). The expression of *CYP1A1*, a known AHR regulated gene, was markedly increased in a concentration-dependent manner in HSPCs by TCDD treatment (Figure 3.17 C), indicating the AHR

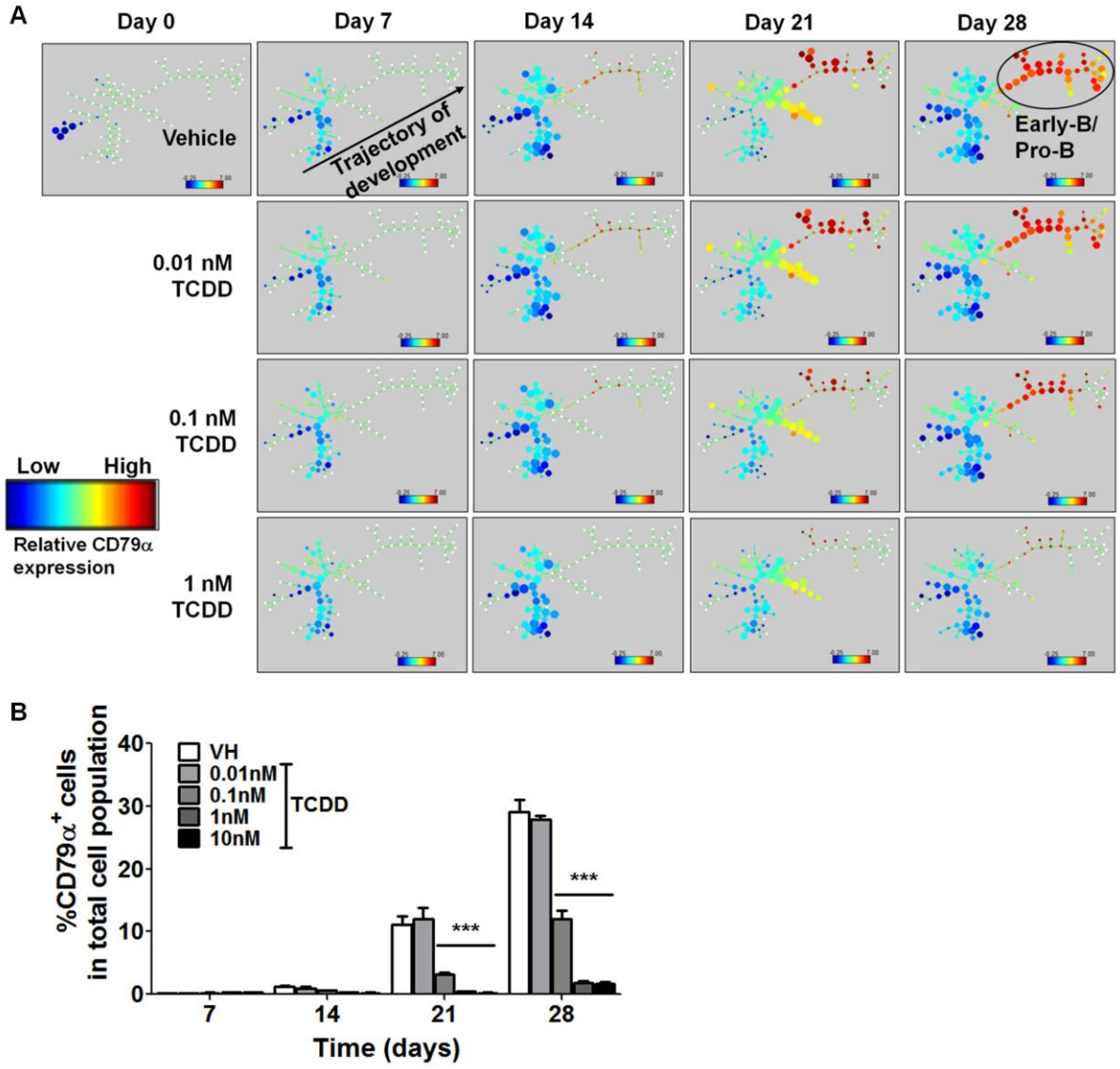


Figure 3.16: TCDD treatment impeded B cell development in feeder free culture

Human CD34^{+} HSPCs were treated with vehicle (VH, 0.02% DMSO) or TCDD (0.01, 0.1, 1 or 10 nM) on day 0 and cultured for up to 28 days. **A)** SPADE visualization of median CD79α levels in clustered groups of cells along the HSPC to B cell developmental trajectory. Cells were clustered into nodes based on the expression of

Figure 3.16 (cont'd)

cell markers CD34, CD10, CD7α and CD19. The size of a node reflects the size of the cell population in that cluster. The nodes of cell clusters were colored according to the expression intensity of CD79α. Panels along columns represent time points, while rows represent treatments, with the first row representing vehicle treatment. Cells progressed chronologically along a lymphopoiesis trajectory with more differentiated cells ($CD79\alpha^{hi}$) towards the right of each panel. This progression is disrupted by TCDD in a concentration-dependent manner, as indicated by a depletion of the early-/pro- B cell subpopulation. **B)** The percentage of $CD79\alpha^{+}$ cells in total cell population was quantified by flow cytometry. Data are presented as mean ± SE of triplicate measurements. ***p <0.001, compared to VH using two way ANOVA with Bonferroni posttest. Data are representative of three independent experiments with similar results.

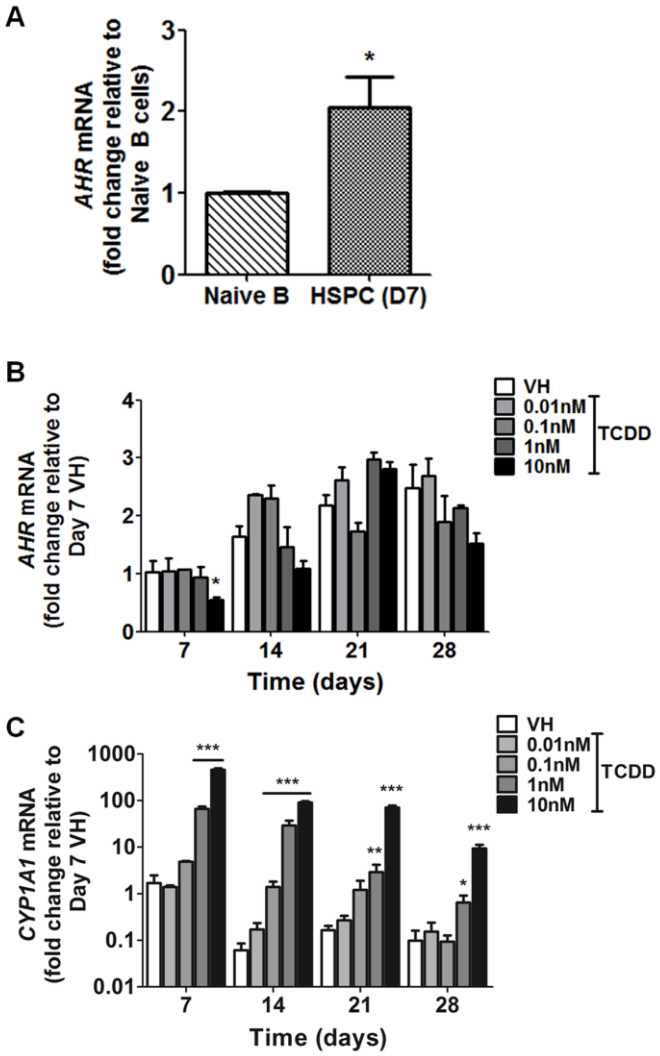


Figure 3.17: AHR expression and CYP1A1 induction in HSPCs

A) *AHR* mRNA levels in human peripheral blood naive B cells and $CD34^+$ HSPCs after 7 days of culture were determined by real-time quantitative PCR and normalized to 18s ribosomal RNA. Data are presented as mean ± SE of triplicate measurements. *p <0.05, compared to naive B cells by t-test after logarithmic transformation.

Figure 3.17 (cont'd)

B,C) Human $CD34^+$ HSPCs were treated with vehicle (VH, 0.02% DMSO) or TCDD (0.01, 0.1, 1 or 10 nM) on day 0 and cultured for up to 28 days. Cells were harvested weekly. The mRNA levels of *AHR* (B) and *CYP1A1* (C) were determined by real-time quantitative PCR and were normalized to 18s ribosomal RNA. Data are presented as mean ± SE for triplicate measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the VH using a two way ANOVA with Bonferroni posttest after logarithmic transformation. Data are representative of two independent experiments with similar results.

signaling pathway to be functional in HSPCs. Next, a structure-activity-relationship experiment was conducted to confirm AHR involvement. Four polychlorinated dibenzo-p-dioxin (PCDD) congeners were used: 1-chlorodibenzo-p-dioxin (MCDD), 2,3,7-trichlorodibenzo-p-dioxin (TriCDD), 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin (HxCDD) and TCDD. The rank order for AHR binding affinity is: MCDD < TriCDD < HxCDD < TCDD (Poland et al. ; Sulentic et al.). The treatment of PCDD congeners (1 nM) on HSPCs resulted in suppression of early-B and pro-B cell generation (Figure 3.18). The magnitude of suppression was correlated with AHR binding affinity for respective congeners, suggesting AHR activation as a key event in TCDD-elicited suppression of B lymphopoiesis. To further confirm AHR involvement, a well-characterized AHR antagonist, CH223191, was utilized. HSPCs were treated with vehicle (0.2% DMSO), CH223191 (CH) (0.3, 1, 3, 10 μM), TCDD (1 nM) or a combination of CH and TCDD (Figure 3.19). Compared to vehicle control (no CH or TCDD), 1 nM TCDD treatment significantly decreased the percentage of early-B cells that emerged from HSPCs, which was attenuated in a concentration-dependent manner by addition of the AHR antagonist (Figure 3.19). Therefore, the results from both the structure-activity-relationship experiments and those utilizing the AHR antagonist demonstrate AHR-mediated involvement in suppression of B lymphopoiesis by TCDD.

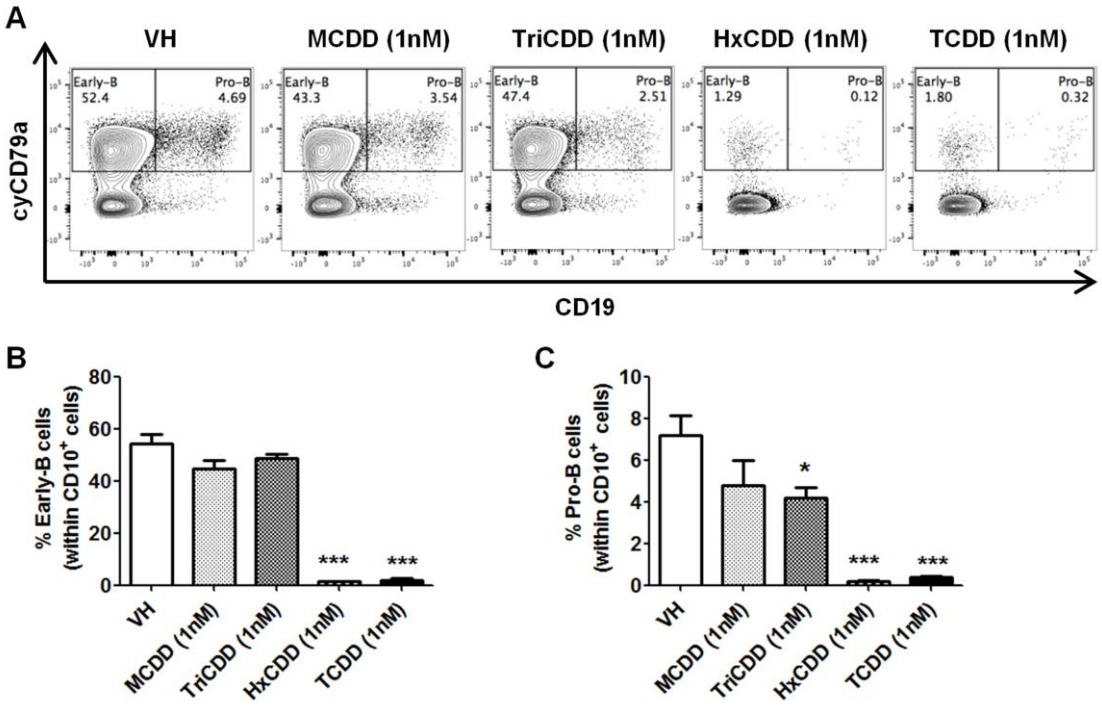


Figure 3.18: Structure-activity-relationship for PCDD-mediated impairment of B lymphopoiesis

Human $CD34^+$ HSPCs were treated with vehicle (VH, 0.02% DMSO) or 1nM of chlorinated dioxin congeners (MCDD, TriCDD, HxCDD or TCDD) on day 0. The rank order for AHR binding affinity is: MCDD < TriCDD < HxCDD < TCDD. Cells were harvested on day 21 and analyzed by flow cytometry. Cells were first gated on $CD10^+$. **A)** The gating scheme of early-B cells ($CD10^+$ $CD79^+$ $CD19^-$) and pro-B cells ($CD10^+$ $CD79^+$ $CD19^+$). **B)** Quantification of early-B and pro-B cell percentages in (A). Data are mean ± SE of triplicate measurements. * $p < 0.05$, *** $p < 0.001$, compared to VH using a one way ANOVA with Dunnett's multiple comparison test. Data are representative of three independent experiments with similar results.

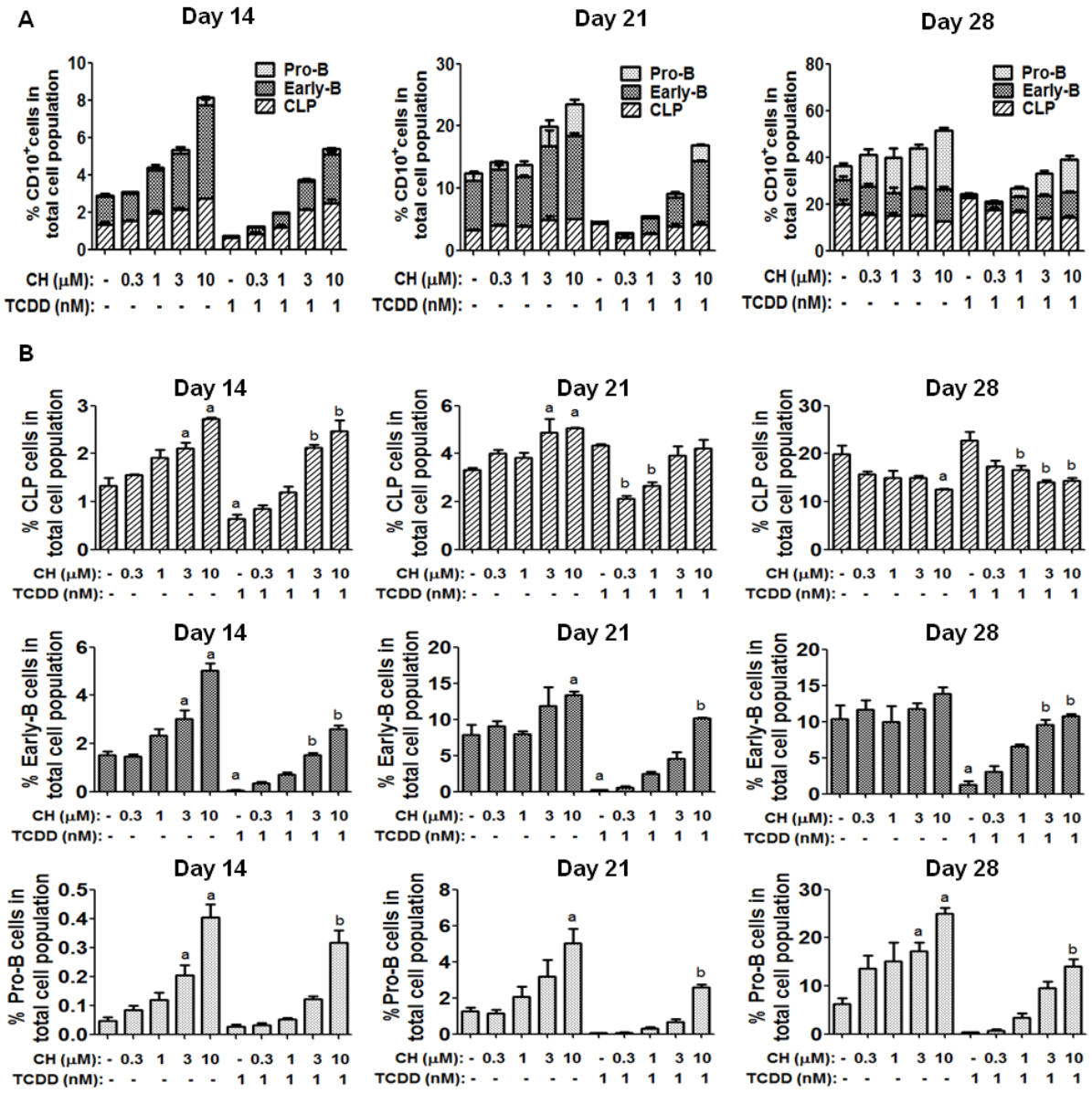


Figure 3.19: AHR antagonist reversed the TCDD-mediated suppression of early-B and pro-B cell generation

Cord blood-derived human $CD34^+$ HSPCs were treated on day 0 by vehicle (0.02% DMSO), AHR antagonist CH223191 (CH) (0.3, 1, 3 and 10 mM), TCDD (1 nM) or combination of CH and TCDD. **A)** The percentage of $CD10^+$ cell population, which

Figure 3.19 (cont'd)

includes common lymphoid progenitors (CLP), early-B and pro-B cells. **B)** The percentage of CLPs ($CD10^+$ $CD79\alpha^-$ $CD19^-$), early-B cells ($CD10^+$ $CD79\alpha^+$ $CD19^-$) and pro-B cells ($CD10^+$ $CD79\alpha^+$ $CD19^+$). Data are mean ± SE of triplicate measurements. a = significant difference compared to vehicle control group, b = significant difference compared to the TCDD (1 nM) treated group, by one way ANOVA with Bonferroni's multiple comparison posttest. Data are representative of two independent experiments with similar results.

3.2 Investigation of the molecular mechanism underlying TCDD-mediated impairment of human B lymphopoiesis

EBF1 and PAX5 expression in HSPCs were reduced by AHR activation

As shown in Figure 3.15, AHR activation by TCDD suppressed the generation of early-B and pro-B cells but not their preceding progenitor CLPs, suggesting that AHR activation impedes B cell lineage specification and commitment. Two important transcription factors governing B cell lineage specification and commitment are EBF1 and PAX5. EBF1 is a critical regulator driving the B cell specification program (Seet et al. 2004; Zhang et al. 2003). EBF1 activates the expression of PAX5. Together, EBF1 and PAX5 direct cell fate choice during lymphopoiesis leading to B cell lineage commitment (Nechanitzky et al. 2013a; Nutt et al. 1999; Schebesta et al. 2007). Gene expression analysis revealed that *EBF1* mRNA levels were increased over time as cells progressed toward B cell specification (Figure 3.20 A, VH groups); however, this up-regulation of *EBF1* was suppressed in the presence of TCDD (Figure 3.20 A). By employing PrimeFlow, we quantified the *EBF1* mRNA levels in individual cells by flow cytometry. First, the intracellular protein level of CD79α and mRNA levels of EBF1 were measured simultaneously (Figure 3.20 B). The co-expression of EBF1 mRNA and cytoplasmic CD79α protein suggests that the expression of *EBF1* is B cell lineage specific. Then, an extended concentration response of TCDD was performed. TCDD treatment decreased the percentage of *EBF1*-expressing cells (Figure 3.20 C,D); however, the average expression level of *EBF1* mRNA in the *EBF1*-expressing cells, as represented by MFI, was not decreased (Figure 3.20 E), demonstrating

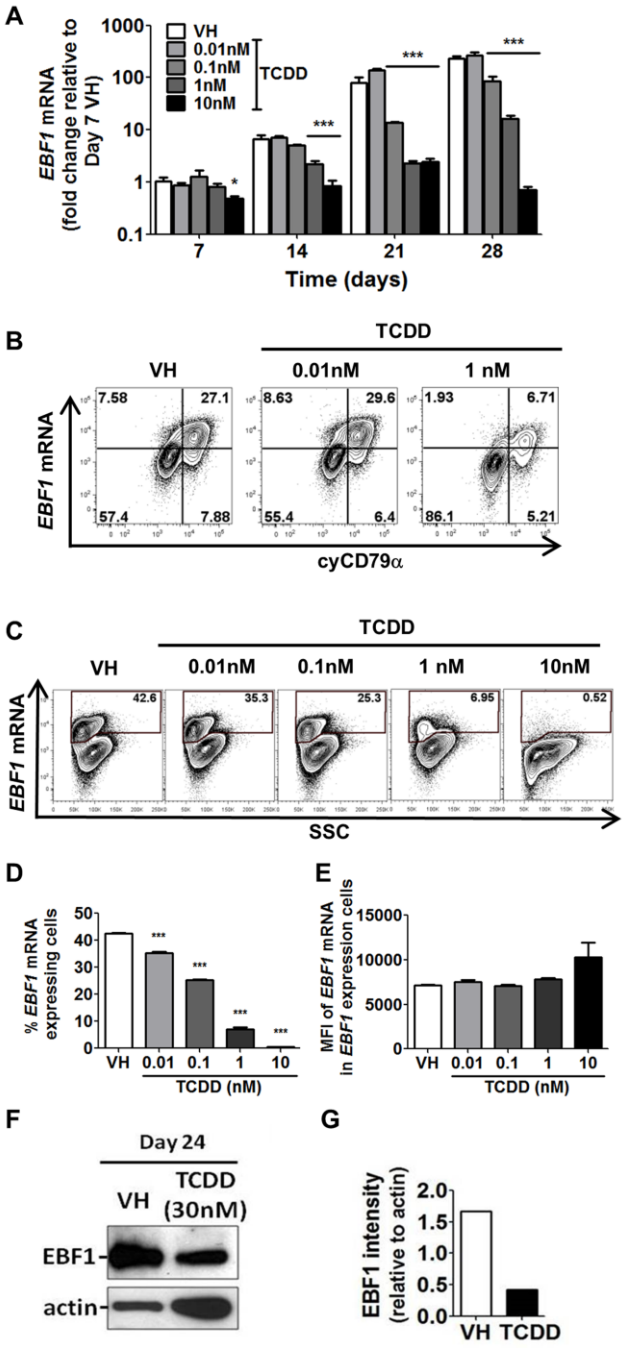


Figure 3.20: TCDD decreased *EBF1* expression

Human CD34^{+} HSPCs were treated with vehicle (VH, 0.02% DMSO) or TCDD (0.01, 0.1, 1 or 10 nM) on day 0. Cells were cultured for up to 28 days and harvested at the

Figure 3.20 (cont'd)

specified time points. **A)** The mRNA levels of *EBF1* were determined by real-time quantitative PCR and were normalized to 18s ribosomal RNA. Data are presented as mean ± SE of triplicate measurements. * p <0.05, ***p <0.001, compared to the VH using a two way ANOVA with Bonferroni posttest after logarithmic transformation. **B)** The PrimeFlow staining of *EBF1* mRNA and intracellular CD79 on day 24. **C)** The PrimeFlow staining of *EBF1* mRNA on day 28 post treatment. **D)** The percentage of the *EBF1* mRNA expressing cells as gated in C. **E)** The mean fluorescence intensity (MFI) of *EBF1* mRNA in *EBF1* mRNA expressing cells as gated in C. Data are presented as mean ± SE of triplicate measurements. ***p <0.001, compared to VH by one way ANOVA using a Dunnett's multiple comparison test. **F)** EBF1 immunoblot on day 24 post treatment by VH or TCDD (30nM). **G)** Quantification of EBF1 protein levels relative to actin in (F). Data are representative of three independent experiments with similar results.

an all-or-none (binary) decrease in *EBF1* mRNA levels by TCDD treatment. The decrease in EBF1 at the protein level was also demonstrated by Western blotting (Figure 3.20 F,G). Given that EBF1 regulates PAX5, the effect of TCDD on PAX5 expression was also investigated. Consistent with EBF1, PAX5 mRNA increased over time, but was suppressed by TCDD treatment (Figure 3.21).

To explore the involvement of the AHR in TCDD-elicited suppression of EBF1 and PAX5, an AHR antagonist was used. Specifically, HSPCs were treated with vehicle (0.2% DMSO), CH223191 (CH) (0.3, 1, 3, 10 μM), TCDD (1 nM) or in combination with CH and TCDD. Compared to vehicle control (no CH or TCDD), 1 nM TCDD treatment significantly decreased EBF1 mRNA; however, the decrease was reversed in a concentration-dependent manner with the addition of increasing amounts of the AHR antagonist (Figure 3.22 A). Likewise, similar effects were observed for PAX5 mRNA (Figure 3.22 B). These findings suggest that AHR activation by TCDD leads to suppression of *EBF1* and *PAX5*.

During B lymphopoiesis, the initiation of the EBF1-PAX5 regulatory axis involves multiple transcription factors that activate EBF1 expression, including ETS1, TCF3 and SPI1 (Roessler et al. 2007). To explore the possibility that the suppression of EBF1 and PAX5 by AHR activation results from the alterations of upstream regulators, we examined the expression of ETS1, TCF3 and SPI1 in the presence of TCDD (Figure 3.23). The time course and concentration response study showed a modest suppression of ETS1 and TCF3 and enhancement of SPI1 by TCDD

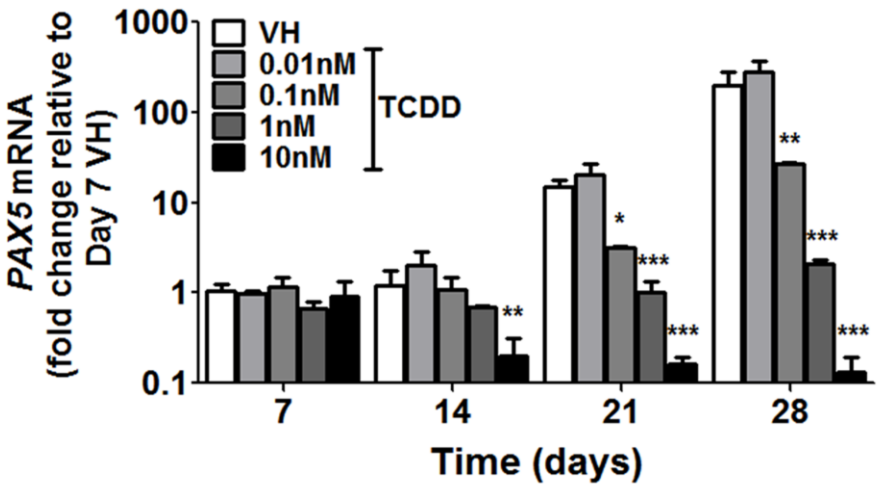


Figure 3.21: TCDD decreased *PAX5* expression

Human CD34^{+} HSPCs were treated with vehicle (VH) or TCDD (0.01, 0.1, 1 or 10 nM) on day 0. Cells were cultured for up to 28 days and harvested at the specified time points. The mRNA levels of *PAX5* were assayed by real-time quantitative PCR and were normalized to 18s ribosomal RNA. Data are presented as mean ± SE of triplicate measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the VH using a two way ANOVA with Bonferroni posttest after logarithmic transformation. Data are representative of three independent experiments with similar results.

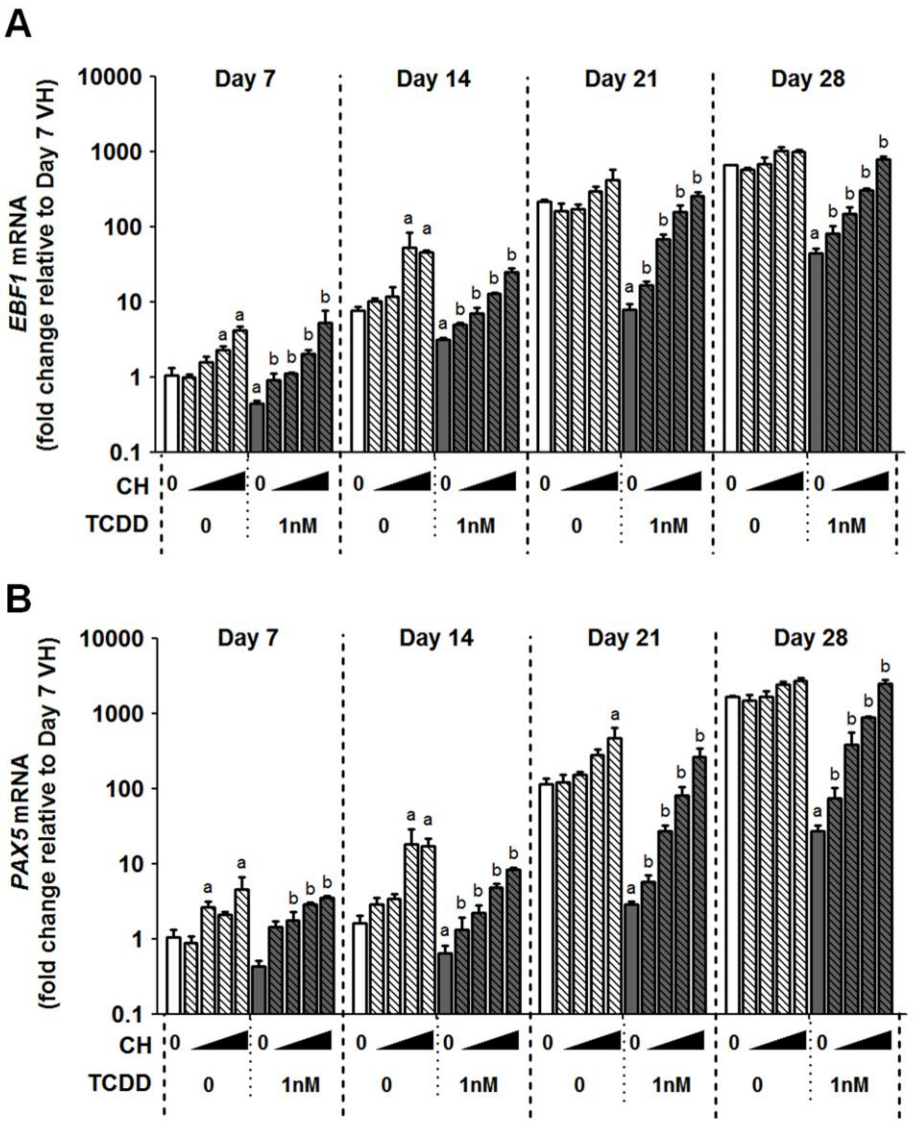


Figure 3.22: AHR antagonist reversed the TCDD-elicited suppression of *EBF1* and *PAX5* expression

Human CD34^{+} HSPCs were treated with vehicle, AHR antagonist CH223191 (CH) (0.3, 1, 3 or 10 M), TCDD (1 nM) or a combination of CH and TCDD on day 0. Cells were cultured for up to 28 days and harvested at the specified time points. The

Figure 3.22 (cont'd)

mRNA levels of *EBF1* **(A)** and *PAX5* **(B)** were determined by real-time quantitative PCR and were normalized to 18s ribosomal RNA. Data are presented as the mean ± SE of triplicate measurements. a = significantly different compared to vehicle control, b = significantly different compared to the TCDD (1 nM) treated group, by two way ANOVA with Bonferroni posttest after logarithmic transformation. Data are representative of two independent experiments with similar results.

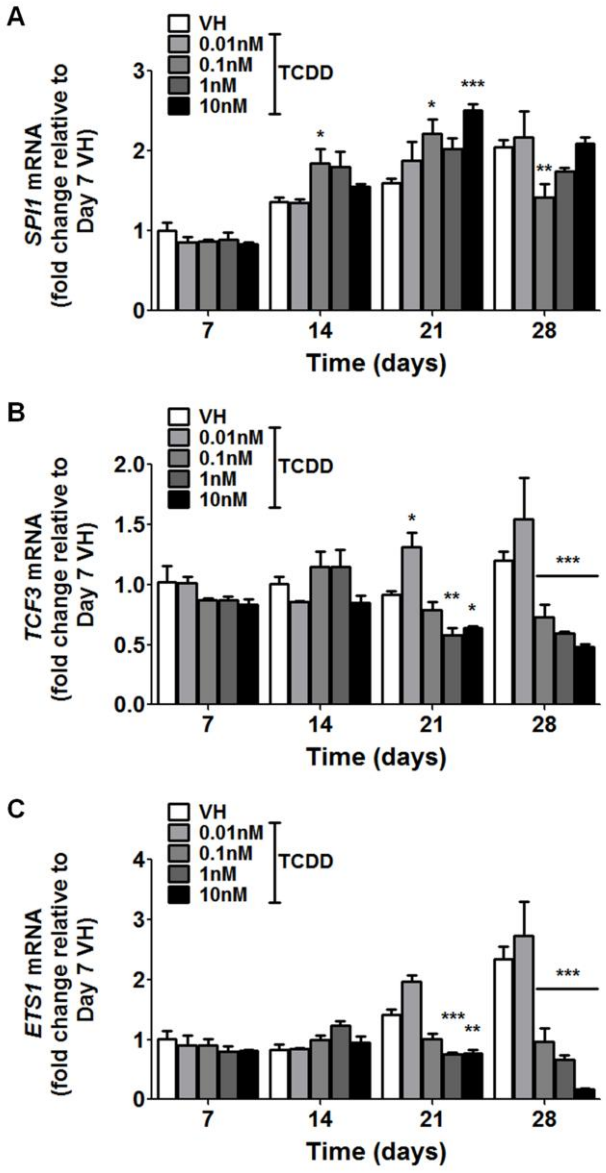


Figure 3.23: The effects of TCDD on the expression of transcription factors that regulate *EBF1*

Human CD34^{+} HSPCs were treated with vehicle (VH) or TCDD (0.01, 0.1, 1 or 10 nM) on day 0. Cells were cultured for up to 28 days and harvested weekly. The mRNA levels of *SPI1* **(A)**, *TCF3* **(B)** and *ETS1* **(C)** were determined by real-time

Figure 3.23 (cont'd)

quantitative PCR and were normalized to 18s ribosomal RNA. Data are presented as the mean ± SE of triplicate measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the VH using a two way ANOVA with Bonferroni posttest after logarithmic transformation. Data are representative of three independent experiments with similar results.

treatment, suggesting that effects on upstream regulators may also contribute, in part, to the suppression of EBF1 expression.

Ligand-activated AHR binds to DREs in EBF1 promoter

EBF1 has been identified as a putative target for the AHR in a genome-wide ChIP-on-chip and gene expression microarray study in a murine cell line (De Abrew et al. 2010). In addition, we have identified 13 putative AHR binding sites (DRE) in the human *EBF1* promoter region (Figure 3.24 A). The matrix similarity scores (MSS) of these DREs were calculated based on the position weight matrix (Sun et al. 2004). All 13 DREs in the *EBF1* promoter have a MSS higher than the threshold score based on experimentally confirmed DREs (Sun et al. 2004). To further explore whether AHR transcriptionally regulates EBF1 expression by direct binding within the *EBF1* promoter, we selected 3 DREs (DRE 4, 6 and 7) with the highest MSS to assess AHR binding using electrophoretic mobility shift assays (EMSA) (Figure 3.24 B) and by EMSA-Western analysis (Figure 3.24 C). Briefly, nuclear protein was isolated from cells treated with vehicle (0.02% DMSO) or TCDD (30 nM), incubated with ^{32}P-labeled (Figure 3.24 B) or unlabeled (Figure 3.24 C) DRE oligomers. The nuclear protein-DNA complex was resolved on a non-denaturing PAGE gel. TCDD-induced protein binding to DRE 4 and 7 was observed (Figure 3.24 B). In addition, the protein-DNA complex for DRE4 and 7 located at the same position as consensus DRE, indicating the binding of AHR to DRE4 and 7. Protein binding to DRE6, as

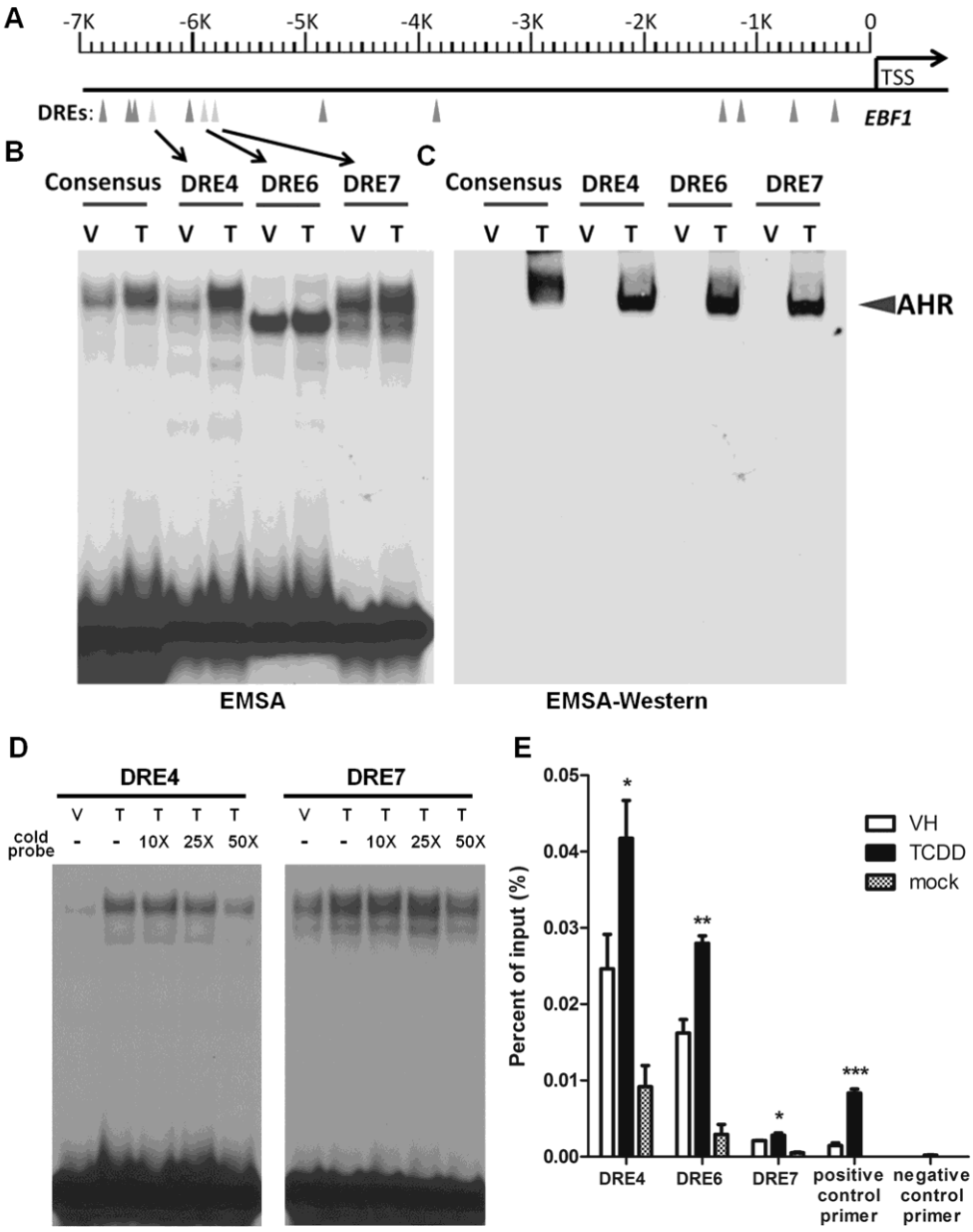


Figure 3.24 DNA binding analysis of the ligand-activated AHR to putative dioxin response elements (DRE) within the *EBF1* promoter

A) Schematic of predicted DRE sites in human *EBF1* gene promoter region. **B,C,D)** Nuclear protein was isolated from HEPG2 cells treated with vehicle (V, 0.01% DMSO)

Figure 3.24 (cont'd)

or TCDD (T, 30nM). **B)** Nuclear protein was incubated with ^{32}P-labeled DRE oligomers. Protein:DNA complexes were resolved on a 4% nondenaturing PAGE gel, dried and visualized by autoradiograph. **C)** Nuclear protein was incubated with unlabeled DRE oligomers. Protein:DNA complexes were resolved on a 4% nondenaturing PAGE gel, transferred to nitrocellulose, and probed with anti-AHR antibody. Arrow indicates specific binding of AHR to DRE oligomers. **D)** Nuclear protein was incubated with ^{32}P-labeled DRE oligomers (DRE4 or DRE7), with addition of unlabeled consensus DRE oligomer at 10, 25 or 50 fold excess relative to the labeled DREs. Protein:DNA complexes were resolved on a 4% nondenaturing PAGE gel, dried and visualized by autoradiograph. Results are representative of three independent experiments. **E)** Human CD34$^+$ HSPCs were cultured as described in method for 28 days. On day 28, cells were treated with vehicle (VH, 0.02% DMSO) or TCDD (1 nM). Three hours post treatment, cells were harvested for chromatin immunoprecipitation (ChIP) analysis. ChIP reactions were performed using either anti-AHR antibody or negative control antibody (mock). DNA primers specific to DRE4, 6 and 7 in EBF1 promoter were used to quantify the enrichment of AHR-bound chromatin using q-PCR. A positive control primer set that amplifies a AHR binding region in CYP1A1 promoter as well as a negative control primer set that amplifies a region in a gene desert on human chromosome 12 were also included. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to VH by one-tailed t-test.

evaluated by EMSA, appeared to be independent of TCDD treatment and located at a different position as compared to DRE4 and 7, which might be due to the binding of nuclear proteins besides AHR to the DNA oligomers. To confirm the presence of AHR, the protein-DNA complex was transferred to a nitrocellulose membrane and probed with anti-AHR antibody (Figure 3.24 C). Consistent with the EMSA results, AHR was detected in the TCDD-induced protein-DNA complex for all three DREs (Figure 3.24 C). In addition, competition EMSA were performed to demonstrate binding specificity (Figure 3.24 D). Nuclear extracts were incubated with ^{32}P-labeled DRE4 or DRE7 with addition of unlabeled (cold) consensus DRE oligonucleotide at 10, 25 or 50 fold excess, relative to the labeled DREs. The DNA-protein binding intensity was induced by TCDD and subsequently decreased by addition of unlabeled consensus DRE oligonucleotide, indicating specific binding of nuclear proteins to DRE4 and 7 (Figure 3.24 D). Moreover, to demonstrate that ligand-activated AHR binding to DREs in EBF1 promoter can occur in HSPCs, chromatin immunoprecipitation (ChIP) analysis was conducted. CD34$^+$ HSPCs were cultured for 28 days and treated with vehicle (VH, 0.02% DMSO) or TCDD (1 nM). ChIP reactions were performed using either anti-AHR antibody or negative control antibody (mock). DNA primers specific to DREs in EBF1 promoter were used to quantify the enrichment of AHR-bound chromatin using q-PCR. TCDD-induced enrichment at DRE4, 6 and 7 in EBF1 promoter indicated AHR binding to these loci in HSPCs (Figure 3.24 E). The consistent findings via EMSA and ChIP demonstrated that the TCDD-activated AHR is able to bind to DREs in the human *EBF1* promoter.

Together with the aforementioned findings of the AHR-mediated decrease in *EBF1* mRNA levels, our study suggests direct transcriptional regulation of EBF1 by AHR activation.

AHR and PAX5 bind to common sites in EBF1 promoter

AHR is a ligand-activated transcription factor that is capable of binding to cognate DNA sequences (i.e., DREs) in the regulatory region of target genes. AHR binding to DRE results in DNA bending, chromatin remodeling, recruitment of a variety of coactivators and corepressors, and alters gene transcription (Beischlag et al. 2008; Hankinson 2005; Nguyen et al. 1999). In addition, AHR binding to DNA might directly interfere with the ability of other transcription factors to bind to their cognate DNA sequences, leading to the disruption of gene transcription (Masten and Shiverick 1995). In line with this notion, we have identified that a prevalent DNA sequence within the consensus PAX5 binding site resembles the core AHR binding site (DRE) (Figure 3.25), suggesting AHR can compete with PAX5 for DNA binding. Indeed, there are 13 putative PAX5 binding sites predicted in the human *EBF1* promoter, among which 5 PAX5 binding sites overlap with DREs (Figure 3.26). As proof of principle, the binding of PAX5 to two of the 5 overlapping sites were confirmed by EMSA and EMSA-Western (Figure 3.27), which occurs at loci -6973 (overlap with DRE1) and -5971 (overlap with DRE7) in the *EBF1* promoter (Figure 3.27 C and D).

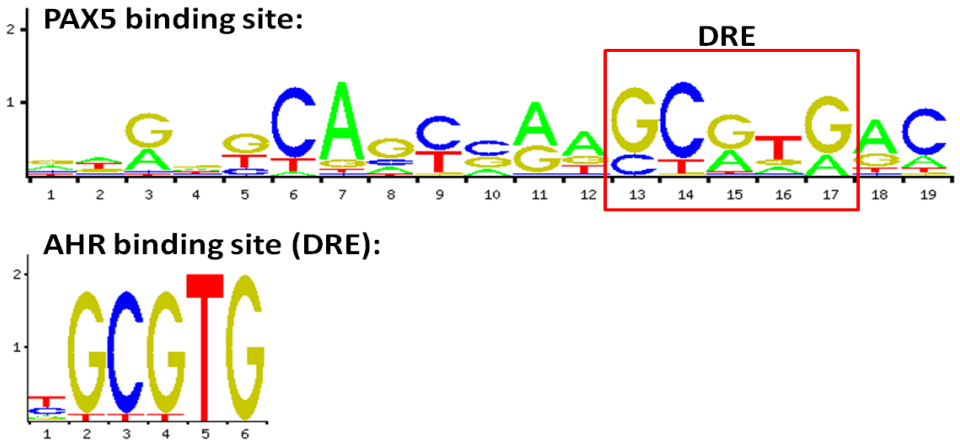
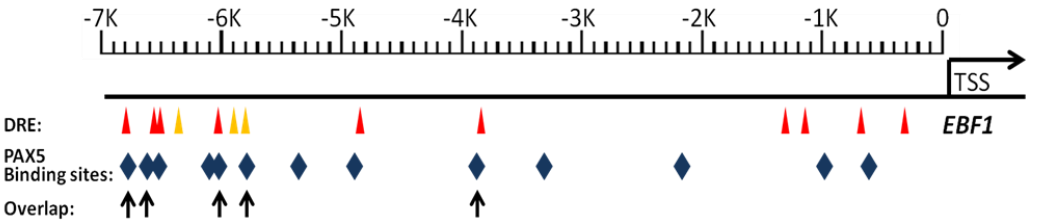


Figure 3.25: Consensus sequence for PAX5 and AHR binding sites

This figure illustrated the sequence logo for PAX5 and AHR binding sites from JASPAR database. The relative sizes of the letters (A, T, G, C) at each position indicate their frequency at each position. The most frequently occurring nucleotides within the boxed section in the PAX5 binding site form an AHR binding site (DRE).



AHR binding sites (DREs)		
ID	Relative Position	MS Score
1	-6794	0.81
2	-6559	0.82
3	-6528	0.79
4	-6371	0.87
5	-6018	0.79
6	-5918	0.85
7	-5789	0.89
8	-4860	0.77
9	-3873	0.76
10	-1312	0.78
11	-1152	0.79
12	-694	0.77
13	-327	0.80

PAX5 binding sites		
Relative position	Relative score	Predicted site sequence
-6793	0.83	AAGGGCAGGCGTGCAGACC
-6554	0.80	TGGTGCACACGGGCGTGAG
-6545	0.82	GTGTGCACCAATGCCTTAC
-6035	0.80	AAGGGCCACCGGGTGGGAT
-6013	0.81	GGATGCCCTAGGGCGTGAT
-5791	0.82	GAGCTCACGCAAGCTTGCC
-5441	0.84	CCGTCCACCCAGCTGTGGC
-4871	0.80	GGGAGTAGCAGGCCAGGGG
-3868	0.82	AAGGGAAGGAAGGCGTGAG
-3352	0.81	CAGGCCAGCAGAGCAGTAG
-2164	0.86	CTGGCCTGCAGTGCATGGC
-982	0.84	CAGAACGGCGGAGCAGGAC
-652	0.81	CACCGCAGCCAATCGCGGC

Figure 3.26: Bioinformatics predicted overlap between AHR and PAX5 binding sites within human *EBF1* promoter

The binding sites for AHR and PAX5 within 7kb upstream of human EBF1 transcription start site (TSS) were identified using JASPAR database. DRE 1, 2, 5, 7 and 9 overlap (indicated by arrows) with highly scored PAX5 binding sites. These PAX5 binding sites contain DRE consensus sequence (red). The binding of AHR to DRE 4, 6 and 7 was experimentally confirmed (orange) (Figure 3.24).

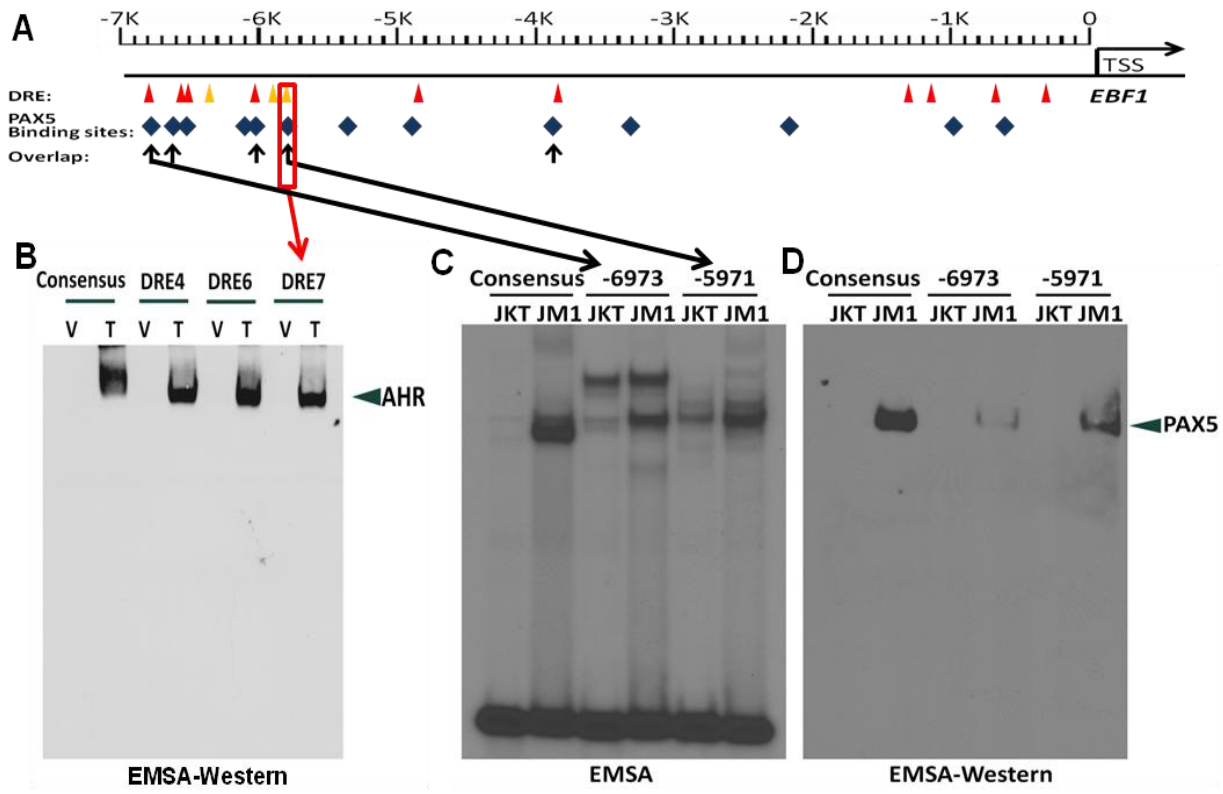


Figure 3.27: EMSA analysis of PAX5 binding to DNA loci that overlap with DRE

A) Schematic of predicted AHR binding sites (DREs) and PAX5 binding sites in human *EBF1* promoter region. **B)** The binding of AHR to DRE 4, 6 and 7 was shown in Figure 3.24 C. **C,D)** Nuclear protein was isolated from JM1 cells (expressing PAX5) and Jurkat cells (do not express PAX5, serve as a negative control). **C)** Nuclear protein was incubated with ^{32}P-labeled PAX5 binding site oligomers. Protein:DNA complexes were resolved on a 4% nondenaturing PAGE gel, dried and visualized by autoradiograph. **D)** Nuclear protein was incubated with unlabeled PAX5

Figure 3.27 (cont'd)

binding site oligomers. Protein:DNA complexes were resolved on a 4% nondenaturing PAGE gel, transferred to nitrocellulose, and probed with anti-PAX5 antibody. Arrow indicates specific binding of AHR to DRE oligomers.

The ability of both AHR and PAX5 to bind to the same locus (-5971 or DRE7) (Figure 3.27 B, C and D) suggests a competition between those two factors for DNA binding, thereby dysregulating *EBF1* transcription.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1 Discussion

In this study, three different culture systems were employed to facilitated *in vitro* human B lymphopoiesis starting from cord blood-derived $CD34^+$ cells. Using these *in vitro* models, we show for the first time that activation of AHR using a high affinity ligand and environmental contaminant, TCDD, impairs human B cell development from HSPCs. Consistent effects of TCDD were observed across all three *in vitro* human B lymphopoiesis models, including: (i) the number of HSPC-derived cells was decreased, which was not due to apoptotic or necrotic cell death; (ii) TCDD decreased the expression of CD34, an phenotypic cell marker of HSPCs; and (iii) the generation of lineage committed B cells from human HSPCs was diminished by TCDD. The involvement of the AHR in the TCDD-elicited effects was demonstrated by structure-activity relationship experiments and in experiments using an AHR antagonist. Subsequent gene expression analysis revealed a significant decrease in the mRNA levels of EBF1 and PAX5, two critical transcription factors directing B cell lineage commitment. Concordantly, binding of the ligand-activated AHR to the putative dioxin response elements in the *EBF1* promoter was demonstrated by electrophoretic mobility shift assays and chromatin immunoprecipitation analysis, suggesting transcriptional regulation of EBF1 by AHR. Taken together, this study for the first time, demonstrates a role for the AHR in regulating human B cell development, and suggests that transcriptional alterations in EBF1 by the AHR are involved in the underlying mechanism.

To mimic the microenvironment of human B lymphopoiesis *in vitro*, human feeder cells (primary human marrow stromal cells) and/or human recombinant cytokines were used to facilitate HSPC to B cell development. The present *in vitro* findings showing that TCDD impaired human B lymphopoiesis are consistent with *in vivo* animal studies (Singh et al. 2009; Thurmond and Gasiewicz 2000), suggesting mechanistic parallels across species. In addition, our studies suggest that TCDD directly affects the HSPCs rather than mediating its effects indirectly through actions on stromal cells. It is noteworthy that the magnitude of TCDD-mediated impairment (i.e. decrease of total number of cells (Figure 3.9), CD34 expression (Figure 3.10) and $CD19^+$ cell number (Figure 3.11)) was more modestly diminished in the stromal cell free culture compared to co-culture, which could be attributable to several factors. One possibility is that TCDD also mediates some effects on the HMSCs, which further impairs B cell development. Although we cannot exclude this possibility, it is unlikely since the HMSCs undergo significant irradiation prior to co-culture with HSPCs and are functionally compromised as evidenced by a complete absence of proliferative capability (Figure 4.1). A second possibility is that under stromal cell-free culture conditions, HSPCs are subjected to supraoptimal concentrations of growth factors present in HMSC conditioned media, which attenuate some of the inhibitory effects produced on HSPCs by TCDD. The latter explanation is supported by our observation that the same number of HSPCs generated more HSPCs in stromal cell-free cultures than in co-cultures (Figure 3.2 and 3.9, vehicle groups). It is worth mentioning that both co-culture and stromal cell free culture contain stromal cell component (i.e., stromal cells and/or growth factors secreted by stromal cells).

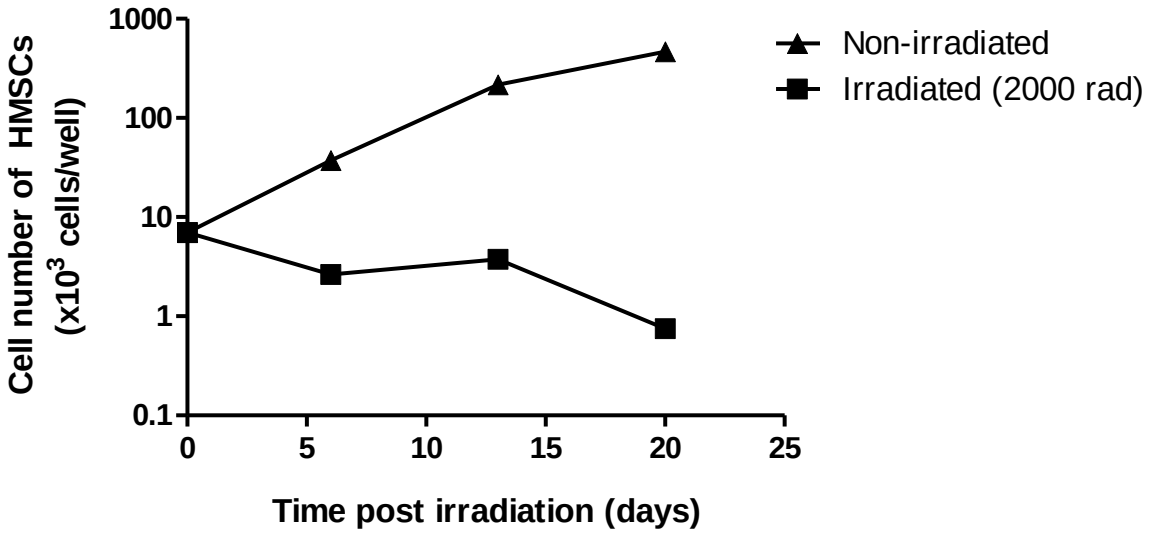


Figure 4.1: Sub-lethal irradiation prevented the proliferation of human marrow stromal cells

Human marrow stromal cells (HMSC) ($7x10^3$ cells/well) were sub-lethally irradiated (2000 rad) and cultured in marrow stromal cell growth medium for up to 20 days. The non-irradiated controls and irradiated cells were harvested at indicated time points and enumerated using a hemocytometer.

Since the influence of stromal cells and their secretory factors are poorly defined, we also employed a feeder-free culture system to explore the direct effects of TCDD on human B lymphopoiesis. Despite the consistency in TCDD-mediated impairment of human B lymphopoiesis across all culture systems, the magnitude of suppression was more profound in the feeder-free culture. Given the apparent differences in presence/absence of stromal cell component in culture systems, the discrepancy in the magnitude of TCDD-mediated effects is not surprising and likely attributable to several factors. Due to the lipophilicity of TCDD, one likely factor is that in addition to binding to $CD34^+$ cells, a portion of TCDD is also bound to stromal cells hence reducing the overall TCDD exposure of HSPCs. A second possibility is that stromal cells or soluble cytokines produced by stromal cells promote B lymphopoiesis and therefore attenuate the suppressive effect of TCDD.

One of the most consistent and profound effects by TCDD in the present study was on the decrease of CD34 expression by HSPCs. CD34 has been identified as a hematopoietic stem cell marker which has been used clinically in HSPC transplantation for more than 20 years. As CD34 expression is restricted on hematopoietic stem and progenitor cells and is absent from terminally differentiated hematopoietic lineages, one possible interpretation is that TCDD drives HSPC more rapidly toward a differentiated state. This abnormal acceleration of development might disrupt the sequential progression required for B cell development and hence result in reduced production of lineage committed B cells. On the other hand, studies have shown that the expression of CD34 associates with the activation status of

HSPCs (Roberts and Metcalf 1995; Tajima et al. 2000). Specifically, quiescent HSPCs express low levels of CD34, whereas activated and proliferating HSPCs express CD34 at a relatively high level. Therefore, decreased CD34 expression could reflect the transition of activated HSPCs into a quiescent stage by TCDD, hence, accounting for suppressed HSPC proliferation. Concordantly, a previous study has shown that *Ahr*-null mice had more activated HSCs (in the G_1 and S phase of cell cycle) compared to WT mice (Singh et al. 2011), supporting the possibility that TCDD activates AHR in HSPCs which leads to reduced cell activation and proliferation. This possibility is also supported by our observation that TCDD reduced the total number of HSPCs via a mechanism not involving cell death.

Activation of the AHR using a high affinity agonist suppressed the generation of early-B and pro-B cells but not their preceding progenitors, CLPs (Figure 3.15), suggesting an arrest during B cell lineage specification and commitment. This interruption of development was visualized using the SPADE algorithm (Figure 3.16), showing that cells failed to progress along the development trajectory to become early-B and pro-B cells. Given the crucial role of EBF1 in the regulatory circuit underlying B cell lineage specification and commitment, we hypothesize that AHR-mediated suppression of EBF1 is involved in the mechanism by which AHR activation suppresses human B lymphopoiesis. The decrease in EBF1 mRNA levels by TCDD during B lymphopoiesis was demonstrated by both qPCR and PrimeFlow analyses. In addition, an AHR antagonist attenuated the TCDD-elicited suppression of EBF1 mRNA levels, suggesting AHR involvement. Interestingly, TCDD decreased EBF1

mRNA levels in an all-or-none (binary) rather than graded mode: i.e., TCDD reduces the proportion of EBF1 mRNA expressing cells in a concentration-dependent manner (Figure 3.20 C, D) rather than decreasing the EBF1 mRNA levels in cells that express EBF1 mRNA (Figure 3.20 E). This binary switch-like behavior of EBF1 expression is not unexpected, as the positive feedback loop between EBF1 and PAX5 forms a bistable memory module, which is a characteristic regulatory motif that governs cell fate decisions during cellular development (Xiong and Ferrell Jr 2003; Zhang et al. 2013).

Within the B lymphopoiesis regulatory circuit, the expression of PAX5 is activated by EBF1 (Zandi et al. 2008), which is consistent with our observation that the up-regulation of EBF1 mRNA precedes that of PAX5 during B cell development (Figure 3.20 A, Figure 3.21 and Figure 3.22, VH groups). In our study, AHR activation suppressed both EBF1 and PAX5. Given that EBF1 and PAX5 act in a hierarchical manner, we speculate the suppression of PAX5 is a consequence of AHR-mediated suppression of EBF1. To explore whether the down-regulation of EBF1 results from AHR-mediated alterations of upstream transcription factors, we examined the expression of ETS1, TCF3 and SPI1, which are known to initiate EBF1 expression during B lymphopoiesis (Roessler et al. 2007). Unlike the responses of EBF1 to AHR activation (Figure 3.20 A), all three transcription factors exhibited modest changes that occurred only in the late stage of the 28 day culture period (Figure 3.23). Hence, we hypothesize that AHR can exert direct transcriptional regulation of EBF1.

It is established that AHR is a ligand-activated transcription factor, which regulates gene expression by binding to DREs in regulatory regions of target genes. Here we demonstrate the binding of the ligand-activated AHR to three putative DREs within the *EBF1* promoter using EMSA and EMSA-Western analysis. EMSA has high sensitivity but falls short in specificity. Bioinformatic predictions suggest that there are multiple putative binding sites for transcription factors besides the AHR, even within the short 17-bp oligonucleotide probe we used in this assay. Indeed, the failure to detect TCDD-induced protein binding to DRE6 is likely the result of other nuclear proteins binding to DRE6 in addition to AHR, which masked visualization of AHR-DRE binding by EMSA. To increase the specificity of detecting AHR-DRE binding, we conducted EMSA-Western analysis. The advantage of EMSA-Western is the ability to identify specific DNA binding proteins using antibodies, which in this case were used to identify the AHR. Due to the specificity of the EMSA-Western assay, AHR-DRE6 binding was detected, which was otherwise masked in EMSA analysis by the binding of other proteins to the DNA probe. Consistent with EMSA and EMSA-Western assays, the ChIP analysis in HSPCs also demonstrated that the ligand-activated AHR is capable of binding to DREs in *EBF1* promoter, suggesting that AHR transcriptionally impairs *EBF1* expression.

AHR exhibits diverse mechanisms of action in regulating gene expression. In addition to the classic pathway of dimerizing with ARNT to influence gene expression, AHR has also been reported to interact with a variety of coactivators and corepressors (Nguyen et al. 1999). The potential transcriptional impairment of EBF1

by AHR might result from interactions with corepressors. Alternatively, AHR binding to promoter regions of EBF1 might directly interfere with the ability of other transcription factors to bind to their cognate DNA sequences, leading to the disruption of *EBF1* transcription (Masten and Shiverick 1995). Indeed, we have identified 5 putative common binding sites for AHR and PAX5 in the *EBF1* promoter (Figure 3.26). In addition, we demonstrated that both AHR and PAX5 can bind to the same locus in the *EBF1* promoter using EMSA and EMSA-Western(Figure 3.27 B, C and D). These findings suggest a competition between AHR and PAX5 for DNA binding, which might contribute to the dysregulation of *EBF1* transcription by TCDD.

To demonstrate that AHR mediated the TCDD-elicited suppression of B cell lineage commitment, we used an AHR antagonist. Interestingly, HSPCs treated with AHR antagonist alone showed an accelerated progression toward B cell lineage (Figure 3.19), suggesting a physiological role of AHR in regulating B lymphopoiesis through endogenous AHR activation. Increasing evidence suggests a physiological/developmental role for the AHR in variety of biological responses, including hematopoiesis (Fernandez-Salguero et al. 1995; Schmidt et al. 1996). Enhanced proliferation of HSCs in $Ahr^{-/-}$ mice (Singh et al. 2011) as well as increased expansion of human HSPCs in the presence of AHR antagonist (Boitano et al. 2010) suggests the existence of endogenous AHR ligands that influence hematopoiesis. The search for endogenous AHR ligands has identified a growing list of compounds, including indigoids, equilenin, tryptophan metabolites, arachidonic acid metabolites and heme metabolites (Nguyen and Bradfield 2008). It is believed that endogenous

AHR activation is controlled via an autoregulatory feedback pathway such that endogenous ligands activate the AHR, which in turn upregulates the expression of cytochrome P450 enzymes to degrade endogenous ligands (Chiaro et al. 2007). As a growing body of evidence suggests that endogenous AHR activation modulates immune responses (Bessede et al. 2014; DiNatale et al. 2010), our study provides new insights into the physiological role of AHR in human B lymphopoiesis.

Here we explored the effects of persistent AHR activation on B lymphopoiesis using TCDD, a stable high affinity ligand and environmental contaminant. Although some of the *in vitro* concentrations of TCDD (i.e., 10 and 30 nM) used in this study are higher than the levels to which the general public is typically exposed, the goal of the present investigation was to utilize TCDD as a mechanistic probe. With that said, significant impairment in the generation of lineage committed early-B and pro-B cells was observed by TCDD treatment at concentrations as low as 0.1nM, representing one of the most sensitive toxic endpoint yet reported with TCDD. It is estimated that 1 nM TCDD is comparable with serum levels observed in exposed individuals after the Sevoso, Italy accident in 1976 (Needham et al. 1999). It is also important to emphasize that in the current study, HSPCs were treated with TCDD only on day 0 with half of the media then being replaced weekly without further addition of TCDD. Hence the concentration of TCDD at the end of the 28-day culture period was reduced over time.

TCDD is a ubiquitous environment contaminant, which exhibits a wide spectrum of toxicity including immunotoxicity (Peterson et al. 1993; Poland and

Knutson 1982). Historically, suppression of humoral immunity has been identified as a very sensitive adverse response to TCDD and dioxin-like compound exposure as evidenced by a decrease in the primary antibody response observed across virtually all animal species evaluated (Holsapple et al. 1991; Sulentic and Kaminski 2011). Epidemiological studies have also shown an association between TCDD exposure and decreased humoral immunity. A cohort study in Dutch children found that high perinatal exposure to dioxin-like compounds was correlated with low vaccine titters and a high incidence of chicken pox and otitis (ten Tusscher et al. 2003). Likewise, a case-control study in Sevoso, Italy, identified a significant association between decreased plasma immunoglobulin G (IgG) levels and increasing TCDD plasma concentration (Baccarelli et al. 2002). Although these dioxin-associated effects on humoral immunity could have arisen from direct effects by dioxin on mature B cells (Lu et al. 2011; Lu et al. 2010; Wood and Holsapple 1993), impairment of B cell developmental processes by TCDD, as suggested by our study, may represent an additional contributing factor responsible for compromised humoral immunity. Due to the relatively short life span of circulating mature B cells (18 days on average in human) (Macallan et al. 2005), the homeostasis of the peripheral B cell population requires life-long replenishment by B lymphopoiesis from HSCs. Therefore, suppression of B lymphopoiesis by TCDD could significantly impair the status of humoral immunity. In addition, TCDD exposure has also been epidemiologically associated with increased incidence of non-Hodgkin's lymphoma and multiple myeloma (Becher et al. 1996; Floret et al. 2003; Kogevinas et al. 1997; Kramarova et al. 1998; Viel et al. 2008). As the disruption of *EBF1* and *PAX5* has been frequently

implicated in human leukemia (Kuiper et al. 2007; Mullighan et al. 2007), the TCDD-elicited decrease of EBF1 and PAX5, as reported in this study, might contribute to the carcinogenic effect of TCDD.

In summary, we show that AHR activation by TCDD suppresses B cell lineage specification and commitment, which was attenuated by an AHR antagonist, suggesting a physiological role of AHR in regulating human B lymphopoiesis. The AHR-mediated suppression of EBF1, together with the binding of ligand-activated AHR to DREs in *EBF1* promoter, suggests that transcriptional regulation of EBF1 is involved in the mechanism by which AHR regulates B lymphopoiesis.

4.2 Future directions

4.1.1 Investigation of the direct transcriptional regulation of EBF1 by AHR

In this study, TCDD treatment significantly decreased the expression of *EBF1* (Figure 3.20). The decrease in *EBF1* mRNA by TCDD was attenuated by addition of AHR antagonist (Figure 3.22), suggesting that AHR mediates alterations in EBF1 expression. In addition, there are 13 putative AHR binding sites (DREs) identified in *EBF1* promoter. Binding of the ligand-activated AHR to three DREs in the *EBF1* promoter was demonstrated by EMSA and ChIP analyses (Figure 3.24). Given that AHR acts as a ligand-activated transcription factor, it is hypothesized that AHR can transcriptionally regulates *EBF1* expression. To test this hypothesis, reporter assays will be conducted to examine whether AHR binding to DREs alters the transcriptional activity of *EBF1* promoter. Specifically, a luciferase reporter vector will be constructed using clusters of DREs identified in the *EBF1* promoter followed by a minimal *EBF1* promoter to drive luciferase gene. In addition, in order to study individual DRE function, a serial of reporter vectors will also be derived from the original vector by sequentially mutating the DREs. All of the reporter vectors will then be transfected into JM1 cells to carry out reporter assays. The transcriptional regulation in the *EBF1* promoter by AHR will be evaluated by measuring luciferase activity in transfected JM1 cells in the presence and absence of TCDD. The JM1 cells were selected as they are human pre-B lymphoblast cells and can serve as a surrogate for primary B precursors. However, the mRNA level of AHR in JM1 cells was found to be extremely low, in fact approximately 8,000 fold lower than in HSPCs (Figure 4.2 A).

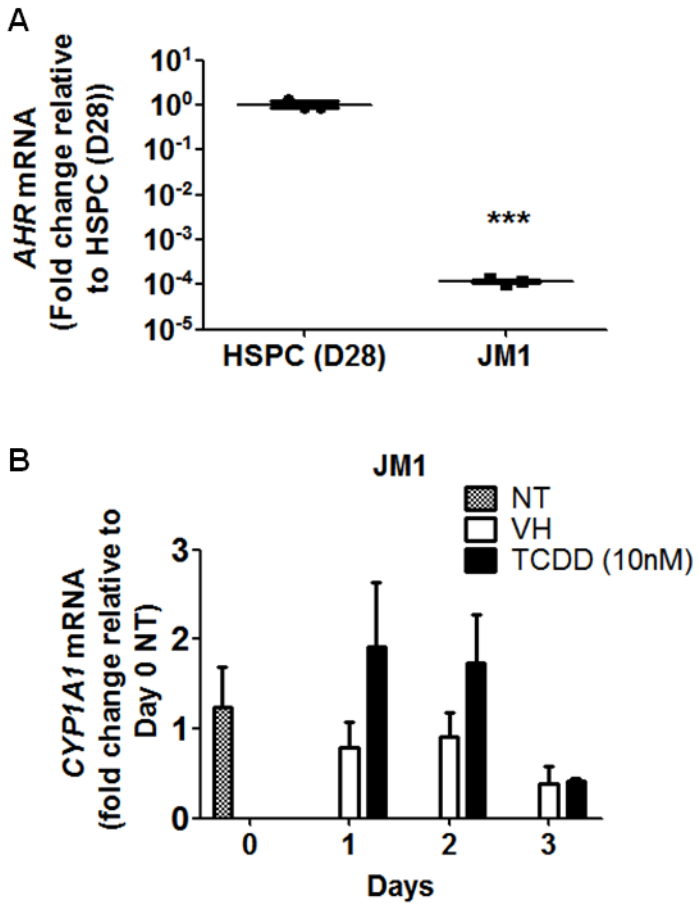


Figure 4.2: *AHR* expression and *CYP1A1* induction in JM1 cells

A) *AHR* mRNA levels in $CD34^+$ HSPCs after 28 days of culture and JM1 cells were determined by real-time quantitative PCR and normalized to 18s ribosomal RNA. Data are presented as mean ± SE of triplicate measurements. ***p <0.001, compared to HSPCs (day 28) by t-test after logarithmic transformation. **B)** JM1 cells were either not treated (NT) or treated with vehicle (VH, 0.02% DMSO) or TCDD (10 nM) on day 0 and cultured for up to 3 days. The mRNA levels of *CYP1A1* were determined by

Figure 4.2 (cont'd)

real-time quantitative PCR and were normalized to 18s ribosomal RNA. Data are presented as mean ± SE for triplicate measurements. No significant difference was detected using a two way ANOVA with Bonferroni posttest after logarithmic transformation. Data are representative of two independent experiments with similar results.

Concordantly, the induction of *CYP1A1* mRNA by TCDD was minimal in JM1 cells (Figure 4.2 B). The low expression level of AHR hinders the ability to conduct reporter assays in JM1 cells. To circumvent this problem, a vector was constructed that contains an AHR-GFP fusion gene driven by a minimal CMV promoter and Tet-operator (Figure 4.3 A). This vector was then delivered into JM1 cells via lentiviral transduction. After puromycin selection and cloning by limiting dilution, a monoclonal transduced JM1 cell population was established. The induced expression of GFP by doxycycline treatment suggests the successful delivery of the AHR-GFP gene into JM1 cells (Figure 4.3 B). With further characterization of AHR expression and the AHR signaling pathway in transduced JM1 cell line, this engineered cell will be used to conduct reporter assays to determine whether AHR exerts direct transcriptional regulation on *EBF1* expression. These assays will provide further molecular insights into the mechanism underlying TCDD-elicited impairment of human B lymphopoiesis.

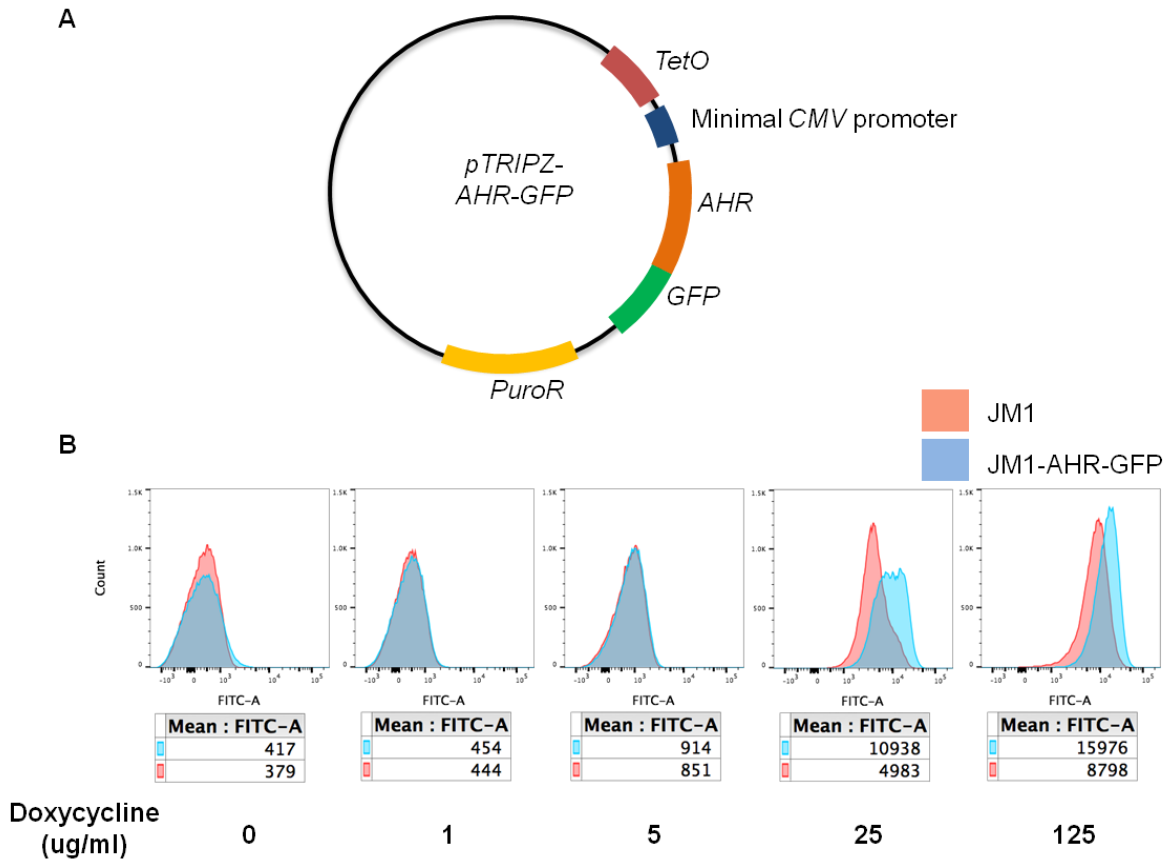


Figure 4.3: Transduction of AHR-GFP gene into JM1 cells

A) The schematic representation of *pTRIPZ-AHR-GFP* vector. The AHR-GFP fusion gene was cloned into pTRIPZ backbone, which can be used for lentiviral transduction. **B)** Transduced JM1 cells (JM1-AHR-GFP, represented in blue) and non-transduced control (JM1, represented in red) were treated with doxycycline (0, 1, 5, 25 or 125 µg/ml) for 24 hours. The expression of GFP was assessed by flow cytometry. The plots represent the histogram of GFP expression in JM1 and

Figure 4.3 (cont'd)

JM1-AHR-GFP cells. The mean of FITC-A represents the mean fluorescence intensity of GFP, which indicates the average expression level of GFP in the cell population.

4.1.2 Explore the impact on B lymphopoiesis by AHR and PAX5 competition

PAX5 is an essential transcription factor in regulating development, function and identity of B cells. In the absence of Pax5, B lymphopoiesis is arrested at the pro-B cell stage (Nutt et al. 1997). Moreover, conditional loss of Pax5 in mature B cells leads to dedifferentiation and conversion of B cells into other hematopoietic cell types (Cobaleda et al. 2007a; Nutt et al. 1999).

During B cell lineage commitment, PAX5 performs an essential role by suppressing 110 non-B cell genes to eliminate alternative cell fate choices (Delogu et al. 2006; Pridans et al. 2008) and activating 170 B cell-specific genes that confer B cell identity (Schebesta et al. 2007). In this study, we have demonstrated that AHR is capable of binding to PAX5 cognate DNA sequence in EBF1 promoter (Figure 3.27), suggesting a competition between AHR and PAX5 for DNA binding. The potential AHR-PAX5 competition would disrupt the ability of PAX5 to properly regulate the transcription of target genes, representing a novel mechanism by which AHR ligands could significantly impair B lymphopoiesis.

To investigate this putative mechanism on a genome-wide scale, ChIP-seq was performed. Specifically, human $CD34^+$ HSPCs were cultured using feeder free culture system for 28 days as described in Method chapter. Cells were then treated with VH (vehicle, 0.02% DMSO) or TCDD (1 nM). After 3 hours, a major portion of the cells from both treatment groups were harvested for ChIP-seq (Active Motif) to evaluate the genome-wide profile of AHR and PAX5 binding in response to TCDD treatment. The rest of the cells from both treatment groups were harvested 24 hours

post-treatment for gene expression analysis to confirm whether the ChIP-seq identified AHR-PAX5 competition would result in subsequent alterations in gene expression. Currently, this project is in the process of ChIP-seq data analysis.

CHAPTER 5: CONCLUDING REMARKS

Historically, immune suppression, especially suppression of B cell mediated immune responses, has consistently been demonstrated as a highly sensitive consequence of exposure to TCDD. Although studies have demonstrated that TCDD impairs the function of already established human mature B cells, the effects of TCDD on human B cell development has not been investigated. This dissertation study for the first time characterizes the suppressive effects of TCDD on human B cell development and provides insights into the underlying molecular mechanisms. In this study, three *in vitro* models of human B cell development were established. Using these models, the effects of TCDD on human B lymphopoiesis were consistently observed, including: 1) decrease in the total number of cells derived from HSPCs via mechanisms not involving cell death; 2) reduced expression of CD34, a cell surface protein demarcating HSPCs; and 3) impeded B cell lineage commitment as evidenced by the decrease in generation of early-B cells and pro-B cells from HSPCs. Mechanistic investigations revealed that TCDD treatment significantly decreased the mRNA level of EBF1 and PAX5, two critical transcription factors directing B cell lineage commitment. Studies using an AHR antagonist demonstrated that AHR mediated the effects of TCDD. In addition, binding of the ligand-activated AHR to the putative dioxin response elements in the *EBF1* promoter was demonstrated by EMSA and ChIP analysis, suggesting transcriptional regulation of EBF1 by AHR. Taken together, this study for the first time, demonstrates that AHR activation by TCDD impairs human B cell development, and suggests that

transcriptional alterations of EBF1 by the AHR are involved in the underlying mechanism.

Epidemiological studies have identified an association between TCDD exposure and decreased humoral immunity in human. Although compromised humoral immunity could have arisen from direct effects by dioxin on mature B cells (Lu et al. 2011; Lu et al. 2010; Wood and Holsapple 1993), impairment of B cell developmental processes by TCDD, as suggested by this study, may represent an additional contributing factor. Due to the relatively short life span of circulating mature B cells (18 days on average in human) (Macallan et al. 2005), the homeostasis of the peripheral B cell population requires life-long replenishment by B lymphopoiesis from HSCs. Therefore, suppression of B lymphopoiesis by TCDD could significantly impair the status of humoral immunity. In addition, TCDD exposure has also been epidemiologically associated with increased incidence of B cell related cancers (Becher et al. 1996; Floret et al. 2003; Kogevinas et al. 1997; Kramarova et al. 1998; Viel et al. 2008). As the disruption of *EBF1* and *PAX5* has been frequently implicated in human leukemia (Kuiper et al. 2007; Mullighan et al. 2007), the TCDD-elicited decrease of EBF1 and PAX5, as reported in this study, might contribute to the carcinogenic effect of TCDD.

This study also provides new insights into the physiological role of AHR in regulating B lymphopoiesis. HSPCs treated with AHR antagonist alone showed an accelerated progression toward B cell lineage specification and commitment(Figure 3.19), which might result from antagonism of endogenous AHR activation. This

observation suggests that AHR activation through endogenous ligand might function as a "brake" to keep the B lymphopoiesis in check and to regulate the homeostasis of peripheral B cell population. In line with this notion, AHR might serve as a therapeutic target to manipulate B lymphopoiesis and humoral immunity.

Animal models have been used extensively in mechanistic immunotoxicological studies. Given the recently reported differences in immune responses between human and mouse (Carninci 2014; Mestas and Hughes 2004; Seok et al. 2013), there is a tendency of using human models as a supplement or replacement of animal models in immunotoxicology studies. In this study, all three established *in vitro* models of human B lymphopoiesis comprise only human cells and human recombinant cytokines to mimic the microenvironment of human B lymphopoiesis. Hence, these models could be used in future developmental immunotoxicology studies of chemicals found in the environment, consumer products and foods.

In summary, this dissertation study for the first time characterizes the impairment of human B cell development by TCDD. Mechanistic studies utilizing multi-faceted approaches suggest that AHR activation disrupts the EBF1-PAX5 axis in the regulatory circuit directing B cell development. Future studies that can provides further molecular insights include: exploring transcriptional regulation of EBF1 by AHR, investigating the impact of AHR-PAX5 competition on B lymphopoiesis, and validating the current *in vitro* findings using a humanized-mouse model.

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