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MECHANISMS FOR 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN-MEDIATED EFFECTS ON CD40 LIGAND-INDUCED ACTIVATION AND EFFECTOR FUNCTION OF PRIMARY HUMAN AND MOUSE B CELLS

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HAITIAN LU

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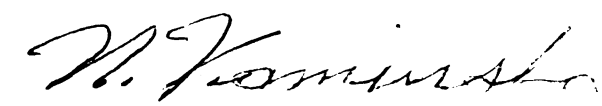

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MECHANISMS FOR 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN-MEDIATED EFFECTS ON CD40 LIGAND-INDUCED ACTIVATION AND EFFECTOR FUNCTION OF PRIMARY HUMAN AND MOUSE B CELLS

By

Haitian Lu

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ABSTRACT

MECHANISMS FOR 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN-MEDIATED EFFECTS ON CD40 LIGAND-INDUCED ACTIVATION AND EFFECTOR FUNCTION OF PRIMARY HUMAN AND MOUSE B CELLS

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Suppression of primary humoral immune responses is one of the most sensitive sequela associated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure in mouse models, with the B cell identified as the primary cellular target. Yet the sensitivity of humoral immunity to TCDD in humans represents an important toxicological data gap. Therefore, the objectives of this investigation were three-fold. The first objective was to establish an *in vitro* polyclonal immunoglobulin M (IgM) response model that comprehensively assesses xenobiotic-mediated effects on the effector function of primary human B cells, for comparison to the mouse. A model comprised of cell surface-expressed CD40 ligand (CD40L) and recombinant cytokines was established to induce robust IgM responses in both human and mouse B cells, mimicking T cell-dependent activation. In addition to antibody production, proliferation and phenotypic changes characteristic of B cell activation and plasmacytic differentiation were also significantly induced. In addition to two well-characterized immunotoxicants, arsenic and benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) were compared in both human and mouse B cells during the establishment of the model. The second objective was to assess the effects of TCDD on primary human and mouse B cells that include induction of known aryl hydrocarbon receptor (AHR)-responsive genes as a measure of early biological responses, and

modulation of the B cell effector function as measured by CD40L-induced IgM response. AHR responsive genes in human B cells exhibited slower kinetics and reduced magnitude of induction by TCDD, when compared to mouse B cells. Evaluation of the IgM response in B cells from 20 donors found 15 donors that exhibited similar sensitivity to suppression by TCDD as mouse B cells, while no suppression was observed in another 5 donors. The third objective was to investigate mechanisms for TCDD-mediated effects on B cells by rigorously assessing several critical stages involved in the activation and plasmacytic differentiation of B cells, using the CD40L activation model. TCDD effects on the expression of plasmacytic differentiation regulators, B lymphocyte-induced maturation protein 1 (Blimp-1) and paired box protein 5 (Pax5), were observed in mouse but not human B cells. TCDD modestly affected proliferation, but profoundly attenuated the activation of human B cells, as evidenced by decreased expression of activation markers CD80, CD86, and CD69. The impaired activation, correlated with decreased cell viability, preventing the progression of B cells toward plasmacytic differentiation. TCDD also caused ubiquitous perturbation of immediate and/or persistent activation of mitogen-activated protein kinases, protein kinase B/Akt, signal transducer and activator of transcription 3, activation protein-1 (c-Jun), and RelA/p65 in human B cells. Collectively, this dissertation research utilized a novel *in vitro* model to demonstrate for the first time that the effector function of primary human B cells was impaired by TCDD, through mechanisms that involve the disruption of B cell activating signals.

DEDICATION

To my wife, Hong, for her unwavering love, support, and confidence in me.
To my parents, Xuanyi and Shengning, for their love, guidance, and dedication to my education.
To my grandparents, Zhongan and Qinglan, for their love and care that nourished me. I will always try my best to make you proud.

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Dr. Sandra O'Reilly helped me irradiate CD40L-L cells almost every week in the last 3 years. Dr. Russell Thomas trained me in microarray studies. Dr. Helen Haggerty introduced me to a research environment in industry. Dr. Scott Loveless, Dr. Lawrence Updyke, Dr. Kristina Chadwick, Dr. Jeanine Bussiere, Dr. Thomas Kawabata, Dr. Joseph Piccotti, and many others educated me about different career paths in toxicology, answered my endless questions, and listened to me when I needed someone to talk to.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
AF	Alexa fluor
AFC	antibody forming cell
AHR	aryl hydrocarbon receptor
Ahr^{b1}	high-resposive *Ahr* allele
Ahr^{d}	low-resposive *Ahr* allele
AHRr	AHR repressor
ALDH3A1	aldehyde dehydrogenase 3 family, member 1
ANOVA	analysis of variance
AP-1	activator protein 1
APC	allophycocyanin
ARA-9	AHR-activated 9
ARNT	aryl hydrocarbon receptor nuclear translocator
$NaAsO_3$	sodium (meta)arsenite
BCL-6	B-cell lymphoma 6
BCR	B cell receptor
Blimp-1	B lymphocyte maturation protein 1
BPDE	benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide
BSA	bovine serum albumin

Ca^{2+}	calcium
CD	cluster of differentiation
CD40L	CD40 ligand
CD40L-L	mouse fibroblast line expressing human CD40L
cDNA	complementary DNA
CFSE	carboxyfluorescein succinimidyl ester
CYP	cytochrome P450
d	day
DLC	dioxin like compound
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
DRE	dioxin responsive element
ELISPOT	enzyme-linked immunospot
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FCM	flow cytometry
FITC	fluorescein isothiocyanate
GSTA1	glutathione S-transferase A1
G>A	guanine to adenine
h	hour
HIV	human immunodeficiency virus

HSP	heat shock protein
IC	intracellular
IC_{50}	half maximal inhibitory concentration
IFNγ	interferon gamma
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IgJ	immunoglobulin joining chain
Igκ	immunoglobulin κ light chain
Igλ	immunoglobulin λ light chain
Igμ	immunoglobulin μ heavy chain
IL	interleukin
IFNγ	interferon gamma
JAK	Janus family kinase
JNK	c-Jun N terminal kinase
LD	lethal dose
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinases
MFI	mean fluorescence intensity
MHC II	major histocompatibility complex class II
min	minute
mRNA	messenger ribonucleic acid
NA	naive

NFκB	nuclear factor kappa B
NQO-1	NAD(P)H dehydrogenase quinone 1
Pac Blue	Pacific Blue
Pax5	paired box protein 5
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxin
PCDF	polychlorinated dibenzofuran
PE	phycoerythrin
PI3K	phosphoinositide 3-kinase
PRDM1	PR domain zinc finger protein 1
Socs2	suppressor of cytokine signaling 2
sRBC	sheep erythrocyte
STAT	signal transducer and activator of transcription
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCR	T cell receptor
Th	helper T cell
TLR	Toll-like receptor
TRAF	tumor necrosis factor receptor associated factor
TSST	toxic shock syndrome toxin

T>C	thymine to cytosine
U	unit
VH	vehicle
XBP-1	X-box binding protein 1

INTRODUCTION

I. Humoral immunity and B cell differentiation

A. The immune system and B cells

The immune system plays a critical role in protecting the host from invading pathogens and is both complex and tightly regulated. It is composed of numerous immune-related organs, cells, and soluble factors. The immune system relies on the highly coordinated activities of these components to distinguish between "non-self" and "self", and eliminate the pathogenic "non-self". The vertebrate immune system has functions that belong to two general categories, innate and adaptive immunity. Innate immunity is a nonspecific host defense mechanism that involves anatomic barriers, inflammation, coagulation, the complement system, and various functions of immune cells such as phagocytosis by macrophages and direct killing by natural killer cells. The innate immune system not only provides the immediate first line of defense upon encounter of pathogens, but also serves as the prelude that in turn activates the adaptive immune system. Unlike the innate immunity, adaptive immunity is highly specific and tailored to maximally eliminate pathogens. It has the abilities to recognize specific pathogens and develop immunological memory against them this enables faster and more efficient attacks on subsequent encounters. Adaptive immunity is carried out by lymphocytes (T cells and B cells) and can be further divided into cell-mediated immunity and humoral immunity. Cell-mediated immunity, carried out by antigen-specific T cells, protects the host against intracellular bacteria, viruses, and cancer by killing the infected or tumor cells. Instead, humoral immunity protects the host against extracellular bacteria, parasites, and pathogenic foreign macromolecules. Humoral immunity is mediated by

differentiated B cells that secrete immunoglobulins (Ig) or antibodies, the effector molecules of humoral immune responses.

B cells may become activated in response to various stimuli, and then differentiate into plasma cells that secrete large amount of antibodies. Antibodies can bind specifically to a pathogen; such binding may prevent the pathogen from entering or damaging host cells, activate the complement system to destroy the pathogen, or recruit other immune cells that can endocytose or kill the pathogen. Antibodies are heterodimers composed of two identical heavy chains and two identical light chains linked by disulfide bonds. Each chain contains a variable region and constant region. The variable regions of both chains determine the specificity of antigen binding, while the constant region of the heavy chain designates five different classes (or isotypes) of antibodies, namely IgM, IgD, IgG, IgA, and IgE, each of which has different biological functions. Two types of light chains are present in mammals, namely the Ig kappa chain (Igκ) and Ig lambda chain (Igλ). IgM is the first antibody produced during a primary immune response, has low affinity but high avidity due to its unique pentameric or hexameric structure, and is particularly effective at complement activation. IgD is primarily of interest in its membrane form since little is known about the role of its soluble form. IgG is predominantly involved in a secondary immune response, has the longest biological half-life, is the only isotype that crosses the placenta, and can directly neutralize pathogens or activate complement or immune cells. IgA is the dominant antibody isotype that contributes to immunity at mucosal surfaces, and a dimeric form accounts for the majority of secretory IgA. IgE is best known for its avidity for receptors on mast cells, and association with allergic response and hypersensitivity reactions and immunity to

parasites. Ig joining chain (IgJ), a conserved polypeptide found in polymeric IgA and secreted pentameric IgM, influences the polymerization of these multimers. Each of the Igs, except for IgJ, can also exist as membrane molecules, known as the B cell antigen receptor (BCR). Antibodies and BCRs of the same class made by an individual B cell have almost identical structures, allowing this B cell to recognize a specific epitope on certain antigen and secrete antibodies against it. One set, or clone, of B cells differs from another in the binding regions of their BCR, the antibodies they secrete and therefore the antigenic epitope they recognize. The highly diverse heavy and light chain variable regions and the pairing of heavy and light chains lead to a large number of structurally distinct Ig molecules. Such a primary repertoire of antibody diversity is sufficiently large so that most epitopes on antigens can be recognized by B cells with the complementary BCR and bound by the antibodies secreted by them.

B. B cell activation and the primary antibody response

The primary antibody response is mounted against a new antigen that has not been experienced by the immune system, and the predominant antibody isotype produced during a primary antibody response is IgM. As mentioned above, B cell activation is the prerequisite for the differentiation into antibody secreting plasma cells, and thus any antibody response, and it may occur independently or dependently of helper T cells (Th). B cells may become activated in a T cell-independent manner by antigens that act on Toll-like receptors (TLR). For instance, bacterial cell wall compound lipopolysaccharide (LPS) activates TLR4, viral genomic single stranded RNA activates TLR7, and bacterial DNA component unmethylated CpG sequences activate TLR9, all of which may activate

B cells and induce antibody responses. Repetitive polymers such as bacterial capsular polysaccharides or viral particles can directly cross-link the BCR.

Most protein antigens, however, are T cell-dependent, in which the help from antigen-specific Th cells is required to elicit the most robust antibody response and the development of immunological memory in B cells. Such T cell-dependent B cell activation and primary antibody response is a multi-step process that involves several key cell types. Upon encounter of a new antigen, antigen presenting cells, primarily dendritic cells, endocytose and process the antigen, and present the antigenic peptides in the context of cell surface-expressed major histocompatibility complex class II (MHC II). These antigenic peptide-bearing dendritic cells then prime those Th cells that express the T-cell antigen receptors (TCR) that recognizes these antigenic peptides. In the meantime, B cells also capture the native antigen with BCR that recognizes the antigen, internalize and degrade it, and return the antigenic peptides bound to MHC II. These antigen-specific B cells then enter the T-cell zone of secondary lymphoid tissues, where they receive help from primed Th cells that recognize the same antigen. The primary cognate interaction between Th cells and B cells is the binding of TCR by the antigenic peptide expressed in the context of MHC II on B cells. The signal through TCR triggers Th cells to become further activated and express both cell surface-bound and secreted effector molecules as contact-dependent and independent stimuli that synergize to activate B cells. The optimal T cell-dependent B activation *in vivo* requires two signals received by B cells, the primary through the engagement of BCR by the native antigen, and the secondary from the activating signals delivered by the Th cells in cognate interaction with B cells.

Upon activation, B cells proliferate, and then either differentiate into short-lived plasma cells, or undergo germinal center reactions to become either memory B cells or long-lived plasma cells. Antibody class switch, which allows B cells to secrete antibodies of other isotypes rather than IgM, may occur in both pathways of B cell differentiation. Somatic hypermutation and affinity maturation also take place during germinal center reactions to ensure the immunological memory is carried by B cells that can respond to certain antigens at the best efficiency upon future encounters (i.e., in a secondary response). Somatic hypermutation significantly expands the repertoire of antibody diversity by rapidly mutating the variable region of the Ig heavy and light chains, although only some of mutations will increase the affinity of antibodies that bind to certain antigen. During affinity maturation, only B cells whose BCR binds to the antigen can be rescued from an active process of apoptosis occurring in germinal center reactions. Therefore, B cells with the most avid BCR have the growth advantage and are selected to dominate the population of responding cells.

Among the surface-expressed effector molecules on activated Th cells, CD40 ligand (CD40L) is a particularly important one. CD40L is a glycoprotein that binds to CD40 that is constitutively expressed on the surface of B cells through development and differentiation (Banchereau *et al.* 1994). The cognate interaction between CD40 on B cells and CD40L on activated Th cells plays important roles in all stages of T cell-dependent primary antibody response *in vivo*. Once a Th cell-B cell contact forms, ligation of CD40 by CD40L, in combination with the signal through BCR, provides a strong stimulating signal to initiate the activation, proliferation, and differentiation of B cells. Upon the ligation by CD40L, CD40 trimerizes and its cytoplasmic domain rapidly

recruits several intracellular proteins that belong to the tumor necrosis factor receptor-associated factor (TRAF) family, including TRAFs 2, 3, 5, and 6 (Bishop and Hostager 2001). TRAFs serve as adapters that transmit the signal through CD40 and contribute to the activation of multiple downstream signaling cascades in B cells, including the nuclear factor κB (NFκB)/Rel pathway, the phosphoinositide 3-kinase (PI3K)/Akt pathway, the mitogen activated protein kinase (MAPK) pathway (i.e., c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 kinase), and the Janus family kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Bishop 2004; Elgueta *et al.* 2009; van Kooten and Banchereau 2000). The DNA binding activity of both NFκB/Rel and activator protein 1 (AP-1) was also induced in B cells stimulated through CD40 (Francis *et al.* 1995). The activation of these signaling pathways, among which extensive cross-talk is commonly observed, likely culminate into an "activated" phenotype in B cells that is characteristic of up-regulated expression of numerous genes including CD80 (B7.1), CD86 (B7.2), MHC II, and intercellular adhesion molecule-1 (Bishop and Hostager 2001). The signaling pathways triggered by ligation of CD40 are diverse, and may consequently contribute to various aspects of B cell responses during a functional humoral immune response. For instance, NFκB activation is found to be required in the up-regulation of co-stimulatory molecule CD80 in B cells (Hsing *et al.* 1997). Activation of NFκB/Rel and PI3K/Akt pathways also contribute to the proliferation and survival of B cells induced by CD40L (Andjelic *et al.* 2000). Moreover, both AP-1 and STAT3 promote the plasmacytic differentiation of B cells, therefore are linked directly with B cell antibody response (Diehl *et al.* 2008; Ohkubo *et al.* 2005; Vasanwala *et al.* 2002) .

The indispensable role of CD40L in T cell-dependent humoral immunity is evidenced by the complete abolishment of T cell-dependent humoral immunity in both humans with genetic alteration of CD40L and CD40L knockout mice (Bishop and Hostager 2003). Since its identification, CD40L has been primarily employed to induce robust activation of primary B cells *in vitro*, in the absence of BCR ligation (thus independently of antigen recognition). In a limited number of previous studies, the ligation of CD40 in primary human B cells with CD40L effectively drives the differentiation into antibody secreting cells that produce IgM, IgG, IgA, or IgE. The proportion of antibody isotypes are subjected to modulation by the specific soluble factors added simultaneously, including IL-2, IL-4, IL-6, IL-10, and IL-21 (Armitage *et al.* 1993; Arpin *et al.* 1995; Ettinger *et al.* 2005; Rousset *et al.* 1992; Spriggs *et al.* 1992). As mentioned above, activated Th cells also secrete various effector molecules, including, but not limited to, interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-10, and IL-21. These cytokines could facilitate B cell antibody responses by ligating their receptors on the surface of B cells, in turn triggering downstream signaling pathways that largely overlap with the ones utilized by CD40.

C. Regulation of plasmacytic differentiation of B cells

B cell plasmacytic differentiation into antibody secreting plasma cells is the basis of any humoral immune response. However, antibody responses that fail to distinguish between self and non-self can be pathogenic and lead to autoimmune diseases. Therefore, B cell plasmacytic differentiation is a tightly regulated process characterized by highly coordinated alterations of various transcription factors including, but not limited to, paired box protein 5 (Pax5), B-cell lymphoma 6 (BCL-6), and B lymphocyte maturation

protein 1 (Blimp-1) (Calame *et al.* 2003; Igarashi *et al.* 2007; Shapiro-Shelef and Calame 2005). During a primary antibody response, B cells differentiate into plasma cells that secrete large quantities of IgM. IgM is typically secreted as a polymer in which individual IgM monomers are joined together by IgJ. Significantly increased expression of genes coding for the IgM heavy chain (*IgH*), light chain (*Igκ*), and IgJ (*IgJ*) is required for high level IgM production and is characteristic of antibody secreting plasma cells. Pax5 plays a critical role in maintaining B cell identity by transcriptional repression of IgH, Igκ, and IgJ (Calame *et al.* 2003). During the plasmacytic differentiation in B cells, Pax5 is progressively decreased to release IgH, Igκ, and IgJ from repression. Another important target of repression by Pax5 is X-box binding protein 1 (XBP-1), a transcription factor required by plasmacytic differentiation (Reimold *et al.* 2001; Reimold *et al.* 1996). Pax5 directly binds to and inhibits the promoter activity of *XBP-1* gene. Once the repression by Pax-5 is relieved, XBP-1 is in turn induced, and subsequently triggers the plasma cell secretory apparatus necessary for the secretion of large amounts of antibodies (Reimold *et al.* 2001). However, the functional deletion of *Pax5* is insufficient to activate B cell plasmacytic differentiation, suggesting the contribution from other regulators to protection of B cell identity (Horcher *et al.* 2001). Indeed, BCL-6 is another transcriptional repressor that induces B cells to maintain an activated state and undergo germinal center reactions while inhibiting differentiation into antibody secreting plasma cells (Fukuda *et al.* 1997; Phan and Dalla-Favera 2004; Phan *et al.* 2005). Pax5 and BCL-6 maintain the B cell phenotype by repressing a critical target protein called Blimp-1, often referred to as the "master regulator" of B cell plasmacytic differentiation (Fairfax *et al.* 2008; Martins and Calame 2008; Nera *et al.* 2006; Reljic *et*

al. 2000; Tunyaplin *et al.* 2004). An indispensable role of Blimp-1 was established by previous studies using mouse models, including the observations that enforced expression of Blimp-1 drives B cells to become plasma cells, and genetic ablation of Blimp-1 prevents B cells from differentiating into antibody secreting plasma cells (Kallies *et al.* 2004; Shapiro-Shelef *et al.* 2003; Turner *et al.* 1994). Although whether functional Blimp-1 is absolutely required in plasmacytic differentiation of human B cells is unknown, elevated expression of Blimp-1 was indeed found to correlate with plasma cell commitment of B cells in normal human lymphoid tissues (Cattoretti *et al.* 2005). Blimp-1 functions predominantly as a transcriptional repressor, directly repressing a variety of targets, among which the two critical targets are Pax5 and BCL-6 (Nutt *et al.* 2007). The repression of Pax5 by Blimp-1 releases the Pax5-mediated repression of XBP-1 and Ig genes. Blimp-1 also represses the transcription of cell cycle regulator c-myc, leading to the cessation of proliferation necessary for plasmacytic differentiation into a post-mitotic state (Lin *et al.* 2000; Lin *et al.* 1997). The reciprocal repressions between positive regulator Blimp-1 and negative regulators Pax5 and BCL-6 comprise a regulatory circuit that ensures the tight regulation of plasmacytic differentiation in activated B cells. Such a regulatory circuit can also be described as a "bistable switch" that exists in one of two stable, mutually exclusive states (Bhattacharya *et al.* 2010; Markevich *et al.* 2004). In the resting mature B cell stage, the expression of Blimp-1 is low. Once B cells become activated, multiple upstream signals may lead to the induction of Blimp-1. For instance, AP-1 is induced in B cells stimulated with CD40L and promotes B cell plasmacytic differentiation by positively regulating Blimp-1 (Francis *et al.* 1995; Ohkubo *et al.* 2005). AP-1 activity can be repressed by BCL-6 through direct protein-protein interaction,

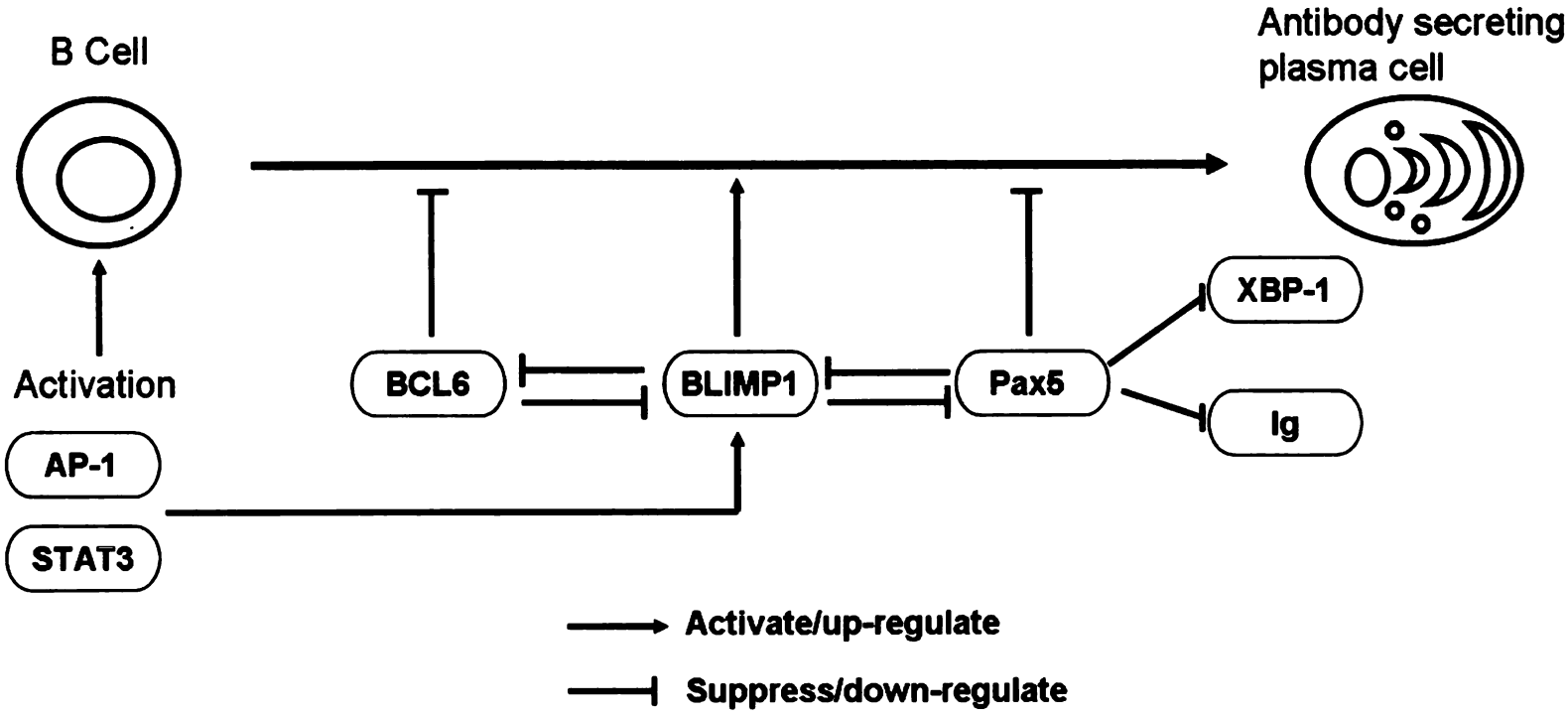


Figure 1. Regulation of B cell plasmacytic differentiation.

contributing to BCL-6-mediated Blimp-1 repression (Vasanwala *et al.* 2002). Signals through CD40 and receptors for various cytokines such as IL-6, IL-10, and IL-21 also induce STAT3 in B cells, which in turn up-regulates Blimp-1 expression and promotes B cell plasmacytic differentiation (Calame 2008; Diehl *et al.* 2008; Schmidlin *et al.* 2009). The rapidly increased Blimp-1 expression leads to the repression of Pax5 and BCL-6 and allows for the overall changes of the gene expression profile in B cells, including a positive feedback that increases Blimp-1 expression itself, which culminates in the plasmacytic differentiation. The critical regulators of B cell plasmacytic differentiation and the coordination and reciprocal regulation among them were summarized in a highly simplified manner in Figure 1.

II. TCDD and AHR

A. General toxicity of TCDD and the AHR pathway

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and dioxin-like compounds (DLCs) belong to a family of structurally related chemical pollutants that are ubiquitously and persistently present in the environment. Chemicals commonly referred to as DLCs are structurally related halogenated aromatic hydrocarbons, including polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBBs). TCDD and DLCs share the characteristic of being highly lipophilic, a property that contributes to their bioaccumulation in the food chain, making diet the primary route of animal and human exposure (Schecter *et al.* 2001). Except when generated in very small amounts during natural combustion and geological processes (e.g., volcano eruption), TCDD and DLCs

were primarily produced as unwanted byproducts in the manufacture of products from chlorinated phenols such as herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and Agent Orange (mixture of butyl esters of 2,4-D and 2,4,5-T). Other sources of TCDD and DLCs include the combustion of chlorinated materials, chlorine bleaching during the manufacture of paper and pulp, and automobile exhaust. TCDD, the prototype of this group, serves as a model compound for studies of biological mechanisms of action and toxicity due to its high biological potency as illustrated by extensive toxicities observed in laboratory animals. Major toxic effects identified in animal models by TCDD include generalized wasting syndrome, lymphoid involution (especially of the thymus), carcinogenesis, endocrine disruption, teratogenesis and embryotoxicity, hepatomegaly and hepatotoxicity, and immunotoxicity (Safe 1986). One interesting feature presented by TCDD is the notable inter-species and inter-strain variability in the effects of TCDD. For instance, the acute toxicity of TCDD, as indicated by the median lethal dose LD_{50}, varies approximately 500 fold between the most sensitive species (guinea pig; 0.6 – 2 μg/kg) and the least sensitive speices (hamster; 5 mg/kg) (Poland *et al.* 1994). The inter-strain variability in the effects of TCDD was first illustrated when studying the induction of activity of drug-metabolizing enzyme cytochrome P450 (CYP) in various mouse strains in response to TCDD. TCDD induced CYP activity in the "responsive" strain C56BL/6 at 10-fold higher potency than in the "non-responsive" strain DBA/2 (Poland and Glover 1974; Poland *et al.* 1974). Further studies identified a genetic locus that determines the sensitivity to TCDD-mediated biochemical and toxicological effects in an autosomal dominant manner in mice, termed the *aryl hydrocarbon*, or *Ah*, locus.

The *Ah* locus encodes a cytosolic protein named the aryl hydrocarbon receptor (AHR), and therefore is later referred to as the gene *Ahr*. AHR is the cellular receptor for TCDD and DLCs, which is believed to mediate the majority of toxicities associated with this compound (Rowlands and Gustafsson 1997). Different *Ahr* alleles have been identified in mouse and encode either high affinity (Ahr^{b1}, as in C57BL/6 mice) or low affinity (Ahr^{d}, as in DBA/2 mice) receptors based on the 10-fold difference in ligand binding affinity (Poland *et al.* 1994). This significant difference was attributed to a polymorphism at codon 375 in the ligand binding domain of the receptor: Ahr^{d} encodes valine at position 375, while Ahr^{b1} encodes alanine (Harper *et al.* 2002). The binding affinity difference was also found to be proportional to the various biochemical and toxic responses caused by exogenous ligands, illustrating the significant role of the AHR, at least in mice. AHR is a ligand-activated transcription factor that belongs to the basic helix-loop-helix family (Burbach *et al.* 1992). In the absence of ligand, AHR is chaperoned in the cytosol by heat shock protein 90 (HSP90), cochaperone p23, and AHR-activated 9 (ARA-9) in the form of a "core complex" (Carver *et al.* 1998; LaPres *et al.* 2000). Other putative binding partners have been suggested but their functional relevance to the AHR signaling pathway remains to be fully elucidated. It is also possible that AHR associates with distinct binding partners in different cell types, leading to cell/tissue-specific responses mediated by the AHR signaling pathway. Upon ligand binding, the AHR cytosolic complex dissociates shedding HSP90 and ARA-9, while translocating into the nucleus, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) to form a heterodimer that acts as a transcription factor. The

ligand-AHR/ARNT complex then binds to defined nucleotide sequences, termed dioxin responsive elements (DRE), located in promoter or enhancer regions of AHR-responsive genes, regulating their transcriptional regulation (Hankinson 1995). The first group of well-characterized AHR-responsive genes, also termed the classic "AHR gene battery", consists of genes that encode phase I and phase II drug metabolizing enzymes such as CYP1A1, CYP1A2, CYP1B1, NAD(P)H dehydrogenase quinone 1 (NQO1), aldehyde dehydrogenase 3 family, member 1 (ALDH3A1), and glutathione S-transferase A1 (GSTA1) (Nebert *et al.* 2000). The enzymes that are regulated by the AHR may play a role in toxic responses by metabolizing certain DLCs into more reactive and/or toxic forms (e.g., benzo[a]pyrene). However, the induction of these enzymes is also considered as a biochemical/adaptive response and are utilized to indicate exposure to TCDD and DLC and activation of the AHR pathway, with CYP1A1 as the most commonly used one. Extensive further studies have identified a large variety of non-metabolic genes that have DRE in their promoter regions, and can be either up-regulated or down-regulated by TCDD through the transcriptional activity of AHR. The modulation of these genes may contribute to the various toxicities observed following TCDD treatment. AHR may also lead to adaptive or toxic responses through cross-talk with other signaling pathways, accounting for TCDD effects that are independent of binding of AHR to DRE. For example, AHR may physically interact with retinoblastoma protein and the Src family kinase c-src, contributing to cell cycle arrest and elevated tyrosine kinase activity, respectively (Enan and Matsumura 1996; Ge and Elferink 1998). AHR may also interact with the NFκB and the estrogen receptor-mediated signaling pathways (Tian *et al.* 1999; Tian *et al.* 1998; Wormke *et al.* 2003).

An interesting question is that in addition to the role of responding to xenobiotics, what physiological roles the phylogenically conserved AHR plays. A wide range of structurally divergent chemicals have been identified as endogenous or dietary AHR ligands, including, but not limited to, tryptophan metabolites, heme metabolites, arachidonic acid metabolites, and natural flavonoids (Denison and Nagy 2003; Nguyen and Bradfield 2008). However, the physiological functions of AHR activation upon binding to these ligands have not been well understood.

B. Immunotoxicity of TCDD and the role of AHR

Immune suppression is among the earliest and most sensitive sequelae of TCDD exposure, and is observed in virtually every animal species studied (Holsapple *et al.* 1991). What drew the attention to the immunotoxic potential of TCDD were the very early findings that exposure to these chemical compounds caused marked thymic involution consisting of the depletion of cortical lymphocytes, for which the mechanisms were shown to involve alterations in thymic epithelial cell differentiation and thymocyte maturation from hematopoietic stem cells (Greenlee *et al.* 1985; Laiosa *et al.* 2003; Staples *et al.* 1998). Cell-mediated immunity is also sensitive to suppression by TCDD, which were most pronounced in either peri- or postnatally exposed animals (Vos *et al.* 1973, 1974). The impairment of cell-mediated immunity by TCDD may be attributed to effects on multiple immune cell subtypes. For instance, TCDD caused a decrease in the activity of cytotoxic T cells and generation of regulatory T cell phenotype (De Krey and Kerkvliet 1995; Funatake *et al.* 2005; Marshall *et al.* 2008). It has also been shown that the functions of antigen presenting dendritic cells may be altered by exposure to TCDD (Bankoti *et al.* 2010a; Vorderstrasse and Kerkvliet 2001). However, one of the best

characterized effects of TCDD on the immune system has been the suppression of the humoral immunity, which was further shown to be a result of direct effects on B cells. A series of distinct experimental findings using mouse models have established B cells as a cellular target highly sensitive to direct actions of TCDD. First, both the T cell-dependent antibody response to sheep erythrocytes (sRBC) and T cell-independent antibody response to LPS and dinitrophenyl (DNP)-ficoll were found to be sensitive to suppression by TCDD (Holsapple *et al.* 1986a). Second, further studies used cell separation/reconstitution techniques to conclusively demonstrate that the B cell is the primary cellular target in suppression by TCDD of T cell-dependent antibody response, by showing that T cell accessory function was modestly affected by TCDD, and T cells derived from TCDD-treated mice were able to reconstitute the helper cell function in the response to a T cell-dependent antigen (Dooley and Holsapple 1988; Dooley *et al.* 1990). Last, IgM secretion by isolated mouse splenic B cells stimulated with anti-Ig plus T cell-derived soluble factors and by CH12.LX mouse B cells stimulated with LPS were suppressed by TCDD in the absence of accessory cells (Sulentic *et al.* 1998; Tucker *et al.* 1986). When other T cell-dependent antigens such as ovalbumin and influenza A virus were used, the suppression of antibody production has been the most consistent endpoint observed in different mouse models (Inouye *et al.* 2003; Mitchell and Lawrence 2003; Warren *et al.* 2000).

AHR was found to be expressed and capable of binding to DREs in mouse splenocytes (Williams *et al.* 1996). mRNA and protein expression, DNA binding, and transcriptional activity of the AHR were up-regulated upon leukocyte activation in the absence of exogenous ligands (Crawford *et al.* 1997). Since immune responses almost

always involve activated immune cells, AHR up-regulation associated with cell activation may contribute, at least in part, to the high sensitivity of immune responses to TCDD. Results from studies that focused on suppression of the humoral immune response established the involvement of AHR in the immunotoxic effects of TCDD. The first line of evidence came from studies in which TCDD-mediated suppression of primary antibody response segregated with the *Ah* locus, with the mouse strains carrying the high affinity AHR being markedly more sensitive than the low affinity AHR-carrying strains (Vecchi *et al.* 1983). These results were corroborated by the positive correlation observed between the affinity for AHR binding and suppression of the primary antibody response by various DLCs (Davis and Safe 1988; Harper *et al.* 1995; Tucker *et al.* 1986). The notion that the immunotoxic action of TCDD is solely mediated through AHR has been challenged by previous studies, which suggested the involvement of AHR may vary upon different conditions of TCDD treatment. In splenocytes isolated from mice with different affinity AHR, TCDD caused comparable suppression of the *in vitro* primary antibody response against both T cell-dependent and T cell-independent antigens (Holsapple *et al.* 1986a). *In vivo* studies also demonstrated that subchronic exposure to 2,7-dichlorodibenzo-p-dioxin, a dioxin congener with minimal binding affinity for the AHR, caused a suppression of the primary antibody response that was comparable to TCDD (Holsapple *et al.* 1986b). Likewise, subchronic exposure also enhanced the suppression of primary antibody response by TCDD in mice carrying the low affinity AHR (Morris *et al.* 1992). Probably the most conclusive evidence that argues for a critical role of AHR in TCDD-mediated disruption of humoral immune response was obtained in studies that utilized immune cells and mice that lack the AHR. Comparing the AHR-expressing

CH12.LX and AHR-deficient BCL-1 mouse B cells, Sulentic *et. al.* found that TCDD suppressed the LPS-induced IgM production from CH12.LX but not BCL-1 cells (Sulentic *et al.* 1998). In AHR-knockout mice, T cell-dependent primary antibody response to sRBC was insensitive to TCDD, while a profound suppression was observed in control animals with the same genetic background but has the high affinity allelic form of AHR (Vorderstrasse *et al.* 2001). The activity of drug metabolizing enzymes regulated by AHR has not been shown to be involved in TCDD-mediated immunosuppression, this could be due to the fact that the expression levels of these enzymes are typically low in hematopoietic cells. Alternatively, it has been generally hypothesized that exposure to TCDD activates AHR, which in turn modulates the expression of various genes that are critical in regulating immune responses. Another alternative to this paradigm is that the AHR may form cell type-specific interactions with critical proteins that regulate immune functions, and such interactions may be perturbed by TCDD binding to the AHR, contributing to immunotoxicity. For example, recent studies suggested that ligand-activated AHR may interact with transcription factor STAT1 in macrophages and T cells, contributing to regulation of inflammatory responses and development of IL-17-producing Th cells (Kimura *et al.* 2009; Kimura *et al.* 2008; Veldhoen *et al.* 2009; Veldhoen *et al.* 2008).

C. TCDD exposure and effects in humans

Human acute exposure to TCDD and DLCs has occurred mostly through environmental exposure accidents. For instance, the exposure of residents of Times Beach, Missouri occurred when dioxin-contaminated waste was accidentally used for the spraying of dirt roads for dust control. In Seveso, Italy, residents were exposed following

an industrial explosion that released TCDD-contaminated chemicals. In Japan and Taiwan, the contamination of rice oil with PCDFs and PCBs led to poisoning incidents (known as "Yusho" in Japan and "Yucheng" in Taiwan). Human exposure to TCDD and DLCs may also be occupational. The most publicly visible case of such exposure is among the Vietnam War veterans who were involved in handling and spraying the defoliant Agent Orange that contained TCDD as a contaminant. It is widely acknowledged that most people are also exposed to TCDD and DLCs through food consumption due to the bioaccumulation of these compounds in the food chain, and the levels of exposure are tens if not hundreds of times lower than in accidents or occupational settings (Schecter *et al.* 2006). Moreover, the levels of TCDD and DLCs have declined over the last several decades both in the environment and in general populations (Hays and Aylward 2003). Nevertheless, due to the ubiquitous and persistent nature of TCDD and DLCs in the environment, and their extensive toxicities observed in laboratory animals, concerns of potential health hazard associated with human exposure still persist.

Our knowledge of human health effects caused by TCDD and DLCs was primarily obtained from occupational and epidemiological, rather than controlled experimental studies. To date, chloracne and hyperkeratosis represent the only adverse human health effects that have been conclusively associated with exposure to TCDD and DLCs. Carcinogenesis, developmental and reproductive abnormalities, neurotoxicity, disorders of the endocrine system, and immune dysfunction have been suggested, although a causal relationship that links them to TCDD and DLCs exposure can not be established based on the data available (Schecter *et al.* 2006). It is intriguing that the

major hallmarks of toxicity observed in sensitive laboratory animals are not prominent in humans with accidental, occupational, and dietary exposure to TCDD and DLCs (Okey 2007). Interestingly, at least in some epidemiological studies, a potential association was observed between exposure to TCDD and DLCs and impairment of humoral immunity, one of the most sensitive endpoints in laboratory animals. Decreased plasma levels of IgG were detected in residents living in areas contaminated by dioxins 20 years after the Seveso, Italy, accident, as well as in Korean veterans exposed to Agent Orange during the Vietnam War (Baccarelli *et al.* 2002; Kim *et al.* 2003). Likewise, Yusho (Yucheng) patients who were accidentally exposed to DLCs were reported to have decreased serum IgM and IgA levels (Lu and Wu 1985; Nakanishi *et al.* 1985). The Yucheng children, examined 16 years after in utero exposure, showed more frequent respiratory and ear infections early in life, although no difference in serum Ig levels was observed (Yu *et al.* 1998). Evidence also comes from studies of general populations. In Dutch preschool and school children, prenatal and lactational exposure to DLCs was correlated with lower antibody levels after primary vaccination and higher prevalence of recurrent middle-ear infections (Weisglas-Kuperus *et al.* 2000; Weisglas-Kuperus *et al.* 1995). A negative correlation was also established between serum IgG level and exposure to DLCs in Flemish adolescents (Van Den Heuvel *et al.* 2002). Despite these results, like other toxic endpoints, the effects of TCDD and DLCs on the immune competence have been difficult to assess and evidence for effects on the humans is sparse. This is likely due to the fact that immune responses are usually transient, and the commonly used clinical immunology markers may not be sufficient to assess the subtle effects associated with exposure. The interpretation of existing data from multiple studies is also challenging

given the high level of variability in methods and failure to control for various potential confounding factors in some studies (Baccarelli *et al.* 2002; Neubert *et al.* 2000). The clinical significance of current findings is largely unknown; however, it is reasonable to speculate that they may reflect a broad alteration of the immune system, which has been suggested to link to other endpoints such as the high incidence of certain cancers (Baccarelli *et al.* 2002).

Even less is known about the role of AHR in potential adverse effects in humans caused by exposure to TCDD and DLCs. When compared with the high affinity AHR in sensitive laboratory rodent species such as the C57BL/6 mouse, human AHR binds to its ligands with relatively low affinity and causes about a 10-fold lower induction of CYP1A1 (Harper *et al.* 2002). This is due to the fact that codon 381 of wild-type human AHR (equivalent to codon 357 in mouse) encodes valine, producing a low affinity receptor (Ema *et al.* 1994). No genetic variation has thus far been identified at codon 381 in humans (Okey *et al.* 2005). Surprisingly few polymorphisms emerged out of extensive search across the human populations that belong to different ethnic groups, suggesting *AHR* may not be highly variant among humans (Okey *et al.* 2005; Rowlands *et al.* 2010). The most widely studied human *AHR* polymorphisms are all located in the transactivation domain at codon 517, 554, and 570 (Harper *et al.* 2002). Studies that aimed at characterizing the functional outcome of these polymorphisms led to complicated and sometimes self-conflicting results. For instance, induced CYP1A1 activity was found correlated with codon 554 polymorphism in one (Smart and Daly 2000) but not the other studies (Cauchi *et al.* 2001; Kawajiri *et al.* 1995; Smith *et al.* 2001). The combinations of polymorphisms at codons 554 and 570 or 517, 554, and 570 both produced an AHR that

failed to induce CYP1A1 (Wong *et al.* 2001b). More studies examined the potential link between polymorphism at codon 554 and adverse health effects including chloracne and various cancers, and no association has been established (Harper *et al.* 2002). On the other hand, no *AHR* polymorphisms have been identified that may account for a wide range of variation in the ligand binding affinity of AHR observed in humans (Harper *et al.* 2002; Nebert *et al.* 2004). Overall, it has been suggested that polymorphisms in the human *AHR* are infrequent and not linked to significant alterations of AHR functions (Okey *et al.* 2005). However, this does not exclude the possibility that exposure to TCDD and DLCs may cause toxicities in humans and a more susceptible subpopulation may exist. For instance, the functional outcome of a known polymorphism in human *AHR* has typically been examined by studies of CYP1A1 induction, which may be confounded by variation in human *CYP* genes and other genes that contribute to stability (e.g., HSP90, ARA9) or function of the AHR (e.g., ARNT). Moreover, accumulating evidence suggest that the binding affinity of AHR and/or the level of induction of the classic AHR gene battery may not be indicative of TCDD toxicity in a given tissue, or the sensitivity difference observed among species. For example, in addition to the ligand-binding domain, human AHR and the high affinity AHR in C57BL/6 mice also have a high degree of divergence in amino acid sequence within their transactivation domain. Recent studies suggested this divergence may lead to differentially recruited coactivator/corepressor complexes, and therefore differentially regulated gene expression (Flaveny *et al.* 2008; Flaveny *et al.* 2010).

III. The B cell as a direct target of TCDD Immunotoxicity

A. TCDD-mediated disruption of B cell plasmacytic differentiation

Humoral immunity, mediated by the production of antibodies, was profoundly impaired by TCDD in adult animals, but not perinatally exposed animals (Holsapple *et al.* 1991). Therefore, TCDD-mediated suppression of antibody response is likely due to its direct effect on the ability of mature B cells to differentiate into a plasmacytic phenotype characteristic of increased levels of antibody production. This notion is further supported by extensive experimental evidence obtained from both *in vitro* and *in vivo* mouse models. TCDD suppressed IgM secretion from isolated splenocytes or splenic B cells stimulated with LPS *in vitro* (Zhang *et al.* 2010). LPS also induced a robust IgM response in mice, which was markedly suppressed the IgM response by TCDD (North *et al.* 2009a). Using flow cytometry, the studies mentioned above also found TCDD decreased the percentage of $CD138^{+}$ intracellular IgM^{high} cells among $CD19^{+}$ B cells, confirming a direct effect of TCDD on B cell differentiation into a phenotype characteristic of plasma cells (North *et al.* 2009a).

The disruption of B cell plasmacytic differentiation by TCDD involves the AHR. The expression of AHR appeared to determine the sensitivity of primary IgM response to TCDD in mouse B cells, as evidenced by the seminal finding that CH12.LX mouse B cells expressed AHR and were remarkably sensitive to TCDD, while BCL-1 cells lacked AHR and were insensitive (Sulentic *et al.* 1998). Although the precise molecular mechanisms by which TCDD impairs the ability of B cells to undergo plasmacytic differentiation into antibody secreting cells remain to be elucidated, significant advances

have been made in a bottom-up and step-wise approach, and have primarily focused on understanding the effects of TCDD on the functions of several critical regulators of the B cell plasmacytic differentiation process. One of the first targets identified in CH12.LX cells was the Ig heavy chain gene that directly regulates the production of IgM. TCDD suppressed Ig μ gene expression by decreasing LPS-induced transcriptional activity of the 3'α Ig heavy chain enhancer (Sulentic *et al.* 2000; Sulentic *et al.* 2004). This effect is AHR-dependent, with TCDD-induced binding of AHR to DRE-like sites identified in the regulatory regions of the 3'α enhancer. As mentioned previously, the expression of IgH, along with Igκ, IgJ, and XBP-1, is repressed by Pax5, a transcription factor that negative regulates the plasmacytic differentiation of B cells. Studies that aimed characterizing the effect of TCDD on regulatory events preceding Ig gene expression demonstrated Pax5 as another target of direct actions by TCDD (Schneider *et al.* 2008; Yoo *et al.* 2004). In both CH12.LX cells and primary mouse splenocytes, LPS down-regulated the mRNA and protein expression of Pax5, and such down-regulation was attenuated by TCDD. In CH12.LX cells, TCDD also prevented LPS-induced decrease of DNA binding activity of Pax5. These observations were made concomitantly with the suppression of Ig gene and XBP-1 expression induced by LPS, confirming a functional outcome of TCDD modulation of Pax5. The reciprocally repressive relationship between Pax5 and another transcription factor Blimp-1 in the context of B cell plasmacytic differentiation led to later studies that focused on characterizing potential effects of TCDD on Blimp-1, primarily using mouse models (North *et al.* 2009a; Schneider *et al.* 2009). TCDD not only suppressed the mRNA and protein expression of Blimp-1 in LPS-activated CH12.LX cells and splenocytes, but also reduced the binding of Blimp-1 to the promoter

of *Pax5* gene. In the same studies, putative DRE-like sites were identified in the regulatory regions of *Blimp-1*, and binding to these DRE-like sites were modulated by TCDD, suggesting a mechanism by which TCDD may alter Blimp-1 expression. Blimp-1 mRNA and protein expression were also induced in splenocytes derived from mice treated with LPS *in vivo*, but were suppressed by co-treatment with TCDD (North *et al.* 2009a). In light of the role of Blimp-1 as the "master regulator" of B cell plasmacytic differentiation required by antibody responses, mostly supported by evidence accumulated in mice, the deregulation of Blimp-1 expression by TCDD could well explain the long-standing observations that the humoral immunity is extremely sensitive to suppression by TCDD. Induction of Blimp-1 in B cells is modulated by the transcriptional activity of numerous upstream transcriptional activators, including AP-1, NFκB, and STAT3 (Calame 2008; Kwon *et al.* 2009). In CH12.LX cells, LPS-induced DNA binding and transcriptional activity of AP-1 (c-Jun), but not NFκB, was reduced by TCDD (Suh *et al.* 2002). Further characterization using the same cell line model revealed that AP-1 binding to the promoter of *Blimp-1* induced by LPS was also suppressed by TCDD, consistent with attenuated Blimp-1 induction upon TCDD treatment in LPS-stimulated CH12.LX cells, primary mouse B cells, and live animals (North *et al.* 2009a; Schneider *et al.* 2009). This series of studies have illustrated the effectiveness of a step-wise approach, and led to the overall hypothesis that TCDD disrupts the plasmacytic differentiation of mouse B cells by deregulating a "B cell plasmacytic differentiation program" that involves coordinated functions of critical regulators AP-1, Pax5, and Blimp-1.

B. TCDD-mediated disruption of B cell early signaling

The potential effect of TCDD on early signaling of B cells was proposed based on results from time of addition studies (Holsapple *et al.* 1986a; Tucker *et al.* 1986), in which the suppression of T cell-independent antibody response induced by LPS and the T cell-dependent antibody response against sRBC only occurred if TCDD was present during the initial 3 h and 24 h, respectively. Similarly, if added 24 h post-LPS activation, TCDD had no effect on IgM secretion from the highly sensitive CH12.LX cells (Crawford *et al.* 2003). Collectively, these results support the existence of a critical window of sensitivity by which TCDD alters early events integral to the activation of B cells, a critical step preceding the IgM reseponse. Subsequent studies focused on B cell activation demonstrated that protein kinase activity, as indicated by global protein phosphorylation, was enhanced by TCDD in isolated mouse B cells (Kramer *et al.* 1987; Snyder *et al.* 1993). TCDD also elevated basal levels of intracellular Ca^{2+} in resting B cells, suggesting the potential of TCDD to interfere with calcium signaling, a required event following B cell activation (Karras *et al.* 1996). Another line of evidence comes from the finding that TCDD suppression of IgM responses induced by LPS or sRBC *in vitro* could be reversed by interferon gamma (IFN-γ) (North *et al.* 2009b; Snyder *et al.* 1993). Interestingly, such reversal by IFN-γ only occurred when added within 2 h post-TCDD treatment, further suggestive of the possibility that TCDD may disrupt early signaling in B cells (North *et al.* 2009b).

Despite numerous research efforts, the direct targets of TCDD during the disruption of B cell activation, and how the link between the B cell activation and suppression of the plasmacytic differentiation remains elusive. A critical stage that

bridges B cell activation with plasmacytic differentiation is proliferation that immediately follows cell activation. Although previous studies found TCDD had little effect on B cell proliferation at concentrations that suppressed antibody responses, TCDD has been demonstrated to alter a critical regulator involved in cell cycle control, specifically the mRNA levels of the cyclin-dependent kinase inhibitor protein, p27kip1, in LPS-activated CH12.LX cells, which may explain a modest decrease in their proliferation (Crawford *et al.* 2003). Another cellular target affected by TCDD during B cell activation is suppressor of cytokine signaling 2 (Socs2), a negative regulator of signaling downstream of various cytokines, including IFN-γ. TCDD induced Socs2 mRNA and protein expression in CH12.LX cells within 4 h post-treatment (Boverhof *et al.* 2004). Very recent studies demonstrated that the toll-like receptor-induced activation (as indicated by phosphorylation) of AKT, ERK, and JNK was impaired by TCDD in CH12.LX cells and primary mouse B cells (North *et al.* 2010). Given the very rapid induction of early signaling events following B cell activation, they may precede AHR binding to DRE and AHR-mediated gene regulation. The perturbation of B cell signaling by TCDD, although not yet linked directly to suppression of plasmacytic differentiation, may lead to the hypothesis that TCDD affects B cell effector function (i.e., antibody production) through both DRE-dependent and –independent mechanisms.

C. Sensitivity of human B cells to TCDD

Compared to the large amount of data accumulated using mouse B cells, little is known about the effect of TCDD on human B cell function. This represents a critical data gap, especially in light of the concerns raised in the context of data extrapolation from mouse to human and the recognition that immunotoxicology investigations conducted

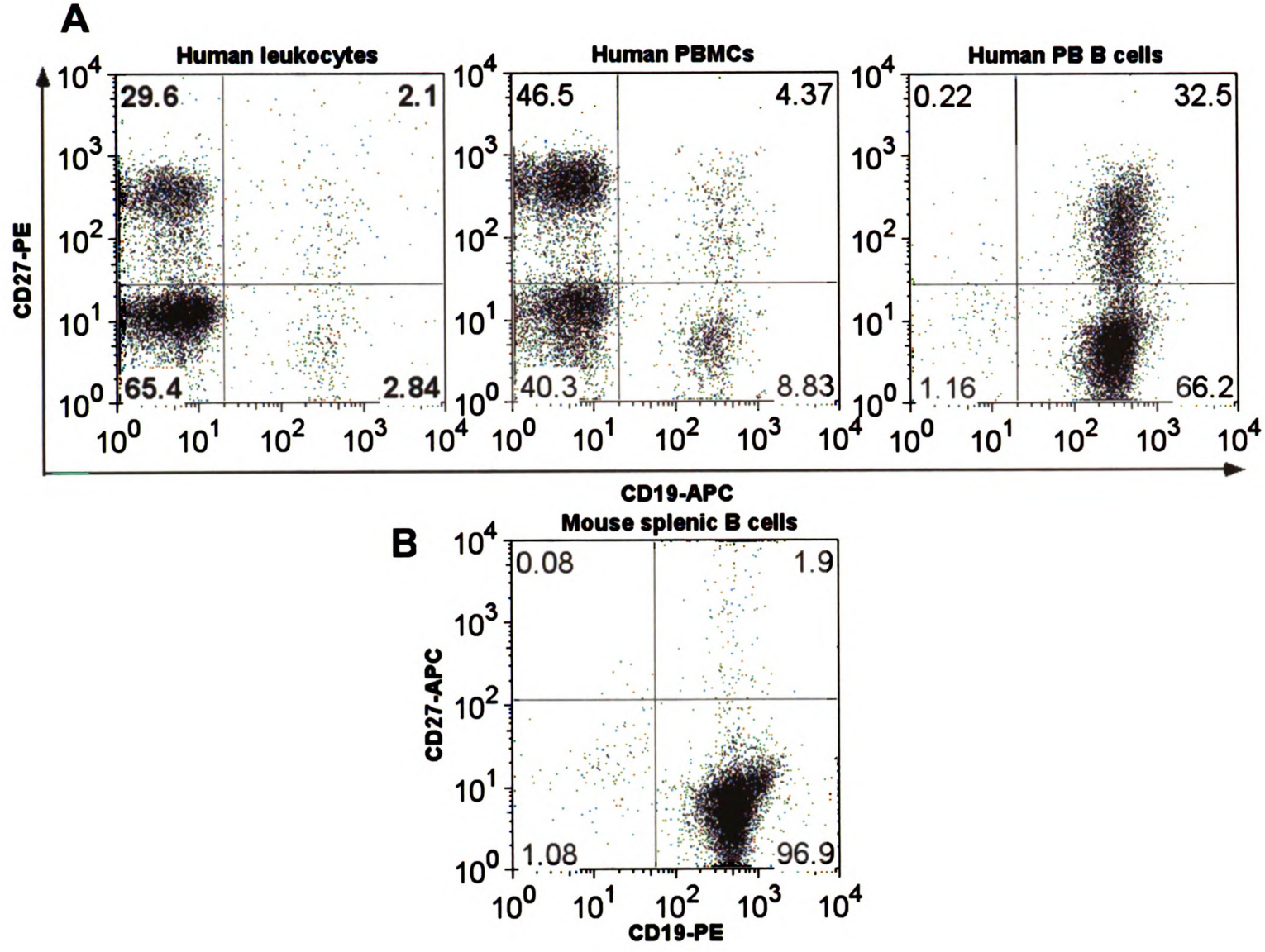
exclusively using animal models may not always be predictive of human toxicity (Selgrade 1999; Vos and Van Loveren 1998). The characterization of TCDD effects on human B cells was initiated by Holsapple *et. al.*, using human tonsillar lymphocytes and B cells as biological models (Wood and Holsapple 1993; Wood *et al.* 1993; Wood *et al.* 1992). The initial studies found TCDD suppressed background, but not pokeweed mitogen-stimulated, proliferation and IgM secretion of human tonsillar lymphocytes (Wood *et al.* 1993; Wood *et al.* 1992). Further studies showed IgG secretion stimulated by LPS plus T cell-replacing factors and IgM secretion stimulated by superantigen toxic shock syndrome toxin (TSST-1) in human B cells were also sensitive to suppression by TCDD (Wood and Holsapple 1993; Wood *et al.* 1993). These studies significantly advanced the field by demonstrating for the first time that exposure to TCDD could affect the function of mature human B cells *in vitro*, an observation suggested by epidemiological studies that provided indirect evidence that TCDD exposure may affect the humoral immunity in humans. However, current data concerning the effects of TCDD on human B cells remain very limited, preventing a comprehensive evaluation of health risk in humans posed by TCDD exposure toward humoral immunity. Even less data are available to understand the mechanisms by which TCDD modulates human B cell functions. AHR was detected in both human primary B cells and B cell lines, and mediated a functional AHR pathway that was induced by exogenous ligands to bind to DRE (Lorenzen and Okey 1991; Masten and Shiverick 1996; Waithe *et al.* 1991). Later studies found AHR up-regulated in the absence of exogenous ligands in activated B cells (Allan and Sherr 2005). In a human B cell line, AHR was also reported to recognize and compete for a Pax5 binding site in the regulatory region of the *CD19* gene, potentially

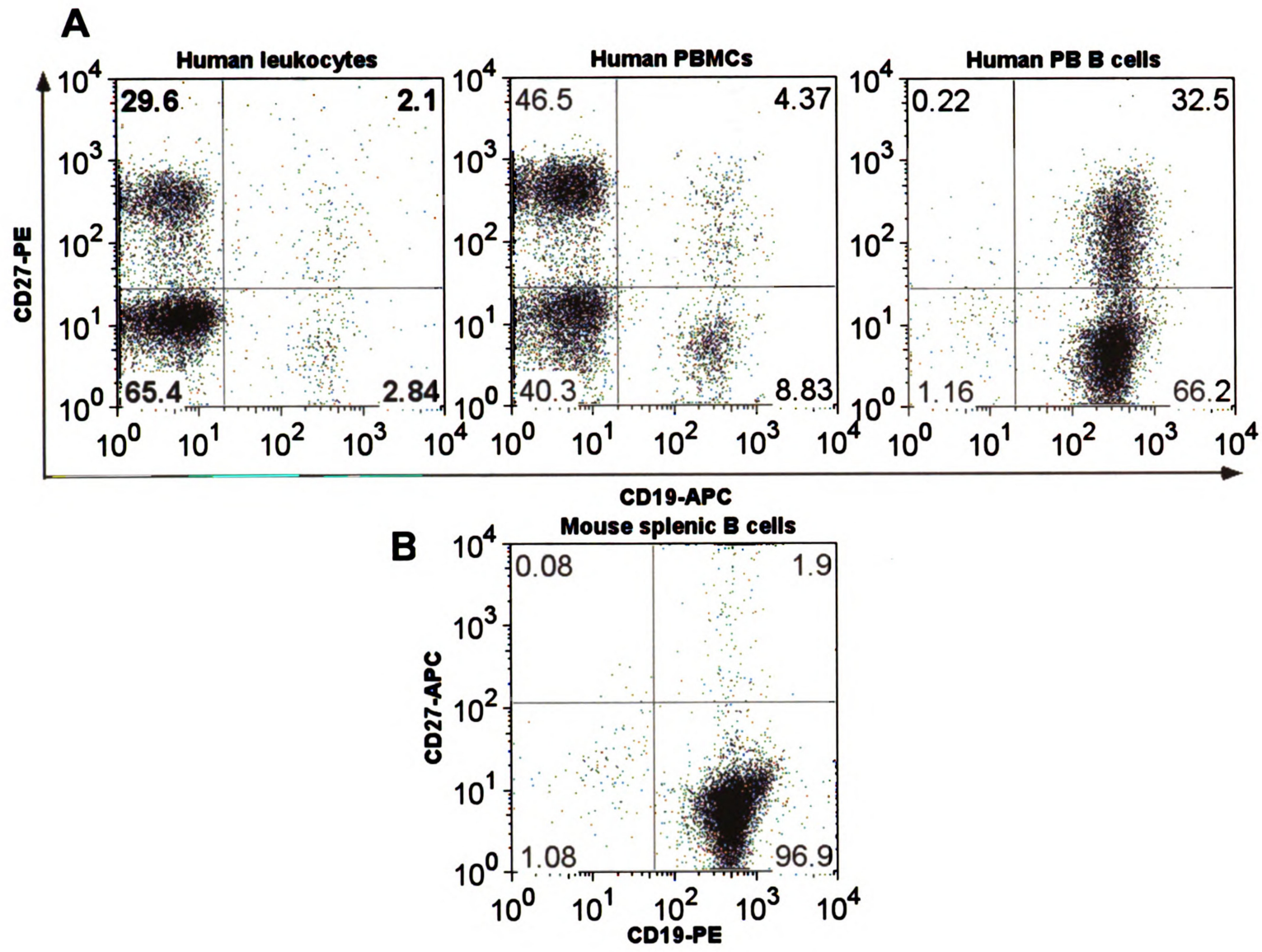
contributing to the decrease of CD19 mRNA expression upon TCDD treatment (Masten and Shiverick 1995). This study represented the first line of evidence that TCDD may alter human B cell function by directly acting through an AHR/DRE paradigm to impair a transcription factor that regulates plasmacytic differentiation. The relevance of this observation to antibody production remains to be investigated.

To date, few studies have assessed the effect of TCDD on human B cell effector functions, and even less investigated in human B cells whether TCDD caused perturbation of plasmacytic differentiation, which has been clearly demonstrated in mouse B cells both *in vitro* and *in vivo*. Historically, the mouse has represented the animal model of choice for immunologists, and the mouse immune system has been the most extensively characterized of any species. Therefore, not surprisingly, the mouse has also been embraced as the primary model for immunotoxicology studies. One critically important assumption for the use of mouse data in human risk assessment is that the mouse and human immune systems are sufficiently similar that xenobiotic actions in the mouse are predictive of effects in the human immune system. However, recent advances in basic immunology suggest many differences exist between mouse and human immune systems at numerous levels, spanning the molecular regulation of effector function to leukocyte composition and distribution (Davis 2008; Mestas and Hughes 2004). It has been realized that when studying signaling pathways or regulation mechanisms, findings in mice can not be readily extrapolated to human leukocytes, including those involving B cells (Schmidlin *et al.* 2009). The unique characteristics of mouse and human immune systems, in addition to the well-documented species differences in sensitivity to TCDD, advocate strongly for extending immunotoxicity studies into human cell models.

Specifically for studying B cell effector function, with technical advancements and a more in-depth understanding of basic biology, *in vitro* activation of primary human B cells to mimic the plasmacytic differentiation pathways has become feasible. One way to activate human B cells to differentiate into antibody secreting plasma cells is the application of CD40L, a molecule critical to T cell-dependent B cell antibody response as described earlier. Both CD40L and CD40 are highly conserved between human and mouse, and human CD40L was found to be interchangeably bio-active in human and mouse B cells (Spriggs *et al.* 1992). Therefore, CD40L may be a valuable tool to study the effect of TCDD on the plasmacytic differentiation of both human and mouse B cells following similar activation.

Figure 2. Phenotypes of human peripheral blood B cells and mouse splenic B cells. (A) Human or (B) Mouse B cells were isolated from peripheral blood mononuclear cells or spleen cells, respectively. Peripheral blood mononuclear cells were enriched from human leukocyte packs by gradient centrifugation using Ficoll. B cell isolation was conducted using MACS B cell isolation kits specific for human or mouse. Cells were assessed using flow cytometry for surface expression of CD19 and CD27 immediately following B cell isolation. Results depicted are fluorescent signals for CD19 and CD27. Percentages of total cells within each quadrant are shown in the corners of each individual plot. These data are from one experiment that is representative of experiments with cells from three human donors and two identical experiments with mouse cells.


A
Human leukocytes
Human PBMCs
Human PB B cells
29.6
2.1
65.4
2.84
46.5
4.37
40.3
8.83
0.22
32.5
1.16
66.2
CD27-PE
CD19-APC
B
Mouse splenic B cells
0.08
1.9
1.08
96.9
CD27-APC
CD19-PE


A
Human leukocytes
Human PBMCs
Human PB B cells
CD27-PE
CD19-APC
29.6
2.1
65.4
2.84
46.5
4.37
40.3
8.83
0.22
32.5
1.16
66.2
B
Mouse splenic B cells
CD27-APC
CD19-PE
0.08
1.9
1.08
96.9

MATERIALS AND METHODS

I. Chemicals

TCDD (99.1% pure) was purchased from Accustandard Inc. (New Haven, CT) as a solution in 100% dimethyl sulfoxide (DMSO). Sodium (meta)arsenite was purchased from Sigma-Aldrich (St Louis, MO). Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) was purchased from National Cancer Institute's Chemical Carcinogen Reference Standards Repository (Kansas City, MO). DMSO was purchased from Sigma-Aldrich.

II. Animals

Virus-free, female C57BL/6 mice (6 weeks of age) were purchased from Charles River (Portage, MI). On arrival, mice were randomized, transferred to plastic cages containing sawdust bedding (five mice per cage), and quarantined for 1 week. Mice were provided food (Purina certified laboratory chow) and water *ad libitum* and were not used for experimentation until their body weight was 17 - 20g. Animal holding rooms were kept at 21 - 24°C and 40 - 60% humidity with a 12-h light/dark cycle. Mice were used in accordance with guidelines set forth by the Michigan State University Institutional Animal Care and Use Committee.

III. Human leukocyte packs

Human leukocyte packs collected from anonymous donors were purchased from the Gulf Coast Regional Blood Center (Houston, TX), and shipped overnight. All donors were screened for HIV and hepatitis at the blood center.

IV. Isolation and culture of human and mouse B cells

Human $CD19^+$ (total) and/or $CD19^+CD27^-$ (naive) B cells were isolated from peripheral blood mononuclear cells enriched from each leukocyte pack by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ). Mouse B cells were isolated from spleen preparations that were made into single-cell suspensions by passage through a 40 μm cell strainer (BD Biosciences, San Jose, CA). Negative selection of human or mouse B cells was conducted using MACS Human B cell, Naive B cell, or Mouse B Cell Isolation Kit following the manufacturer's protocols (Miltenyi Biotec, Auburn, CA) and as described previously (Lu *et al.* 2009; Schneider *et al.* 2008). In brief, human peripheral blood mononuclear cells or mouse spleen cells were first subjected to incubation with a cocktail of biotin-conjugated antibodies specific for all the non-B cell subtypes (i.e., granulocytes, T cells, natural killer cells, dendritic cells, monocytes, macrophages, erythroid cells, etc.), followed by additional incubation with anti-biotin antibodies conjugated to magnetic beads. In all steps, cells were maintained in phosphate buffered saline (PBS) at pH 7.2 containing 0.5% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid (EDTA) and kept at 4 °C. Typically, $2 - 6 \times 10^7$ naive B cells or $6 - 10 \times 10^7$ total B cells were obtained from each human leukocyte pack, and $3 - 4 \times 10^7$ B cells were obtained from each mouse spleen. The purity of isolated B cells was assessed using flow cytometry by enumerating the percentage of $CD19^+$ or $CD19^+CD27^-$ cells. In all cases, the purity of isolated B cells was routinely found $\geq 95\%$. Approximately 60% of isolated human peripheral blood total B cells were $CD27^-$ (naive),

while ≥ 98% of mouse splenic B cells were naive (Figure 2). Isolated human or mouse B cells were cultured in Iscove's Modified Dulbecco's medium (Invitrogen) supplemented with 10% human AB serum (Invitrogen) or Roswell Park Memorial Institute-1640 (RPMI) medium (Invitrogen) supplemented with 10% heat-inactivated bovine calf serum (HyClone, Logan, UT), 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 50 μM of 2-mercaptoethanol. In all cases cells were cultured at 37°C in 5% CO_2.

V. Cell line

The stably-transfected mouse fibroblast line expressing human CD40L (CD40L-L cell) was a generous gift from Dr. David Sherr (Boston University School of Public Health). CD40L-L cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% bovine calf serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 50 μM of 2-mercaptoethanol, and 1x HT supplement (Invitrogen).

VI. Gene expression analysis

Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. RNA was reverse-transcribed into cDNA using the High Capacity Archive cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The expression of target genes was determined by TaqMan real-time PCR using ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Relative steady state mRNA levels of the target genes were calculated and normalized to the endogenous reference, 18S ribosomal RNA using

the ΔΔCT method (Jan *et al.* 2002). All primers were purchased from Applied Biosystems and listed in Appendix A. Probe for the 18S ribosomal RNA primer is VIC-MGB, while probe for all the other primers is FAM-MGB.

VII. Enzyme-linked immunospot (ELISPOT) assay

ELISPOT assay was performed using MultiScreen-HA filter plates (Millpore, Billerica, MA). ELISPOT wells were coated with purified anti-human (BD Biosciences, San Jose, CA) or mouse (Sigma-Aldrich, St. Louis, MO) IgM antibody overnight at 4°C. Plates were washed with PBS containing 0.1% Tween-20 (Sigma-Aldrich) and water, and then blocked with PBS containing 5% bovine serum albumin for 30 min at 37°C. Cells were harvested, washed, diluted to appropriate densities, and incubated in the ELISPOT wells in regular culture medium for 16 - 20 h at 37°C in 5% CO_2. Prior to the incubation, viability of the cells was assessed by the pronase activity assay. In brief, cells were incubated with pronase that lysed the dead cells. Cells were then counted on a particle counter and the density was adjusted accordingly prior to incubation in the ELISPOT wells. Cells were removed from the wells, and plates were washed as described above and incubated with biotin-conjugated anti-human or mouse IgM antibody (Sigma-Aldrich) for 1.5 h at 37°C. Plates were washed as described above and incubated with streptavidin-horseradish peroxidase (Sigma-Aldrich) for 1.5 h at 37°C. Plates were washed as described above, and spots were developed using the aminoethylcarbazole Staining Kit (Sigma-Aldrich). Data were collected and analyzed using the CTL ImmunoSpot system (Cellular Technology Ltd., Shaker Heights, OH).

VIII. Flow cytometry (FCM) analysis

The antibodies used in FCM analysis were listed in Appendix B. When necessary, Live/Dead Fixable Dead Cell Stain Kit (red or near-infrared dye, Invitrogen) was used to stain dead cells in a protein-free buffer (1 x Hank's Balanced Salt Solution (HBSS; Invitrogen), pH 7.4) per the manufacturer's protocol prior to all the other staining steps. Surface Fcγ receptors was blocked by incubating mouse cells with anti-mouse CD16/CD32 or human B cells with human AB serum (Invitrogen), prior to staining of surface and intracellular antigens. In all cases, cells were kept on ice in all steps. If not assessed on the same day, cells were frozen in bovine calf serum containing 10% DMSO at -20°C or -80°C until FCM analysis. Staining for intracellular antigens was typically conducted on the same day followed immediately by FCM analysis. When antibodies conjugated to tandem dyes (i.e., PE/Cy5 and PE/Cy7) were used, staining was conducted in the dark. The amount of antibodies used varied in staining of each specific antigen and required preliminary experiments, including antibody titration. In all cases, cells were assessed on a FACS Calibur or a FACS CantoII cell analyzer (BD Biosciences) and analyzed using FlowJo (Tree Star, Ashland, OR) or Kaluza (Bechman Coulter, Miami, FL) software.

Staining of surface antigens was conducted in single cell suspension in FCM buffer (HBSS (Invitrogen) containing 1% bovine serum albumin (BSA, Calbiochem) and 0.1% sodium azide (Sigma), pH 7.6). When multiple antigens were stained simultaneously, all the antibodies were typically pre-diluted in FCM buffer at appropriate amounts prior to addition to the cells. Staining was conducted by incubation with

antibodies for 30 min. Cells were then washed with FCM buffer, and fixed by incubation in the CytoFix fixation buffer (BD Biosciences) for 15 min.

For staining of intracellular IgM, cells were incubated with anti-mouse or human IgM not conjugated to any fluorophore to block surface IgM. Cells were permeablized by incubation in the Perm/Wash buffer (BD Biosciences) for 30 – 60 min, and then stained in the same solution. For staining of intracellular transcription factors, cells were permeablized by incubation in FCM buffer containing 0.5% saponin (Calbiochem, San Diego, CA) for 30 – 60 min, and then stained in the same solution. For studies of cell proliferation, isolated human or mouse B cells were labeled by incubation with 5 μM of carboxyfluorescein succinimidyl ester (CFSE) or Violet dye (CellTrace Cell Proliferation Kits, Invitrogen) at the density of 5×10^6 cell/ml following manufacturer's protocols. The labeled cells were washed in complete culture medium, and then adjusted to the desired cell density prior to culture. For intracellular staining of phosphorylated antigens, isolated B cells were equilibrated in the 37°C water bath for 2 – 4 h prior to experimentation. Cells were treated with TCDD and the activating stimuli, and then returned to the water bath to incubate for appropriate time periods. If cells were stimulated for longer than 60 min, the typical cell culture conditions were applied in the incubator at 37°C with 5% CO_2. Cells were fixed by adding 1.5 – 3 % paraformaldehyde, diluted directly in cell culture from the 32% stock (electron microscopy grade, Electron Microscopy Sciences, Hartfield, PA). Cells were incubated in 37°C for 15 min and then pelleted down. To permeablize the cells, ice cold 99.9% methanol was added drop-wise to the cell pellet while vortexing at medium speed. Permeablization was completed by

further incubation in methanol at 4°C for 15 min. Cells were stored in methanol at -80°C until staining with antibodies specific for phosphorylated epitopes.

IX. Statistical analysis

Graphpad Prism 4.00 (Graphpad Software, San Diego, CA) was employed for all statistical analysis. The mean ± S.E.M. was determined for each treatment group in the individual experiments, and error bars indicate the S.E.M. of each group. Homogeneous data were evaluated by one-way analysis of variance (ANOVA), and Dunnett's two-tailed *t*-test was used to compare treatment groups with the VH control when significant differences ($p < 0.05$) were observed. When two variables were involved (e.g., different time points vs. treatment groups), two-way ANOVA was used to assess the significance of differences among groups. For data expressed as either fold-change or percent, logarithmic transformation was conducted prior to statistical analysis as described previously (Kaplan *et al.* 2010). IC_{50} values were calculated from non-linear regression of data using the sigmoidal concentration response curve.

EXPERIMENTAL RESULTS

I. Establishment of the CD40L-depedent IgM response model

A. CD40L-induced IgM responses from primary human and mouse B cells

CD40L in combination with T cell-replacing cytokines induce the differentiation of primary human B cells into either memory B cells or antibody secreting cells. The initial series of studies aimed to establish and optimize the CD40L-induced IgM responses in parallel for mouse and human primary B cells. The general strategy was to establish B cell activation and culture conditions as similar as possible to facilitate comparisons of xenobiotic modulation between the two species. The magnitude of the IgM response was measured by enumerating IgM secreting cells by ELISPOT. Due to the limited number of B cells that can be obtained from each human leukocyte pack, a 96-well format was adopted as the primary experimental platform for human B cells. Human CD40L was utilized to activate both mouse and human B cells since it is highly conserved between the two species (Spriggs *et al.* 1992). Cell surface-expressed CD40L was selected over recombinant CD40L as it is membrane-bound, and hence more closely mimics the CD40L expression by activated T cells. In preliminary studies, we also observed greater consistency in inducing IgM responses using CD40L-L cells when compared to recombinant CD40L with both mouse and human B cells (data not shown). Prior to co-culture with B cells, CD40L-L cells were gamma ray irradiated to prevent proliferation. This CD40L-dependent model includes two culture phases, the first is in the presence of CD40L-L cells (4 d for human, 3 d for mouse), while the second is in the absence of CD40L-L cells (3 d for both). These assay conditions are based on previous

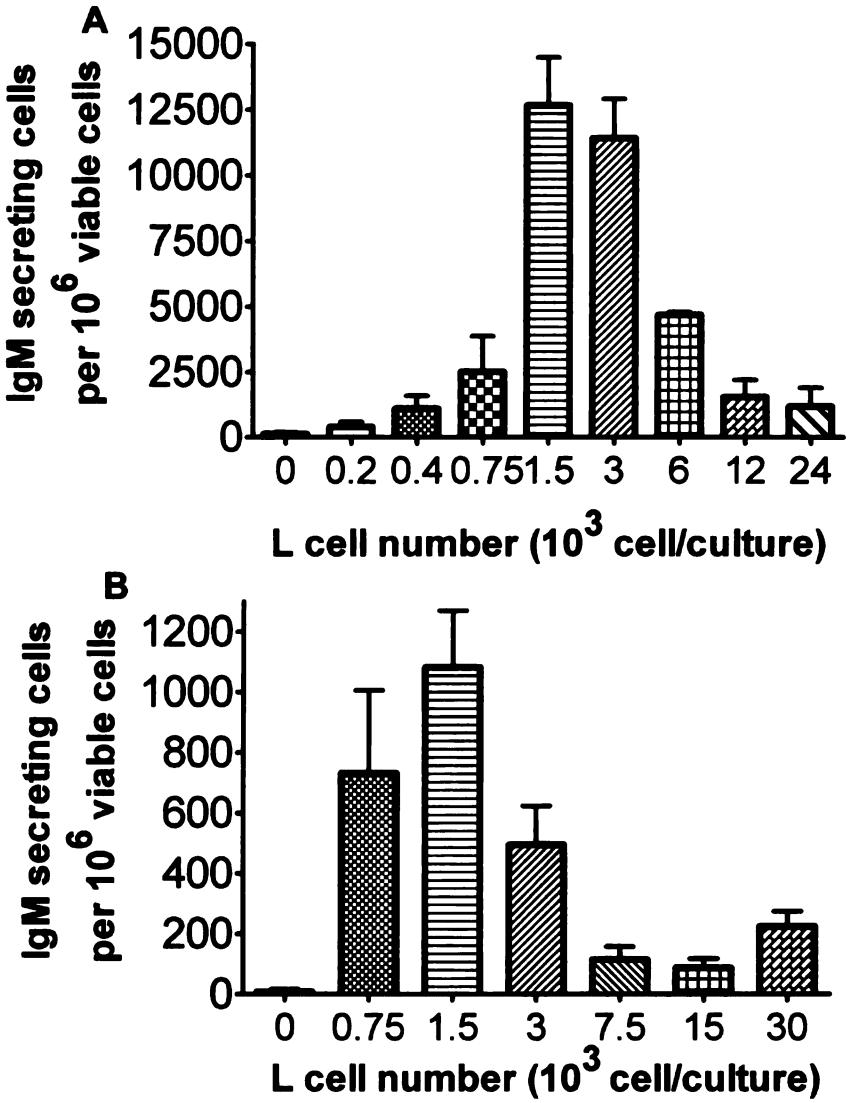


Figure 3. Effect of CD40L stimulation level on the human B cell IgM response. (A) Total or (B) naive human B cells (1.5 x 10^5 cell/culture) were co-cultured with irradiated CD40L-L cells at indicated numbers per culture in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 4 d, and further cultured for additional 3 d without CD40L-L cells. In both (A) and (B), cells were harvested on Day 7 to enumerate IgM secreting cells by ELISPOT. Cell number was determined by a particle counter, and cell viability was assessed using the pronase activity assay. These data are from two experiments, each of which is representative of two separate experiments with at least four replicates per group. Data in panel A and panel B were obtained using total and naive B cells, respectively, from two different donors in two separate experiments. Data are presented as mean ± S.E.M. from four experimental replicates per group.

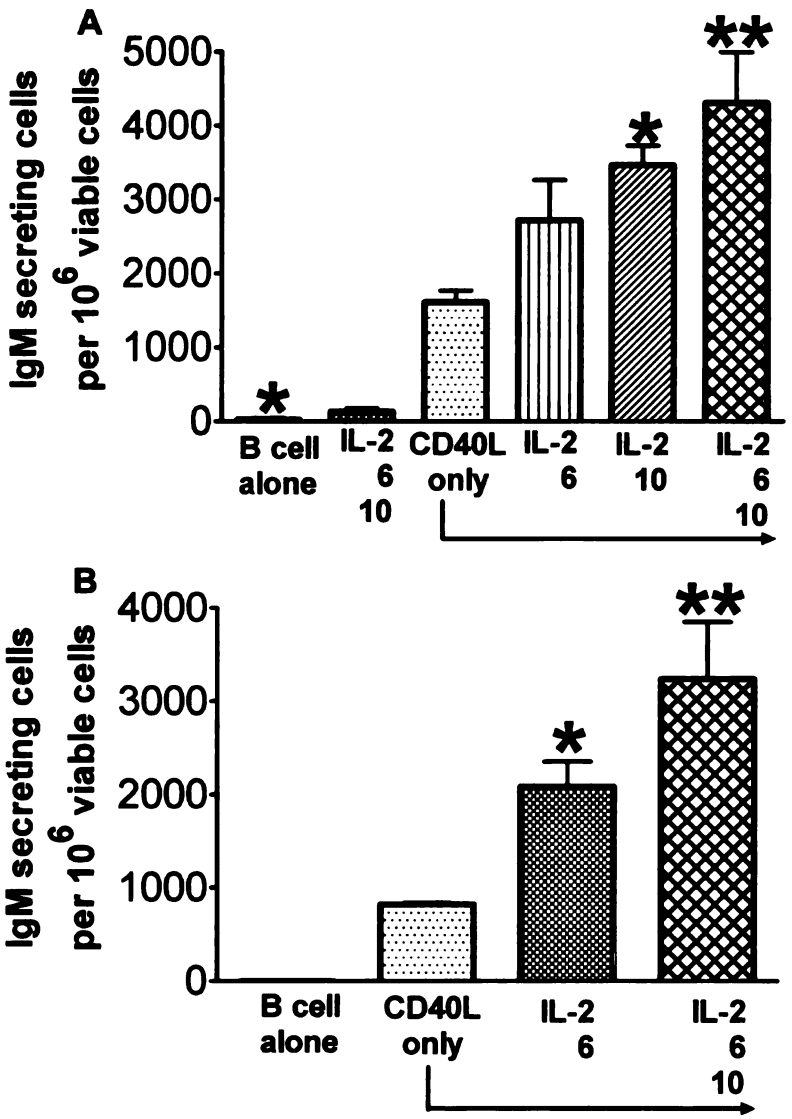


Figure 4. Effect of cytokines on the human B cell IgM response. (A) Total or (B) naive human B cells (1.5×10^5 cell/culture) were co-cultured with irradiated CD40L-L cells (3×10^3 cell/culture) for 4 d, and further cultured for additional 3 d without CD40L-L cells. Cytokines were added as indicated to each individual group (IL-2: 10 U/ml; IL-6: 100 U/ml; IL-10: 20 ng/ml). Cells were harvested on Day 7 to enumerate IgM secreting cells by ELISPOT. Cell number was determined using a particle counter, and cell viability was assessed using the pronase activity assay. *, $p < 0.05$, **, $p < 0.01$, compared with CD40L-L cell only group. These data are from two experiments, each of which is representative of two separate experiments (B cells from two donors) with at least four replicates per group. Data are presented as mean ± S.E.M. from four experimental replicates per group.

findings from our laboratory, showing that when human B cells were cultured with CD40L-L cells for the duration of the culture period, the IgM response was significantly decreased. This is also consistent with a previous finding that showed extended exposure to CD40L drives more B cells to become memory cells rather than antibody secreting cells (Arpin *et al.* 1995). Preliminary kinetic studies suggested that, at least for human B cells, extension of either culture phase (to 5 d) did not increase the magnitude of response. Under our experimental conditions, 1.5 – 3 x 10^3 CD40L-L cell/culture induced the strongest IgM responses in human total B cells, while 1.5 x 10^3 CD40L-L cell/culture led to the strongest response in naive B cells (Figure 3). Different combinations of cytokines have been previously reported to induce antibody responses using human B cells (Armitage *et al.* 1993; Arpin *et al.* 1995; Ettinger *et al.* 2005; Kindler and Zubler 1997). In our studies, recombinant human IL-2, IL-6, and IL-10, when combined with CD40L stimulation, induced a robust IgM response in both naive and total human B cells (Figure 4). The mouse CD40L-dependent model was established using a similar approach (data not shown). Although addition of IL-10 did not further enhance the magnitude of mouse IgM response (data not shown), it was still included to maintain similar culture conditions for both human and mouse B cells. The CD40L-dependent B cell IgM response model is illustrated in Figure 5.

To determine the inherent variability in the responsiveness of human B cells to the CD40L induced IgM response, naive and total human B cells from multiple human donors were assessed. To date, relatively consistent IgM responses in naive B cells from 22 human donors and total human B cells from 12 human donors have been observed using the optimized CD40L activation conditions (Figure 6). When both naive and total

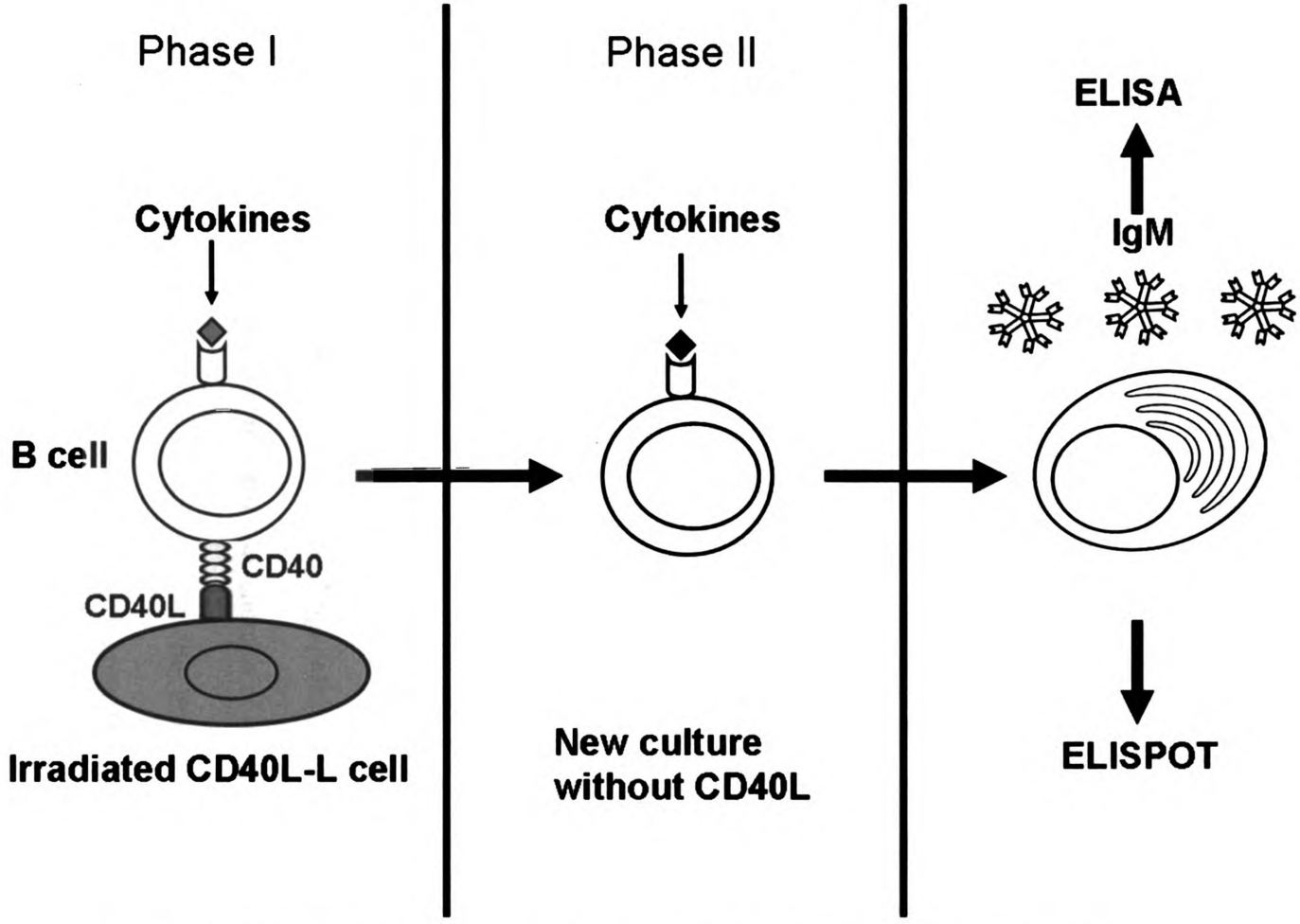


Figure 5. The CD40L-dependent activation model for human and mouse B cells.

B cells from the same donor were assayed, the naive B cells yielded lower overall IgM responses than observed using total B cells in all donors. This observation is consistent with previous studies in which naive B cells were found to be less responsive than memory B cells to CD40L-induced differentiation into antibody secreting cells (Kindler and Zubler 1997; Neron *et al.* 2005). It is important to emphasize that in future investigations, only naive human B cells, as assessed by $CD27^-$, were used rather than the entire peripheral blood B cell pool. The rationale for excluding memory B cells is that greater than 95% of splenic B cells in C57BL/6 mice possess a phenotype that is close to naive human peripheral blood B cells ($CD27^-$) (Figure 2). Thus, naive human B cells were used exclusively in order to make meaningful comparisons between mouse and human B cells.

B. CD40L-induced proliferation and phenotypic changes during B cell IgM response

Plasmacytic differentiation of B cells into antibody secreting cells is preceded by two critical events: activation and proliferation. Engagement of CD40 triggers the activation and proliferation of B cells *in vitro (*Banchereau *et al.* 1994*)*. Kinetic studies were conducted using flow cytometry to investigate the proliferative responses and expression of activation markers in mouse and naive human B cells induced using the CD40L activation model. CFSE labeling was used to examine the proliferation of B cells. As shown in Figure 7, CD40L in combination with IL-2, IL-6, and IL-10 induced cell divisions of both human B cells and mouse B cells, as indicated by the dilution of CFSE signal. By Day 5, the majority of viable human B cells had gone through cell divisions.

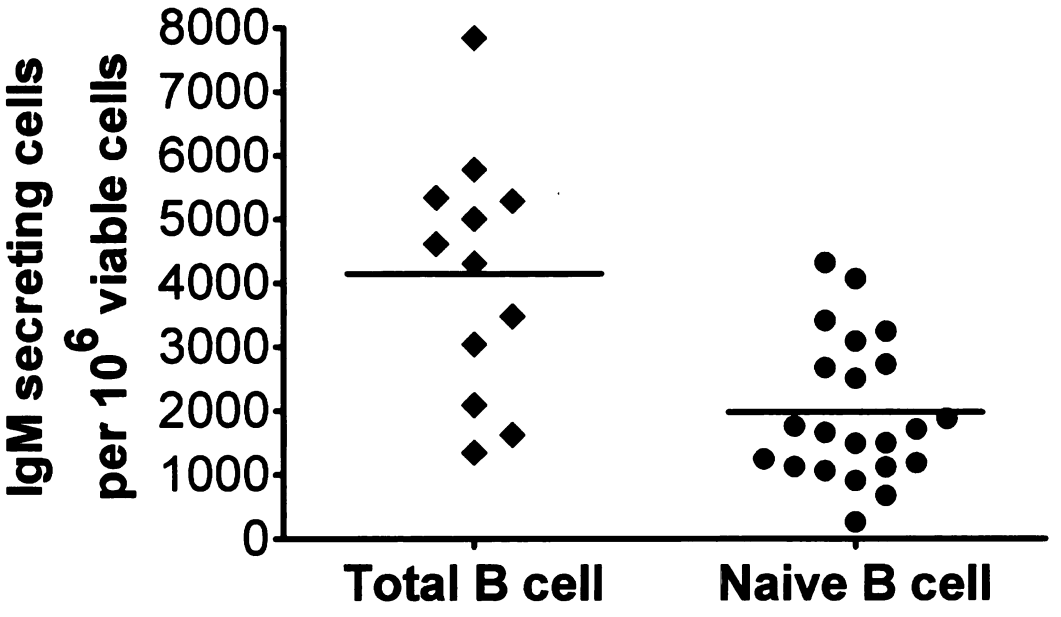


Figure 6. Donor-to-donor variability in the CD40L-induced IgM response. Total or naive human B cells were co-cultured with irradiated CD40L-L cells (1.5 or 3 x 10^3 cell/culture) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 4 d, and further cultured for additional 3 d without CD40L-L cells. Cells were harvested on Day 7 to enumerate IgM secreting cells by ELISPOT. Cell number was determined using a particle counter, and cell viability was assessed using the pronase activity assay. Total B cells from 12 donors and naive B cells from 22 donors were assessed.

Figure 7. CD40L-induced proliferation of human and mouse B cells. (A) Naive human B cells or (B) mouse B cells were labeled with CFSE and then co-cultured at 1.5 x 10^5 cell/culture (human) or 1.5 x 10^6 cell/culture (mouse) with irradiated CD40L-L cells (human: 1.5 x 10^3 cell/culture; mouse: 5 x 10^4 cell/culture) in the presence of recombinant human or mouse IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for up to 5 d, and CD40L stimulation was removed on Day 4 (human) or Day 3 (mouse). Cells were harvested at the indicated time points and assessed by flow cytometry for the fluorescence of CFSE. Dead cells were identified by staining with Live/Dead near-infrared (mouse) or red (human) Staining Kit, and are not included in this analysis. These data are from one experiment that is representative of two separate experiments (for human data, each separate experiment used B cells from one individual donor) with three replicates per time point. Results shown are concatenated from three experimental replicates included one separate experiment.

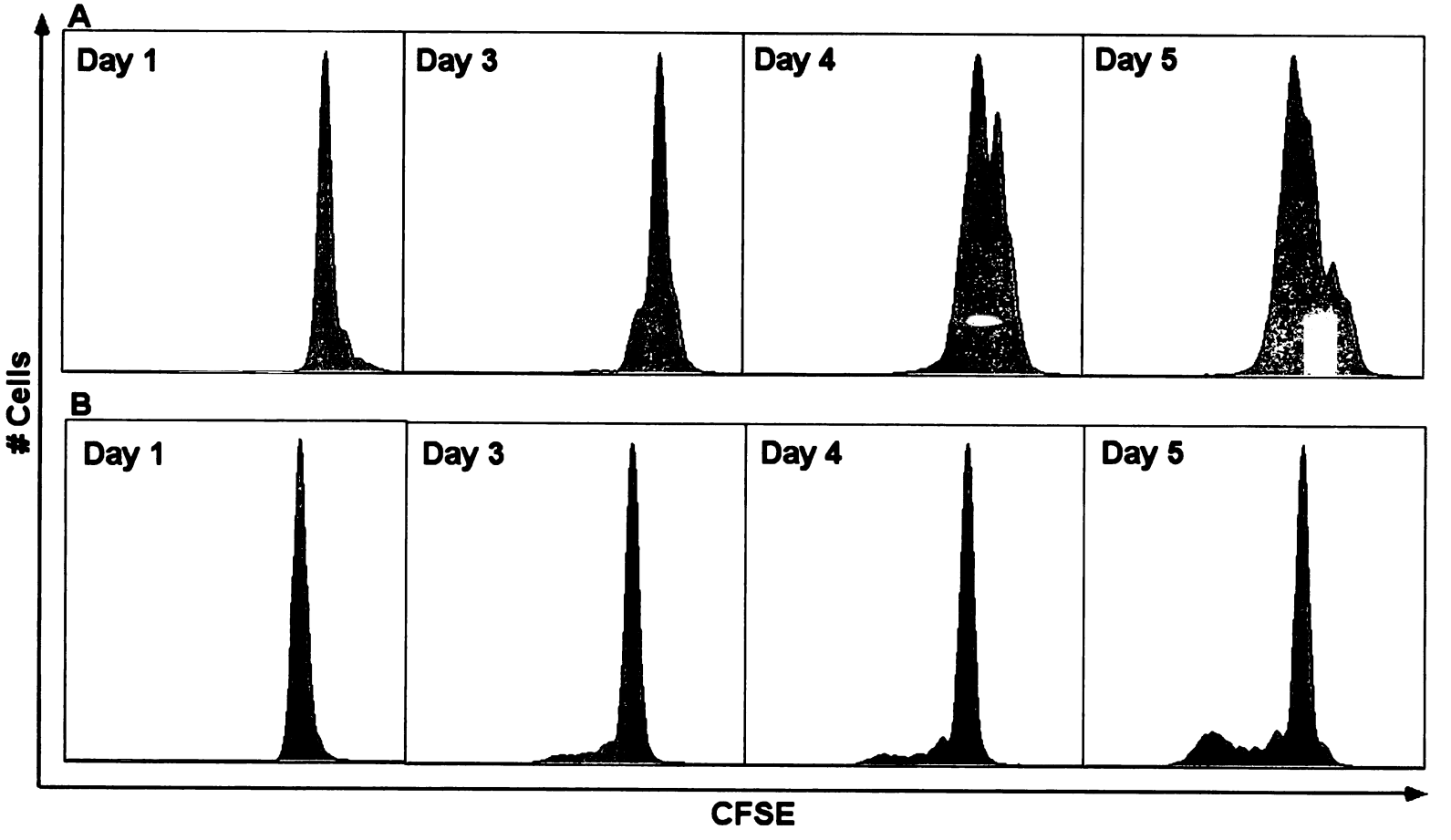
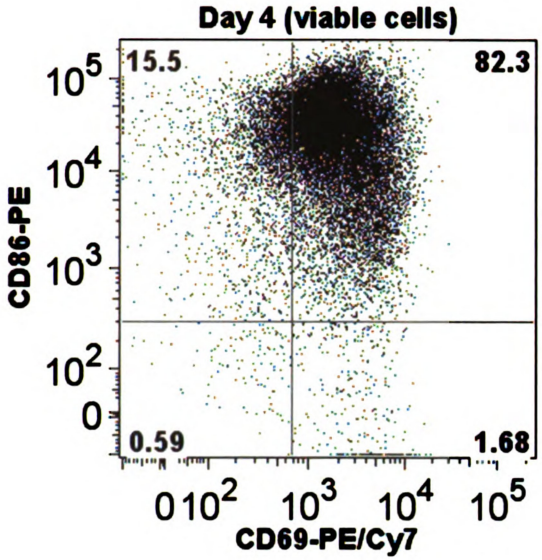

A
Day 1
Day 3
Day 4
Day 5
B
Day 1
Day 3
Day 4
Day 5
Cells
CFSE

Figure 8. CD40L-induced activated phenotype in human B cells. Naive human B cells (1.5×10^5 cell/culture) were co-cultured with irradiated CD40L-L cells (1.5×10^3 cell/culture) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for up to 6 d (Day 6 data not shown), and CD40L stimulation was removed on Day 4. Cells were harvested at indicated time points and assessed by flow cytometry for surface expression of CD86 and CD69. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit. Results depicted are fluorescent signals for CD86, CD69, and the Live/Dead stain. Total cells, both viable and non-viable, are included in the dot plots unless indicated otherwise (the very left plot). Percentages of cells within each quadrant are shown in the corners of each individual plot. These data are from one experiment that is representative of two separate experiments (for human data, each separate experiment used B cells from one individual donor) with three replicates per time point. Results shown are concatenated from three experimental replicates included one separate experiment.


Day 4 (viable cells)
15.5
82.3
0.59
1.68
CD86-PE
CD69-PE/Cy7

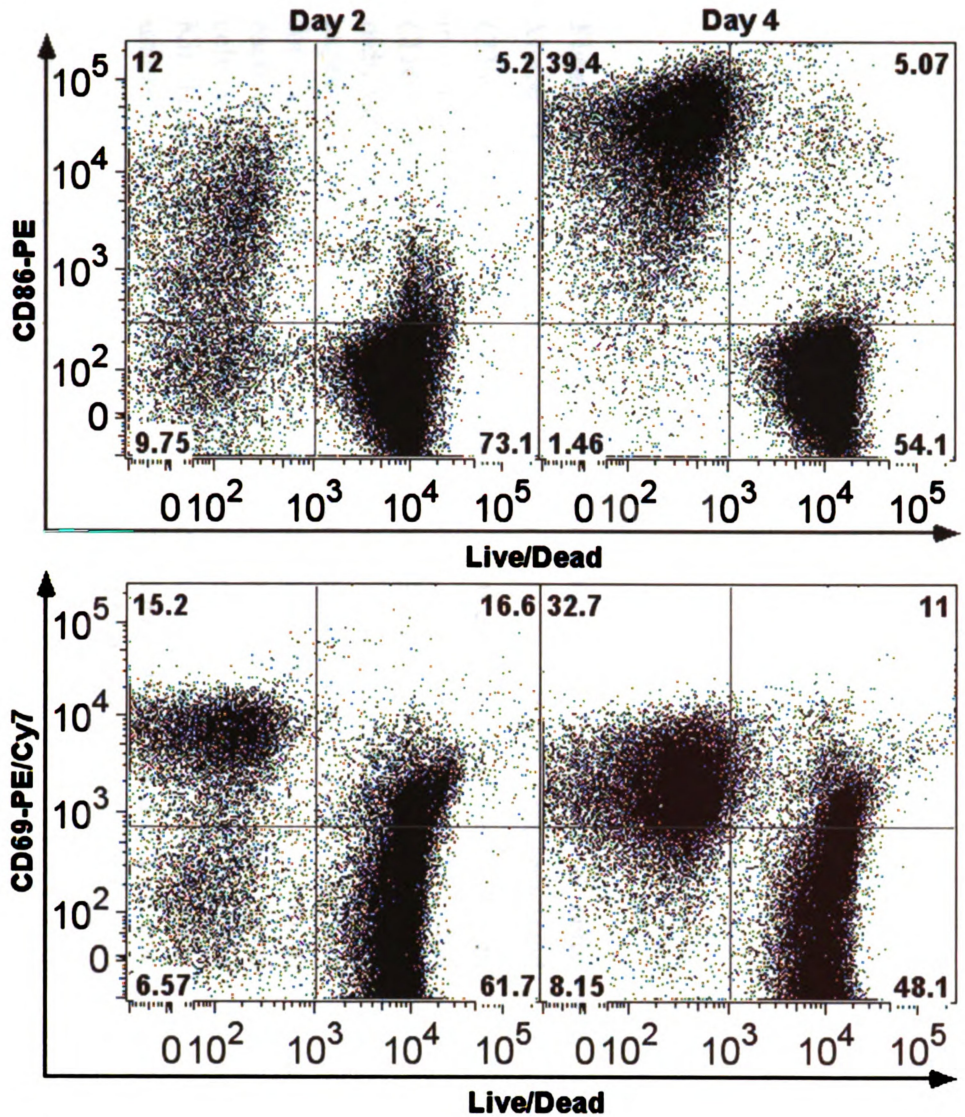
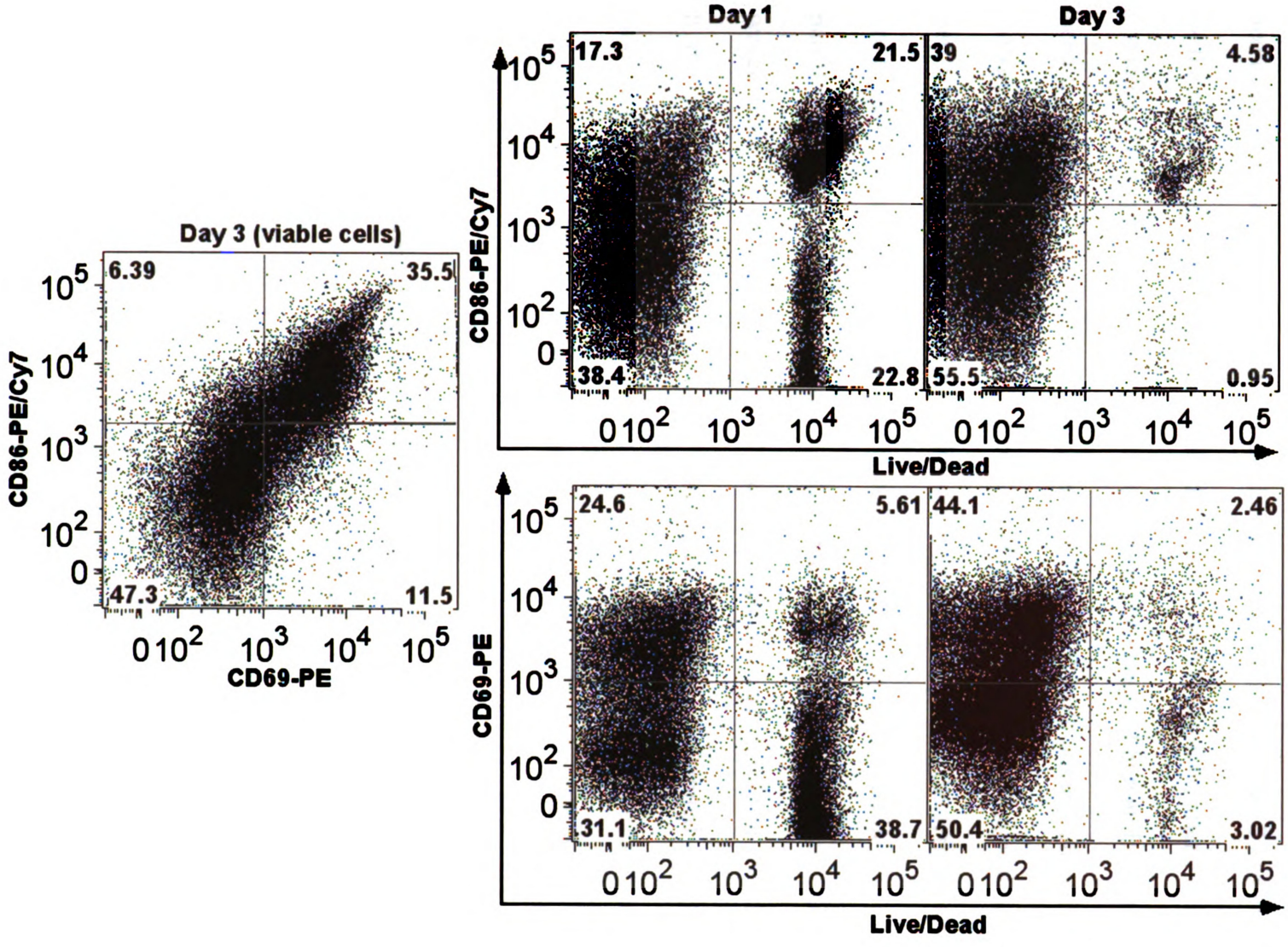

Day 2
Day 4
12
5.2
39.4
5.07
9.75
73.1
1.46
54.1
CD86-PE
15.2
16.6
32.7
11
6.57
61.7
8.15
48.1
CD69-PE/Cy7
Live/Dead
Live/Dead

Figure 9. CD40L-induced activated phenotype in mouse B cells. Mouse B cells (1.5×10^6 cell/culture) were co-cultured with irradiated CD40L-L cells (5×10^4 cell/culture) in the presence of recombinant mouse IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml), and CD40L stimulation was removed on Day 3. Cells were harvested at the indicated time points and assessed by flow cytometry for surface expression of CD86 and CD69. Results depicted are fluorescent signals for CD86, CD69, and the Live/Dead stain. Percentages of cells within each quadrant are shown in the corners of each individual plot. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit. Total cells, both viable and non-viable, are included in the dot plots unless indicated otherwise (the very left plot). These data are from one experiment that is representative of two separate experiments (for human data, each separate experiment used B cells from one individual donor) with three replicates per time point. Results shown are concatenated from three experimental replicates included one separate experiment.


Day 3 (viable cells)
6.39
35.5
47.3
11.5
CD86-PE/Cy7
CD69-PE
Day 1
Day 3
17.3
21.5
38.4
22.8
39
4.58
55.5
0.95
CD86-PE/Cy7
Live/Dead
24.6
5.61
31.1
38.7
44.1
2.46
50.4
3.02
CD69-PE
Live/Dead

Interestingly, in constrast to human cells, only a small population of viable mouse B cells started cell division at Day 3, and underwent further divisions between Day 3 and Day 5, while the majority of viable cells did not divide even at Day 5. Therefore, after excluding non-viable cells, it appeared that a significantly greater percentage of human B cells were induced to proliferate than mouse B cells. Once activated, B cells possess an activated phenotype characteristic of increased expression of a number of surface molecules, also termed activation markers, including CD86 and CD69. As shown in the left panels of Figure 8 and 9, expression of CD86 and CD69 was significantly induced in both naive human B cells and mouse B cells 2 and 1 d following CD40L stimulation, respectively. However, when the expression profile of activation markers was viewed in correlation with the viability of cells, human and mouse B cells again exhibited significant phenotypic differences. At Day 4, greater than 80% of viable human cells exhibited an activated phenotype ($CD86^{+}CD69^{+}$), with less than 1% of viable cells being non-activated ($CD86^{-}CD69^{-}$) (Figure 8 right panel; cells positive for the Live/Dead stain were not viable). In other words, the vast majority of cells that expressed neither CD86 nor CD69 were not viable. The same observation was made at Day 6 (data not shown). Whereas approximately 50% of total mouse B cells did not exhibit an activated phenotype as indicated by the absence of CD86 or CD69 expression, but the majority remained viable 3 d and 5 d after activation with CD40L (Figure 9 right panel; Day 5 data not shown). The plasmacytic differentiation of B cells could be further characterized by an antibody secreting plasma cell phenotype, which can be characterized the up-regulation of intracellular IgM expression and a plasma cell marker CD138 (syndecan-1) (Klein *et al.* 2003). As shown in the upper panels of Figure 10 and 11, expression of

Figure 10. Plasmacytic differentiation of CD40L-activated human B cells. Naive human B cells (1.5×10^5 cell/culture) were co-cultured with irradiated CD40L-L cells (1.5×10^3 cell/culture) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml), and CD40L stimulation was removed on Day 4. Cells were harvested at indicated time points and assessed by flow cytometry for intracellular expression of IgM and surface expression of CD138. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit. Results depicted in the histograms (upper) are fluorescent signals for intracellular IgM (left) and CD138 (right). Only viable cells are included in the histograms. Results depicted in the dot plot (bottom) are fluorescent signals for intracellular IgM and the Live/Dead stain. Percentages of cells within each quadrant are shown in the corners of the dot plot. Total cells, both viable and non-viable, are included in the dot plots. These data are from one experiment that is representative of two separate experiments (for human data, each separate experiment used B cells from one individual donor) with three replicates. Results shown are concatenated from three experimental replicates included one separate experiment.

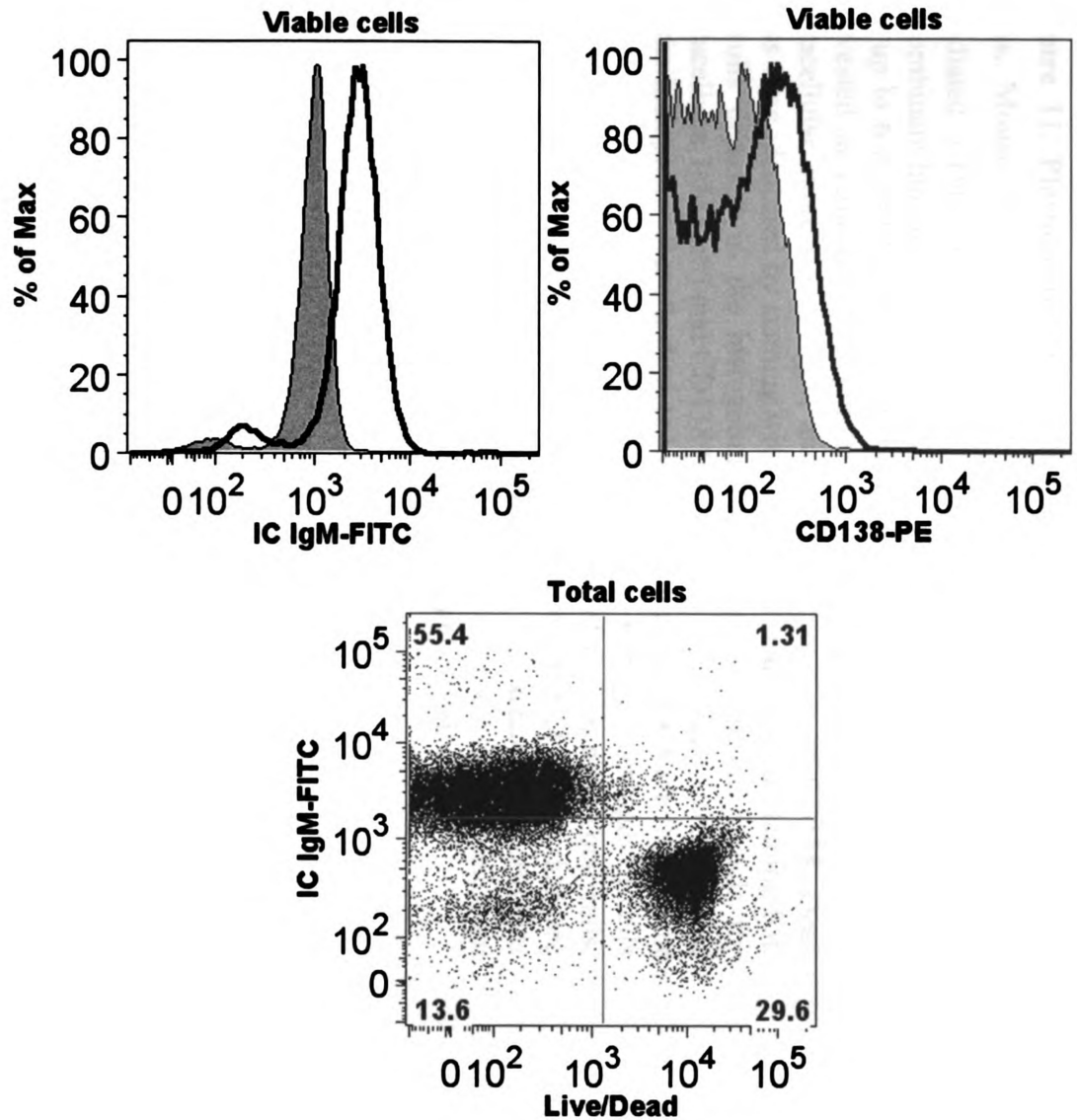
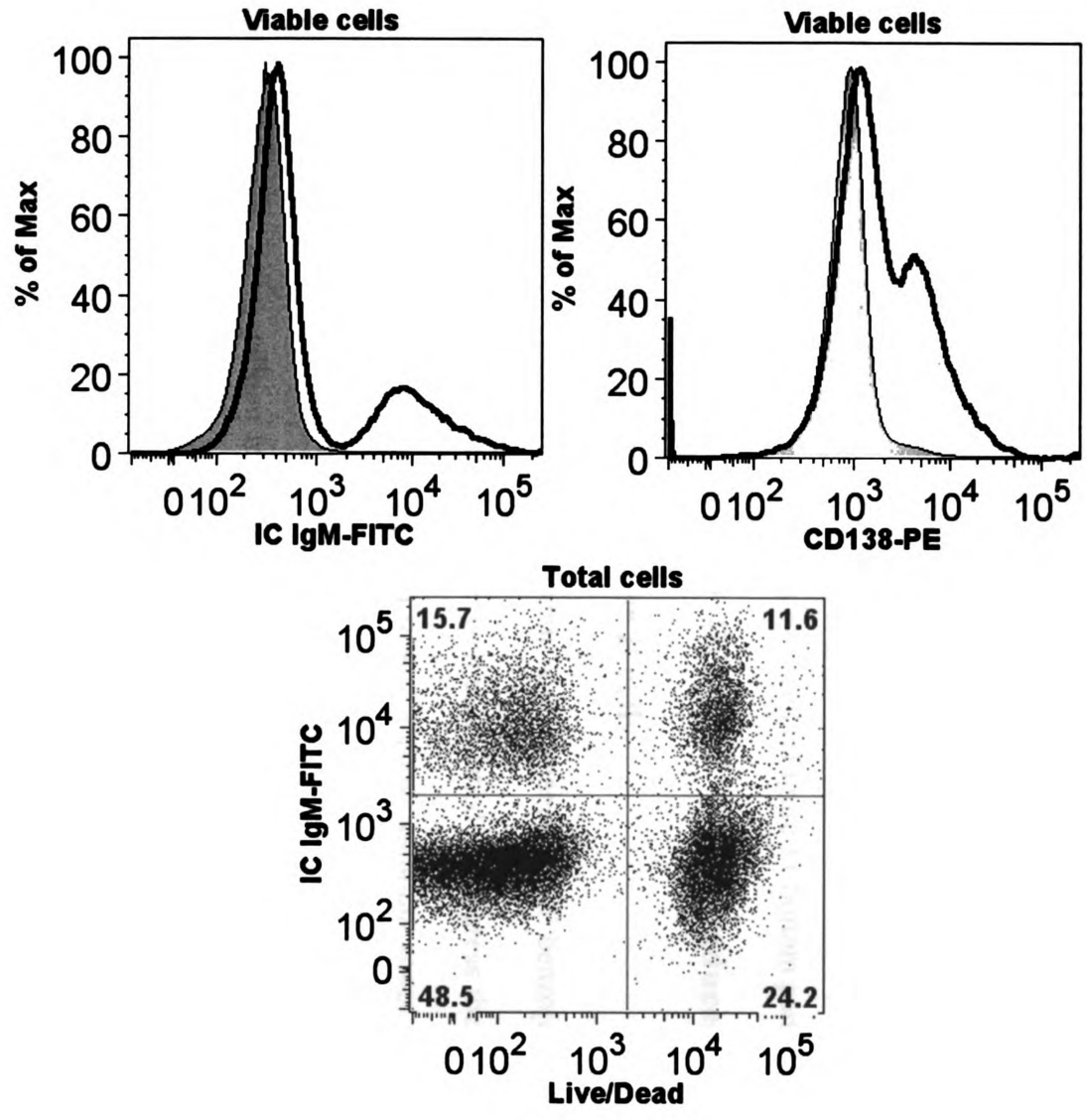

Viable cells
% of Max
IC IgM-FITC
Viable cells
% of Max
CD138-PE
Total cells
55.4
1.31
13.6
29.6
IC IgM-FITC
Live/Dead

Figure 11. Plasmacytic differentiation of CD40L-activated mouse B cells. Mouse B cells (1.5×10^6 cell/culture) were co-cultured with irradiated CD40L-L cells (5×10^4 cell/culture) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for up to 6 d, and CD40L stimulation was removed on Day 3. Cells were harvested at indicated time points and assessed by flow cytometry for intracellular expression of IgM and surface expression of CD138. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit. Results depicted in the histograms (upper) are fluorescent signals for intracellular IgM (left) and CD138 (right). Only viable cells were included in the histograms. Results depicted in the dot plot (bottom) are fluorescent signals for intracellular IgM and the Live/Dead stain. Percentages of cells within each quadrant are shown in the corners of the dot plot. Total cells, both viable and non-viable, are included in the dot plot. These data are from one experiment that is representative of two separate experiments with three replicates per time point. Results shown are concatenated from three experimental replicates included one separate experiment.


Viable cells
% of Max
IC IgM-FITC
Viable cells
% of Max
CD138-PE
Total cells
IC IgM-FITC
Live/Dead
15.7
11.6
48.5
24.2

intracellular IgM and surface CD138 was up-regulated in both viable human and mouse cells at Day 7 and Day 6, respectively, indicating an antibody secreting plasma cell phenotype. However, phenotypic differences were still observed between human and mouse B cells. In human B cells, the majority of viable cells expressed elevated level of IgM expression, while the majority of cells that did not were not viable (Figure 10 bottom panel). In contrast, only part of the viable mouse B cells exhibited elevated expression of intracellular IgM and surface CD138, while the majority of viable cells still expressed both at the levels that were similar to non-activated B cells (Figure 11 bottom panel).

C. Suppression by arsenic and BPDE of CD40L-induced IgM responses in human and mouse B cells

The immunosuppressive effects of arsenic and metabolites of benzo[a]pyrene have been widely studied in mice, and both are well established to suppress the *in vitro* mouse anti-sRBC antibody response (Salas and Burchiel 1998; Selgrade 1999). Arsenic was first identified as a major contributor to the suppression of both the anti-sRBC antibody responses using gallium arsenide *in vitro* and *in vivo* in mice (Sikorski *et al.* 1991). BPDE was reported to be an ultimate metabolite of benzo[a]pyrene that directly caused immunosuppression (Kawabata and White 1989). However, very few studies to date have assessed the potential immunosuppressive effect exerted by arsenic or BPDE on the effector function of primary human leukocytes, including B cells. Following the establishment of the CD40L activation model, the effect of arsenic and BPDE on the IgM response in human B cells induced by CD40L was investigated, and also compared to mouse splenic B cells. The concentration range selected for both compounds were based on previous published studies in which suppression of the mouse anti-sRBC antibody

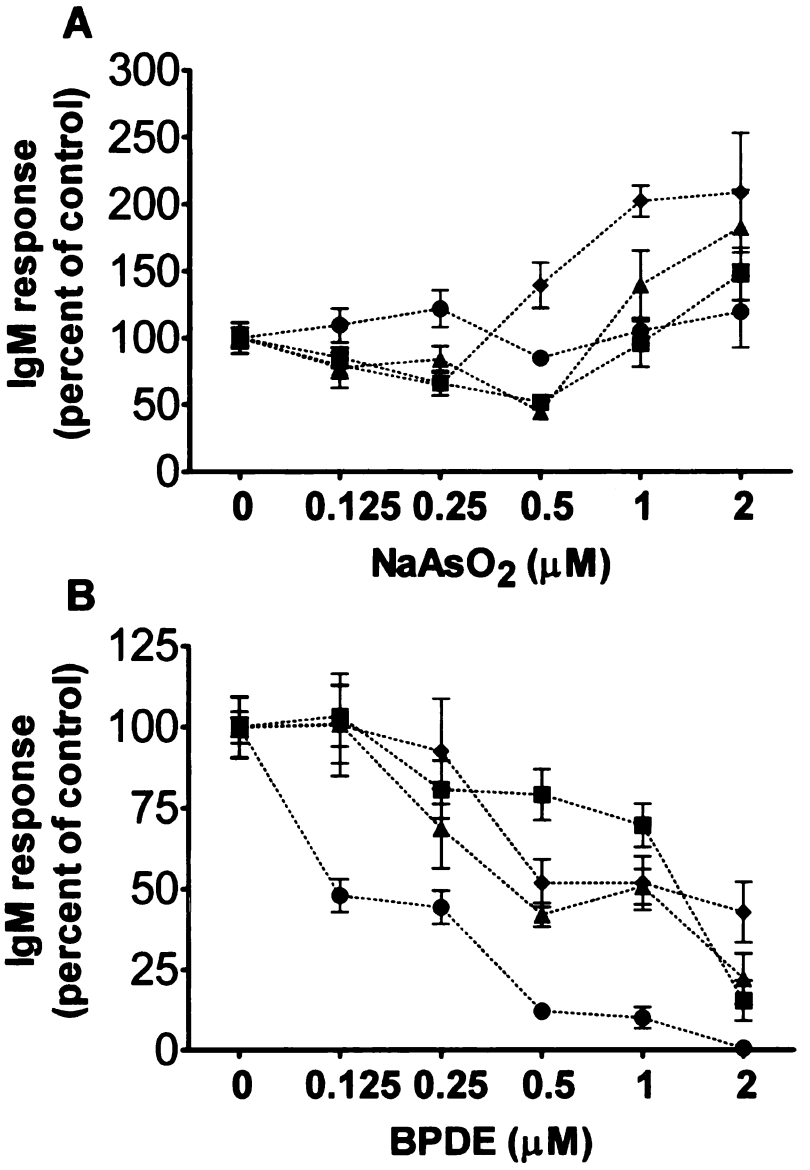


Figure 12. Effect of Arsenic and BPDE on the CD40L-induced IgM response from human B cells. Naive human B cells (1.5×10^5 cell/culture) were treated with (A) $NaAsO_2$ or (B) BPDE at indicated concentrations or vehicle (VH) and then co-cultured with irradiated CD40L-L cells (1.5×10^3 cell/culture) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 4 d, and further cultured for additional 3 d without CD40L-L cells. Cells were harvested on Day 7 to enumerate IgM secreting cells by ELISPOT. Cell number was determined using a particle counter, and cell viability was assessed using the pronase activity assay. Data were normalized to the VH-treated group (100%) and presented as percent of control with six replicates per group. B cells from four human donors were assessed. Each symbol in the line graph represents one individual human donor. Data are presented as mean ± S.E.M. from three experimental replicates per group.

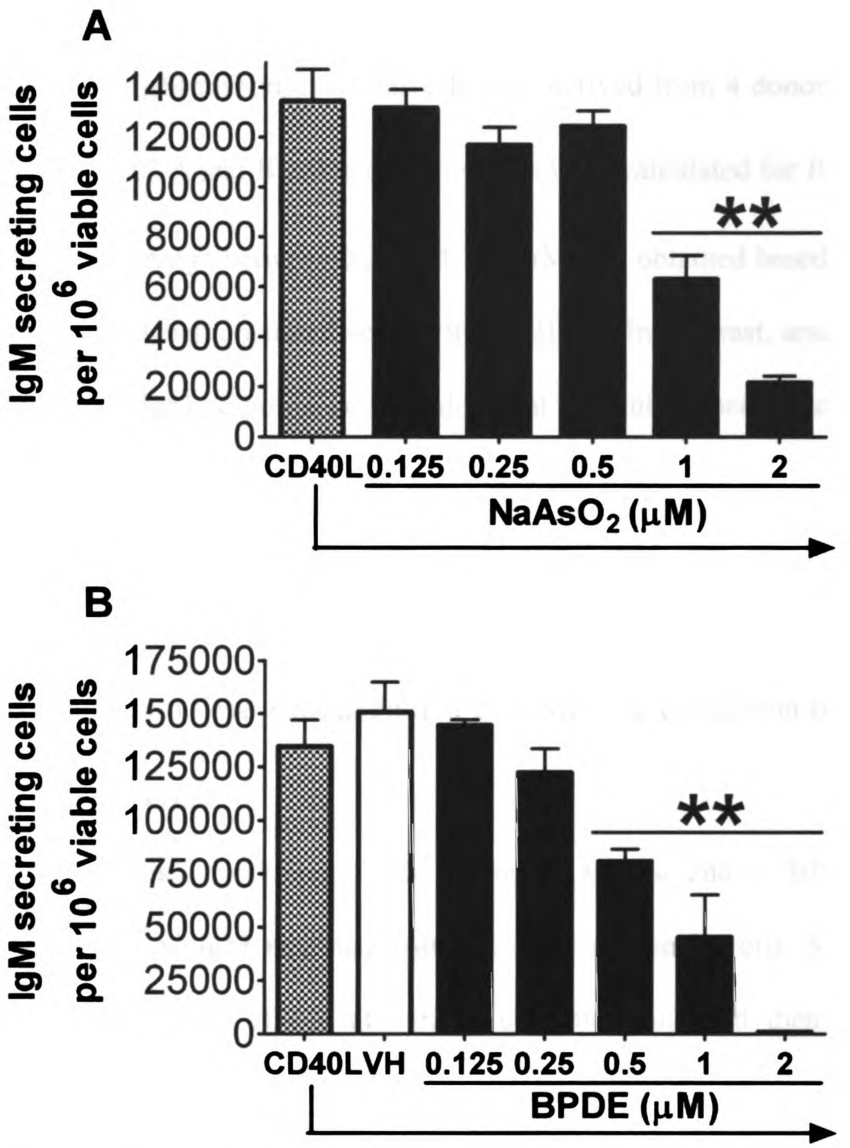


Figure 13. Effect of Arsenic and BPDE on the CD40L-induced IgM response from mouse B cells. Mouse B cells (1.5 x 10^6 cell/culture) were treated with (A) $NaAsO_2$ or (B) BPDE at indicated concentrations or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (5 x 10^4 cell/culture) in the presence of recombinant mouse IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 3 d, and further cultured for additional 3 d without CD40L-L cells. Cells were harvested on Day 6 to enumerate IgM secreting cells by ELISPOT. Cell number was determined using a particle counter and the viability was assessed using the pronase activity assay. **, $p < 0.01$, compared with CD40L group (arsenic) or VH-treated group (BPDE). These data are from one experiment that is representative of two separate experiments with four experimental replicates per treatment group. Data are presented as mean ± S.E.M. from four replicates per group.

response was observed (Kawabata and White 1989; Sikorski *et al.* 1991). Arsenic and BPDE modulated the IgM responses of naive B cells derived from 4 donors, as assessed by ELISPOT (Figure 12 A and B). When IC_{50} values were calculated for BPDE for each donor, a concentration range between 0.26 and 1.36 μM was obtained based on data from 4 donors whose B cells demonstrated sensitivity to BPDE. In contrast, arsenic-mediated modulation of human IgM response is quite different. Arsenic appeared to be modestly suppressive of the human IgM response at lower concentrations (0.25 – 0.5 μM), but had no or an enhancing effect at concentrations above 1 μM, the concentrations that significantly suppressed the mouse B cell IgM response induced by CD40L activation. The lack of concentration-dependent inhibition precluded the calculation of IC_{50} values for arsenic for human B cells.

Studies were also conducted to determine if arsenic and/or BPDE similarly impaired CD40L-induced IgM response using isolated mouse B cells. Mouse B cells were treated with arsenic or BPDE at various concentrations and then activated by CD40L in the presence of IL-2, IL-6, IL-10. The total number of IgM secreting cells was enumerated by ELISPOT. As illustrated in Figure 13 A and B, the frequency of IgM secreting cells in the total pool of viable cells was significantly decreased by arsenic at 1 and 2 μM and BPDE at concentrations between 0.5 and 2 μM. The overall profile of BPDE-mediated suppression of the IgM responses from naive human B cells was similar to those observed in mouse B cells. The IC_{50} was calculated as 0.97 μM for arsenic and as 0.55 μM for BPDE for mouse B cells. One μM of arsenic was also the lowest concentration found to suppress the *in vitro* mouse anti-sRBC antibody response

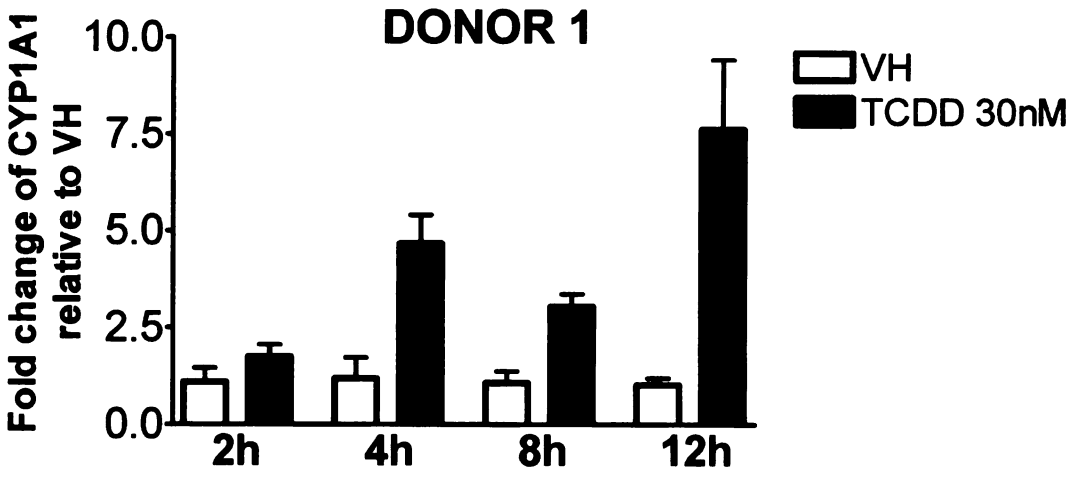
(Sikorski *et al.* 1991).

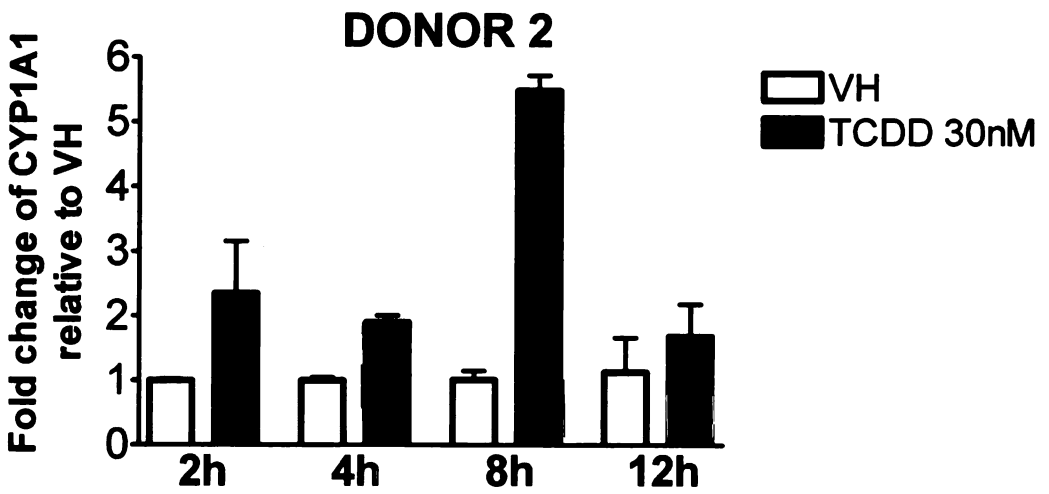
II. TCDD effects on B cells: induction of AHR-responsive genes and modulation of the IgM response

A. Expression kinetics of AHR-responsive genes in TCDD-treated B cells

CYP1A1 is expressed in human PBMCs and can be further induced by treatment with TCDD or 3-methylcholanthrene (Nohara et al. 2006; Yamamoto et al. 2004). In the present study, naive human B cells were isolated from 3 individual donors, treated with vehicle (VH) or 30 nM TCDD, and incubated for various time periods to obtain the expression kinetics of AHR-responsive genes induced by TCDD. Due to the limited number of B cells recovered from each leukocyte pack, the NA (no treatment) group was only included in one of three donors. In preliminary studies that used TCDD over a broad range of concentrations (0.3 – 30 nM), a wide variety of well-characterized AHR-responsive genes were measured in human B cells treated with TCDD, and either not detected (CYP1A2, ALDH3A1, and GSTA1), or not significantly induced by TCDD (NQO1) (data not shown). In mouse B cells, neither CYP1A2 nor ALDH3A1 was found to be expressed, and NQO1 was not significantly induced by TCDD (data not shown). Therefore, the present studies focused on CYP1A1, CYP1B1, AHR repressor, and TIPARP, which were consistently detected and induced by TCDD in B cells from multiple human donors. CYP1A1 and CYP1B1 were induced by TCDD more robustly, as opposed to AHR repressor and TIPARP in naive human B cells (Figure 14 - 17). Moreover, the four genes demonstrated distinct expression kinetics with the induction of

Figure 14. Time-dependent induction of CYP1A1 gene expression by TCDD in human B cells. Naive human B cells (1×10^6/ml) were treated with 30 nM of TCDD or vehicle (VH) for 2, 4, 8, and 12 h. Total RNA was isolated, and steady state mRNA levels of CYP1A1 were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold-change compared to the VH-control group at the same time point. Data from three individual donors are presented. Data are presented as mean ± S.E.M. from three experimental replicates per group.


DONOR 1
Fold change of CYP1A1 relative to VH
10.0
7.5
5.0
2.5
0.0
2h
4h
8h
12h
VH
TCDD 30nM


DONOR 2
Fold change of CYP1A1 relative to VH
6
5
4
3
2
1
0
2h
4h
8h
12h
VH
TCDD 30nM

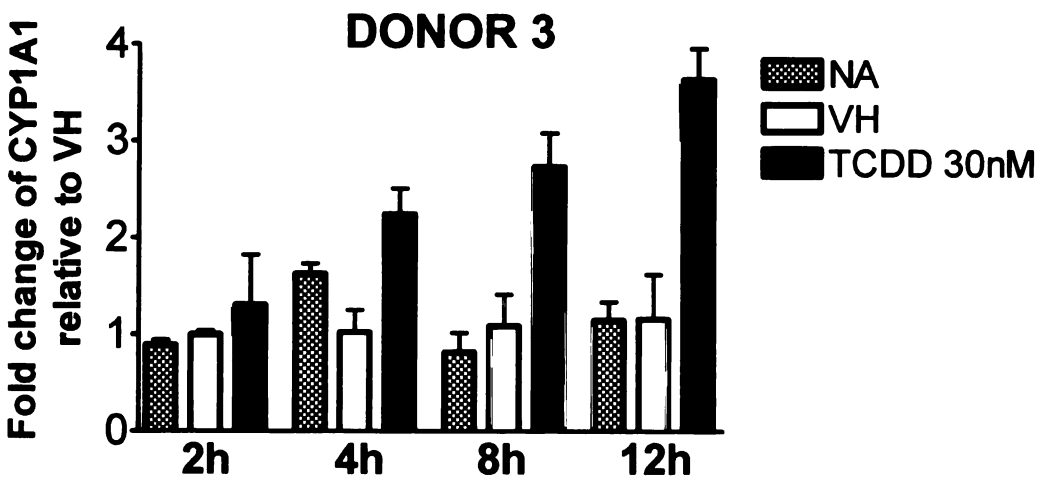

DONOR 3
Fold change of CYP1A1 relative to VH
4
3
2
1
0
2h
4h
8h
12h
NA
VH
TCDD 30nM

Figure 15. Time-dependent induction of CYP1B1 gene expression by TCDD in human B cells. Naive human B cells (1 x 10^6/ml) were treated with 30 nM of TCDD or vehicle (VH) for 2, 4, 8, and 12 h. Total RNA was isolated, and steady state mRNA levels of CYP1A1 were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold-change compared to the VH-control group at the same time point. Data from three individual donors are presented. Data are presented as mean ± S.E.M. from three experimental replicates per group.

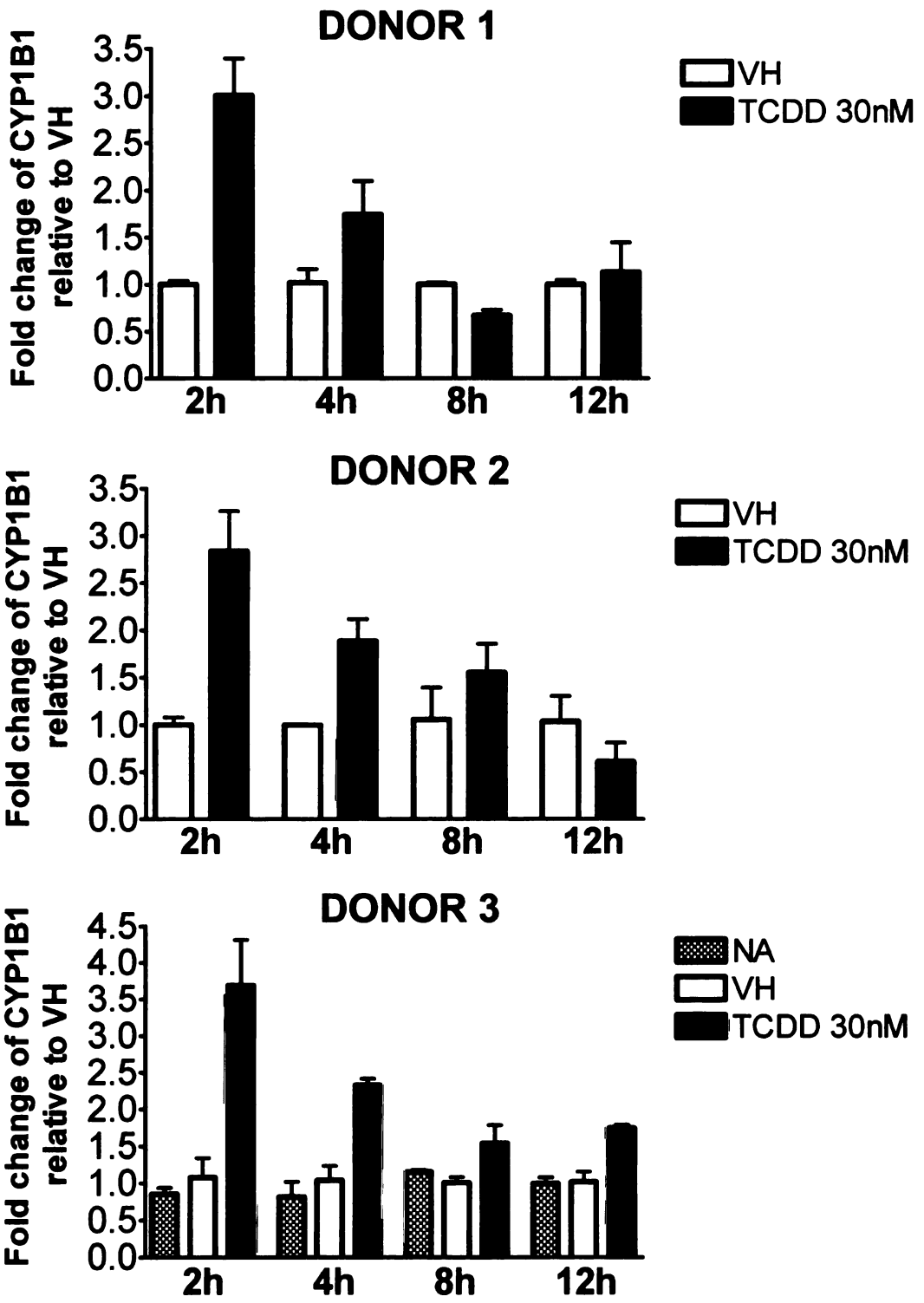

DONOR 1
Fold change of CYP1B1 relative to VH
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2h
4h
8h
12h
VH
TCDD 30nM
DONOR 2
Fold change of CYP1B1 relative to VH
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2h
4h
8h
12h
VH
TCDD 30nM
DONOR 3
Fold change of CYP1B1 relative to VH
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2h
4h
8h
12h
NA
VH
TCDD 30nM

Figure 16. Time-dependent induction of AHR repressor gene expression by TCDD in human B cells. Naive human B cells (1 x 10^6/ml) were treated with 30 nM of TCDD or vehicle (VH) for 2, 4, 8, and 12 h. Total RNA was isolated, and steady state mRNA levels of AHR repressor were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold-change compared to the VH-control group at the same time point. Data from three individual donors are presented. Data are presented as mean ± S.E.M. from three experimental replicates per group.

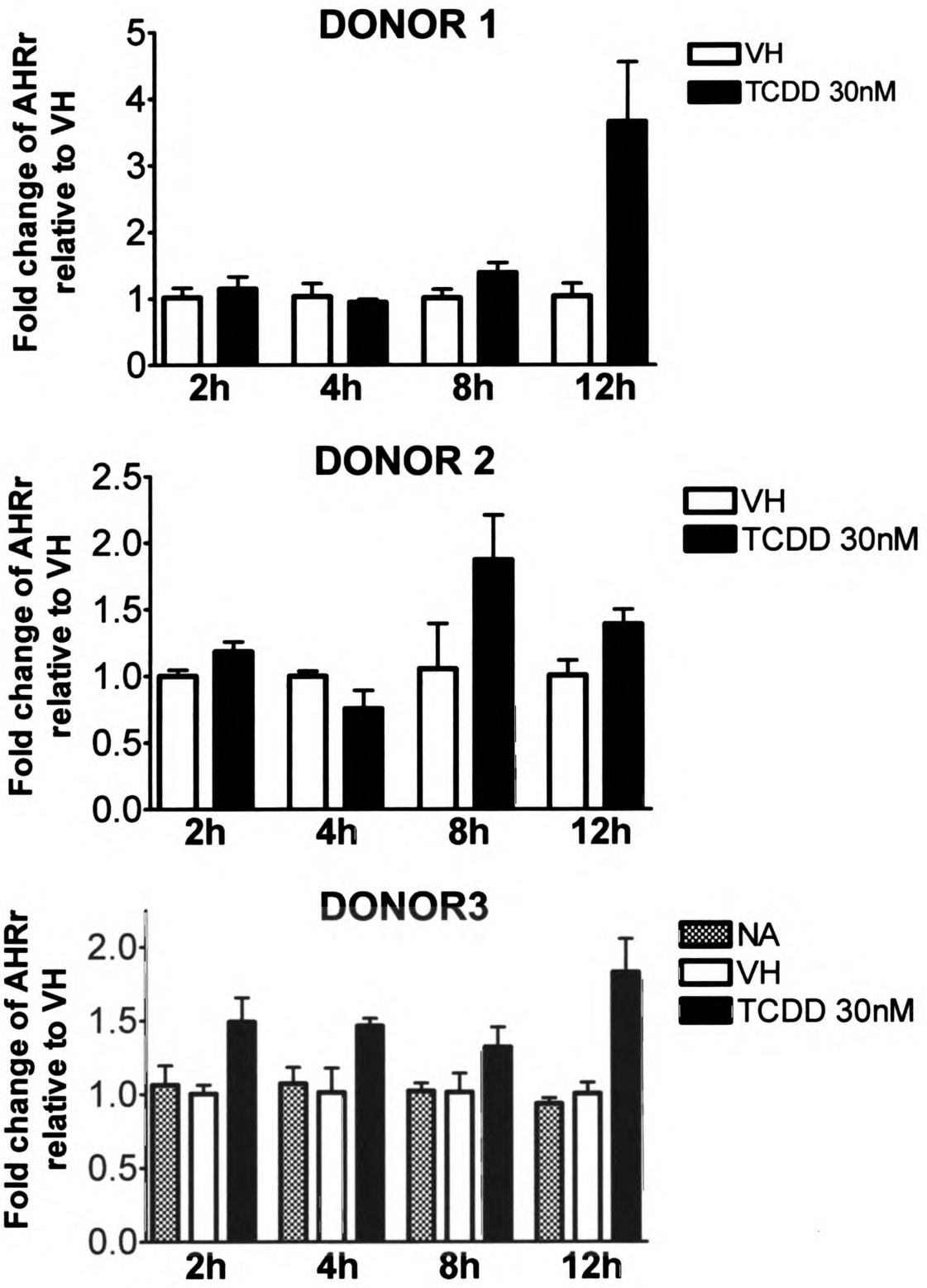

DONOR 1
Fold change of AHRr relative to VH
5
4
3
2
1
0
2h
4h
8h
12h
VH
TCDD 30nM
DONOR 2
Fold change of AHRr relative to VH
2.5
2.0
1.5
1.0
0.5
0.0
2h
4h
8h
12h
VH
TCDD 30nM
DONOR3
Fold change of AHRr relative to VH
2.0
1.5
1.0
0.5
0.0
2h
4h
8h
12h
NA
VH
TCDD 30nM

Figure 17. Time-dependent induction of TIPARP gene expression by TCDD in human B cells. Naive human B cells (1 x 10^6/ml) were treated with 30 nM of TCDD or vehicle (VH) for 2, 4, 8, and 12 h. Total RNA was isolated, and steady state mRNA levels of AHR repressor were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold-change compared to the VH-control group at the same time point. Data from three individual donors are presented. Data are presented as mean ± S.E.M. from three experimental replicates per group.

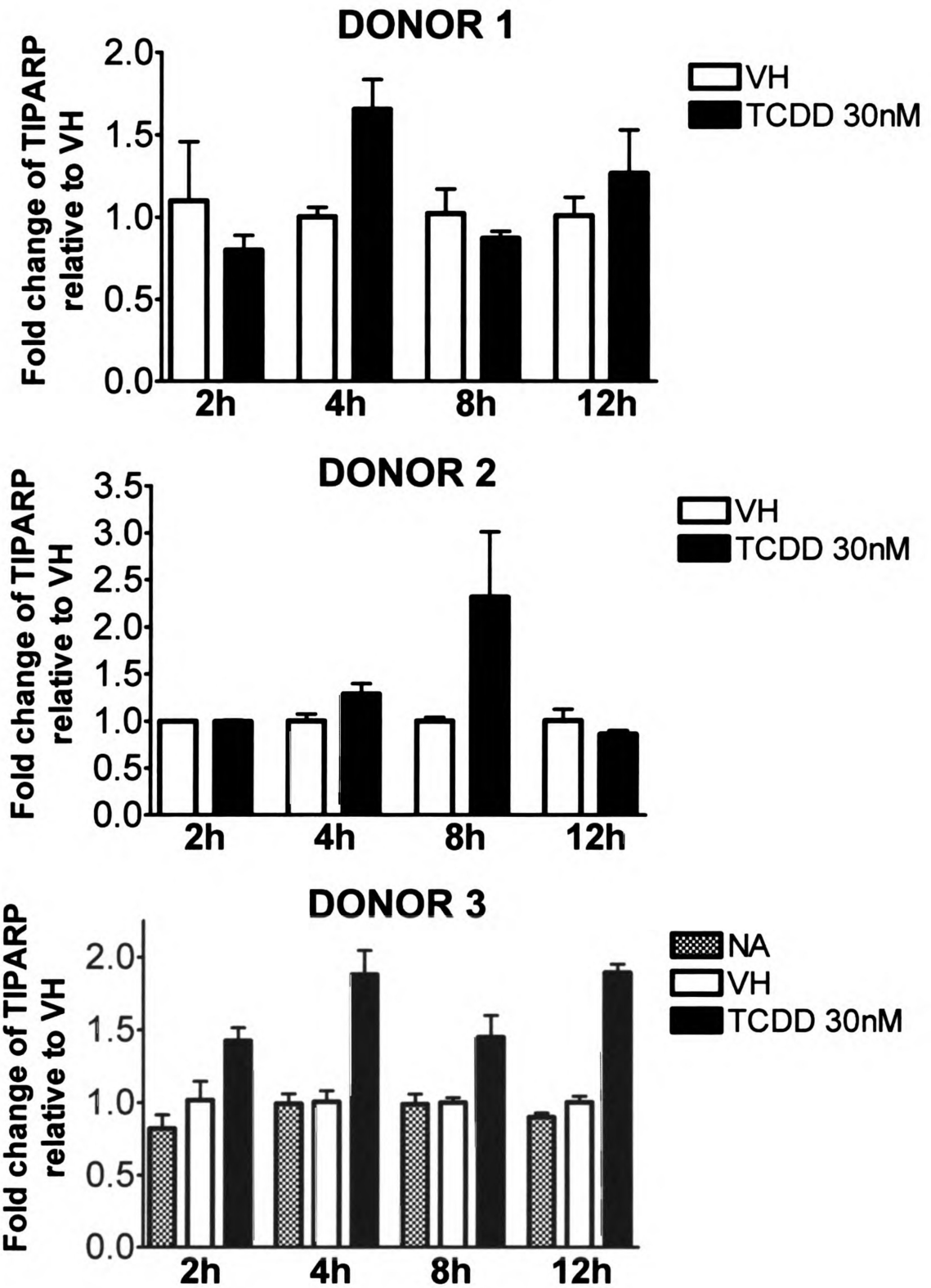
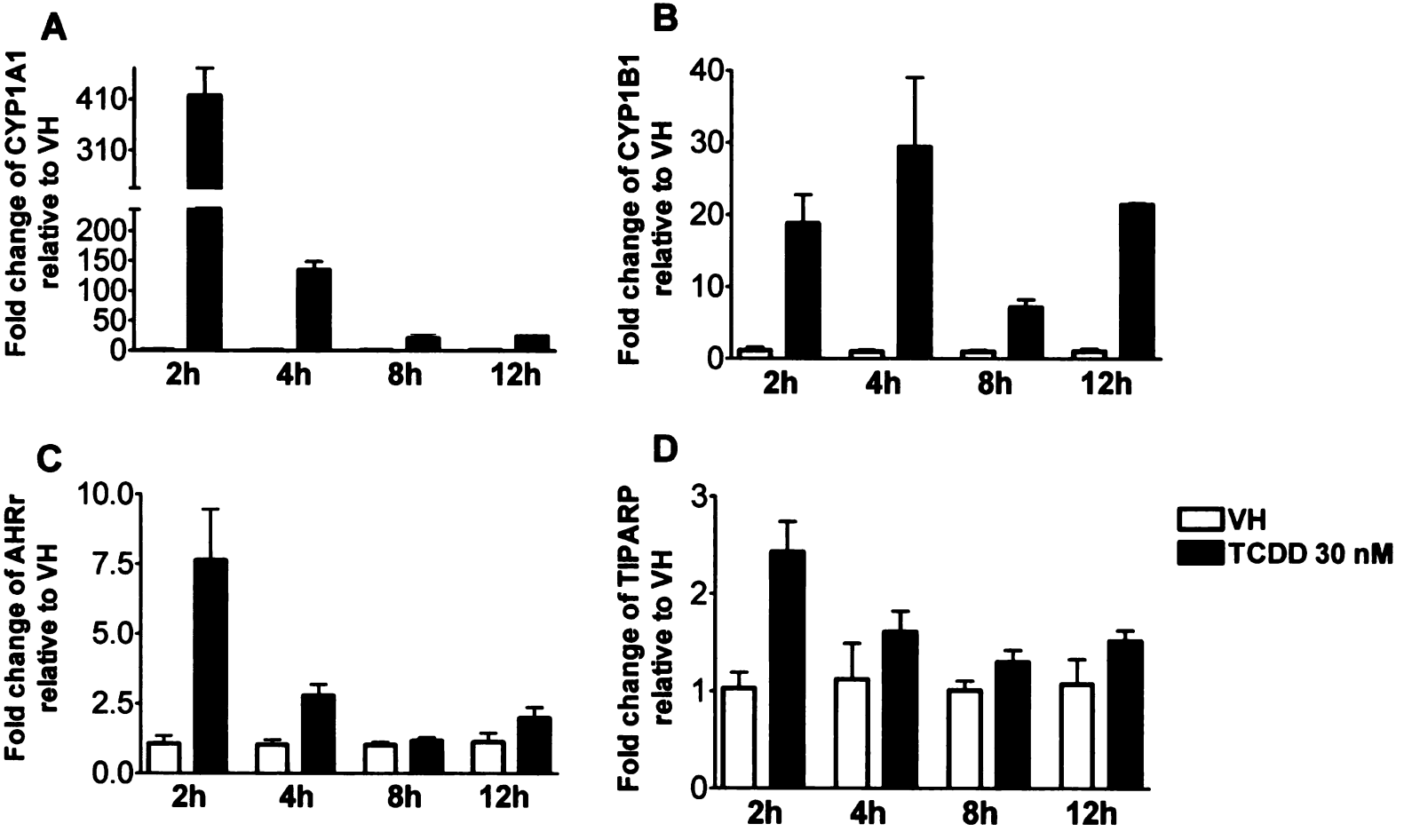

DONOR 1
Fold change of TIPARP relative to VH
2.0
1.5
1.0
0.5
0.0
2h
4h
8h
12h
VH
TCDD 30nM
DONOR 2
Fold change of TIPARP relative to VH
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2h
4h
8h
12h
VH
TCDD 30nM
DONOR 3
Fold change of TIPARP relative to VH
2.0
1.5
1.0
0.5
0.0
2h
4h
8h
12h
NA
VH
TCDD 30nM

Figure 18. Time-dependent induction of AHR-responsive gene expression by TCDD in mouse B cells. Mouse B cells (1 x 10^6/ml) were treated with 30 nM of TCDD or vehicle (VH) for the indicated time periods. Total RNA was isolated, and steady state mRNA levels of (A) CYP1A1, (B) CYP1B1, (C) AHR repressor, and (D) TIPARP were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold-change compared to the VH-control group at the same time point. Data are representative of two separate experiments with three experimental replicates per group. Data are presented as mean ± S.E.M. from three replicates per group.


A
Fold change of CYP1A1 relative to VH
410
310
200
150
100
50
0
2h
4h
8h
12h
B
Fold change of CYP1B1 relative to VH
40
30
20
10
0
2h
4h
8h
12h
C
Fold change of AHRr relative to VH
10.0
7.5
5.0
2.5
0.0
2h
4h
8h
12h
D
Fold change of TIPARP relative to VH
3
2
1
0
2h
4h
8h
12h
VH
TCDD 30 nM

CYP1B1 being more rapid and less sustained over the time course compared to CYP1A1 and AHR repressor. A similar time course study was conducted using mouse B cells. In contrast to the expression kinetics in naive human B cells, all four genes responded more rapidly in mouse B cells, as indicated by the peak time of induction, and with greater magnitude of induction, as exhibited by the fold change compared to the VH control (Figure 18). In mouse B cells, the induction of CYP1A1, AHR repressor, and TIPARP peaked at 2 h and declined at later time points, while CYP1B1 exhibited the greatest magnitude of induction at 2 h, but also showed biphasic expression kinetics between 4 h and 12 h. When the fold induction was compared among the four genes, CYP1A1 was the most highly induced gene by TCDD in both naive human and mouse B cells.

B. Concentration-dependent induction of AHR-responsive genes in TCDD-treated B cells

No study to date has characterized the concentration-dependent expression of AHR-responsive genes in human B cells treated with TCDD for comparison to a TCDD-responsive mouse strain such as the C57BL/6 mouse. For these experiments the induction of AHR-responsive gene expression was measured at the peak time of induction for human and mouse B cells over an extensive range of TCDD concentrations spanning from 0.1 to 30 nM. CYP1A1 and AHR repressor were genes of choice since they were induced consistently in human B cells derived from multiple donors, and their expression followed similar kinetics in both human and mouse B cells. 12 h and 2 h were selected as the peak time for CYP1A1 and AHR repressor in human and mouse B cells, respectively, based on results from kinetic studies described above. As shown in Figures 19 and 20, both genes were induced in human and mouse B cells treated with TCDD in a

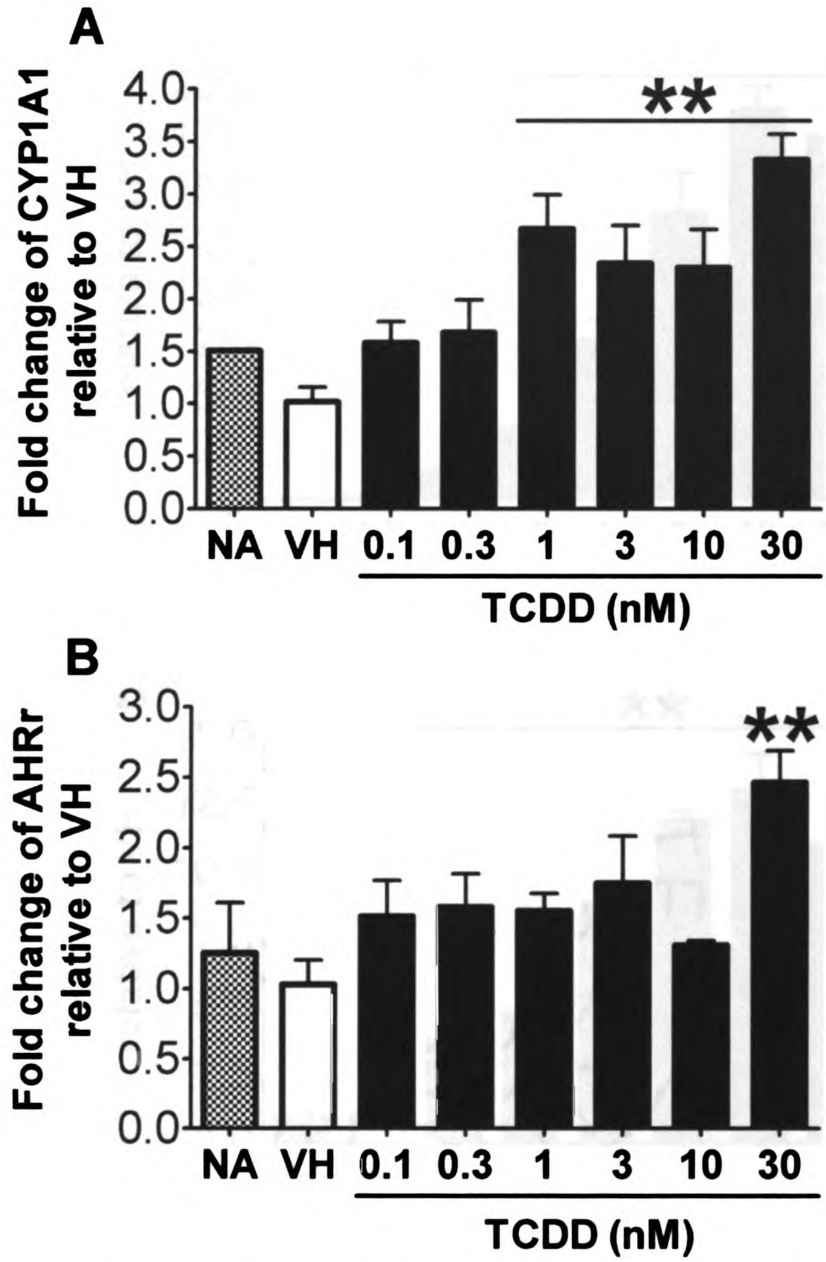


Figure 19. Concentration-dependent induction of CYP1A1 and AHR repressor expression by TCDD in human B cells. Naive human B cells (1 x 10^6/ml) were treated with TCDD at indicated concentrations or vehicle (VH) for 12 h. Total RNA was isolated, and steady state mRNA levels of (A) CYP1A1, and (B) AHR repressor were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold change compared to the VH-control group. **, $p < 0.01$, compared with VH-control group. These data are from one experiment that is representative of three separate experiments using B cells isolated from three individual donors. Data are presented as mean ± S.E.M. from three replicates per group.

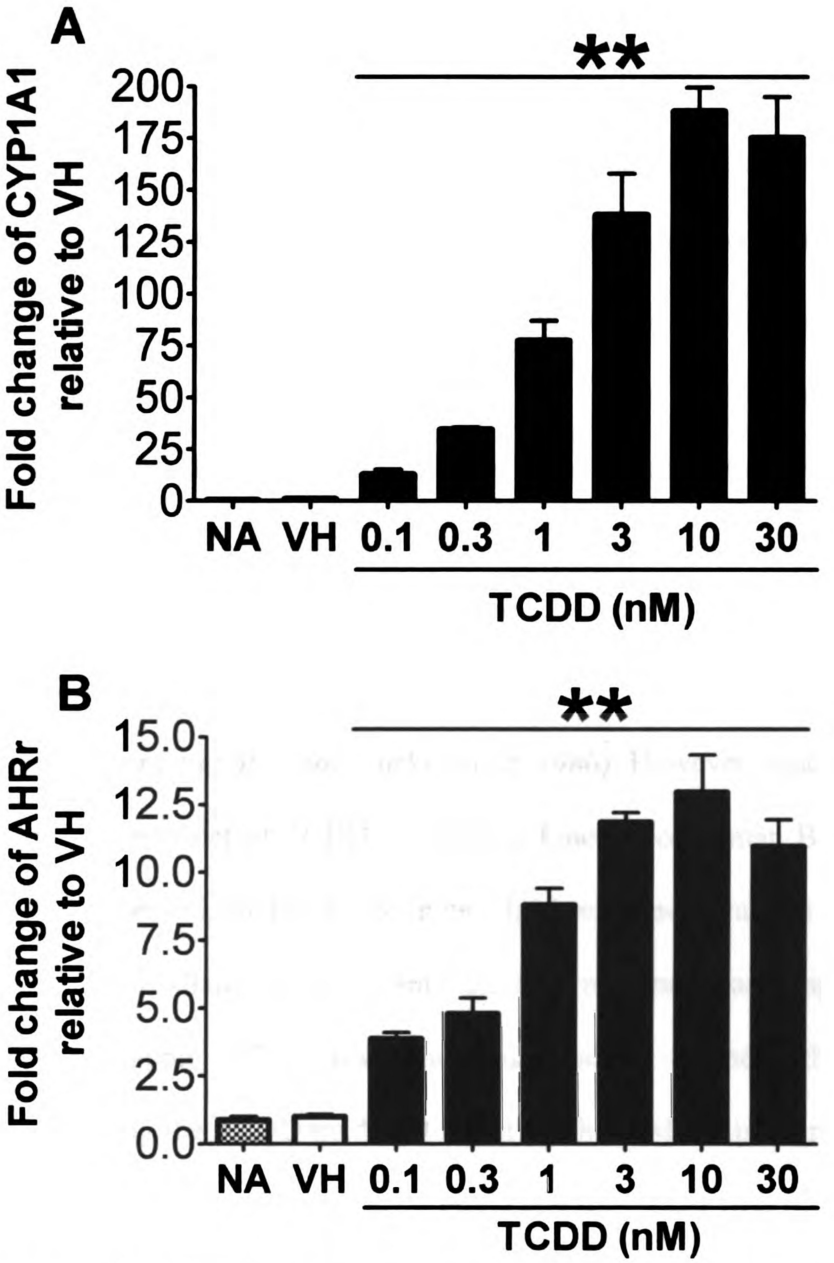


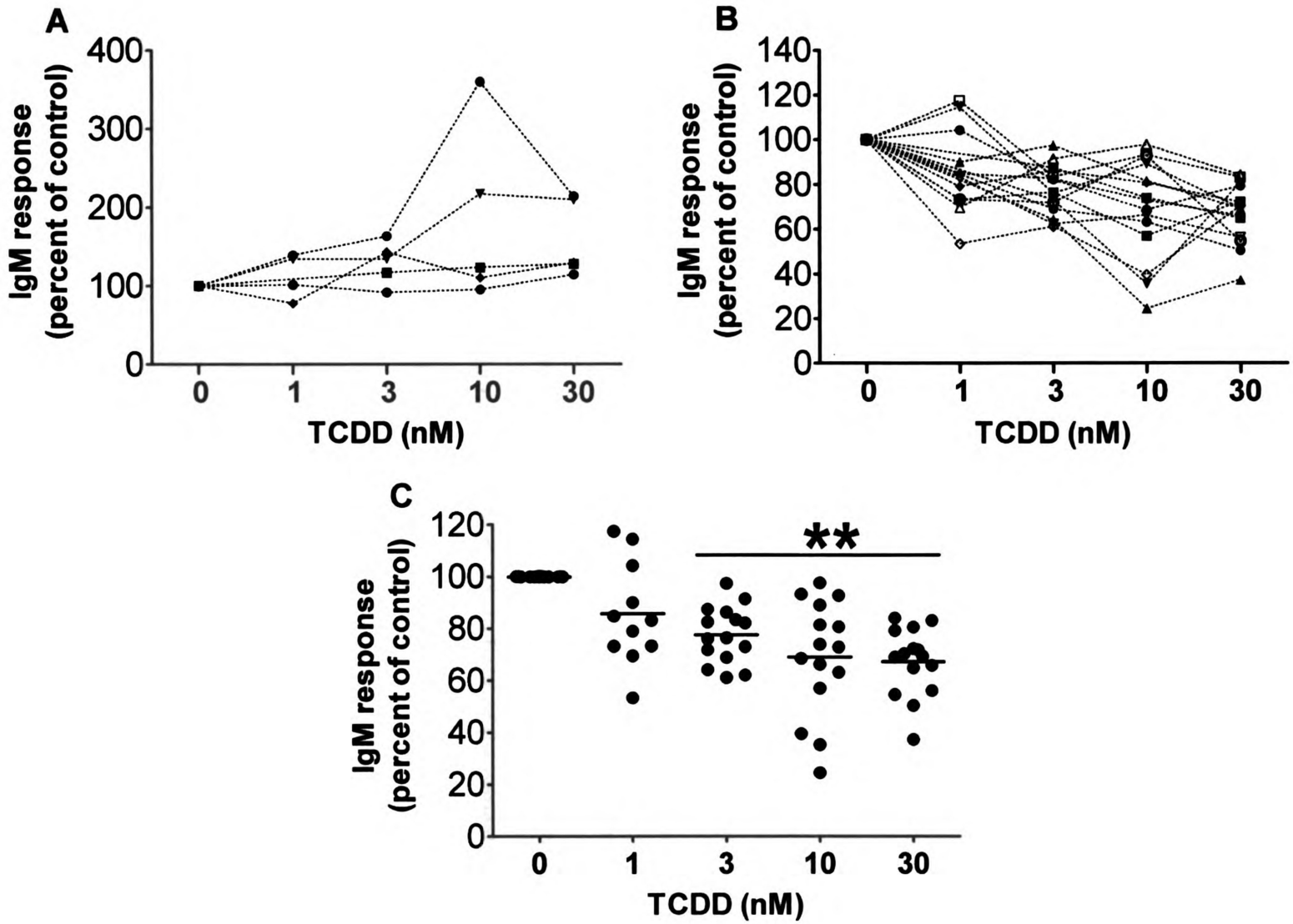
Figure 20. Concentration-dependent induction of CYP1A1 and AHR repressor expression by TCDD in mouse B cells. Mouse B cells (1 x 10^6/ml) were treated with TCDD at indicated concentrations or vehicle (VH) for 2 h. Total RNA was isolated, and steady state mRNA levels of (A) CYP1A1, and (B) AHR repressor were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold change compared to the VH-control group. **, $p < 0.01$, compared with VH-control group. Data are from one experiment that is representative of two separate experiments with three experimental replicates per group. Data are presented as mean ± S.E.M. from three replicates per group.

concentration-dependent manner. When the magnitude of induction was compared between human and mouse B cells, it appeared that both CYP1A1 and AHR repressor were induced to a greater magnitude in mouse B cells than in human B cells.

C. TCDD effects on CD40L-induced IgM responses of B cells

In a number of previous studies, TCDD has been shown to suppress the T cell dependent anti-sRBC IgM antibody response both in mice and cultured mouse splenocytes (Holsapple *et al.* 1986a; Smialowicz *et al.* 1994; Vecchi *et al.* 1980). It was also well established that the B cell is a sensitive cellular target of TCDD during the suppression of the T cell-dependent antibody response against sRBC, consistent with the findings that the effector function of B cells is sensitive to TCDD in the absence of other accessory cell types (Sulentic *et al.* 1998; Tucker *et al.* 1986). However, much less data are available to assess the effect of TCDD on effector function of human B cells. The first studies using the superantigen TSST-1 to induce IgM response in human tonsillar B cells in the presence of irradiated T cells found the IgM response was suppressed by TCDD (Wood and Holsapple 1993). This dissertation research extended these initial investigations by assessing the TCDD-mediated effect on the IgM antibody response in naive human B cells isolated from the peripheral blood and stimulated with the CD40L-L dependent B cell activation model established as described above. B cells isolated from multiple human donors were assayed as the donor to donor variability in sensitivity to TCDD was expected. Also due to the difference among donors with respect to the magnitude of each donor's control response, the IgM responses in all treatment groups were normalized to percentage of the VH-treated group (presented as 0 nM of TCDD) for B cells from each donor. The results from 20 donors are presented in Figure 21. For

Figure 21. Effect of TCDD on the CD40L-induced IgM response in human B cells from twenty donors (A, "non-responsive"; B, "responsive"; C, pooled "responsive" donors). Naive human B cells (1 x 10^6/ml) were treated with TCDD at indicated concentrations or VH, and then cultured with irradiated CD40L-L cells (1.5 - 3 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 4 days, and CD40L stimulation was removed on Day 4. Cells were cultured for another 3 days prior to being harvested on Day 7 to enumerate IgM secreting cells by ELISPOT. Cell number was determined by a particle counter, and cell viability was assessed using the pronase activity assay. Data were normalized to no TCDD group (100%) and presented as percent of control. B cells from multiple human donors were assessed. **, $p < 0.01$, compared with the VH-treated group (TCDD 0 nM).


A
IgM response
(percent of control)
400
300
200
100
0
0
1
3
10
30
TCDD (nM)
B
IgM response
(percent of control)
140
120
100
80
60
40
20
0
0
1
3
10
30
TCDD (nM)
C
IgM response
(percent of control)
120
100
80
60
40
20
0
**
0
1
3
10
30
TCDD (nM)

some donors, limited concentrations of TCDD were tested due to the limited number of B cells recovered. Figure 21 A shows data from 5 donors whose Bcells were either not affected by TCDD at all concentrations tested and in two donors an actual enhancement in IgM response by TCDD treatment at higher concentrations. B cells from 2 these donors were treated with even higher concentrations of TCDD 50 and 100 nM, and still no sensitivity was observed at those concentrations (data not shown). In Figure 21 B, data are included from 15 donors whose B cells exhibited some level of suppression of the IgM response by TCDD. In Figure 21 C, results from the 15 donors were pooled such that each of the 15 "responsive" donors was considered as one biological replicate in one "experiment", ultimately consisting of a total of 15 replicates. Using this approach, 3, 10 and 30 nM of TCDD significantly suppressed the CD40L-induced IgM response in a concentration-related manner in B cells isolated from "responsive" donors. The two distinct "phenotypes" observed among B cells from different donors are intriguing and the mechanistic basis for this remains to be elucidated. In light of the critical role of the AHR in mediating TCDD toxicities including the immunotoxicity on B cells, the inherent differences within the *AHR* gene among humans may contribute to the different B cell-specific phenotypes observed in the present study. In preliminary studies that aimed at characterizing such potential differences, we sequenced the exons of the *AHR* from three 'responsive' donors and three 'non-responsive' donors among all the donors we assayed thus far. Interestingly, two of the three non-responsive donors were found to possess previously characterized polymorphisms within the exons of the *AHR*: one has 132 T>C in codon 44 of exon 2 encoding part of the basic-helix-loop-helix domain, and the other has 1661 G>A in condon 554 of exon 10 encoding the transactivation domain

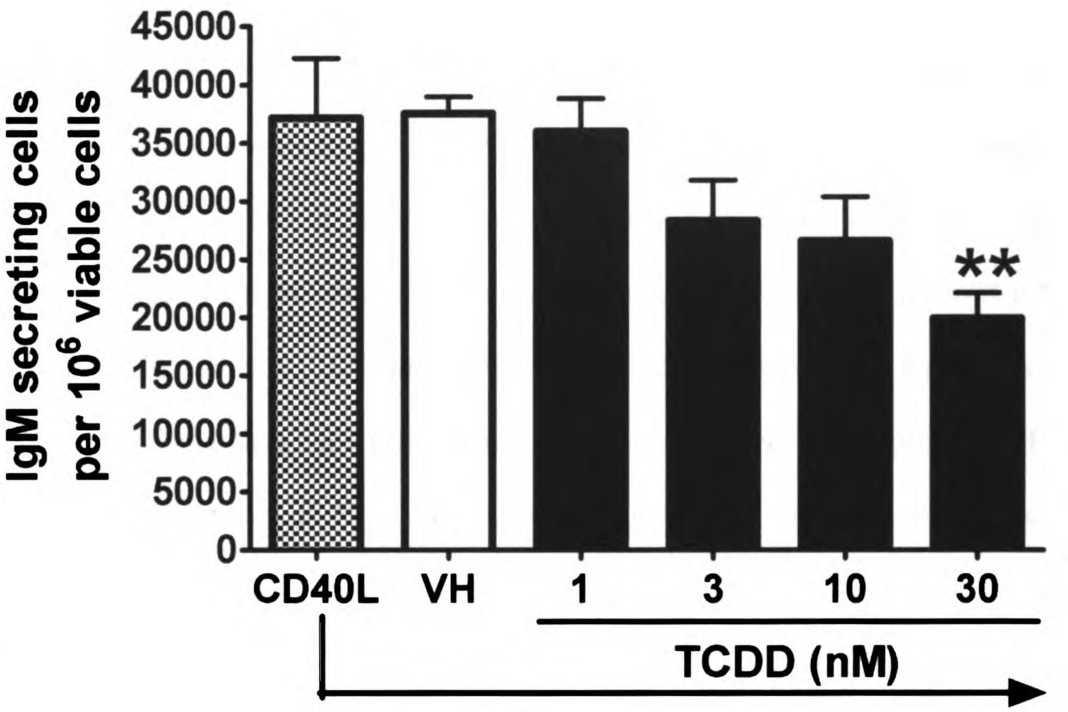


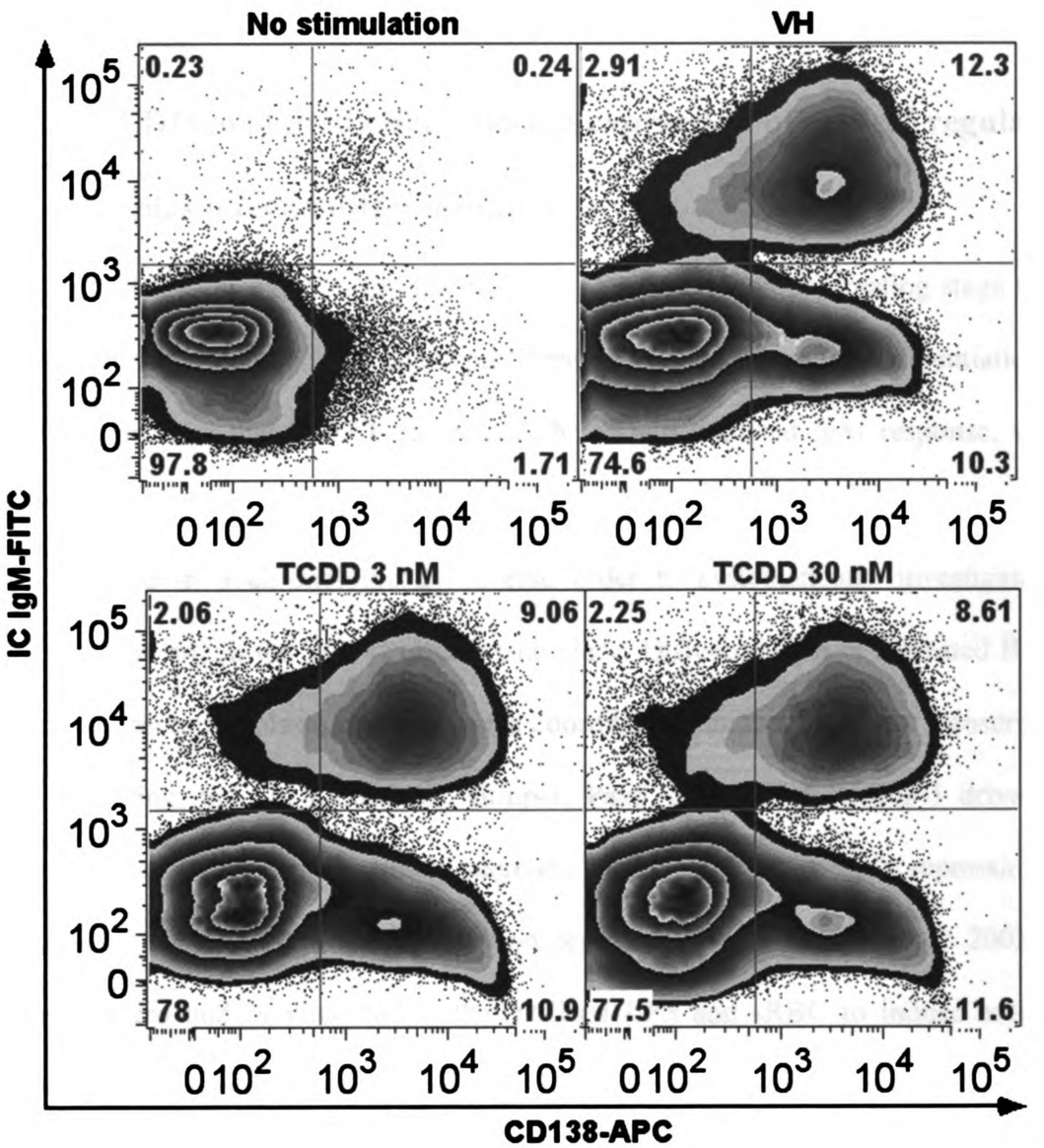
Figure 22. Effect of TCDD on the CD40L-induced IgM response in mouse B cells. Mouse B cells (1 x 10^6/ml) were treated with TCDD at concentrations indicated or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (5 x 10^4 cell/well) in the presence of recombinant mouse IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 3 days. Cells were transferred to new plates in the absence of CD40L-L cells and cultured for additional 3 days before being harvested for ELISPOT analysis of IgM secreting cells. Cell number was determined by a particle counter, and cell viability of cells was determined using the pronase activity assay. **, $p < 0.01$, compared to the VH-treated group. Data are from one experiment that is representative of three separate experiments with at least three experimental replicates per group. Data are presented as mean ± S.E.M. from three replicates per group.

(Harper *et al.* 2002). By contrast, no polymorphisms were identified in the exons of the *AHR* from the.three responsive donors.

As shown in Figure 22, the CD40L-induced IgM antibody response in mouse B cells, as measured by ELISPOT that enumerates the IgM secreting cells, was suppressed by TCDD treatment in a concentration-dependent manner with 30 nM of TCDD decreasing the response to approximately 50% of the VH control group. The magnitude of suppression by TCDD at 30 nM is similar to the suppression of *in vitro* antibody response observed in sRBC-stimulated mouse splenocytes (Holsapple *et al.* 1986a; North *et al.* 2009b). Intriguingly, TCDD did not suppress the IgM response in a previous study that stimulated isolated B cells with fixed and activated Th cells in the presence of IL-2, IL-4, and IL-5 (Karras *et al.* 1996). This discrepancy is likely due to the different culture conditions that may lead to varied levels and/or characteristics of the activation signals received by B cells.

Plasma cells, differentiated from activated B cells during primary antibody responses, exhibit a distinct phenotype that is characteristic of a high level of intracellular IgM and the expression of surface molecule CD138 (syndecan-1). Using flow cytometry, the expression of IgM in the cytosol and CD138 on the surface was measured simultaneously on a single-cell basis. As shown in Figure 23 and Table 1 (Page 96), the CD40L activation model induced a IgM secreting plasma cell phenotype as indicated by significantly increased percentage of $IgM^{high}CD138^{+}$ cells and the mean fluorescent intensity (MFI) of intracellular IgM in the entire cell population. Altogether, these data clearly demonstrated that TCDD impaired the ability of mouse B cells to differentiate into plasma cells that produce high levels of IgM and exhibit a $CD138^{+}$ phenotype

Figure 23. Effect of TCDD on the CD40L-induced antibody secreting plasma cell phenotype from mouse B cells. Mouse B cells (1 x 10^6/ml) were treated with 1 (not shown), 3, 10 (not shown), or 30 nM of TCDD, or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (5 x 10^4 cell/well) in the presence of recombinant mouse IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 3 d. Cells were transferred to new plates in the absence of CD40L-L cells and cultured for additional 3 d. Cells were harvested right after isolation (Day 0, no stimulation) on Day 6 (CD40L + VH, CD40L + TCDD 3nM, and CD40L + TCDD 30 nM) assessed by flow cytometry for surface expression of CD138 and intracellular expression of IgM. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit, and are not included in this analysis. Results depicted are fluorescent signals for intracellular IgM and CD138. Percentages of viable cells within each quadrant are shown in the corners of each individual plot. These data are from one experiment that is representative of two separate experiments with three replicates per group. The data shown are concatenated sample from three experimental replicates.


No stimulation
VH
TCDD 3 nM
TCDD 30 nM
0.23
0.24
97.8
1.71
2.91
12.3
74.6
10.3
2.06
9.06
78
10.9
2.25
8.61
77.5
11.6
IC IgM-FITC
CD138-APC
0
10^2
10^3
10^4
10^5

following activation with CD40L in combination with cytokines.

III. TCDD effects on critical stages of the plasmacytic differentiation of B cells

A. TCDD-mediated effects on the expression of critical regulators of plasmacytic differentiation

Multiple stages exist during the progress of a B cell from the resting stage to the differentiated stage, namely activation, proliferation, and plasmacytic differentiation. In light of the findings that TCDD suppressed the CD40L-induced IgM response, which represents the ultimate biological consequence of the series of events listed above, studies were conducted in a chronologically reverse order to systematically investigate the critical stages at which the TCDD effects take place. Differentiation of activated B cells into antibody secreting plasma cells requires coordinated action of multiple transcription factors, including, but not limited to, Blimp-1, Pax5, and BCL-6. Blimp-1 drives the differentiation of B cells into antibody secreting plasma cells by directly repressing the expression of negative B cell differentiation regulator Pax5 (Calame *et al.* 2003). In previous *in vitro* and *in vivo* studies that utilized LPS and sRBC to induce antibody responses, TCDD was shown to impair the mRNA and protein expression of Blimp-1 in mouse B cells, suggesting Blimp-1 is a potential target of TCDD during the suppression of primary antibody responses in mice (North *et al.* 2009a; Schneider *et al.* 2009). Based on the sensitivity of CD40L-induced IgM responses of human B cells to the suppression by TCDD as described above, studies were conducted to investigate whether the TCDD

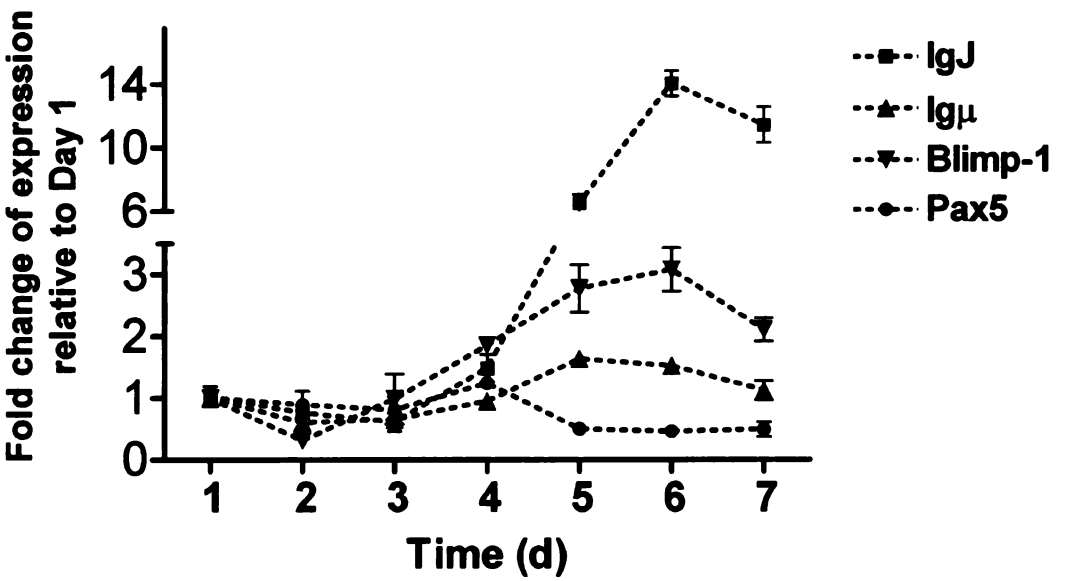
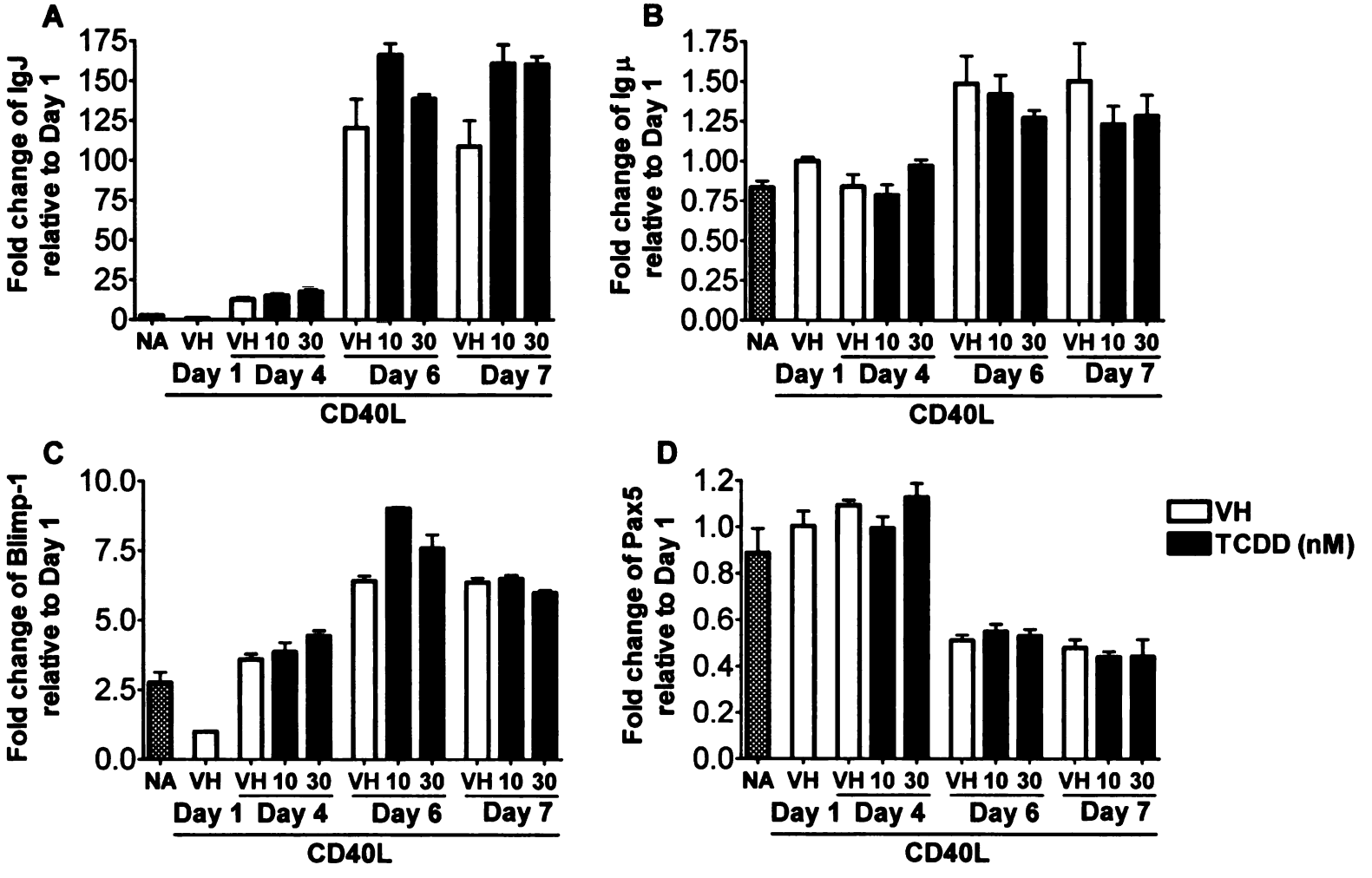


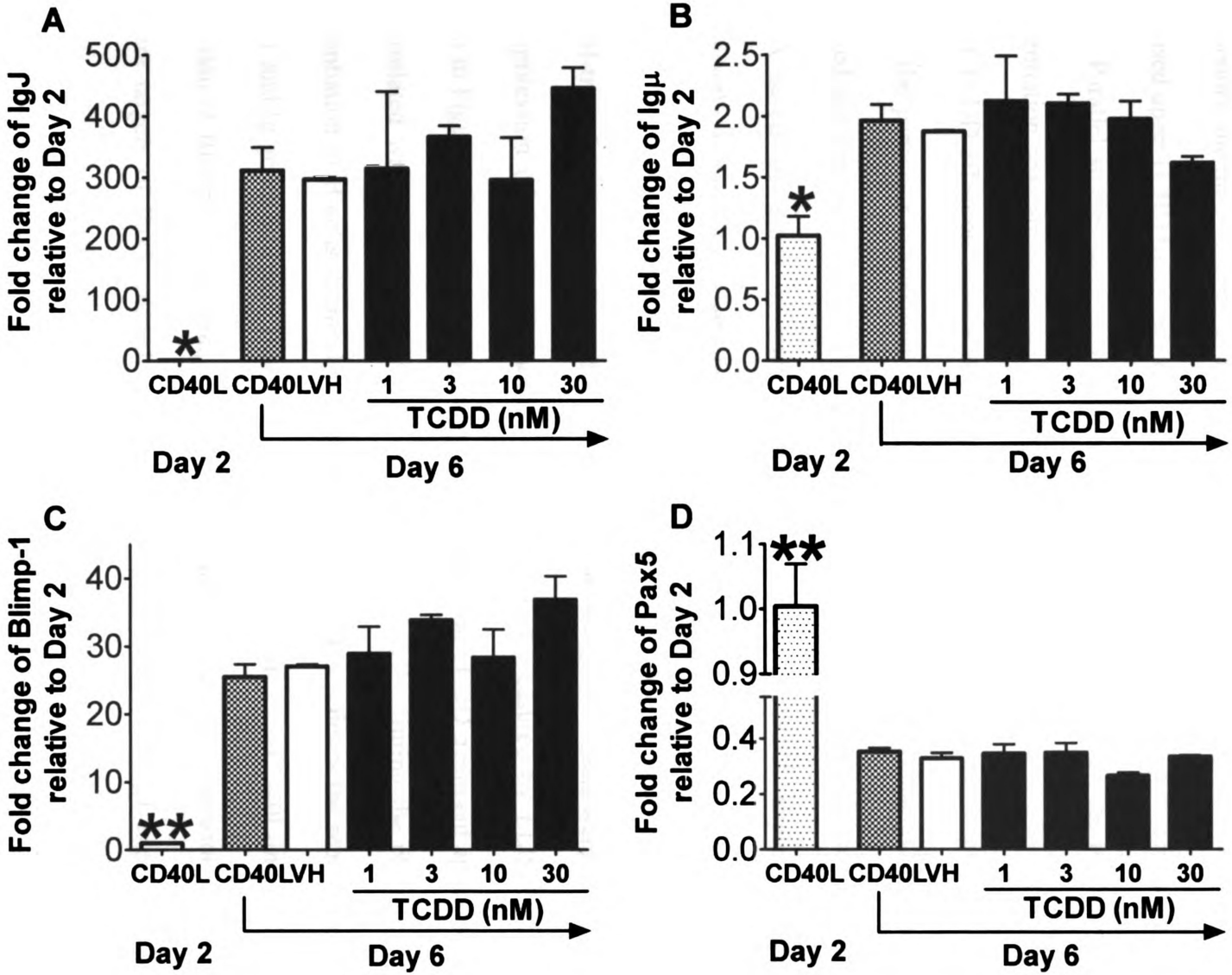
Figure 24. Expression kinetics of plasmacytic differentiation regulators in human B cells activated with CD40L. Naive human B cells (1 x 10^6/ml) were treated co-cultured with irradiated CD40L-L cells (1.5 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 4 d. Cells were transferred to new plates in the absence of CD40L-L cells on Day 4, and cultured for additional 3 days. Cells were harvested on Day 1, 2, 3, 4, 5, 6, and 7, total RNA was isolated, and steady state mRNA levels of IgJ, Igμ, Blimp-1, and Pax5 were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold change compared to Day 1. Data are from one experiment that is representative of two separate kinetic experiments (each used B cells from one individual donor) with three experimental replicates per group. Data are presented as mean ± S.E.M. from three replicates per group.

Figure 25. Effect of TCDD on the CD40L-induced mRNA expression of plasmacytic differentiation regulators in human B cells. Naive human B cells (1 x 10^6/ml) were treated with TCDD at concentrations indicated or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (1.5 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 4 days. Cells were transferred to new plates in the absence of CD40L-L cells on Day 4, and cultured for additional 3 days. Cells were harvested on Day 1, 4, 6, and 7, total RNA was isolated, and steady state mRNA levels of (A) IgJ, (B) Igu, (C) Blimp-1, and (D) Pax5 were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold change compared to the VH treated group on Day 1. Data are from one experiment that is representative of two separate experiments (each experiment used B cells from one individual donor) with three experimental replicates per group. Data are presented as mean ± S.E.M. from three replicates per group.


A
Fold change of IgJ relative to Day 1
175
150
125
100
75
50
25
0
NA VH VH 10 30 VH 10 30 VH 10 30
Day 1 Day 4 Day 6 Day 7
CD40L
B
Fold change of Ig μ relative to Day 1
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
NA VH VH 10 30 VH 10 30 VH 10 30
Day 1 Day 4 Day 6 Day 7
CD40L
C
Fold change of Blimp-1 relative to Day 1
10.0
7.5
5.0
2.5
0.0
NA VH VH 10 30 VH 10 30 VH 10 30
Day 1 Day 4 Day 6 Day 7
CD40L
D
Fold change of Pax5 relative to Day 1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NA VH VH 10 30 VH 10 30 VH 10 30
Day 1 Day 4 Day 6 Day 7
CD40L
VH
TCDD (nM)

effect observed on human B cell IgM responses was also due to the disrupted expression of critical molecules that regulate plasmacytic differentiation. The first series of experiments aimed at obtaining the expression kinetics of these critical plasmacytic differentiation regulators, as relatively less is known concerning the transcriptional regulation of plasmacytic differentiation of human B cells. As shown in Figure 24, the expression of IgJ, Ig heavy mu chain (Igμ), and Blimp-1 were increased while Pax5 was decreased in a time-related manner, and similar expression kinetics was observed in B cells from multiple human donors (data not shown), suggesting that the CD40L activation consistently induced plasmacytic differentiation of human B cells into antibody secreting cells. The expression kinetics also demonstrated that the plasmacytic differentiation program most likely initiated between Day 3 and Day 4, and the expression pattern only became apparent after Day 4. The next series of studies focused on characterizing the effect of TCDD on the mRNA expression of critical regulators involved in plasmacytic differentiation in human B cells. Experiments were also conducted in parallel using B cells from the same donors to assess whether the IgM response was suppressed by TCDD, and data described below were obtained from donors whose B cells exhibited sensitivity to suppression of IgM response by TCDD. TCDD at concentrations that suppressed the IgM response (10 and 30 nM) of B cells from multiple donors as described above did not exhibit significant effects on the mRNA levels of IgJ, Igμ, Blimp-1, and Pax5 at any of the time points assessed (Figure 25). The concentration-related effect of TCDD was also investigated at the time point when the mRNA expression of IgJ, Igμ, and Blimp-1 peaked, using B cells from another donor. Again, no significant effect of TCDD was observed at all concentrations tested (1 – 30 nM) (Figure 26). Notably, in B cells from

Figure 26. Concentration-dependent effect of TCDD on the CD40L-induced mRNA expression of plasmacytic differentiation regulators in human B cells. Naive human B cells (1×10^6/ml) were treated with TCDD at concentrations indicated or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (1.5×10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 4 days. Cells were transferred to new plates in the absence of CD40L-L cells on Day 4, and cultured for additional 3 days. Cells were harvested on Day 6, total RNA was isolated, and steady state mRNA levels of (A) IgJ, (B) Igu, (C) Blimp-1, and (D) Pax5 were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold change compared to the CD40L group on Day 2. *, $p < 0.05$, **, $p < 0.01$, compared to the VH-treated group on Day 6. Data are from one experiment that is representative of two separate experiments (each experiment used B cells from one individual donor) with three experimental replicates per group. Data are presented as mean ± S.E.M. from three replicates per group.


A
Fold change of IgJ relative to Day 2
500
400
300
200
100
0
*
CD40L
CD40LVH
1
3
10
30
TCDD (nM)
Day 2
Day 6
B
Fold change of Igμ relative to Day 2
2.5
2.0
1.5
1.0
0.5
0.0
*
CD40L
CD40LVH
1
3
10
30
TCDD (nM)
Day 2
Day 6
C
Fold change of Blimp-1 relative to Day 2
40
30
20
10
0
**
CD40L
CD40LVH
1
3
10
30
TCDD (nM)
Day 2
Day 6
D
Fold change of Pax5 relative to Day 2
1.1
1.0
0.9
0.4
0.2
0.0
**
CD40L
CD40LVH
1
3
10
30
TCDD (nM)
Day 2
Day 6

both donors, the mRNA levels of Igμ, specific for the expression of IgM, were modestly decreased upon TCDD treatment, although the effects were not statistically significant.

Parallel studies were conducted to assess the TCDD effect on the plasmacytic differentiation program in mouse B cells activated with CD40L, and also to ensure the lack of TCDD effect on plasmacytic differentiation regulators in human B cells was not due to the particular stimuli that were used to activate the cells. Mouse B cells were activated and harvested at Day 1 (not activated) and Day 5 (differentiated) to assess the mRNA levels of genes of interest. At Day 5, Blimp-1 mRNA expression was significantly up-regulated when compared to Day 1 (Figure 27). Treatment with TCDD caused a concentration-dependent decrease of Blimp-1 mRNA levels when compared to the VH-treated group, and the profile of concentration-dependent effects were similar to the suppression of the CD40L-induced IgM response as measured by ELISPOT. As shown in Figure 27, decreased expression of Blimp-1 upon TCDD treatment appeared to be correlated with increased mRNA level of Pax5 in B cells. During the plasmacytic differentiation of B cells, Blimp-1 represses Pax5 and in turn relieves the repression on XBP-1 and Ig genes by Pax5. Treatment with TCDD attenuated the CD40L-induced up-regulation of Blimp-1 expression, and might consequently alter the expression of genes that are targets of Pax5. Indeed, the up-regulation of XBP-1 and IgJ expression in CD40L-activated B cells was also attenuated by TCDD (Figure 27). To confirm the altered mRNA levels also lead to changes on the level of protein expression, multiparametric flow cytometry was employed to assess the intracellular expression of Blimp-1 and Pax5, along with the surface expression of plasma cell marker CD138. At Day 6, a sub-population of cells exhibited a $CD138^{+}$ phenotype and also possessed

Figure 27. Effect of TCDD on the CD40L-induced mRNA expression of plasmacytic differentiation regulators in mouse B cells. Mouse B cells (1×10^6/ml) were treated with TCDD at concentrations indicated or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (5×10^4 cell/well) in the presence of recombinant mouse IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 3 days. Cells were transferred to new plates in the absence of CD40L-L cells on Day 3, and cultured for additional 2 days. Cells were harvested on Day 1 and Day 5, total RNA was isolated, and steady state mRNA levels of (A) Ig J chain, (B) Blimp-1, (C) Pax5, and (D) XBP-1 were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. Data are presented as fold change compared to the CD40L group on Day 1. *, $p < 0.05$, **, $p < 0.01$, compared to the VH-treated group on Day 5. Three experimental replicates were included in each group. Data are from one experiment that is representative of two separate experiments, in which mRNA levels of IgJ, Blimp-1, Pax5, and XBP-1 were modulated by TCDD. Data are presented as mean ± S.E.M. from three replicates per group.

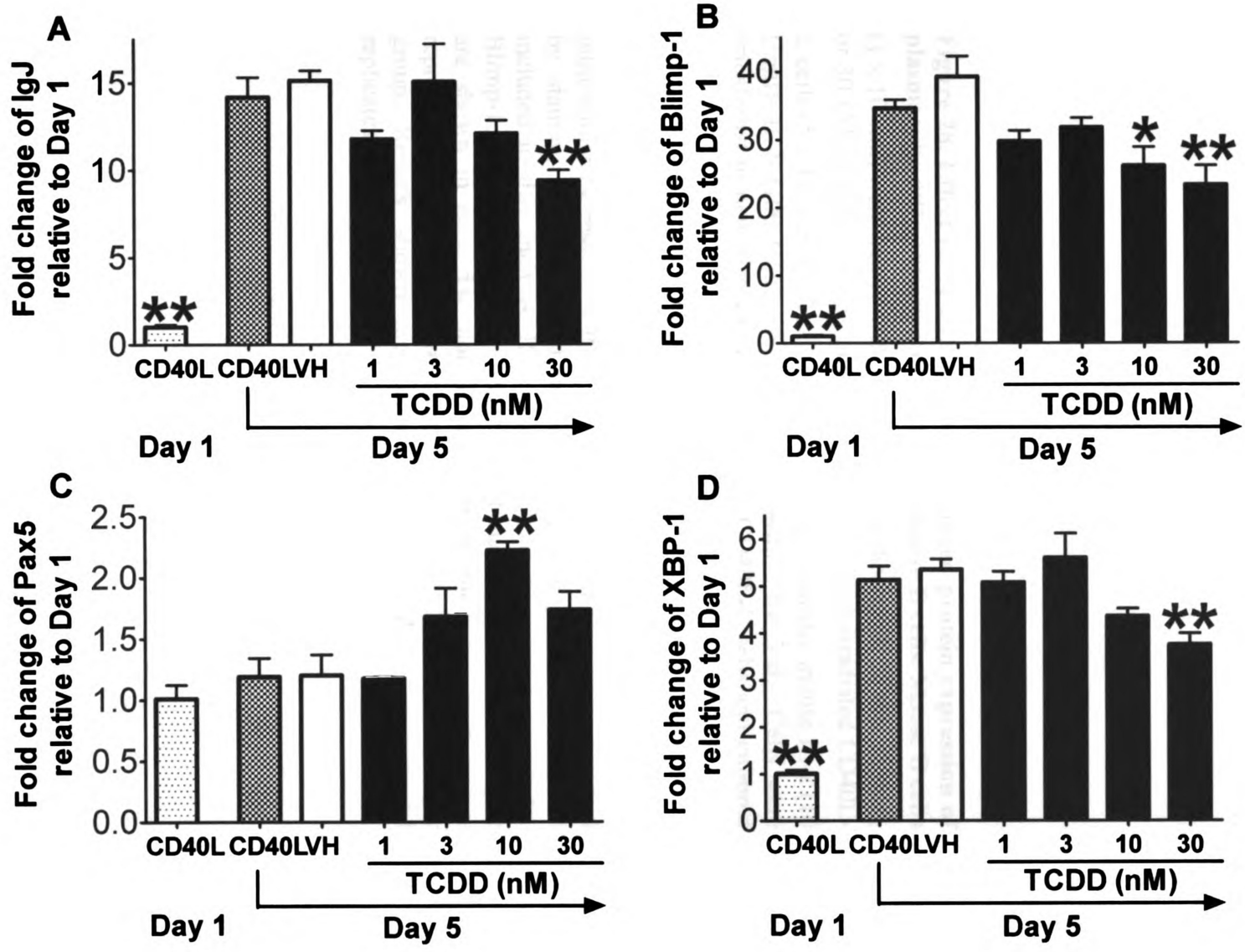

A
Fold change of IgJ relative to Day 1
15
10
5
0
**
CD40L
CD40LVH
1
3
10
30
TCDD (nM)
Day 1
Day 5
B
Fold change of Blimp-1 relative to Day 1
40
30
20
10
0
**
*
**
CD40L
CD40LVH
1
3
10
30
TCDD (nM)
Day 1
Day 5
C
Fold change of Pax5 relative to Day 1
2.5
2.0
1.5
1.0
0.5
0.0
**
CD40L
CD40LVH
1
3
10
30
TCDD (nM)
Day 1
Day 5
D
Fold change of XBP-1 relative to Day 1
6
5
4
3
2
1
0
**
**
CD40L
CD40LVH
1
3
10
30
TCDD (nM)
Day 1
Day 5

Figure 28. Effect of TCDD on CD40L-induced protein expression of plasmacytic differentiation regulators in mouse B cells. Mouse B cells (1×10^6/ml) were treated with TCDD at 1(not shown), 3, 10 (not shown), or 30 nM, or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (5×10^4 cell/well) in the presence of recombinant mouse IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 3 d. Cells were transferred to new plates in the absence of CD40L-L cells and cultured for additional 3 d. Cells were harvested right after isolation (Day 0, naive), and on Day 2 (not shown) and Day 6 (CD40L + VH, CD40L + TCDD 3 nM, and CD40L + TCDD 30 nM), and then assessed by multiparametric flow cytometry for surface expression of CD138 and intracellular expression of Blimp-1 and Pax5. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit, and are not included in this analysis. Results depicted are fluorescent signals for Blimp-1, Pax5, and CD138. Percentages of viable cells within each gate are shown in each dot plot. Data are from one experiment that is representative of two separate experiments with three replicates per group. Results shown are concatenated from three experimental replicates.

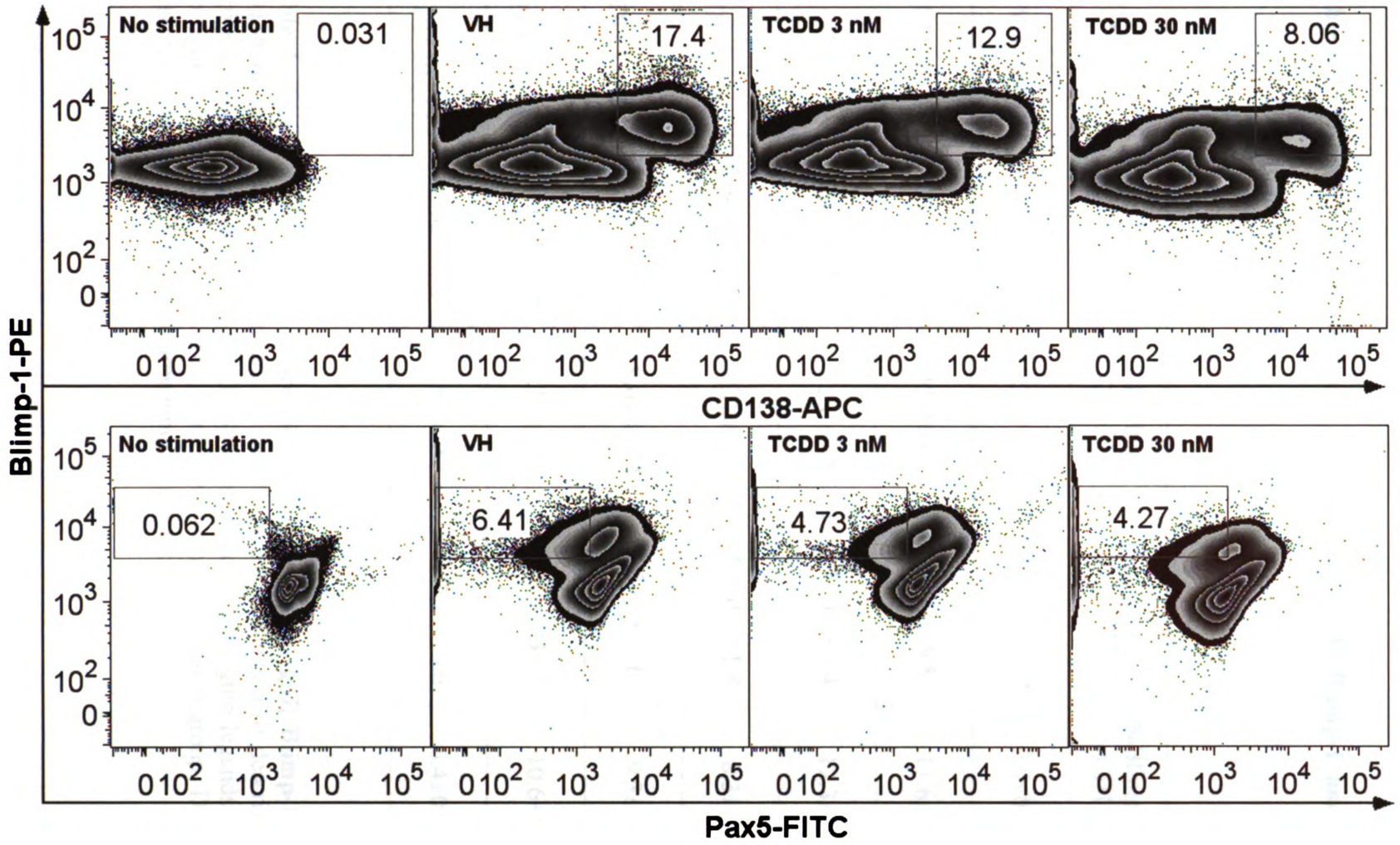

No stimulation
0.031
VH
17.4
TCDD 3 nM
12.9
TCDD 30 nM
8.06
No stimulation
0.062
VH
6.41
TCDD 3 nM
4.73
TCDD 30 nM
4.27
Blimp-1-PE
CD138-APC
Pax5-FITC

Table 1. TCDD effect on the expression of intracellular IgM, Blimp-1, and CD138 in mouse B cells

Group	MFI IgM	%IgMhigh CD138+	MFI Blimp-1	%Blimp-1high CD138+
NA (Day 0)	397.0±5.6**	0.2±0.0**	1660.0±27.1**	0.0±0.0**
CD40L	2703.0±141.3	11.8±0.5	2933.0±39.8	11.6±0.9
+VH	3130.0±47.2	12.0±0.2	2973.0±202.4	12.3±1.4
+ TCDD 1 nM	2539.0±169.8*	10.1±0.6*	2736.0±111.8	8.3±1.0
+ TCDD 3 nM	2493.0±167.4*	9.0±0.1**	2754.0±22.6	9.3±0.8
+ TCDD 10 nM	2448.0±161.0*	9.1±0.1**	2478.0±236.7	10.6±1.7
+ TCDD 30 nM	2152.0±158.8*	8.5±0.5**	1827.0±116.6**	6.4±0.5**

Table 1. TCDD effect on the expression of intracellular IgM, Blimp-1, and CD138 in mouse B cells. Summary of data from experiments also described in Figure 23 and 28. Experimental details were provided in the figure legends of the two figures. *, $p < 0.05$, **, $p < 0.01$, compared to the VH-treated group. Data are presented as mean ± S.E.M. from three replicates per group.

higher expression of Blimp-1 and lower expression of Pax5 as shown in Figure 28 and Table 1, treatment with TCDD significantly impaired formation of such a differentiated phenotype, indicated by decrease in the percentage of $CD138^{+}Blimp\text{-}1^{high}$ cells, $Blimp\text{-}1^{high}Pax5^{low}$ cells, and the MFI of Blimp-1 in the entire cell population. These data suggested that TCDD-mediated suppression of the plasmacytic differentiation of CD40L-activated B cells into IgM secreting cells involved alteration of the expression of critical regulators Blimp-1 and Pax5. In previous studies using LPS-activated CH12.LX mouse B cells, Blimp-1 binding to the *Pax5* promoter was attenuated by TCDD treatment, suggesting that TCDD-mediated modulation of Pax5 expression is likely resulted from its effect on Blimp-1 (Schneider *et al.* 2009). It also remains to be investigated whether TCDD directly modulates the expression of Blimp-1, or it is secondary to certain effect occurring upstream of Blimp-1.

B. TCDD-mediated effects on CD40L-induced proliferation and activation of B cells

Activated B cells undergo several cell divisions prior to committing to a fate of plasma cell differentiation. Proliferation not only amplifies the magnitude of the antibody response by expansion of precursor differentiated cells, but may also be a requirement for plasmacytic differentiation and serves a regulatory role (Tangye and Hodgkin 2004). Robust proliferation of both human and mouse B cells was induced by the CD40L activation model described above. Therefore, the effect of TCDD on proliferation of naive human B cells and mouse B cells was also characterized in response to CD40L activation. Figure 29 A demonstrated the profile of naive human B cells that underwent

multiple divisions following stimulation with CD40L, which led the dilution of the proliferation dye and therefore the decrease in its fluorescent signal. Illustrated in Figure 29 B are the percentages of total viable cells that underwent certain number of divisions. TCDD treatment had no significant effect on the total number of divisions. In the group treated with TCDD, slightly more cells were present with fewer divisions and likewise fewer cells progressed to the later divisions, although the effect was modest. Studies were conducted using B cells from three different donors; despite varied levels of proliferation in vehicle-treated cells, an absence of significant TCDD effects was a consistent observation. In studies conducted in parallel using mouse B cells, fewer mouse B cells were induced by CD40L to undergo proliferation, when compared to human B cells (as indicated by more mouse B cells present in division 0) (Figure 30 A). Similar to human B cells, TCDD did not significantly affect CD40L-induced mouse B cell proliferation (Figure 30 B). Based on these results, it can be concluded that the TCDD-mediated suppression of CD40L-induced IgM response observed in both mouse and human B cells can not be attributed to decreased proliferation. These results are also consistent with previous studies in which mouse and human B cell proliferation, as measured indirectly by [^{3}H] thymidine incorporation, was found to be relatively refractory to TCDD (Allan *et al.* 2006; Dooley and Holsapple 1988; Holsapple *et al.* 1986a; Wood and Holsapple 1993).

The induction of plasmacytic differentiation is dependent on, and preceded by, the activation of B cells as a consequence of upstream signals through the engagement of BCR, CD40, or TLRs (Calame 2008). The activation status of B cells is commonly characterized by the expression of various activation markers. In LPS-stimulated mouse

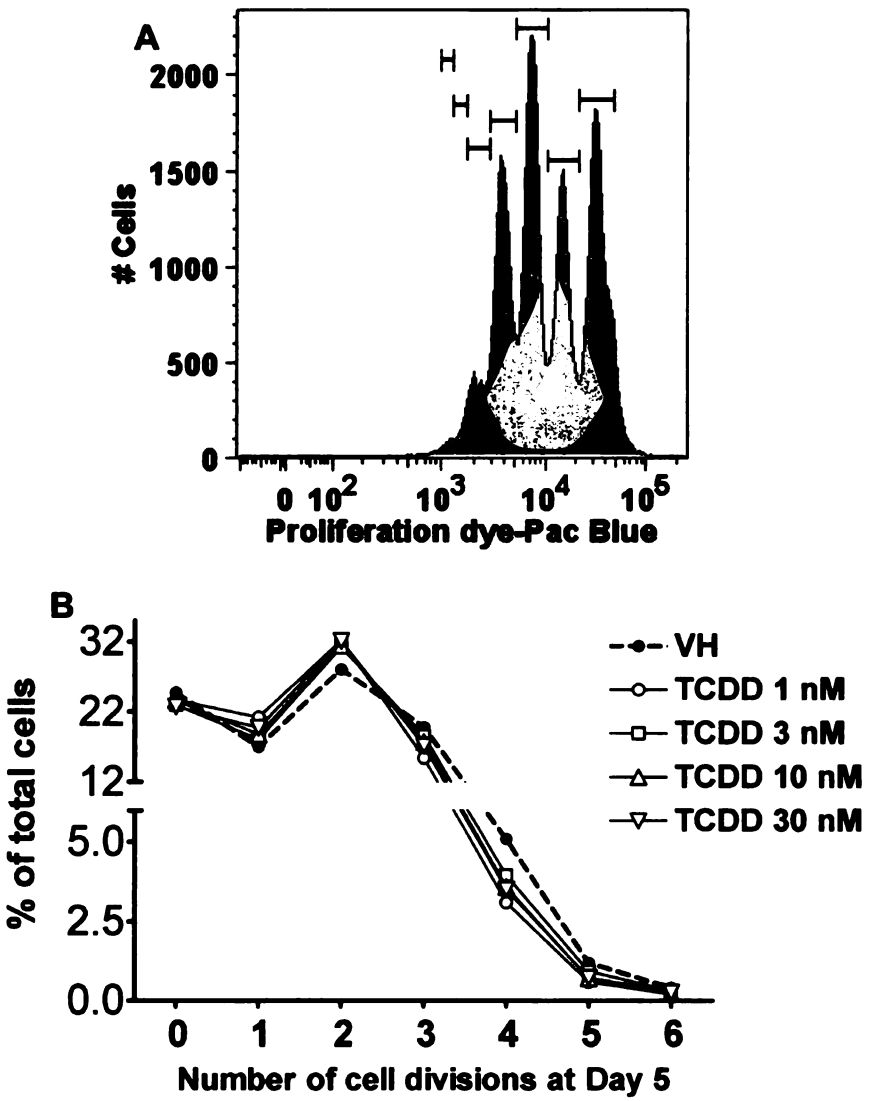


Figure 29. Effect of TCDD on the CD40L-induced proliferation of human B cells. Naive human B cells (1 x 10^6/ml) were labeled with the proliferation dye, treated with 1, 3, 10, or 30 nM of TCDD, or vehicle (VH, 0.05% DMSO), and then co-cultured with irradiated CD40L-L cells (1.5 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 4 d, and CD40L stimulation was removed on Day 4. Cells were cultured for one additional day prior to being harvested on Day 5, and assessed by flow cytometry for fluorescence of the proliferative dye. Dead cells were identified by staining with the Live/Dead near-infrared Dead Cell Staining Kit, and are not included in this analysis. (A) Histogram concatenated from the no treatment group; each bracket indicates a group of cells that undergo certain number of divisions. (B) Percentage of cells with different number of divisions. Data are presented as mean from three replicates per group.Data are from one experiment that is representative of two separate experiments (each experiment used B cells from one individual donor), with three experimental replicates included per group.

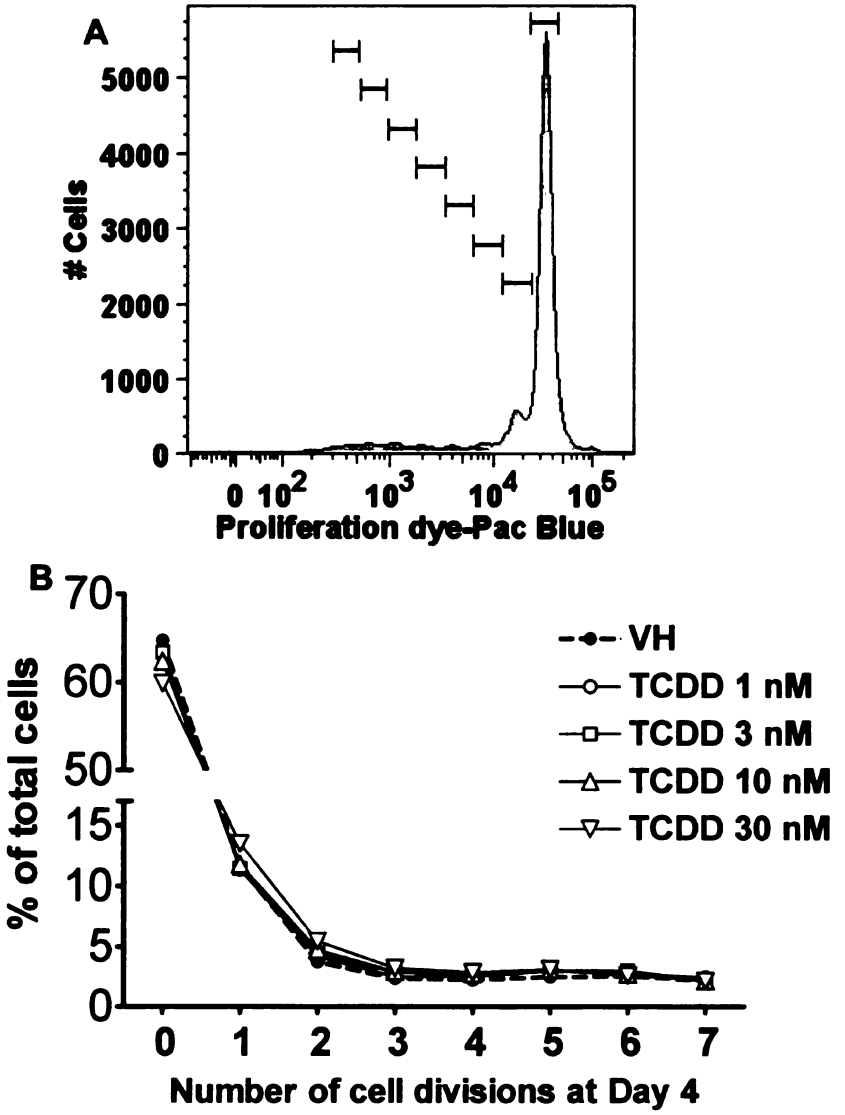


Figure 30. Effect of TCDD on the CD40L-induced proliferation of mouse B cells. Mouse B cells (1 x 10^6/ml) were labeled with the proliferation dye, treated with 1, 3, 10, or 30 nM of TCDD, or vehicle (VH, 0.05% DMSO), and then co-cultured with irradiated CD40L-L cells (5 x 10^4 cell/well) in the presence of recombinant mouse IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 4 days, and CD40L stimulation was removed on Day 3. Cells were cultured for one additional day prior to being harvested on Day 4, and assessed by flow cytometry for fluorescence of the proliferative dye. Dead cells were identified by staining with the Live/Dead near-infrared Dead Cell Staining Kit, and are not included in this analysis. (A) Histogram concatenated from the no treatment group; each bracket indicates a group of cells that undergo certain number of divisions. (B) Percentage of cells with different number of divisions. Data are presented as mean from three replicates per group.Data are from one experiment that is representative of two separate experiments, with three experimental replicates included per group.

B cells, treatment with TCDD attenuated the up-regulation of CD80, CD86, CD69, and MHC Class II (North *et al.* 2010). However, no study to date has investigated whether TCDD treatment impairs the activation of human B cells. To this end, naive human B cells were activated with the CD40L model, and the expression of CD80, and CD86, and CD69 was assessed at time points Day 1, 2, and 3 using multiparametric flow cytometry. MHC II, another common activation marker for B cells, was not included in the panel as multiple haplotypes for MHC II exist in humans, and measuring the expression of MHC II requires prior knowledge about the particular haplotype of each human donor. The time course was selected to represent the entire activation phase preceding the plasmacytic differentiation phase. As shown in Figure 31, the expression of all three activation markers was enhanced throughout the time course, indicating robust induction of an "activated phenotype" in CD40L-activated human B cells. Treatment with TCDD at 10 and 30 nM significantly attenuated the up-regulation of all three activation markers in viable cells, with the most profound effect occurring on Day 3 (Figure 31). In another study using B cells from a different donor, which were on Day 3 after treatment with TCDD (1 – 30 nM), the CD40L-induced CD80, CD86, and CD69 expression in viable cells was suppressed by TCDD in a concentration-dependent manner (Figure 32). It has been described in Chapter I of the Experimental Results that the vast majority of $CD86^-$ $CD69^-$ (not activated) human B cells were not viable at Day 4 in culture (Figure 8). Indeed, TCDD treatment was found to not only down-regulate the average expression of activation markers in viable B cells, but also decreased the percentage of viable $CD80^+CD86^+$ $CD69^+$ (fully activated) cells and increased the percentage of non-viable

Figure 31. Effect of TCDD on the CD40L-induced expression of activation markers in human B cells. Naive human B cells (1 x 10^6/ml) were treated with 10 or 30 nM of TCDD, or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (1.5 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 3 days. Cells were harvested right after isolation (Day 0, naive), and on Day 1, 2, and 3, and then assessed by multiparametric flow cytometry for surface expression of CD80, CD86, and CD69. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit, and are not included in this analysis. **, $p < 0.01$, compared to the VH-treated group at each time point. Three experimental replicates were included per group. Data are presented as mean ± S.E.M. from three experimental replicates per group.

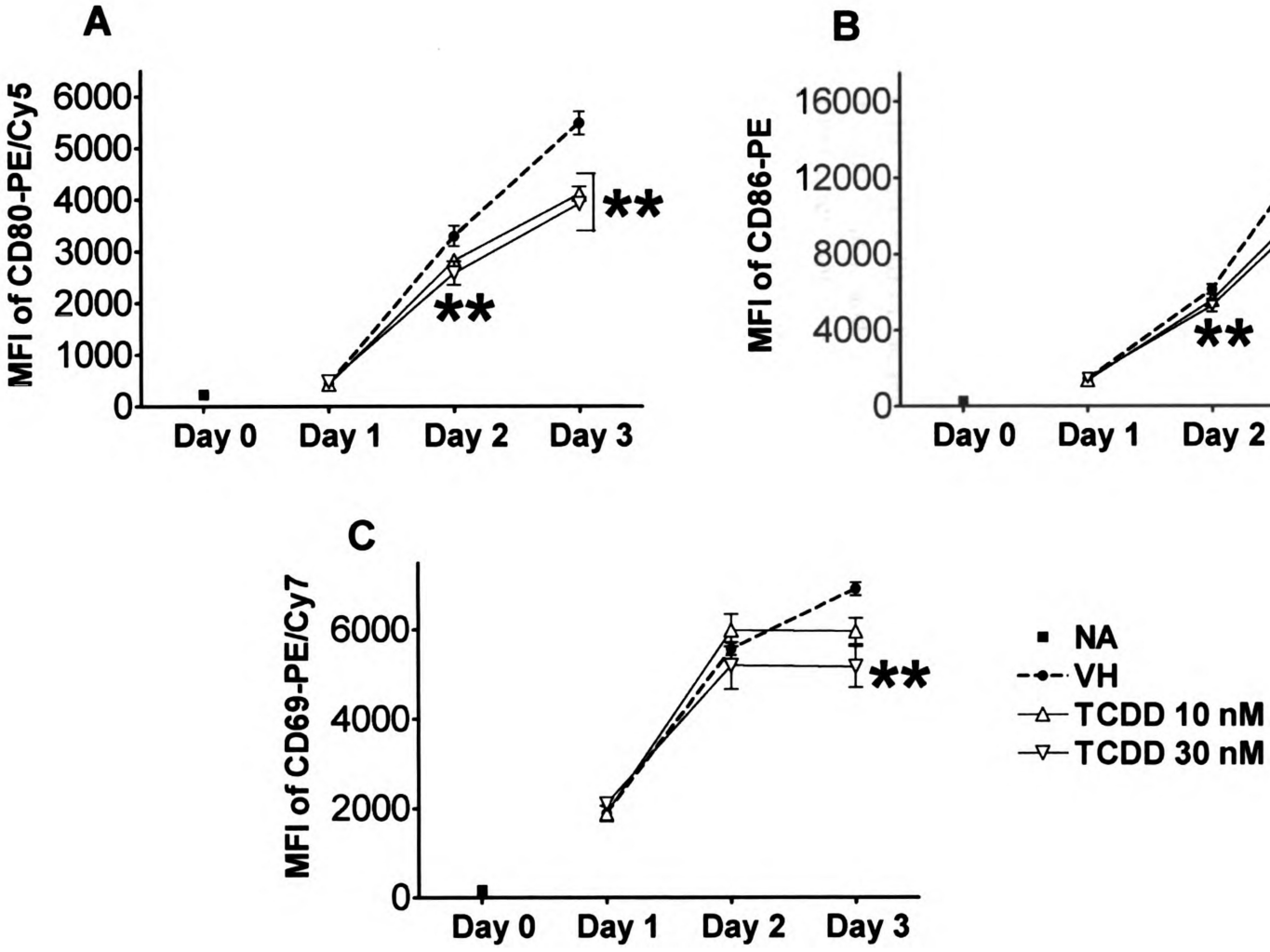
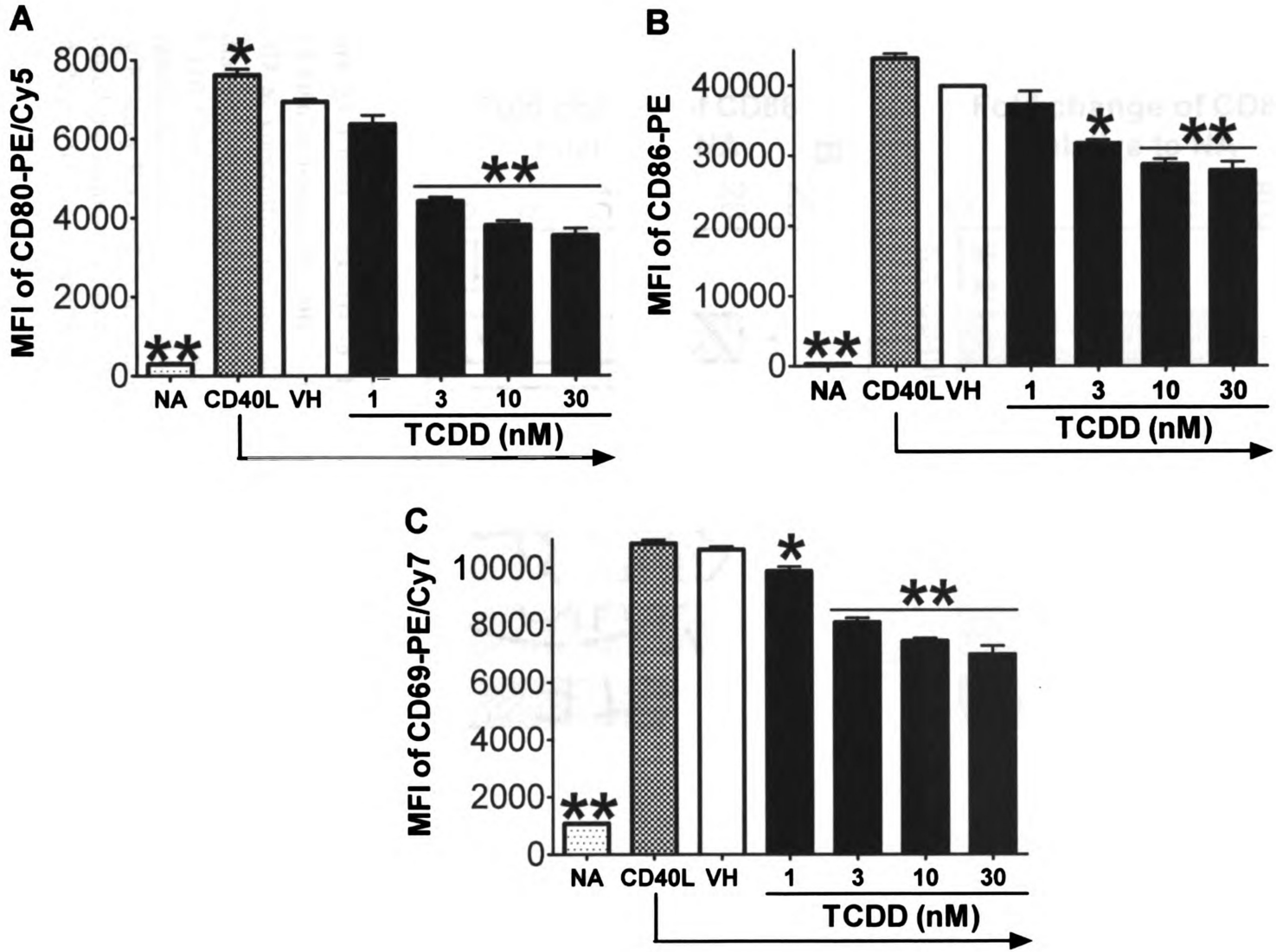

A
MFI of CD80-PE/Cy5
6000
5000
4000
3000
2000
1000
0
Day 0
Day 1
Day 2
Day 3
**
**
B
MFI of CD86-PE
16000
12000
8000
4000
0
Day 0
Day 1
Day 2
Day 3
**
**
C
MFI of CD69-PE/Cy7
6000
4000
2000
0
Day 0
Day 1
Day 2
Day 3
**
NA
VH
TCDD 10 nM
TCDD 30 nM

Figure 32. Concentration-dependent effect of TCDD on the CD40L-induced expression of activation markers in human B cells. Naive human B cells (1×10^6/ml) were treated with TCDD at concentrations indicated, or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (1.5×10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 3 days. Cells were harvested right after isolation (Day 0, naive) and on Day 3, and then assessed by multiparametric flow cytometry for surface expression of CD80, CD86, and CD69. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit, and are not included in this analysis. *, $p < 0.05$, **, $p < 0.01$, compared to the VH-treated group. Three experimental replicates were included per group. Data are presented as mean ± S.E.M. from three experimental replicates per group.


A
MFI of CD80-PE/Cy5
8000
6000
4000
2000
0
*
**
**
NA
CD40L
VH
1
3
10
30
TCDD (nM)
B
MFI of CD86-PE
40000
30000
20000
10000
0
**
*
**
NA
CD40L
VH
1
3
10
30
TCDD (nM)
C
MFI of CD69-PE/Cy7
10000
8000
6000
4000
2000
0
**
*
**
NA
CD40L
VH
1
3
10
30
TCDD (nM)

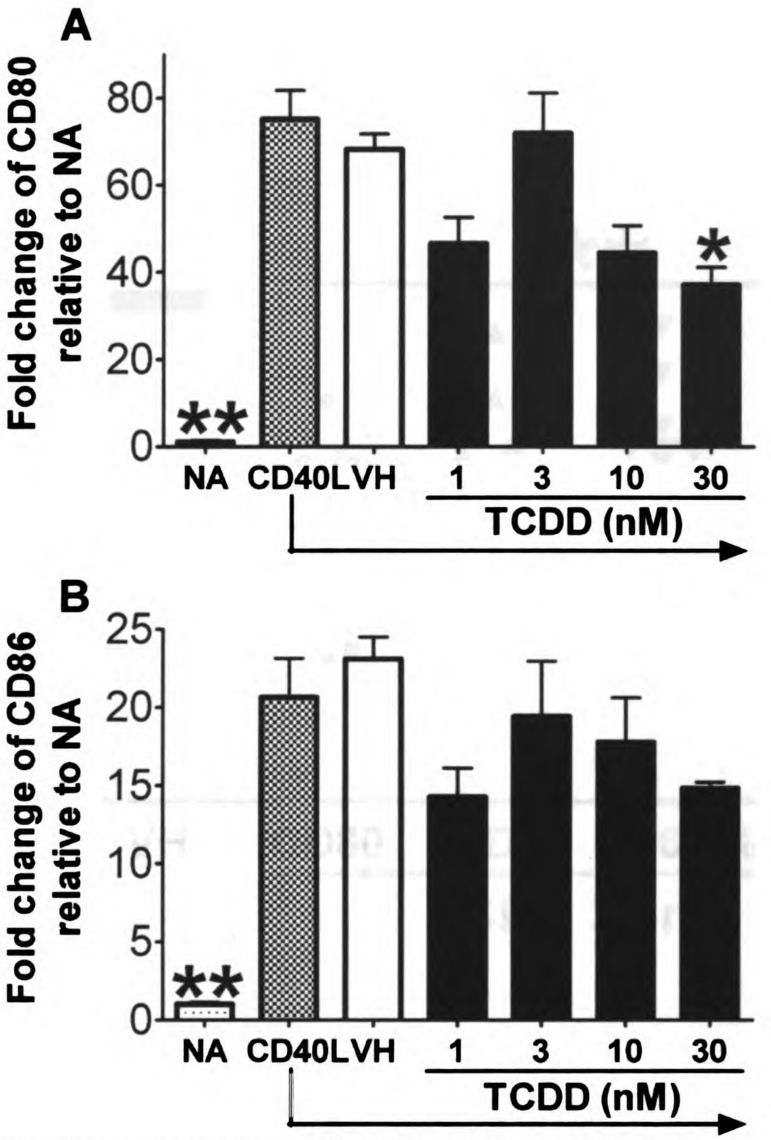


Figure 33. Effect of TCDD on the CD40L-induced mRNA expression of CD80 and CD86 in human B cells. Naive human B cells (1 x 10^6/ml) were treated with TCDD at concentrations indicated, or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (1.5 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 3 days. Cells were harvested right after isolation (Day 0, naive) and on Day 3, total RNA was isolated using RNeasy Kit, and steady state mRNA levels of (A) CD80 and (B) CD86 were measured by Taqman real-time PCR and normalized to endogenous 18S ribosomal RNA. *, $p < 0.05$, compared to the VH-treated group. Three experimental replicates were included per group. Data are from one experiment that is representative of two separate experiments (each experiment used B cells from one individual donor), in which decreased mRNA levels of CD80 and CD86 were observed upon TCDD treatment. Data are presented as mean ± S.E.M. from three experimental replicates per group.

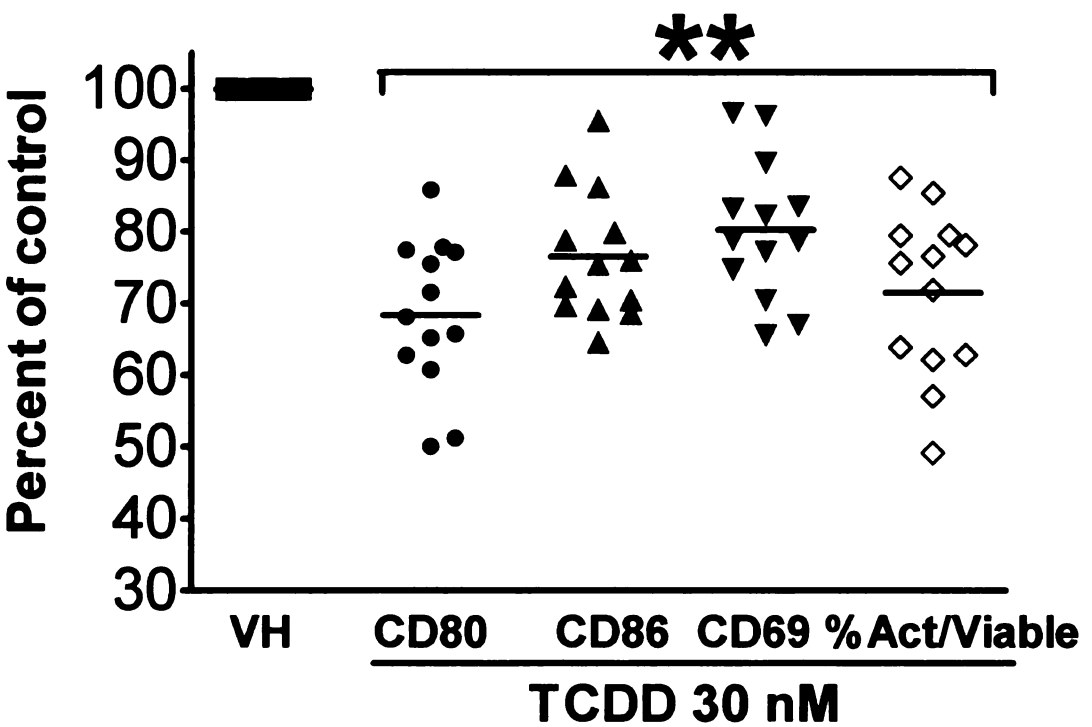
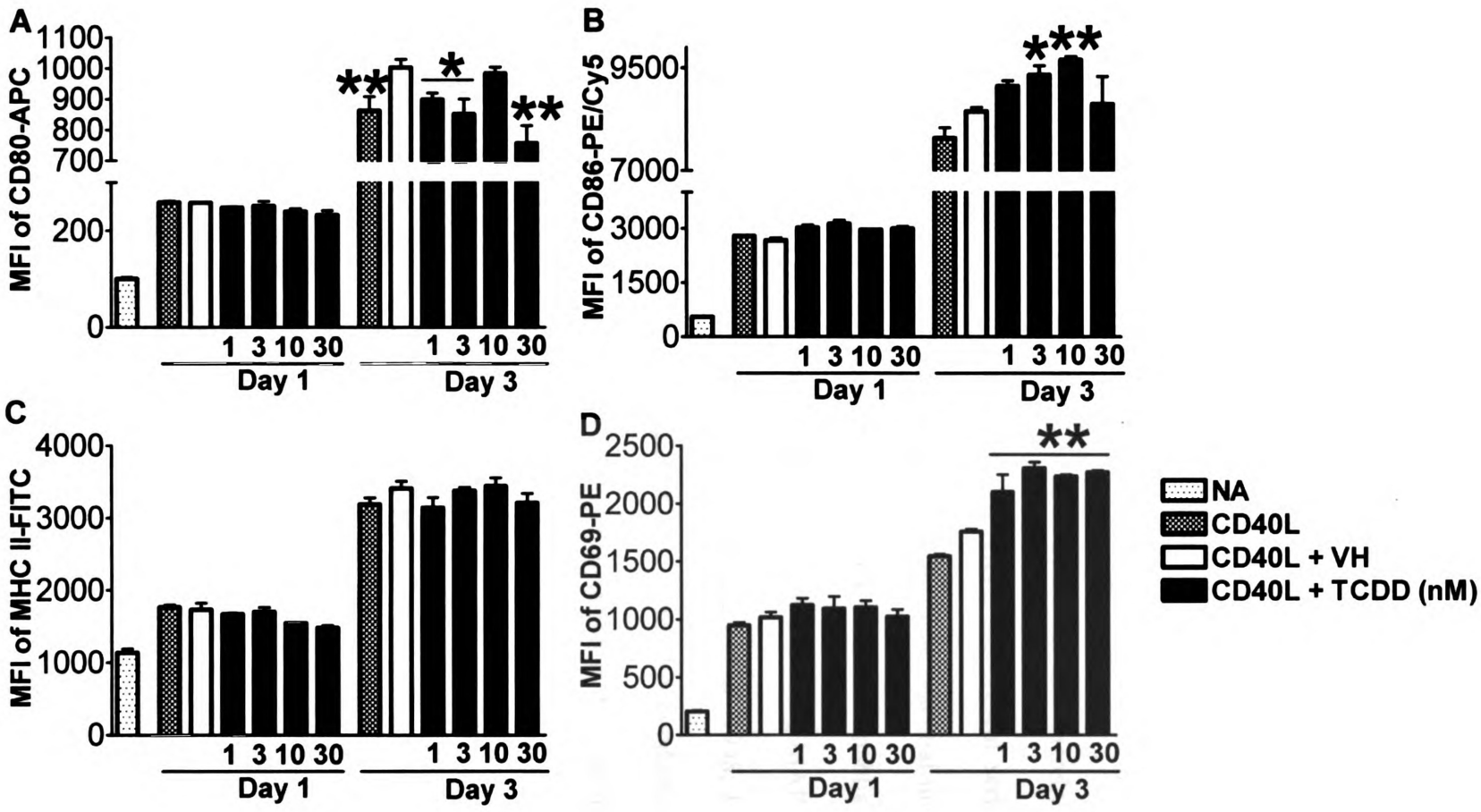


Figure 34. Effect of TCDD on the CD40L-induced activated phenotype in human B cells from thirteen donors. **, $p < 0.01$, compared with the VH-treated group. Data were normalized to the VH-treated group (100%) and presented as percent of control with at least three replicates per group. Included in the graph are mean fluorescence intensity comparisons for CD80, CD86, and CD69, and the percentages of cells that were viable and also expressed all three activation markers. These percentages were obtained using the Boolean gating technique of the FlowJo software. Each dot represents data from one individual donor. Data are presented as mean from three experimental replicates per group.

$CD80^{-}CD86^{-}CD69^{-}$ (not activated) cells in the total cell pool, starting on Day 2 and becoming more pronounced on Day 3. TCDD affected the protein expression of CD80 and CD86, at least in part, at the level of transcription, since mRNA levels of *CD80* and *CD86* were modestly decreased in TCDD-treated human B cells (Figure 33). The decreased expression of CD80, CD86, and CD69 was consistently observed in B cells from 13 donors. In Figure 34, data were summarized as one human donor as an experimental replicate. As shown in Figure 34, the expression of CD80 appeared to be the most affected, and the decreased activation marker expression was correlated with lower cell viability upon TCDD treatment. Studies were conducted in parallel to investigate whether TCDD also affected CD40L-induced activation of mouse B cells. Robust and time-related induction of CD80, CD86, CD69, and MHC II expression was observed (Figure 35), and TCDD treatment did not lead to significant effects on expression of any of the activation markers in viable cells on Day 1. On Day 3, the expression of CD80 was significantly decreased upon TCDD treatment, while the expression of both CD86 and CD69 was increased in viable cells (Figure 35). Consistent with what was described in Chapter I of the Experimental Results section (Figure 9), a substantial population of mouse B cells (approximate 40%) were $CD80^{-}CD86^{-}CD69^{-}$ (non-activated), yet still viable at Day 3. TCDD treatment had no effect on the percentage of viable mouse $CD80^{+}CD86^{+}CD69^{+}$ (fully activated) B cells in the total B cell pool on either Day 1 or Day 3.

Figure 35. Effect of TCDD on the CD40L-induced expression of activation markers in mouse B cells. Mouse B cells (1 x 10^6/ml) were treated with TCDD at concentrations indicated, or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (5 x 10^4 cell/well) in the presence of recombinant mouse IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 3 days. Cells were harvested right after isolation (Day 0, naive (NA)) and on Day 1 and 3, and then assessed by multiparametric flow cytometry for surface expression of CD80, CD86, MHC class II, and CD69. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit, and are not included in this analysis. *, $p < 0.05$, **, $p < 0.01$, compared to the VH-treated group at each time point. Data are from one experiment that is representative of two separate experiments with three experimental replicates per group. Data are presented as mean ± S.E.M. from three experimental replicates per group.


A
MFI of CD80-APC
1100
1000
900
800
700
200
0
**
*
**
1 3 10 30
1 3 10 30
Day 1
Day 3
B
MFI of CD86-PE/Cy5
9500
7000
3000
1500
0
***
1 3 10 30
1 3 10 30
Day 1
Day 3
C
MFI of MHC II-FITC
4000
3000
2000
1000
0
1 3 10 30
1 3 10 30
Day 1
Day 3
D
MFI of CD69-PE
2500
2000
1500
1000
500
0
**
1 3 10 30
1 3 10 30
Day 1
Day 3
NA
CD40L
CD40L + VH
CD40L + TCDD (nM)

C. TCDD-mediated effects on the early signaling events in CD40L-activated human B cells

Activation of B cells with CD40L and cytokines is likely the end result of signals from multiple pathways downstream of CD40 and cytokine receptors exerting a biological response. TCDD attenuated CD40L-induced up-regulation of CD80, CD86, and CD69 expression in activated human B cells, suggesting TCDD may perturb the early signaling events induced by CD40L and/or cytokines and thereby causing the decrease in expression of activation markers. Therefore, the present series of studies focused primarily on human B cells, and sought to investigate potential mechanisms by which TCDD perturbs B cell activation, with an emphasis on well-established signaling pathways downstream of CD40 and the cytokine receptors involving MAPK, PI3K/Akt, JAK/STAT, and NFκB/Rel (Berberich *et al.* 1994; Francis *et al.* 1998; Hanissian and Geha 1997; Sutherland *et al.* 1996). Parallel studies were also conducted to confirm TCDD-mediated suppression of activation marker expression in B cells from the same donors. Soluble recombinant human CD40L and cytokines were utilized to activate human B cells, as the activation of the signaling pathways of interest is not only rapid but also could be transient following cell activation. Phospho-specific antibodies were used to specifically assess the expression level of certain phosphorylated forms that directly reflect the activation of each signaling molecule. In previous studies using the same technique, TCDD was found to impair the immediate activation of ERK, JNK, and Akt in mouse B cells activated through TLRs (North *et al.* 2010). In the present study, CD40L and cytokines robustly induced activation of ERK, p38, and Akt between 15 and 30 min following activation, while the activation of JNK was relatively modest (15 min data not

shown). Interestingly, some basal level activity of p38 and Akt was observed in resting B cells in the absence of cell activation. Treatment with TCDD prior to activation with CD40L and cytokines attenuated the activation of ERK, p38, and Akt in a concentration-related manner at 30 min. 30 nM TCDD significantly affected all three kinases, while activation of JNK was the least affected (Table 2). Viewed in a multiparametric manner, TCDD profoundly diminished the population of activated cells that simultaneously expressed elevated levels of two or more phosphorylated kinases (Figure 36, 37, and 38). For instance, TCDD at 30 nM decreased the percentage of cells that expressed elevated levels of phosphorylated ERK, JNK, p38, and Akt by approximately 50% (compared to VH-treated group).

The JAK/STAT pathway is also induced in B cells by signaling through CD40 and the cytokine receptors. Seven STAT family members have been identified thus far, and although signaling through CD40 primarily activates STAT3, a given cytokine may lead to the activation of multiple STATs. For example, IL-2 activates STAT3 and STAT5, while IL-6 and IL-10 activates STAT1 and STAT3 (Diehl *et al.* 2008; Hirano *et al.* 2000; Scheeren *et al.* 2005). Upon activation, STAT proteins become phosphorylated, dimerize to form homo- or heterodimers, and then function as transcription factors (Calo *et al.* 2003). No study to date has directly assessed the effect of TCDD on the JAK/STAT pathway in B cells. CD40L and cytokines strongly induced the activation of STAT1, STAT3, and STAT5 at both 15 and 30 min post treatment, although the level of activation was slightly lower at 30 min, suggesting the immediate activation is transient (15 min data not shown). Activation of STAT1 was less profound when compared with STAT3 and STAT5 (15 min data not shown). When treated with TCDD prior to

Table 2. TCDD effect on the expression of phosphorylated ERK, JNK, Akt, and p38 in human B cells at 30 min

Group	MFI P-ERK	MFI P-JNK	MFI P-Akt	MFI P-p38
NA	180.0±0.6**	177.0±13.5	483.3±10.2	598.0±24.2**
CD40L	327.3±22.8	221.7±7.7	467.0±10.3	848.0±37.1
+VH	330.0±4.7	207.0±17.0	452.7±33.6	881.3±13.9
+ TCDD 1 nM	317.3±3.2	196.7±10.4	422.7±16.2	812.0±13.1
+ TCDD 3 nM	281.3±4.9*	196.7±7.0	402.7±6.3	747.7±19.7**
+ TCDD 10 nM	295.0±6.1	180.7±15.2	422.0±13.6	748.7±21.8**
+ TCDD 30 nM	278.0±12.9*	188.0±9.6	369.3±19.7*	701.0±27.5**

Table 2. TCDD effect on the expression of phosphorylated ERK, JNK, Akt, and p38 in human B cells at 30 min. Summary of data from experiments described in Figure 36, 37 and 38. Experimental details were provided in the figure legends of the three figures. Data included in this table are presented as mean ± S.E.M. from three experimental replicates per group. *, $p < 0.05$, **, $p < 0.01$, compared to the VH-treated group.

Figure 36. Effect of TCDD on the CD40L-induced immediate activation of ERK and JNK in human B cells at 30 min. Naive human B cells (1 x 10^6/ml) were equilibrated at 37°C for 3-4 h, treated with 3, 10 and 30 nM of TCDD, or vehicle (VH), and then stimulated with recombinant human CD40L (200 nM), human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 30 min. Cells were fixed with 1.5% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by multiparametric phosflow for intracellular expression of phosphorylated ERK and JNK. Results depicted are fluorescent signals for phosphorylated ERK and JNK. Percentages of cells within each quadrant are shown in the corner of each individual plot. Data are from one experiment that is representative of three separate experiments (each experiment used B cells from one individual donor) with three replicates per group. Results shown are concatenated from three experimental replicates.

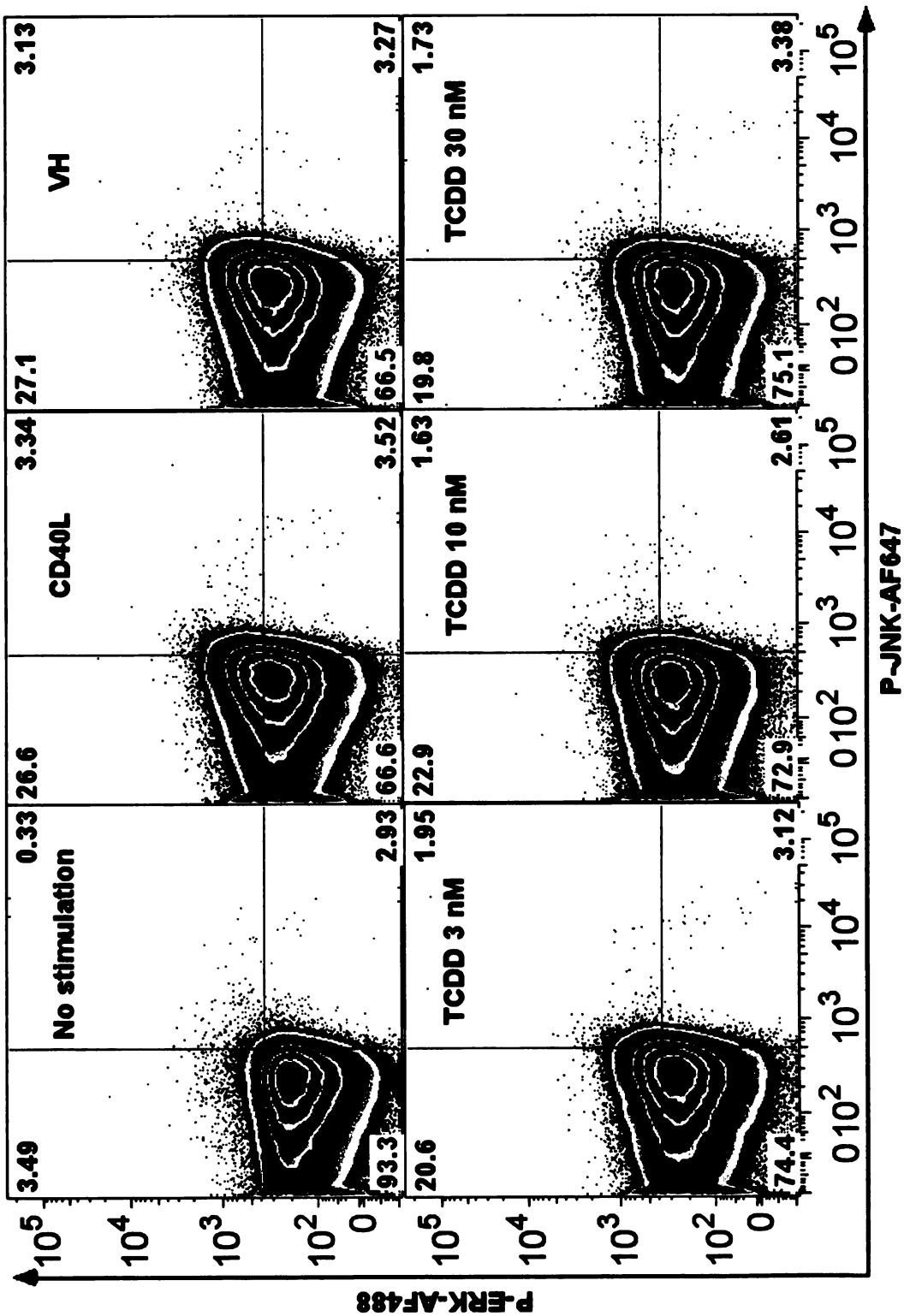
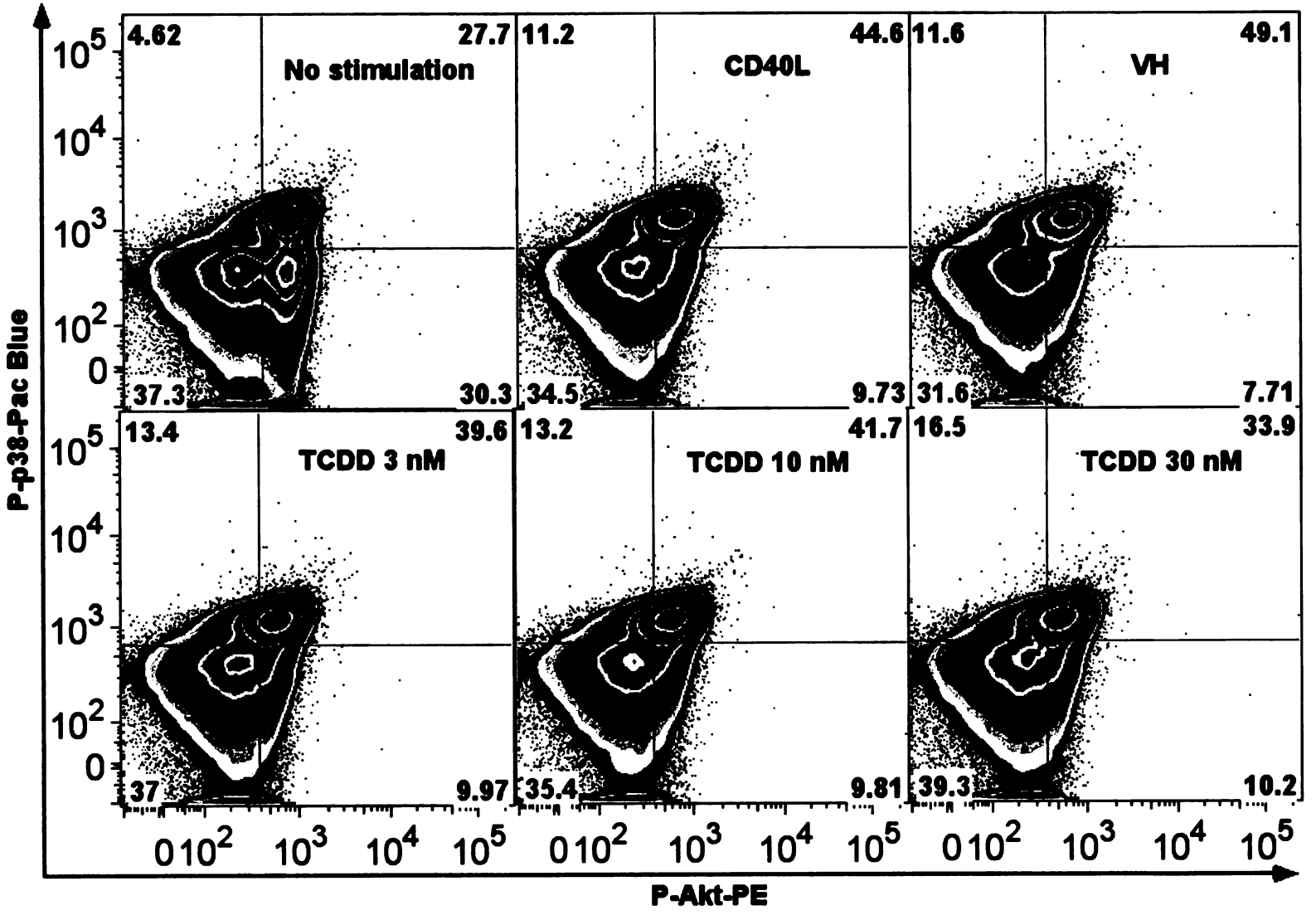

No stimulation
CD40L
VH
TCDD 3 nM
TCDD 10 nM
TCDD 30 nM
3.49
0.33
26.6
3.34
27.1
3.13
93.3
2.93
66.6
3.52
66.5
3.27
20.6
1.95
22.9
1.63
19.8
1.73
74.4
3.12
72.9
2.61
75.1
3.38
P-ERK-AF488
P-JNK-AF647

Figure 37. Effect of TCDD on the CD40L-induced immediate activation of p38 and Akt in human B cells at 30 min. Naive human B cells (1 x 10^6/ml) were equilibrated at 37°C for 3-4 h, treated with 3, 10 and 30 nM of TCDD, or vehicle (VH), and then stimulated with recombinant human CD40L (200 nM), human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 30 min. Cells were fixed with 1.5% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by multiparametric phosflow for intracellular expression of phosphorylated p38 and Akt. Results depicted are fluorescent signals for phosphorylated p38 and Akt. Percentages of cells within each quadrant are shown in the corner of each individual plot. Data are from one experiment that is representative of three separate experiments (each experiment used B cells from one individual donor) with three replicates per group. Results shown are concatenated from three experimental replicates.


4.62
27.7
No stimulation
11.2
44.6
CD40L
11.6
49.1
VH
37.3
30.3
34.5
9.73
31.6
7.71
13.4
39.6
TCDD 3 nM
13.2
41.7
TCDD 10 nM
16.5
33.9
TCDD 30 nM
37
9.97
35.4
9.81
39.3
10.2
P-p38-Pac Blue
P-Akt-PE
0 10^2 10^3 10^4 10^5

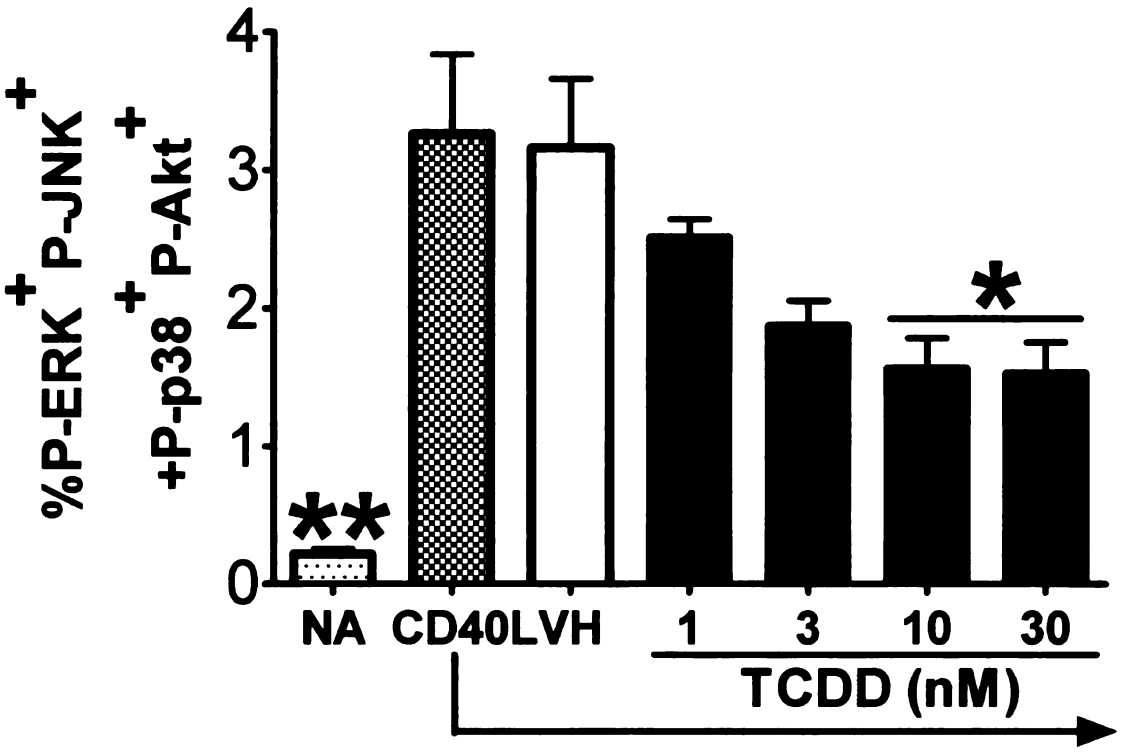


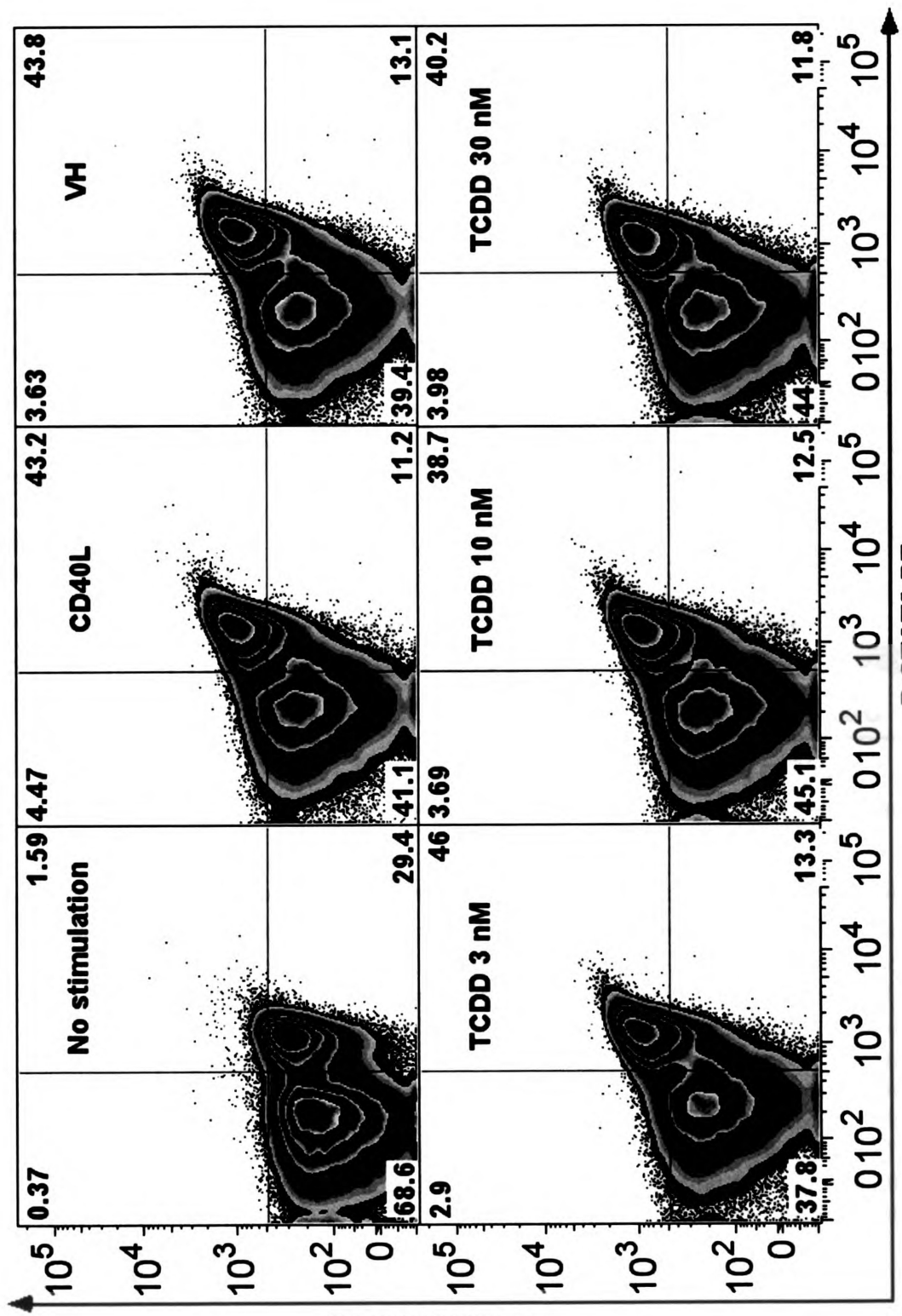
Figure 38. Effect of TCDD on the CD40L-induced immediate MAPK and Akt signaling in human B cells at 30 min. Naive human B cells (1 x 10^6/ml) were equilibrated at 37°C for 3-4 h, treated with 3, 10 and 30 nM of TCDD, or vehicle (VH), and then stimulated with recombinant human CD40L (200 nM), human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 30 min. Cells were fixed with 1.5% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by multiparametric phosflow for intracellular expression of phosphorylated Akt and p38. Three replicates were included per group. The percentages of cells that expressed phosphorylated ERK, JNK, p38, and Akt were obtained using the Boolean gating technique of the FlowJo software. *, $p < 0.05$, **, $p < 0.01$, compared to the VH-treated group. Data are presented as mean ± S.E.M. from three experimental replicates per group.

Table 3. TCDD effect on the expression of phosphorylated STAT1, STAT3, and STAT5 in human B cells at 30 min

Group	MFI of P-STAT1	MFI of P-STAT3	MFI of P-STAT5
NA	127.7±4.2**	162.0±13.0**	453.0±16.5**
CD40L	222.7±2.9	559.7±8.7	849.3±53.0
+VH	213.3±4.9	551.0±21.3	856.0±62.2
+ TCDD 1 nM	208.0±2.0	508.3±18.2	843.0±33.7
+ TCDD 3 nM	216.0±4.5	556.7±24.3	820.7±29.7
+ TCDD 10 nM	196.3±4.0*	494.7±14.1	751.3±33.5
+ TCDD 30 nM	198.3±4.9	510.0±13.1	674.3±57.1*

Table 3. TCDD effect on the xpression of phosphorylated STAT1, STAT3, and STAT5 in human B cells at 30 min. Summary of data from experiments described in Figure 39 and 40. Experimental details were provided in the figure legends of the three figures. Data included in this table are presented as mean ± S.E.M. from two experimental replicates per group. *, $p < 0.05$, **, $p < 0.01$, compared to the VH-treated group.

Figure 39. Effect of TCDD on the CD40L-induced immediate activation of STAT3 and STAT5 in human B cells at 30 min. Naive human B cells (1 x 10^6/ml) were equilibrated at 37°C for 3-4 h, treated with 3, 10 and 30 nM of TCDD, or vehicle (VH), and then stimulated with recombinant human CD40L (200 nM), human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 30 min. Cells were fixed with 1.5% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by multiparametric phosflow for intracellular expression of phosphorylated STAT3 and STAT5. Results depicted are fluorescent signals for phosphorylated STAT3 and STAT5. Percentages of cells within each quadrant are shown in the corner of each individual plot. Data are from one experiment that is representative of three separate experiments (each experiment used B cells from one individual donor) with three replicates per group. Results shown are concatenated from three experimental replicates.


No stimulation
CD40L
VH
TCDD 3 nM
TCDD 10 nM
TCDD 30 nM
0.37
1.59
4.47
43.2
3.63
43.8
68.6
29.4
41.1
11.2
39.4
13.1
2.9
46
3.69
38.7
3.98
40.2
37.8
13.3
45.1
12.5
44
11.8
P-STAT3-AF647
P-STAT5-PE

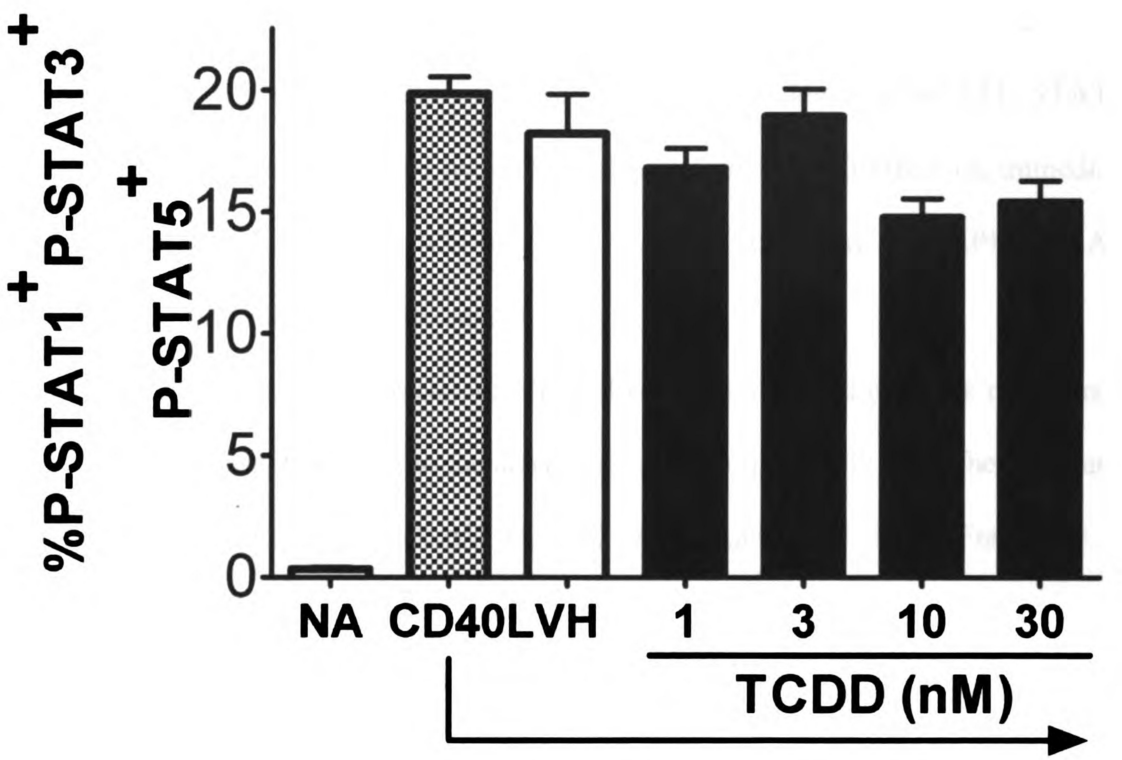


Figure 40. Effect of TCDD on the CD40L-induced immediate STAT signaling in human B cells at 30 min. Naive human B cells (1 x 10^6/ml) were equilibrated at 37°C for 3-4 h, treated with 3, 10 and 30 nM of TCDD, or vehicle (VH), and then stimulated with recombinant human CD40L (200 nM), human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 30 min. Cells were fixed with 1.5% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by multiparametric phosflow for intracellular expression of phosphorylated STAT1, STAT3, and STAT5. Three replicates were included per group. The percentages of cells that expressed phosphorylated STAT1, STAT3, and STAT5 were obtained using the Boolean gating technique of the FlowJo software. Data are presented as mean ± S.E.M. from three experimental replicates per group.

activation, a modest decrease in the expression levels of phosphorylated STAT1, STAT3 and STAT5 was observed, consistent with impairment of cell activation by TCDD (Table 3). The TCDD effect on expression level of phosphorylated STAT1 was slightly less profound when compared with STAT3 and STAT5. As shown in Figure 39 and 40, the same population of activated B cells that expressed elevated levels of STAT1, STAT3, and STAT5 seemed most affected by TCDD, although the TCDD effect on immediate STAT signaling was in general less profound than the activation of MAPK and Akt (approximately 10-15% vs. 50% decrease).

Another critical signaling cascade downstream of CD40 and cytokine receptors is the NFκB/Rel pathway that leads to the activation of transcriptionally active heterodimers p65 (RelA)/p50 (NFκB1) or RelB/p52 (NFκB2) (Berberich *et al.* 1994; Francis *et al.* 1995; Lapointe *et al.* 1996). In previous studies using both human primary B cells and B cell lines, activation through CD40 led to NFκB binding activity that involved p65 as one subunit of the heterodimer (Berberich *et al.* 1994; Lapointe *et al.* 1996; Zarnegar *et al.* 2004). To characterize the potential effects of TCDD on the NFκB/Rel pathway, phosphorylation (i.e., activation) of p65 was used as an indicator for its activation in the present study, as it is a critical subunit of NFκB1 heterodimer that confers strong transcriptional activation (Baeuerle and Henkel 1994). Activation of p65 was significantly induced between 15 and 30 min in human B cells following stimulation with CD40L plus IL-2, IL-6, and IL-10, and the level of activation declined after 30 min (data not shown). Treatment with TCDD at 30 nM prior to stimulation attenuated the induction of p65 phosphorylation at 30 min in a concentration-related manner, with 10 and 30 nM of TCDD causing the most profound suppression (Figure 41). Collectively, this series of

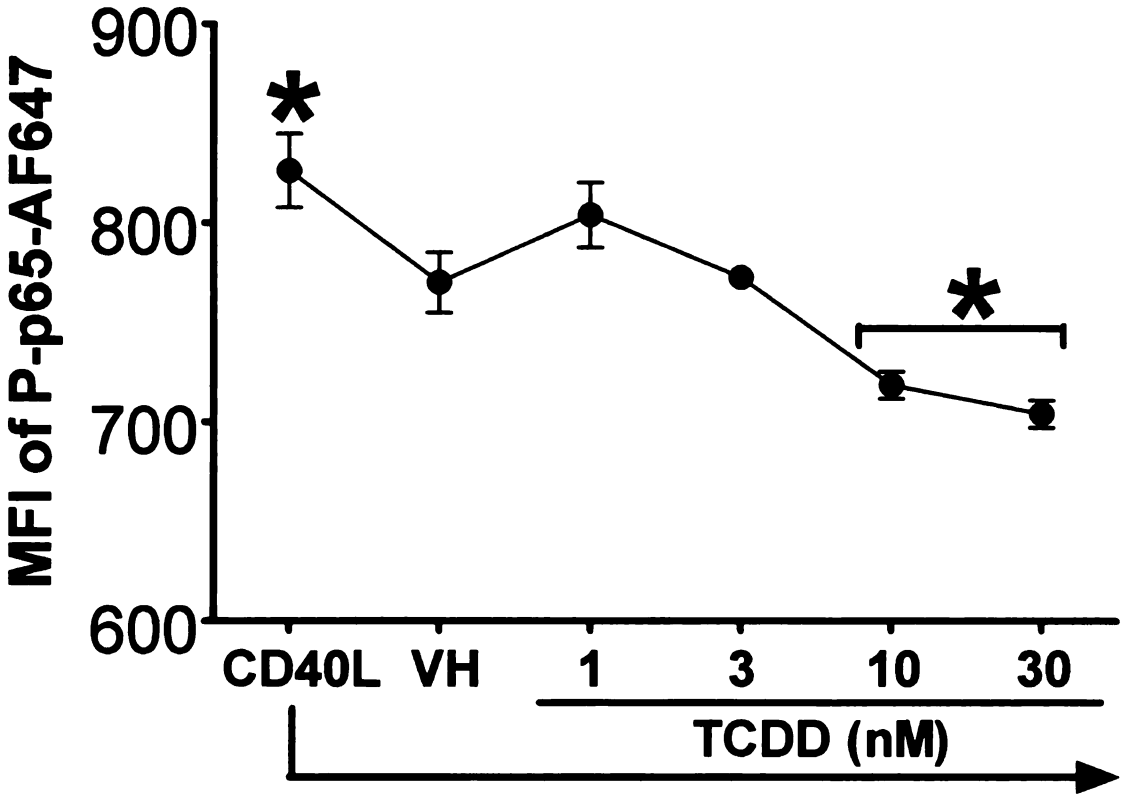


Figure 41. Effect of TCDD on the CD40L-induced immediate activation of p65 in human B cells at 30 min. Naive human B cells (1 x 10^6/ml) were equilibrated at 37°C for 3-4 h, treated with 3, 10 and 30 nM of TCDD, or vehicle (VH), and then stimulated with recombinant human CD40L (200 nM), human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 30 min. Cells were fixed with 1.5% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by phosflow for intracellular expression of phosphorylated p65. Results are presented as mean fluorescence intensity for phosphorylated p65. *, $p < 0.05$, compared to the VH-treated group. Data are from one experiment that is representative of two separate experiments (each experiment used B cells from one individual donor) with three replicates per group. Data are presented as mean ± S.E.M. from three experimental replicates per group.

studies demonstrated that TCDD treatment caused rather ubiquitous effects by attenuating the activation of MAPK, STAT, and p65. Most of the effects were relatively modest, but were consistently observed in B cells from several individual human donors, in which cell activation was also impaired by TCDD (Figure 42).

In the studies described above in which recombinant CD40L was employed to mimic signals received by B cells after coming in contact with CD40L-L cells, treatment with TCDD was found to perturb the immediate early signaling. However, it also stands to reason that the activity of certain key transcription factors needs to be sustained to maintain the activated phenotype of B cells and further contribute to the initiation of plasmacytic differentiation. Therefore, the present series of studies aimed at investigating potential effects of TCDD on the sustained activation of transcription factors AP-1, STATs, and NFκB in B cells activated with CD40L. The activities of these transcription factors are induced by signaling through CD40 and cytokine receptors (Bishop and Hostager 2001; Francis *et al.* 1998; Karras *et al.* 1997), regulated by the upstream signaling pathways that involve MAPK and PI3K/Akt (Andjelic *et al.* 2000; Calo *et al.* 2003; Eferl and Wagner 2003), and have been shown to play critical roles in regulating B cell activation and plasmacytic differentiation (Corcoran 2005; Diehl *et al.* 2008; Ohkubo *et al.* 2005; Vasanwala *et al.* 2002). On Day 1 and 2 following co-culture with CD40L-L cells in the presence of IL-2, IL-6, and IL-10, elevated levels of activated/phosphorylated c-Jun (a typical component of heterodimer AP-1), STAT3, and p65 were observed in a sub-population of B cells (data not shown). Interestingly, only the activation of STAT3, but neither STAT1 nor STAT5, was sustained following activation with CD40L and cytokines, consistent with previously reported findings (Diehl *et al.* 2008). Treatment

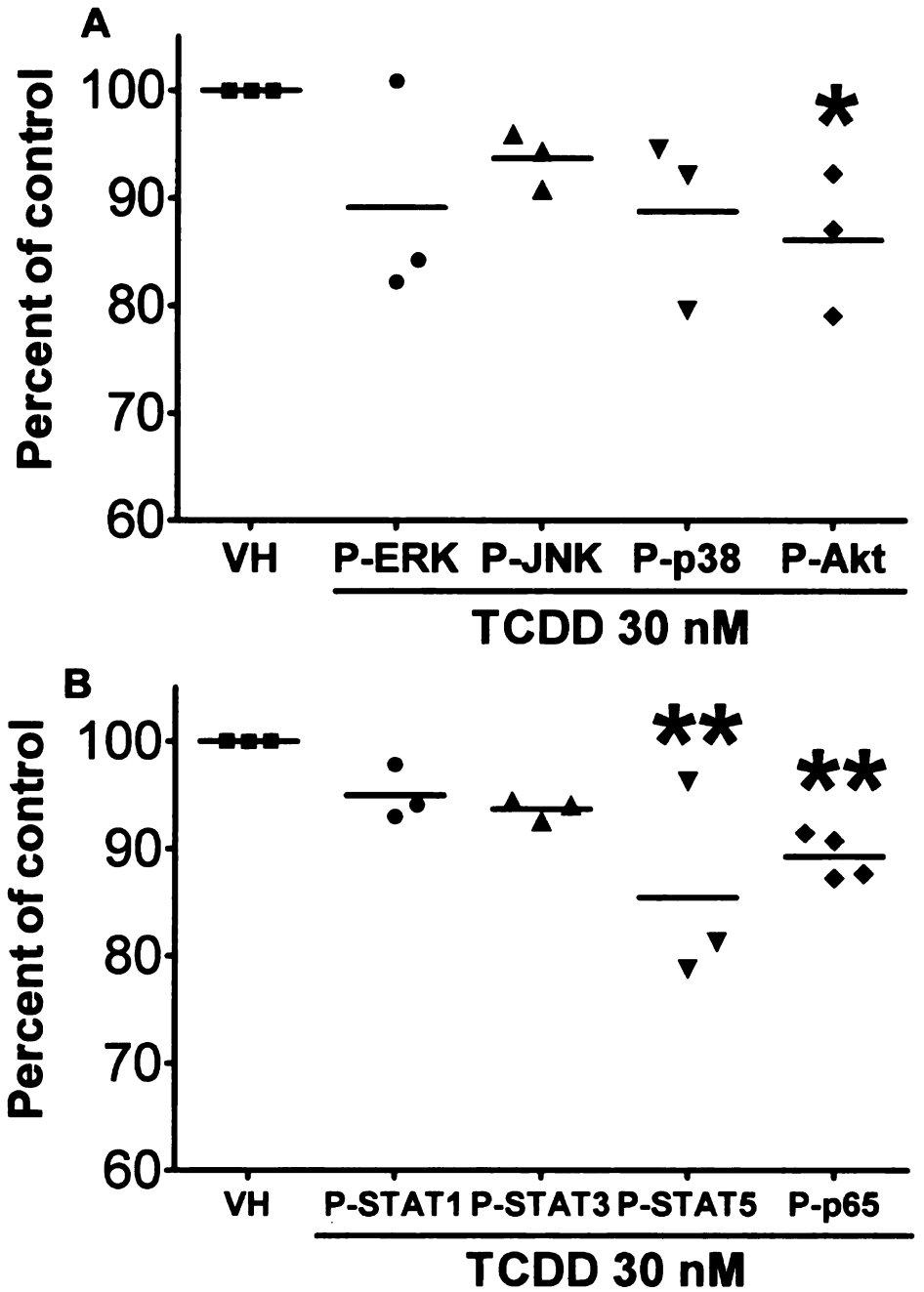


Figure 42. Summary of TCDD Effect on the CD40L-induced immediate activation of MAPK, Akt, STAT, and p65 in human B cells from multiple donors at 30 min. *, $p < 0.05$, **, $p < 0.01$, compared with the VH-treated group. Data were normalized to the VH-treated group (100%) and presented as percent of control with at least two replicates per group. Included in the graph are mean fluorescence intensity comparisons for phosphorylated (A) ERK, JNK, p38, Akt, (B) STAT1, STAT3, STAT5, and p65. Each dot represents data from one individual donor. Data are presented as mean. from at least two experimental replicates per group.

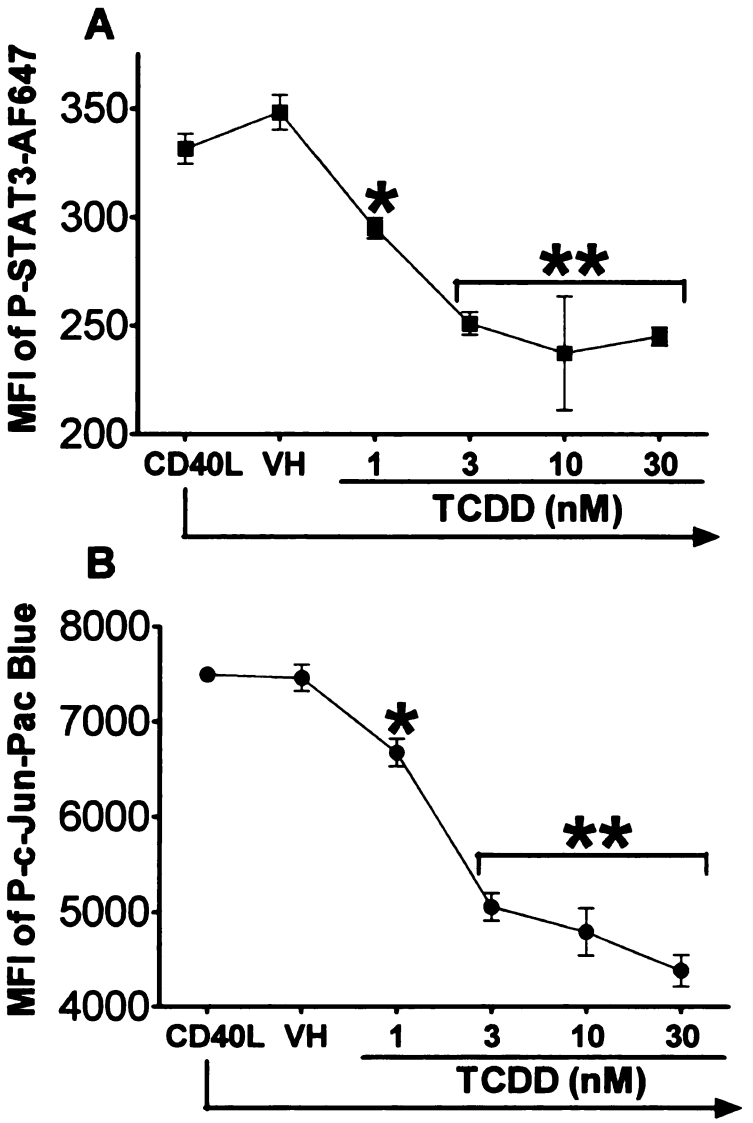
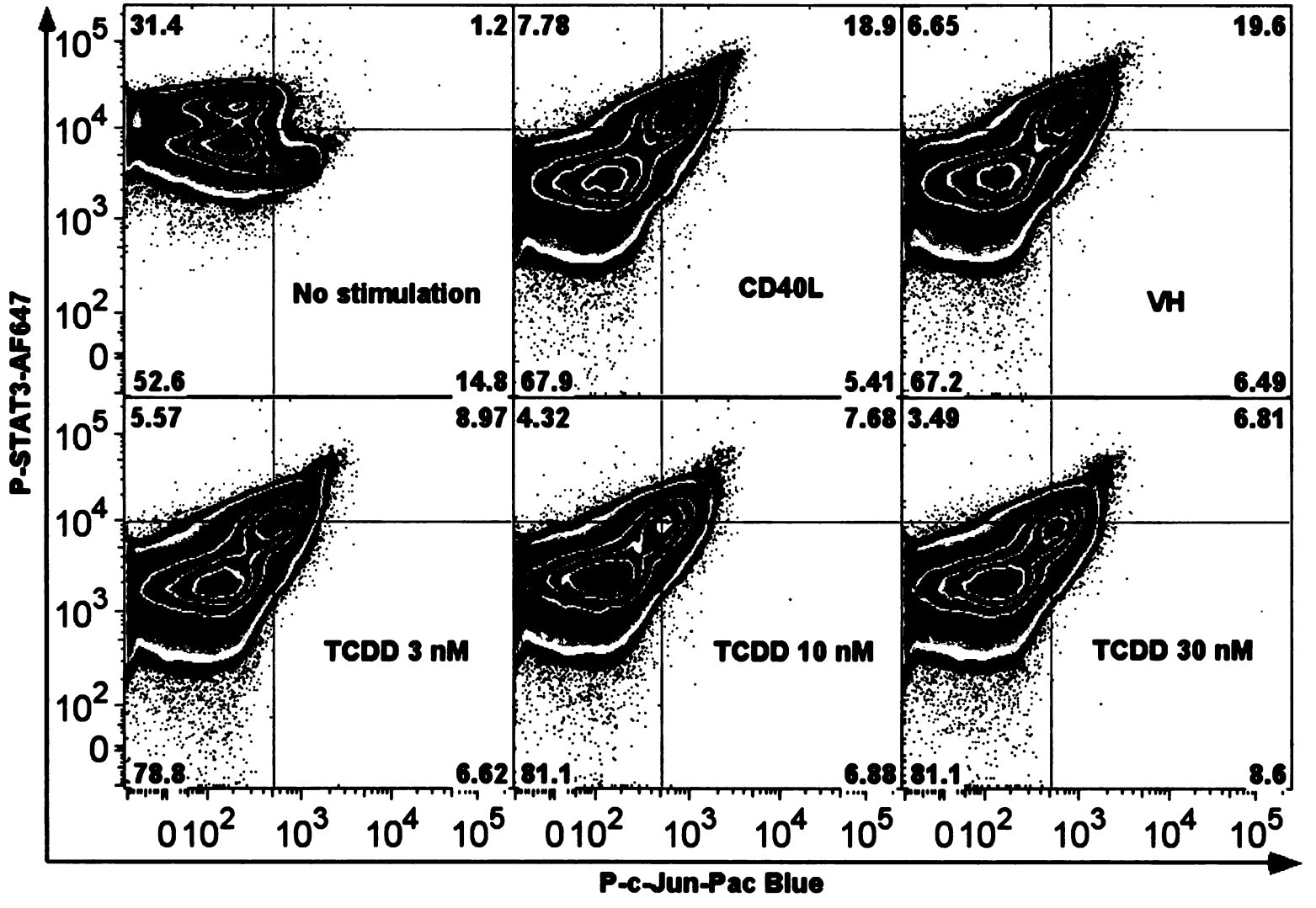


Figure 43. Effect of TCDD on the CD40L-induced sustained expression of phosphorylated STAT3 and c-Jun in human B cells on Day 2. Naive human B cells (1 x 10^6/ml) were treated with TCDD at concentrations indicated, or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (1.5 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 2 d. Cells were harvested following equilibration right after isolation (Day 0, naive) and on Day 2, Cells were fixed with 3% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by multiparametric phosflow for intracellular expression of (A) phosphorylated STAT3 and (B) c-Jun. Results are presented as mean fluorescence intensity for phosphorylated STAT3 and c-Jun. *, $p < 0.05$, **, $p < 0.01$, compared to the VH-treated group. Data are from one experiment that is representative of two separate experiments (each experiment used B cells from one individual donor) with three replicates per group. Data are presented as mean ± S.E.M. from three experimental replicates per group.

Figure 44. Effect of TCDD on the CD40L-induced sustained activation of STAT3 and c-Jun in human B cells on Day 2. Naive human B cells (1 x 106/ml) were treated with TCDD at concentrations indicated, or vehicle (VH), and then co-cultured with irradiated CD40L-L cells (1.5 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 2 d. Cells were harvested following equilibration right after isolation (Day 0, naive) and on Day 2, Cells were fixed with 3% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by multiparametric phosflow for intracellular expression of phosphorylated STAT3 and c-Jun. Results depicted are fluorescent signals for phosphorylated STAT3 and c-Jun. Percentages of cells within each quadrant are shown in the corner of each individual plot. Three replicates were included per group, and the results shown are concatenated from three experimental replicates.


31.4
1.2
52.6
14.8
No stimulation
7.78
18.9
67.9
5.41
CD40L
6.65
19.6
67.2
6.49
VH
5.57
8.97
78.8
6.62
TCDD 3 nM
4.32
7.68
81.1
6.88
TCDD 10 nM
3.49
6.81
81.1
8.6
TCDD 30 nM
P-STAT3-AF647
P-c-Jun-Pac Blue
0
10^2
10^3
10^4
10^5

with TCDD did not affect the activation of c-Jun and STAT3 at Day 1, but attenuated the activation of both transcription factors at Day 2 (data not shown). Using B cells from another human donor, the TCDD effects on activation of c-Jun and STAT3 were also found to be concentration-related (Figure 43). When the activation of c-Jun and STAT3 were analyzed in a correlative manner, the same sub-population of cells expressed elevated levels of both phosphorylated c-Jun and STAT3, most likely representing the cells that maintained activated phenotype, and this population was significantly diminished upon TCDD treatment (Figure 44). Interestingly, similar to c-Jun and STAT3, TCDD also decreased the expression of phosphorylated p65 on Day 2, but not Day 1 (Figure 45). The effect of TCDD treatment on the activation of p65 was also found to be concentration-related using B cells from another donor (Table 4).

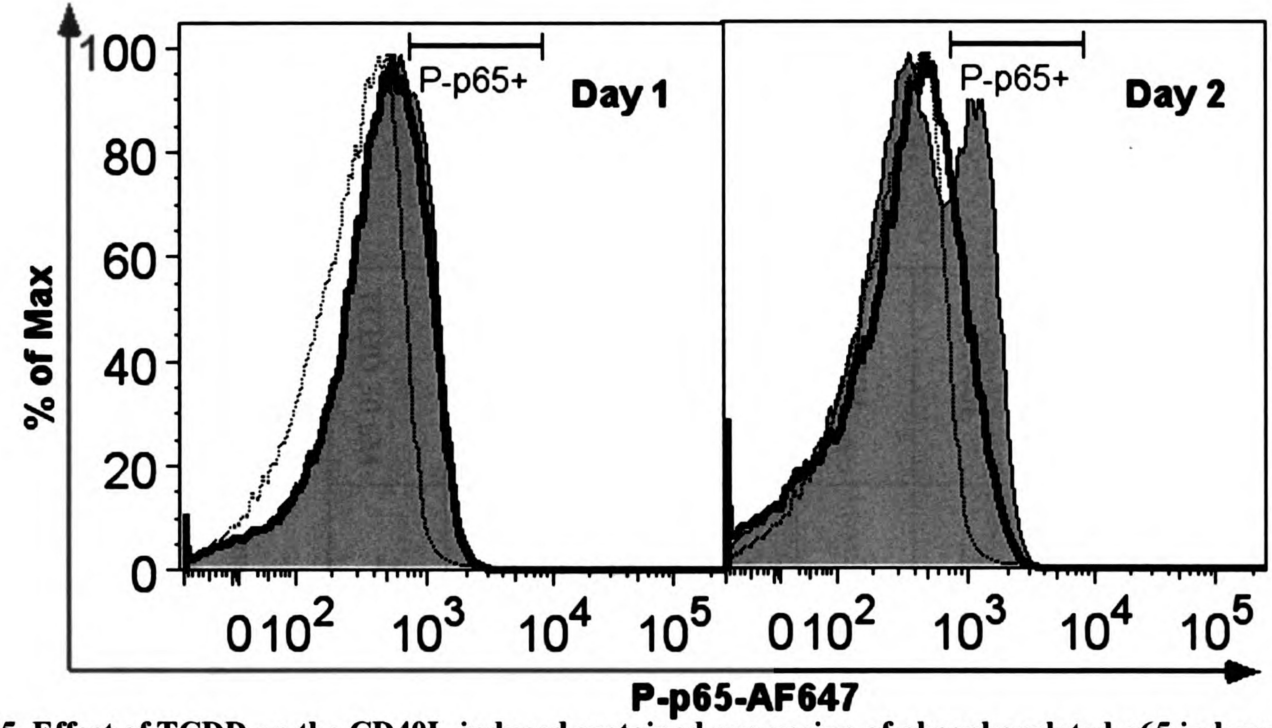


Figure 45. Effect of TCDD on the CD40L-induced sustained expression of phosphorylated p65 in human B cells on Day 2. Naive human B cells (1 x 10^6/ml) were treated with TCDD at 30 nM (solid heavy line), or vehicle (VH) (shaded area), and then co-cultured with irradiated CD40L-L cells (1.5 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 1 or 2 d. Cells were harvested following equilibration right after isolation (Day 0, naive, dotted thin line) and on Day 1 or 2, Cells were fixed with 3% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by phosflow for intracellular expression of phosphorylated p65. Results are presented as histograms that indicate the fluorescent signals for phosphorylated p65. Data are from one experiment that representative of two separate experiments (each experiment used B cells from one individual donor) with three experimental replicates per group. Results shown are concatenated from three experimental replicates.

Table 4. TCDD effect on sustained expression of phosphorylated p65 in human B cells on Day 2

Group	MFI of P-p65
NA	237.0±10.1
CD40L	257.7±10.3
+VH	262.3±8.8
+ TCDD 1 nM	264.7±7.1
+ TCDD 3 nM	280.3±6.1
+ TCDD 10 nM	256.0±6.1
+ TCDD 30 nM	235.0±7.4

Table 4. TCDD effect on sustained expression of phosphorylated p65 in human B cells on Day 2. Naive human B cells (1 x 10^6/ml) were treated with TCDD at indicated concentrations, and then co-cultured with irradiated CD40L-L cells (1.5 x 10^3 cell/well) in the presence of recombinant human IL-2 (10 U/ml), IL-6 (100 U/ml), and IL-10 (20 ng/ml) for 2 d. Cells were harvested following equilibration right after isolation (Day 0, naive) and on Day 2, Cells were fixed with 3% paraformaldehyde and permeablized with ice-cold 99% methanol. Cells were then assessed by phosflow for intracellular expression of phosphorylated p65. Results were presented as mean fluorescent intensity for phosphorylated p65. Data are presented as mean ± S.E.M. from three experimental replicates per group.

DISCUSSION

I. *In vitro* activation and IgM response model using primary human B cells

Recognition that the immune system is sensitive to modulation by a diverse collection of agents and environmental factors was a major impetus for the development of strategies to identify potential human immunotoxicants. Arguably the most successful effort toward this end was the development of an immunotoxicology tiered testing approach that evolved from studies during the 1980s. The approach, which continues to be used worldwide, consists of well-defined immunological measurements and immune function assays that assess various aspects of immune competence (i.e., innate, humoral and cell-mediated immunity) primarily in mice and rats (Luster *et al.* 1988). In spite of the wide adoption of the rodent-based immunotoxicology tiered approach, concerns persist whether studies conducted in rodent models reliably predict human risk. This dissertation research investigated the application of a novel model system that directly assesses the capacity of a xenobiotic to alter the effector function of primary human B cells.

CD40L has rarely been used as an activation model to characterize xenobiotic-induced suppression of the B cell antibody response, especially in humans. Lipopolysaccharide (LPS), a widely used polyclonal stimulator for mouse B cells, does not induce a significant antibody response in primary human B cells in which the expression of TLR4 is absent (Muzio *et al.* 2000). In Chapter I of the Experiment Results section, the application of CD40L was explored as an activation stimulus as it mimics the activating signals B cells receive during a T cell-dependent antibody response, although

the signal through BCR is bypassed. An added advantage in using the CD40L model described here is that the experimental conditions for activating primary human and mouse B cells are similar, allowing for parallel studies that potentially address inter-species difference in sensitivity to xenobiotics. As described above, the CD40L model was optimized by characterizing a number of experimental conditions. The magnitude of CD40L stimulation is a critical factor that contributes to the outcome of B cell activation *in vitro (Neron et al. 2005)*. Extensive investigations were conducted to identify the optimal number of CD40L-L cells and duration of the co-culture period that induces the most robust IgM response in mouse and human B cells. In addition to providing contact-dependent stimulation, activated Th cells also secrete cytokines to promote B cell differentiation. IL-2, IL-6, and IL-10 were included in the CD40L model, as they have been identified in previously published studies as critical T cell-derived cytokines that promote IgM secretion (Arpin *et al.* 1995; Banchereau *et al.* 1994; Burdin *et al.* 1995). IL-4, which also drives B cell differentiation, was not included in our study since IL-4, when combined with other B cell stimuli, can induce isotype switching to IgE (Gascan *et al.* 1991; Splawski and Lipsky 1994). Although it is certain that additional cytokines other than IL-2, IL-6 and IL-10 may further contribute to B cell differentiation, there is significant functional overlap among various groups of cytokines due, at least in part, to common intracellular signaling pathways. Therefore, although the model could certainly be further refined, results described above clearly suggest that IL-2, IL-6, and IL-10, when combined with CD40L stimulation, induced robust IgM responses in both human and mouse B cells.

Donor-to-donor variability poses one of the most significant challenges in using human primary tissues as biological models, including in immunotoxicity evaluations. Based on our anticipation that significant inherent variability between donors would be observed with respect to the magnitude of the control IgM responses by CD40L in total B cells and naive B cells, 12 and 22 donors, respectively, were assayed. In spite of the variations observed, the background responses in human total as well as naive B cells were of a magnitude to allow assessments of biological activity and immunotoxic potential of xenobiotics. It is important to emphasize that variability between donors with respect to the magnitude of their control IgM response becomes inconsequential since each donor also serves as their respective comparative control (i.e., CD40L stimulation in the presence versus absence of xenobiotic). Another significant limitation in using human peripheral blood B cells experimentally is the number of B cells that can be obtained from each donor, since typically B cells comprise approximately 3-5% of circulating leukocytes, with around 60% of total B cells being naive B cells as opposed to pre-activated memory B cells. To circumvent this obstacle, a 96-well plate format was adapted for the human CD40L model, which allows at least 200 naive B cell or 300 total B cell replicates from one leukocyte pack. The model in its present form can be used to evaluate 2-3 agents at extensive concentration ranges using B cells from a single donor, which can be repeated in multiple donors to assess the sensitivity to any individual agent.

B cell activation and proliferation are two critical stages prior to plasmacytic differentiation. It has already been demonstrated that immunotoxic agents may disrupt early activation and/or the proliferation of B cells (Allan *et al.* 2006; Salas and Burchiel 1998). Moreover, various immunosuppressants utilized therapeutically, such as

rapamycin and mycophenolic acid, directly target lymphocyte proliferation. Studies described above demonstrated that following activation with CD40L-L cells in combination with appropriate cytokines, both proliferation and an activated phenotype were induced in primary mouse and naive human B cells. Therefore, in addition to measurements of IgM response, the CD40L model can also be used to dissect the stage(s) of B cell progression from initial activation to the plasmacytic differentiation, identifying the critical stage(s) when xenobiotic-mediated modulation occurs. Another critical observation out of this set of studies was that human and mouse B cells exhibited distinct patterns of responses following activation with the CD40L model. The expression of surface activation markers is widely accepted as a hallmark of B cell activation (Corcoran 2005). In human B cells only the activated cells survived past Day 4, consistent with the observation that the vast majority of viable cells had undergone several rounds of proliferation by Day 5 and expressed elevated levels of IgM and CD138 at Day 7. In contrast, a substantial number of mouse B cells that expressed neither CD86 nor CD69 were still viable at Day 5, although they did not proliferate or express higher levels of IgM and CD138 at Day 7. These results suggest that when B cells are stimulated using the CD40L model established and employed in the present study, human B cells have to become fully activated to maintain viability, while mouse B cells may survive even in the absence of apparent activation and proliferation. Such a discrepancy between B cells from the two species may also have important implications in interpretation of the data concerning the response of B cells to TCDD obtained using the same model. For instance, if the TCDD-mediated effect occurs during the activation stage of human B cells, it may not be reflected by measurements made at time points later than Day 4, as those human B

cells that are not appropriately activated due to TCDD treatment may not survive in culture.

To further characterize the CD40L model, two well-characterized immunotoxicants shown to suppress the mouse anti-sRBC IgM response were investigated using primary mouse and human B cells. Specifically, arsenic and BPDE were demonstrated to suppress the *in vitro* mouse anti-sRBC IgM response, and it was suggested that their immunosuppressive effects involved alterations of B cell function (Kawabata and White 1989; Salas and Burchiel 1998; Sikorski *et al.* 1991). Results described above confirmed that both arsenic and BPDE directly impair mouse B cell function as assessed by the CD40L-induced IgM response. Direct addition of BPDE also suppressed the CD40L-induced IgM response in naive human B cells over a comparable concentration range. Interestingly however, arsenic displayed a distinctly different profile of activity between mouse and human B cells. No effect was observed on the CD40L-induced IgM response at lower doses, which in general was then increased at the highest arsenic concentrations due to a decrease in viable cells per culture. Collectively, this limited study demonstrated that although the mouse was predictive for BPDE-mediated humoral immune suppression in human B cells, the mouse was not predictive for arsenic-mediated modulation in human B cells. These results emphasize the longstanding concern that the mouse immune system may not always serve as an accurate human surrogate. Moreover, the ability to readily obtain human peripheral blood cells represents a unique opportunity to directly investigate whether certain agents have the capability to suppress plasmacytic differentiation and/or the IgM responses in human primary B cells.

Although evaluation of B cell function was the primary focus, one can envision that the CD40L activation model described above could be utilized in conjunction with several additional immune function parameters of T cell and innate immunity. In doing so, human leukocytes could be the basis of a standardized assay battery, although not as extensive as the National Toxicology Program immunotoxicology tiered testing approach, to directly assess immunotoxicity in human leukocytes. In fact, an analysis of National Toxicology Program immunotoxicology historical data showed that when three different assays were used in combination, such as the mouse anti-sRBC IgM response, immunophenotyping of leukocyte subpopulations by flow cytometry, and the natural killer cell activity assay, the results were remarkably predictive of immunotoxicity in the mouse as compared to the complete battery of assays (Luster *et al.* 1992). The importance of assay systems using primary human leukocytes that could serve as an adjunct to rodent-based screening is further supported by the differences in toxicity observed between mouse and human B cells to arsenic, which is one of the more extensively characterized immunotoxicants.

II. TCDD activated the AHR and modulated the IgM response in human B cells

Previous studies, mostly conducted in mice, have demonstrated that TCDD is a potent immunotoxicant capable of producing numerous immune perturbations including profound suppression of the primary antibody response (Holsapple et al. 1991). Due to similarities between the mouse and human immune system, TCDD-mediated immunotoxicity in mice has raised serious concerns pertaining to effects on immune

competence in humans, including suppression of humoral immunity. This notion has been further supported by studies of a limited number of cohorts where an association was observed between exposure to TCDD and/or DLCs and altered circulating antibody levels (Baccarelli et al. 2002; Kim et al. 2003; Lu and Wu 1985; Nakanishi et al. 1985; Weisglas-Kuperus et al. 2000). It is important to emphasize this epidemiologic association has been, at best, suggestive due to numerous confounding factors; the most serious being the absence of information concerning levels of exposure to these chemicals. Due to these and numerous other confounding factors coupled with technical limitations of studying immune function in primary human leukocytes, little is known about the immunotoxicology of TCDD in humans. This dissertation research for the first time evaluated the direct effects of TCDD on the effector function of human peripheral blood B cells. Results described in Chapter II of the Experimental Results section showed that although well characterized genes within the "AHR-responsive gene battery" were markedly less sensitive to induction by TCDD in human B cells than in mouse B cells, suppression of the IgM antibody response by TCDD was comparable in sensitivity between human "responders", and the C57BL/6 mouse. Equally noteworthy, 5 out of the 15 donors evaluated in this study, termed "non-responders", exhibited no suppression of the IgM responses, even at high TCDD concentrations.

In studies characterizing the time- and concentration-dependent AHR-responsive gene expression profiles induced by TCDD, the expression kinetics of CYP1A1, AHR repressor, and TIPARP were slower in human B cells when compared to B cells from C57BL/6 mice. CYP1B1 was an exception, since it was induced most robustly by TCDD as early as 2 h post treatment in both human and mouse B cells. These results were

consistent with the expression kinetics of CYP1A1 in mouse and human mononuclear cells treated with TCDD (Nohara et al. 2006). Likewise the overall magnitude of induction for AHR responsive genes in human B cells was not as pronounced as in mouse B cells. For example, at the time of peak expression for both CYP1A1 and the AHR repressor, the fold induction by TCDD was modest in human B cells compared to mouse B cells. Importantly, a number of notable differences between the human and mouse AHR have been identified, which may contribute to the observed differential effects. For instance, due to a difference in amino acid sequence identified within the N-terminal ligand binding domain at residue A375V, the human AHR has approximately 10-fold lower relative affinity for TCDD than the AHR^b allele carried by the C57BL/6 mouse (Ema et al. 1994). Likewise, the human AHR and the C57BL/6 AHR share limited sequence homology within their transactivation domains (Flaveny *et al.* 2010). In addition, the mouse and human AHRs differentially recruit LXXLL coactivator motif proteins (Flaveny et al. 2008). Collectively, one or more of the aforementioned distinctions between the human and mouse AHR may account for the differences observed here in the induction kinetics and magnitude of TCDD responsive genes in B cells. The expression profiles of the AHR-responsive genes described above reflect the level of AHR activation and the general transcriptional activity of AHR induced by TCDD, as opposed to being directly linked to altered B cell function.

Although peripheral blood leukocytes represent one of the most accessible human tissues available for research, there have been few immunotoxicological assessments of xenobiotics using human leukocytes and likewise very few investigating the immunotoxicity of TCDD using human primary leukocytes. Significant challenges

including, donor-to-donor variability, limitation in the number of leukocytes that can be obtained from a given donor, and the technical obstacles in inducing specific immunological responses using human leukocytes have been the limiting factors for these types of investigations. Studies by Wood and Holsapple were the first to investigate the effects of TCDD on human primary B cells derived from tonsils (Wood and Holsapple 1993; Wood et al. 1993; Wood et al. 1992). However, a common concern pertaining to the use of tonsils as the source of leukocytes is that they are rarely obtained from healthy donors. With this modest caveat, these studies demonstrated that when using the superantigen TSST-1 to induce IgM response in human tonsillar B cells in the presence of irradiated T cells, the IgM response was suppressed by TCDD (Wood and Holsapple 1993). Moreover, the suppression of the TSST-1-induced antibody response was not associated with a decrease by TCDD in [^{3}H]-thymidine incorporations suggesting that B cell proliferation was unaffected.

This dissertation research extended the initial investigations of TCDD on human B cells by Wood and Holsapple and provided a number of new and important insights. While the IgM responses in naive B cells from the fifteen human donors exhibited various degrees of sensitivity to TCDD, perhaps most interesting was the observation that five of these donors showed no suppression of the IgM response. In fact, B cells from two of the five donors were subjected to exceptionally high concentrations of TCDD, up to 50 and 100 nM. The mechanistic basis for this lack of responsiveness to suppression by TCDD of the IgM response in these three donors is unclear but it is tempting to speculate that it is due, in part, to polymorphisms in the *AHR*. In humans, a surprisingly small number of sequence variations, or polymorphisms, have been identified within the *AHR*,

and convincing associations between these polymorphisms and phenotypes as demonstrated by AHR-mediated responses are yet to be established (Harper *et al.* 2002). In the studies described above, polymorphisms were identified in the coding regions of the *AHR* in two out of the three human donors whose B cells were insensitive to TCDD. One of the two polymorphisms identified, the codon 554 G>A change led to the AHR protein that failed to induce *CYP1A1* induction *in vitro* in response to TCDD when combined with another two *AHR* polymorphisms (Wong *et al.* 2001a). Moreover, identification of five out of fifteen donors who were refractory to suppression of the IgM response, at least under the assay conditions used here, suggests that this phenotype is not particularly rare. When the IgM response data were pooled for the remaining twelve "responsive" donors and expressed as percent of control, even with the level of variability observed among human donors, TCDD treatment at 10 and 30 nM significantly suppressed the IgM response in human B cells.

Interestingly, in light of the diminished magnitude of induction by TCDD of AHR battery genes in human B cells compared to mouse, the finding that there was similar sensitivity to suppression of the IgM responses by TCDD between B cells from C57BL/6 mice and "responsive" donors was surprising. One possible interpretation for this lack of concordance between TCDD-mediated AHR-responsive gene induction and suppression of B cell function is that suppression of the IgM response, although dependent on the AHR, may involve events that are not solely dependent on transcriptional regulation. It is also intriguing that in a recent study using genetically engineered mice expressing the human AHR, a greater number of genes functionally clustered around cell cycle regulation and the immune response were differentially regulated after TCDD treatment

than in mice expressing the mouse AHR (Flaveny *et al.* 2010). Conversely, in mice expressing the mouse AHR a greater number of genes functionally clustered around metabolism and membrane transport were differentially regulated after TCDD treatment than in mice expressing the human AHR.

In summary, results from this dissertation research provided novel comparisons between primary human and C57BL/6 mouse B cells. These data are especially important given the concerns raised in the context of data extrapolation from rodents to the human. In fact it has been well recognized that immunotoxicological investigations conducted exclusively using animal models may not always be predictive of human toxicity (Selgrade 1999; Vos and Van Loveren 1998). This point was illustrated by the results described above that arsenic markedly suppressed the CD40L-induced IgM response in mouse but not human B cells. Therefore, in light of the uncertainties associated with cross-species differences in toxicity, this B cell activation model is useful for in-depth investigations into the mechanisms by which TCDD modulates the IgM response in human B cells.

III. TCDD disrupted human B cell activation by perturbing early signaling

A significant step toward understanding the immunotoxic effect of TCDD was the establishment of the B cell as its primary cellular target in the suppression of antibody response in mice (Dooley and Holsapple 1988; Tucker *et al.* 1986). Since then, both *in vitro* and *in vivo* mouse models have been employed to investigate the mechanisms by which TCDD impairs B cell function, primarily focusing on B cell differentiation into

antibody secreting plasma cells. Despite the extensive research efforts, the detailed mechanisms responsible for the TCDD effects on B cells are only starting to emerge. TCDD-mediated deregulation of B cell to plasma cell differentiation involves several key transcription factors, including AP-1, Pax5, and Blimp-1. The observed impairment of the regulators could be, at least in part, attributed to the perturbation of immediate early signaling events (North *et al.* 2009a, 2010; Schneider *et al.* 2008; Schneider *et al.* 2009; Suh *et al.* 2002; Yoo *et al.* 2004). In most of these studies, the TLR4 agonist, LPS, was utilized as a polyclonal activator to induce plasmacytic differentiation of mouse B cells. In this dissertation research that utilized CD40L and cytokines, both mRNA and protein expression levels of B cell differentiation regulators Blimp-1 and Pax5 was found to be impaired by TCDD (Figure 27 and 28). These results not only demonstrated for the first time that TCDD disrupts the plasmacytic differentiation program in isolated mouse B cells stimulated with signals that mimic T cell-dependent activation, but also provided additional evidence to support a common mechanism of TCDD action in suppressing the effector function of B cells, at least in mice. In naive human B cells activated with CD40L and cytokines, critical B cell differentiation regulators, including Blimp-1 and Pax5, followed expression kinetics characteristic of the B cell plasmacytic differentiation process that initiates within a time window at Day 4 post activation (Figure 24). However, no significant differences in the expression of these differentiation regulators were observed upon treatment of human B cells with TCDD from multiple human donors that concomitantly demonstrated sensitivity to suppression of the IgM response by TCDD. These intriguing findings might represent important differences between human and mouse B cells in regard to potential mechanisms of action of TCDD in modulating the

IgM responses, and could arise from several potential scenarios. For instance, it is possible that in human B cells, TCDD has one or multiple molecular targets that are different from those observed in mouse B cells, such as the critical plasmacytic differentiation regulator Blimp-1. Another plausible explanation is that the proximal targets for TCDD in human and mouse B cells are similar but lead to differential effects on relatively late events associated with plasmacytic differentiation, as a consequence of phenotypic differences between the mouse and human B cell responses to CD40L activation. Such potential differences have been illustrated by results presented in Chapter I Section B of the Experimental Results (Figure 8 and 9) and discussed previously in Chapter I of the Discussion. In brief, in the case of early activation being disrupted by TCDD, such an effect may be reflected in the later measurements that focus on the plasmacytic differentiation program in mouse B cells, as a substantial number of mouse B cells were found to be alive but exhibited neither an activated nor a differentiated phenotype. In sharp contrast, human B cells that did not exhibit an activated phenotype also did not maintain viability at Day 4 and late time points, therefore the TCDD effect on activation might not be measurable beyond Day 4.

Cell activation and proliferation are two indispensable steps for a resting B cell to progress toward differentiation into an antibody secreting cell during an immune response *in vivo*, and these steps are also recapitulated and assessed in B cells activated *in vitro* (Schmidlin *et al.* 2009). Therefore, this dissertation research assessed the potential effects of TCDD on the proliferation and activation of both human and mouse B cells following activation with CD40L. Similar between human and mouse B cells is their insensitivity to significant modulation of CD40L-induced proliferation by TCDD. In fact,

to our knowledge this is the first time that the effects of TCDD on B cell proliferation have been directly assessed, as opposed to prior indirect measurements such as DNA synthesis, although these results support the same conclusion that B cell proliferation is not significantly affected by TCDD.

It has been shown that TCDD perturbed early activation of mouse B cells stimulated with TLR agonists (North *et al.* 2010). In human B cells activated with CD40L, the surface expression of activation markers CD80, CD86, and CD69 were significantly decreased upon treatment with TCDD (Figure 34). These results have several important implications. First, they demonstrated for the first time that TCDD, when added *in vitro*, disrupts the activation of human B cells induced by signals mimicking T-cell dependent stimulation. Second, they serve as indirect evidence for potential TCDD effects on the early signaling events occurring in human B cells stimulated with the CD40L activation model, causing disruption in activation. Last, these results strongly suggested that human B cells whose activation was impaired by TCDD did not survive in culture to later time points, when plasmacytic differentiation initiates and alterations in the expression of critical regulators can be measured (Day 4; Figure 24). As a consequence, the expression of plasmacytic differentiation regulators such as Blimp-1 and Pax5 appeared to be un-affected by TCDD in human B cells, as only a modest proportion of the cells that were affected by TCDD during early activation were present to be assayed at later time points. In mouse B cells, rather than disrupting the overall cell activation, TCDD altered the expression profile of activation markers induced by CD40L, with a specific suppressive effect on CD80. Interestingly, in both the human studies described above and previous studies using LPS-stimulated mouse B cells, CD80

appeared to be most profoundly affected by TCDD among all the activation markers assessed (Figure 34) (North *et al.* 2010). The implication of such preferential effect of TCDD on CD80 remains to be investigated, although it may suggest certain up-stream signaling events that are particularly sensitive to TCDD.

In studies that characterized the TCDD effects on immediate and persistent signaling in human B cells activated in the CD40L model, multiple targets of TCDD were identified. Most previous studies have assessed the effect of TCDD on the MAPK signaling in resting cells, which have shown activation of MAPK by TCDD (Park *et al.* 2005; Tan *et al.* 2002; Weiss *et al.* 2005). In human B cells activated with CD40L and cytokines, activation of MAPK ERK, JNK, and p38, as well as Akt, the downstream effector of PI3K, was rapidly induced between 15 and 30 min following activation, and such induction was attenuated when cells were treated with TCDD. These results are consistent with previous findings that TCDD suppressed the activation of ERK, JNK, and Akt in mouse B cells activated through TLRs (North *et al.* 2010), suggesting the effects of TCDD on MAPK and PI3K/Akt activation may be independent of the activating stimulus for B cells.

JAK/STAT pathways were also found to be affected by TCDD in human B cells activated with CD40L and cytokines. The activation of STAT 1, 3, and 5 appeared to be targeted by TCDD. To our knowledge, the results from this dissertation research represent one of the first efforts to investigate the potential effect of TCDD on the activation of the JAK/STAT pathway. The NFκB/Rel pathway is one of the major effectors downstream of the signal through CD40 (Dadgostar *et al.* 2002; Zarnegar *et al.* 2004). In the present study, activation of p65 in human B cells activated with CD40L and

cytokines was suppressed by TCDD in a concentration-related manner. These results are interesting, when considered together with previous findings that the AHR physically interacts with p65, causing mutual suppression of activity between the two transcription factors (Tian *et al.* 1999). TCDD was also found to suppress the transcriptional activity of p65 induced by TLR agonists in mouse dendritic cells (Bankoti *et al.* 2010b). It is tempting to speculate that the suppressive effect of TCDD on p65 activation observed in activated human B cells was due, at least in part, to mechanisms that involves interaction between the AHR and p65. The relative ubiquitous effects of TCDD on the activation of multiple pathways suggest the presence of a common target upstream of MAPK, STATs, and NFκB/Rel in the signaling cascades by which TCDD acts on. An alternative explanation is that the primary targets of TCDD include the signaling molecules that exhibited sensitivity; however, the initial TCDD effect is amplified rapidly due to the extensive crosstalk between signaling pathways. As an example, it has been well established that the activities of both STAT and NFκB molecules can be regulated by one or multiple MAPKs (Decker and Kovarik 2000; Schulze-Osthoff *et al.* 1997).

The perturbation of immediate signaling by TCDD in human B cells, as evidenced by the decreased activation of MAPK, STAT3 proteins, and p65, is a very proximal event as opposed to the TCDD effects on cell activation observed at time points Day 2 and 3. Under co-culture conditions with CD40L-L cells, B cells require a certain period of time to establish interactions with CD40L-L cells, and such interactions almost certainly do not occur for all the B cells simultaneously. Therefore, what the studies using recombinant CD40L recapitulated is likely to be signaling events that continue through the initial stage of the co-culture. The activation of c-Jun, STAT3, and p65 was well

sustained at Day 2, and such activation was significantly decreased in B cells treated with TCDD prior to the co-culture. The effects of TCDD on c-Jun, STAT3, and p65 could be, at least in part, attributed to the disruption of MAPK signaling, as MAPKs are well known to regulate all the three transcription factors (Chang and Karin 2001; Decker and Kovarik 2000). Moreover, Akt activates the NFκB pathway that involves p65 (Madrid *et al.* 2000; Ozes *et al.* 1999), therefore decreased expression of activated p65 at Day 2 could be resulted from attenuation of immediate Akt activation casued by TCDD. Cooperation between STAT3 and NFκB, as well as STAT3 and c-Jun in regulating their downstream target genes (Grivennikov and Karin 2010; Shuai 2000), as shown previously, may also lead to the amplification of the TCDD effects observed on the activation of these transcription factors.

Taken together, the effects of TCDD on activation of the multiple signaling pathways induced by CD40L and cytokines may have significant impact on the function of B cells. First, the TCDD-mediated perturbation of immediate and persistent signaling in human B cells activated with CD40L may in turn lead to decreased expression of CD80, CD86, and CD69 upon TCDD treatment. CD69 expression in activated lymphocytes is regulated by AP-1, while CD80 expression induced in CD40L-induced B cells requires NFκB (Castellanos *et al.* 1997; Hsing and Bishop 1999). Moreover, CD69 is a downstream target subjected to regulation by ERK (Daniel *et al.* 2007; Richards *et al.* 2001). Beyond the role of being indicative of the activated phenotype, CD80 and CD86 expression also represent another critical function of B cells that provides co-stimulatory signals required for the optimal activation of Th cells (Greenwald *et al.* 2005). Second, Akt, NFκB, and STAT3, once activated, have all been linked to the promotion of cell

survival (Andjelic *et al.* 2000; Calo *et al.* 2003). Therefore, decreased activities of these pro-survival proteins, when coupled with the impairment of activation, may lead to the phenotype in TCDD-treated human B cells that is characteristic of cell death correlated with a lack or suboptimal activation stimulus. Last, several of the kinases and transcription factors shown to be affected by TCDD as described above have important functional relevance to the regulation of B cell plasmacytic differentiation and antibody responses. Genetic deletion of the kinase MEKK1, the upstream activator of JNK and p38, led to defective CD40-dependent activation of JNK and p38, as well as impaired T cell-dependent antibody response (Gallagher *et al.* 2007). The activation of MAPK family members induced by B cell activating signals was later shown to regulate the expression of BCL-6, a critical regulator of B cell differentiation (Batlle *et al.* 2009). It has also been proposed that ERK, which down-regulates the activity of BCL-6, serves as a "molecular switch" that regulates plasmacytic differentiation (Rui *et al.* 2006). The induction of transcription factors AP-1, STAT3, and NFκB are also critical events in activated B cells, as they all get repressed by BCL-6, activate Blimp-1, and promote the progression toward plasmacytic differentiation and Ig production (Calame 2008; Diehl *et al.* 2008; Li *et al.* 2005; Niu *et al.* 2003; Ohkubo *et al.* 2005; Reljic *et al.* 2000; Vasanwala *et al.* 2002).

IV. Concluding remarks

Results presented in this dissertation research represent a systematic approach to answer a fundamental question despite many years of research investigating TCDD Immunotoxicology. Especially, does exposure to TCDD adversely affect human B cell

function? The focus was the T cell-dependent IgM response, one of the most sensitive endpoints observed in rodent models. Freshly isolated naive human B cells were chosen as the primary cell model, as they most closely reflect the resting B cells *in vivo* that have never experienced antigenic stimulation, as opposed to memory B cells, which have experienced antigen stimulation, or immortalized B cell lines, most of which are either lymphomas, or pre-activated and virally transformed cell lines.

To induce a robust IgM response in human primary B cells *in vitro*, a polyclonal activation model is required, due to the limited number of precursor B cells that bear a BCR that is specific for any given antigen. Based on the previous knowledge about the critical roles of CD40L and cytokines expressed by Th cells in humoral immune responses, an *in vitro* model comprised of a fibroblast line expressing human CD40L plus direct addition of recombinant Th-associated cytokines IL-2, IL-6, and IL-10 was established and optimized to consistently induce measurable IgM responses in primary human B cells derived from a number of human donors, as well as splenic B cells from the C57BL/6 mice. In addition to the IgM responses, increased expression of lymphocyte activation markers and cell proliferation are also induced by the CD40L-dependent activation model, providing powerful tools that can be applied for mechanistic immunotoxicological studies, which are aimed at investigating potential effects of xenobiotics on different stages during the progression from a resting B cell to a differentiated plasma cell and /or memory B cell. To our knowledge, studies presented in this dissertation research are the first to capitalize on an *in vitro* CD40L-dependent model to investigate the mechanisms for xenobiotic-mediated effects on B cell

Table 5. Different phenotypes observed between human peripheral blood B cells and mouse splenic B cells activated using the CD40L model

Stages of B cell differentiation		Human	Mouse
Activation	General phenotype	<30% viable cells don't express any activation markers at Day 2, <1% at Day 4	>40% viable cells don't express any activation markers between Day 1 and Day 5
	TCDD effect	CD69↓, CD80↓, CD86↓↓, %activated and viable↓	CD69↑, CD80↓, CD86↑, %activated and viable→
Proliferation	General phenotype	> 70% total viable cells proliferated at Day 5, >60% divided 2-4 times	< 20% total viable cells proliferated at Day 5, same group of cells kept dividing
	TCDD effect	Very modest; slightly more cells underwent 2 divisions and less at 4 divisions	No noticeable effect
Plasmacytic differentiation	General phenotype	Blimp-1↑↑, IgJ↑↑, Igμ↑, Pax5↓↓, BCL-6↓ ELISPOT: 0.2% viable cells were high IgM secreting cells FCM: < 15% viable cells were low intracellular IgM; modest increase of CD138	Blimp-1↑↑, IgJ↑↑, Pax5↓, XBP-1↑, BCL-6↓ ELISPOT: 3% viable cells were high IgM secreting cells FCM: > 45% viable cells were low intracellular IgM; profound increase of CD138
	TCDD effect	No significant change on Blimp-1, IgJ, Igμ, and Pax5 30-40% suppression of IgM response, non-responders observed	Blimp-1↓, IgJ↓, Pax5↑, XBP-1↓ 40% suppression of IgM response

effector function. Moreover, interesting phenotypic differences were observed between human and mouse B cells responding to comparable activation conditions (Table 5). Activated with CD40L, human and mouse B cells appeared to make distinct decisions during the progression toward antibody secreting cells. Human B cells, if not sufficiently activated did not maintain their viability in culture. In contrast, a substantial number of mouse B cells were viable through the entire culture period, in the absence of exhibiting a fully activated phenotype as assessed by CD80, CD86, and CD69 expression. This phenotypic difference has clear implications in the interpretation of data concerning later events as discussed above. Although the environmental contaminant TCDD was the focus of this dissertation, application of the CD40L activation model that was established and characterized here can certainly be extended along three potential avenues. First, it can be utilized to assess potential effects of other xenobiotics on the effector function of B cells. For those compounds that have been shown to suppress T cell-dependent humoral immunity, the CD40L model may not only confirm direct effects on B cells, but also characterize potential differences in sensitivity between human and mouse B cells. Our preliminary validation studies using arsenic and BPDE clearly illustrated this particular point. Second, with different combinations of Th cell-derived cytokines, signaling through CD40 might drive naive human B cells to undergo isotype switching and secrete other Ig isotypes (Banchereau *et al.* 1994). For example, IgE, which is induced by addition of IL-4, may be of interest to the immunotoxicology community because of its role in mediating allergic responses (Gould *et al.* 2003). The CD40L model can be used to test whether certain xenobiotics may exacerbate allergic responses by inducing B cells to secrete more IgE. Last, in light of the historical success of the tiered testing approach

and increasing interest in advancing *in vitro* toxicity testing, the CD40L model could be included as a B cell effector function assay as part of a panel of standardized human leukocyte-based assays for assessing the functions of multiple immune cell subtypes *in vitro*.

As a continuation of the early studies by Holsapple *et. al.*, this dissertation sought to examine the biological consequences produced by TCDD on primary human B cells. Toward this end, the induction of AHR-responsive gene expression and the suppression of the B cell IgM response, an approach that directly compared human and mouse B cells was utilized. My studies demonstrated that the magnitude of suppression by TCDD of the CD40L-induced IgM responses was similar between B cells from the "responsive" human donors and a sensitive mouse strain, while at the same time the level of AHR activation, as indicated by the induction of AHR-responsive genes, was vastly different. The dissociation between AHR activation and toxic effect on B cell effector function observed in the present studies is important, as it suggests that responsiveness of the canonical AHR pathway may not predict the sensitivity to certain toxic endpoint of TCDD, especially when cross-species extrapolation is required. Indeed, in addition to the well-known difference in binding affinity to its ligand, the human and mouse AHR are highly divergent in their transactivation domains and regulate the expression of numerous different genes (Flaveny *et al.* 2010). Moreover, although extensive evidence suggests the TCDD effects on B cells are AHR-dependent, whether the transcriptional activity of the AHR is required for the majority of these effects remains unknown. Alternative mechanisms may involve physical interaction between the AHR and other proteins that play critical roles in cellular functions, and such protein-protein interaction may be

tissue- and/or cell type-specific. Another interesting finding was the identification of two previously reported polymorphisms in the *AHR* gene from two out of the three human donors whose B cells exhibited no sensitivity to TCDD. No conclusion can yet be drawn, due to the very small sample size and the scarcity of previous data concerning the biological outcome of these polymorphisms. Added to the challenge is when using cells from anonymous human donors whose background information are largely unavailable, a vast number of potential variables can potentially influence the sensitivity of leukocytes to xenobiotic treatment, and therefore meaningful data interpretation and comprehensive evaluation in this case of the potential correlation between genetic variations in the *AHR* and sensitivity of B cells to TCDD in humans is not possible. Very recently, the human AHR was successfully expressed ectopically in a human B cell line SKW6.4 that otherwise lacks the AHR, rendering it responsive to TCDD as assessed by suppression of the IgM response (Kaminski laboratory, unpublished results). One can envision this model as an opportunity to rigorously assess the impact of genetic variations in the *AHR* by expressing different forms of the *AHR* in SKW6.4 cells, each of which carries a known polymorphism.

Extensive research efforts in both *in vitro* and *in vivo* mouse models have started unraveling the molecular mechanisms responsible for the TCDD-mediated effects on B cell effector function. TCDD was found to markedly impair the plasmacytic differentiation by affecting both positive regulators (i.e., AP-1, Blimp-1, and XBP-1) and negative regulators (i.e., Pax5 and BCL-6) of this process. An integrated approach that combines both laboratory experimentation and computational modeling led to the hypothesis that TCDD increases the threshold for a bistable switch to be "turned on" and

capable of directing a B cell to the fate of plasmacytic differentiation (Bhattacharya *et al.* 2010). This hypothesis is well supported by observations in LPS-activated mouse B cells that treatment with TCDD decreased the number of differentiated mouse B cells and increased the number of non-differentiated B cells, which were viable and measurable at the end of culture. Concordantly, LPS also activated a sub-population of mouse B cells to exhibit an activated phenotype, while the non-activated cells were still viable (North *et al.* 2010). These findings were largely repeated in this dissertation research by using CD40L as another stimulus to activate B cells, therefore suggesting the mouse B cell response to activation and the subsequent effects of TCDD are independent of any particular stimulus. However, the point at which the AHR signaling cascade interacts with B cell signaling appeared to precede the initiation of plasmacytic differentiation in human B cells. At Day 4, the vast majority of human B cells that did not express the activation markers were not viable in culture, indicating the "switch" in human B cells occurs at the stage of cell activation, and it influences cell survival rather than differentiation into antibody secreting cells, which is in contrast to what was observed in the mouse. Moreover, TCDD appeared to affect the decision making of human B cells at such a critical "switch" point, that it profoundly impaired the activation of human B cells, as evidenced by decreased expression of CD80, CD86, and CD69. The early decision making in human B cells, which is clearly affected by TCDD, poses significant challenges to a comprehensive assessment of the TCDD effect on the plasmacytic differentiation at later stages as there are significantly fewer cells that are affected by TCDD remaining to study compared to mouse B cells. TCDD treatment decreased the percentage of IgM^{high} cells in the total viable cell pool, as measured by ELISPOT, suggesting TCDD also affects the

plasmacytic differentiation process in those cells that have passed the earlier point of deciding whether to survive. However, it is worthwhile to note that only 0.1 – 0.2 % of viable cells were considered high IgM secretors by ELISPOT, while flow cytometric analysis showed that the majority of viable human B cells expressed higher intracellular IgM levels when compared to naive cells. Therefore, even if TCDD does have an effect on such a minority of cells, it is highly unlikely that such an effect can be readily studied in the whole population. Future studies to extend the novel findings made in this dissertation, will likely focus on understanding the molecular mechanisms by which TCDD impairs the activation of human B cells. Another interesting potential avenue for future research is to investigate the broader biological consequence of TCDD-mediated effects on the expression of B7 molecules CD80 and CD86, as they provide co-stimulatory signals to T cells during the T cell-B cell interaction, which are crucial for optimal activation of T cells. It will be interesting to learn whether B cells with decreased CD80 and CD86 expression, due to TCDD treatment, also poorly co-activate T cells, leading to impaired T cell responses.

Impaired cell activation observed in human B cells treated with TCDD led us to investigate whether TCDD affects multiple signaling cascades downstream of the B cell activation, in this case CD40L and cytokine treatment. A systematic approach was applied to demonstrate for the first time that TCDD perturbed the immediate and persistent activation of multiple signaling pathways in human B cells activated with signals that mimic T cell-dependent activation. The relatively rapid and ubiquitous TCDD effects are intriguing, but may also provide indirect evidence to further understand the general mechanisms of action of TCDD. For instance, the TCDD effects on

immediate signaling of B cells were observed within 30 min following cell activation in the present study, consisent with previously reported results in mosue B cells (North *et al.* 2010). Such proximal effects likely precede the transcriptional activity of the AHR, but rather support the hypothesis that upon ligand binding, the cytosolic AHR may interact with certain adapter proteins or signaling molecules that are very upstream of the major effector signaling pathways. An alternative hypothesis is that TCDD may disrupt the signaling events downstream of CD40 by altering the reduction-oxidation reaction (redox) status in B cells. The induction of JNK, p38, Akt, and NFκB in B cells activated through CD40 was found to be dependent on the presence of endogenous reactive oxygen species (ROS) (Lee *et al.* 2007), whereas ROS added exogenously inhibited B cell activation through CD40 (Liu *et al.* 2007). Interestingly, TCDD is well known to modulate redox status by altering ROS levels in various tissues and cell types (Kopf and Walker 2010; Lin *et al.* 2007; Shertzer *et al.* 1998; Slezak *et al.* 2002; Slezak *et al.* 2000). Another line of evidence came from previous studies in which TCDD affected mitochondria function, the major source of ROS in most mammalian cell types, in an AHR-dependent manner (Forgacs *et al.* 2010; Shertzer *et al.* 2006). Future studies are needed to test these hypotheses in the B cell, as it represents a highly sensitive target of TCDD.

In summary, significant advances have been made not only addressing the fundamental question whether TCDD affects humoral immunity in humans, but also extending the mechanistic investigation of TCDD immunotoxicity on B cells from mouse to human. Establishment and novel applications of an *in vitro* model overcame the previously existing challenges associated with assessing primary human B cell functions *in vitro*. In combination with utilization of new techniques including ELISPOT and

multiparametric flow cytometry, it has led to a series of new findings that have significantly added to our knowledge of TCDD mechanisms of action, especially on human B cells. The potential species-specific mechanisms revealed, as summarized in Table 5, provide useful references for human risk assessment, and argue for the value of conducting toxicology studies using human-derived cells and materials. Some critical findings in this dissertation research, as discussed previously, have opened new avenues for future exploration.

APPENDICES

APPENDIX A. TaqMan Primers

Gene	Species	Catalog #	Ref Seq
18S	m, h	4319413E	X03205.1
AHR	Human	Hs00169233_m1	NM_001621.4
AHR	Mouse	Mm01291777_m1	NM_013464.4
AHRR	Human	Hs01005075_m1	NM_020731.3
AHRR	Mouse	Mm00477443_m1	NM_009644.2
ALDH3A1	Human	Hs00167469_m1	NM_000691.4 NM_001135167.1 NM_001135168.1
ALDH3A1	Mouse	Mm00839312_m1	NM_007436.2 NM_001112725.1
Blimp-1 (PRDM1)	Human	Hs00153357_m1	NP_001189.2 NP_878911.1
Blimp-1 (PRDM1)	Mouse	Mm00476128_m1	NM_007548.3
CD69	Human	Hs00156399_m1	NM_001781.2
CD80	Human	Hs00175478_m1	NM_005191.3
CD86	Human	Hs99999104_m1	NM_175862.3 NM_006889.3
CYP1A1	Human	Hs00153120_m1	NM_000499.3
CYP1A1	Mouse	Mm00487217_m1	NM_001136059.1
CYP1A2	Human	Hs01070374_m1	NM_000761.3
CYP1A2	Mouse	Mm00487224_m1	NM_009993.3
CYP1B1	Human	Hs00164383_m1	NM_000104.3
CYP1B1	Mouse	Mm00487229_m1	NM_009994.1
GSTA1	Human	Hs00275575_m1	NM_145740.3
IgJ	Human	Hs00376160_m1	NM_144646.3

IgJ	Mouse	Mm00461780_m1	NM_152839.2
Igμ	Human	Hs00378435_m1	N/A
Igμ	Mouse	Mm01718956_m1	N/A
NQO-1	Human	Hs00168547_m1	NP_000894.1 NP_001020604.1 NP_001020605.1
NQO-1	Mouse	Mm00500821_m1	NM_008706.5
PAI-1	Human	Hs00167155_m1	NP_000593.1 NP_001158885.1
PAI-1	Mouse	Mm00435858_m1	NM_008871.2
Pax5	Human	Hs00277134_m1	NM_016734.1
Pax5	Mouse	Mm00435501_m1	NM_008782.2
TIPARP	Human	Hs00296054_m1	NM_015508.3
TIPARP	Mouse	Mm00724822_m1	NM_178892.5
XBP-1	Mouse	Mm00457359_m1	NM_013842.2

APPENDIX B. Antibodies

Target	Fluoro-phore	Host	Reactivity	Type	Supplier
Blimp-1	PE	Goat	m, r, h	polyclonal	Santa Cruz Biotech
CD16/32	None	Rat	Mouse	monoclonal, 2.4G2	BD Biosciences
CD138	APC	Mouse	Human	monoclonal, MI14	BD Biosciences
CD138	PE	Mouse	Human	monoclonal, DL-101	BD Biosciences
CD138	APC	Rat	Mouse	monoclonal, 281-2	BD Biosciences
CD19	APC	Rat	Human	monoclonal, HIB19	BD Biosciences
CD19	APC	Rat	Mouse	monoclonal, 1D3	BD Biosciences
CD19	PE/Cy7	Mouse	Human	monoclonal, HIB19	Biolegend
CD19	PE	Rat	Mouse	monoclonal, 1D3	BD Biosciences
CD19	PE/Cy7	Rat	Mouse	monoclonal, 6D5	Biolegend
CD27	PE	Mouse	Human	monoclonal, O323	Biolegend
CD27	PE	Armenian Hamster	Mouse	monoclonal, LG3A10	BD Biosciences
CD69	PE/Cy7	Mouse	Human	monoclonal, FN 50	Biolegend
CD69	PE	Armenian Hamster	Mouse	monoclonal, H1.2F3	Biolegend
CD80	PE/Cy5	Mouse	Human	monoclonal, 2D10	Biolegend
CD80	APC	Armenian Hamster	Mouse, Dog	monoclonal, 16-10A1	Biolegend
CD86	PE	Mouse	Human, non-human primates	Monoclonal, IT2.2	Biolegend
CD86	PE/Cy7	Rat	Mouse	monoclonal, GL-1	Biolegend
IgM	FITC	Mouse	Human	monoclonal, MHM-88	Biolegend
IgM	FITC	Rat	Mouse	monoclonal, Il/41	BD Biosciences

IgM	none	Mouse	Human	monoclonal, MHM-88	Biolegend
IgM	none	Rat	Mouse	monoclonal, Il/41	BD Biosciences
MHC Class II $I\text{-}A^b$	FITC	Mouse	Mouse	monoclonal, AF6-120.1	Biolegend
Pax5	AF405	Goat	m, r, h	polyclonal	Santa Cruz Biotech
Pax5	FITC	Goat	m, r, h	polyclonal	Santa Cruz Biotech
Phosphorylated Akt (S473/S473)	PE	Mouse	m, h	monoclonal, M89-61	BD Biosciences
Phosphorylated c-Jun (S63)	AF647	Mouse	m, r, h	monoclonal, KM-1	Santa Cruz Biotech
Phosphorylated ERK1/2 (T202/Y204)	AF488	Rabbit	m, h	monoclonal, D13.14.4E	Cell Signaling
Phosphorylated JNK (T183/Y185)	AF647	Mouse	m, r, h	monoclonal, G9	Cell Signaling
Phosphorylated p38 (T180/Y182)	PE	Mouse	m, r, h	monoclonal, 36/p38	BD Biosciences
Phosphorylated NFκB p65 (S529)	AF647	Mouse	Human	monoclonal, K10-895.12.50	BD Biosciences
Phosphorylated STAT1 (Y701)	AF488	Mouse	m, h	monoclonal, 4a	BD Biosciences
Phosphorylated STAT3 (Y705)	AF647	Mouse	Human	monoclonal, 4/P-STAT3	BD Biosciences
Phosphorylated STAT5 (Y694)	PE	Mouse	Human	monoclonal, 47	BD Biosciences

APPENDIX C. CD molecules

CD molecules	Functional implication in the dissertation
CD16/32	Fc gamma receptor for IgG
CD138	Plasma cell marker
CD19	Total B cell marker
CD27	Antigen-experienced (memory) cell marker
CD40	Receptor for CD40 ligand expressed on B cells
CD40 Ligand	Ligand expressed on activated T cells that ligates CD40
CD69	Activation marker for lymphocytes
CD80	Activation marker for B cell; co-stimulatory molecule for T cell
CD86	Activation marker for B cell; co-stimulatory molecule for T Cell

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