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THE ROLE OF PAIRED BOX 5, B LYMPHOCYTE-INDUCED MATURATION PROTEIN-1 AND ACTIVATION PROTEIN-1 IN THE SUPPRESSION OF B CELL DIFFERENTIATION BY 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN

presented by

Dina Schneider

has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Pharmacology and Toxicology

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1/16/08

Date

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By

Dina Schneider

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

2008

ABSTRACT

THE ROLE OF PAIRED BOX 5, B LYMPHOCYTE-INDUCED MATURATION PROTEIN-1 AND ACTIVATION PROTEIN-1 IN THE SUPPRESSION OF B CELL DIFFERENTIATION BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent environmental contaminant. The majority of TCDD-mediated toxicities are believed to be mediated through the aryl hydrocarbon receptor (AHR). AHR is a transcription factor acting through dioxin response elements (DREs) located in regulatory regions of numerous genes. Alterations in gene expression because of AHR-DRE interactions have been postulated to result in toxicity. The primary humoral (IgM) response in B cells is markedly suppressed by TCDD. However, the exact mechanism of TCDD-mediated suppression of the IgM response remains to be elucidated. We hypothesized that TCDD impairs the IgM response to bacterial lipopolysaccharide (LPS) by interfering with terminal B cell differentiation program. The objective of the present studies was to identify the molecular mechanism whereby TCDD impairs terminal differentiation in B cells. Surface markers major histocompatibility complex (MHC) class II, cluster of differentiation 19 (CD19) and syndecan-1 were altered by TCDD treatment in LPS-activated CH12.LX cells, a murine B cell line, during a 72 h culture period indicating suppression of differentiation by TCDD. In addition, x-box protein-1 (XBP-1), a

transcription factor critically involved in the IgM secretion, was suppressed by TCDD. Furthermore, transcription factor Pax5, a repressor of terminal differentiation, was downregulated by LPS, whereas TCDD attenuated the LPS-induced downregulation of Pax5. Blimp-1, an important upstream transcriptional repressor of Pax5 was induced by LPS and suppressed by TCDD at the mRNA level. This finding is in agreement with the TCDD-mediated suppression of Blimp-1 DNA-binding activity in the Pax5 promoter. Electrophoretic mobility shift assay (EMSA) of three putative activator protein -1 (AP-1) response elements found in the mouse B lymphocyte-induced maturation protein - 1 (Blimp-1) promoter demonstrated an increase in AP-1 binding in LPS-activated cells, which was attenuated in the presence of TCDD. By contrast, DRE-like sites DRE-75 and DRE-107 identified in the Blimp-1 promoter, and DRE-506 site identified in the paired box 5 (Pax5) promoter, exhibited no specific inducible protein binding, suggesting no impact on the dysregulation of Pax5 and Blimp-1 by TCDD. In summary, these results demonstrate that TCDD impaired terminal B cell differentiation in concordance with the suppression of a critical differentiation pathway through AP-1, Blimp-1 and Pax5. The contribution of the current studies to the field of immunotoxicology is in demonstrating that the suppression of the IgM response in B cells by TCDD is (1) a result of impaired B cell differentiation program, (2) is mediated, in part, by TCDD interference with an early B cell activation event, and (3) likely involves genomic and non-genomic AHR-mediated mechanisms.

DEDICATION

I dedicate this work to my parents, Ilya Schneider and Aneta Schneider who always believed in me and nourished my desire to learn.

ACKNOWLEDGEMENTS

First, I would like to acknowledge my mentor, Dr. Norbert Kaminski for his guidance and expertise, for creating the unique learning environment in which supervision and independence are intricately balanced, for his advice, and for teaching me to be a good scientist.

The members of my dissertation guidance committee, Dr. John LaPres, Dr. Patricia Ganey and Dr. Jay Goodman for their responsiveness, attention to my progress and useful suggestions.

Dr. Jay Goodman and Dr. Amy Bachman for their assistance in the Pax5 promoter methylation studies.

Bob Crawford, for technical assistance in the first experiments I performed towards my dissertation project and helpful suggestions for many other experiments that followed.

My office mate and true friend, Dr. Barbara Kaplan, for always being there for me.

Kimberly Hambleton, a true friend and colleague, for our daily conversations and emotional support.

The former and present postdoctoral fellows in our laboratory; Dr. Wei Zhang, Dr.

YingYing Wang, Dr. Alison Springs and Dr. Alejandra Manzan, who taught me much about research and life.

My former and present lab mates and friends, Dr. Gautham Rao, Dr. Cheryl Rockwell, Natasha Snider, Dr. John Buchweitz, Colin North, Priya Raman, Peer Karmaus, Haitian Lu and Thitirat Ngaotepprytaram for being a part of my everyday lab existence and contributing to the challenging and the stimulating environment in which we all thrive, year after year.

And many other individuals who, in one way or another, helped me to get through the last five years – your help is greatly appreciated.

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7-AAD	7-amino-actinomycin D
ABTC	2,2 -azino-di(3-ethylbenzthiazolin-sulfonate)
Ag	antigen
AHH	aryl hydrocarbon hydroxylase
AHR	aryl hydrocarbon receptor
AHRR	aryl hydrocarbonP receptor repressor
AIP	AHR-interacting protein
ANOVA	analysis of variance
AP-1	activator protein-1
APC	antigen presenting cell
APC	allophycocyanin
ARA9	AHR-associated protein 9
ARNT	aryl hydrocarbon receptor nuclear translocator
ATF	activating transcription factor
BCL-6	B cell lymphoma factor-6
BCR	B cell receptor
bHLH	basic helix-loop-helix
Blimp-1	B lymphocyte-induced maturation protein-1
BLNK	B cell linker protein
BRDG-1	B cell downstream signaling 1
BSAP	B cell-specific activation protein

BssHII	*Bacillus stearothermophilus* H3
BstUI	*Bacillus stearothermophilus* U458
CIITA	MHC class II transcriptional activator
CAT	chloramphenicol acetyl transferase
CBP	CREB-binding protein
CREB	cAMP response element
CD	cluster of differentiation
CDC2	cyclin-dependent kinase 2
CYP450	cytochrome P450
2,4-D	2, 4-dichlorophennoxyacetic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNP	dinitrophenol
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
DRE	dioxin response element
Dhx38	DEAH (Asp-Glu-Ala-His) box polypeptide 38
DHFR	dihydrofolate reductase
E2F	eucariotic factor
EDTA	ethylenediamine tetraacetic acid
EGF	epithelial growth factor
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay

EROD	ethoxyresorufin-O-deethylase
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ERSE	ER stress element
FITC	fluorescein
FO	lymphoid follicles
G0	gap 0 phase
G1	gap 1 phase
GADD	growth arrest and DNA damage
GLUT4	glucose transporter 4
GVH	graft versus host
HAH	halogenated aromatic hydrocarbon
HAT	histone aminotransferase
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGRg	human groucho gene
HIF-1α	hypoxia-inducible factor 1 alpha
HLA	human lymphocyte antigen
Hox	homeobox
HpaII	*Haemophilus parainfluenzae*
hsp	heat shock protein
ICAM	intracellular adhesion molecule
Ig	immunoglobulin

IgJ	immunoglobulin joining chain
Igκ	immunoglobulin light kappa chain
IgH	immunoglobulin heavy chain
IgM	immunoglobulin M
INF	interferon
IL	interleukin
JNK	jun-amino terminal kinase
KGF	keratinocyte growth factor
LFA	lymphoid function-associated antigen
LPS	lipopolysaccharide
MAPK	mitogen-associated protein kinase
mb-1	membrane glycoprotein-1
MHC	major histocompatibility complex
M.SssI	methyltransferase from *Spiroplasma* strain MQ1
MyoD	myogenic factor D
MZ	marginal zone
MZL	marginal zone lymphoma
NEPACCO	Northeast Pharmaceutical and Chemical Corporation
NF-κB	nuclear factor kappa B
Oct-2	octamer transcription factor 2
PAGE	Polyacrylamide gel electrophoresis
PAMS	periartheriolar marginal shealth
PAS	PER-ARNT-SIM

Pax5	paired box gene 5
PCR	polymerize chain reaction
PE	phycoerythrin
PER	periodictyl
PFC	plaque-forming cell
p27Kip1	cyclin dependent kinase inhibitor p27
PKC	protein kinase C
PR	proline-rich
Prdm-1	positive regulatory domain binding factor 1
Prmt5	protein arginine methyltransferase 5
RAG	recombinase activating gene 1
Rb	retinobalstoma protein
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcriptase polymerase chain reaction
RCL1	RNA terminal phosphate cyclase-like 1
S	synthesis phase
SAGA	Spt-Ada-Gcn5-acetyltransferase
SAR	structure-activity relationship
SCID	severe combined immunodeficiency
SOCS2	suppressor of cytokine signaling 2
SRC-1	steroid receptor co-activator 1
SIM	simple-minded
SP	1-specificity protein-1

SRBC	sheep red blood cell
2,4,5-T	2, 4, 5-trichlorophenoxyacetic acid
Th	T helper cell
TBP	TATA box-binding protein
TCDD	2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin
TCR	T cell receptor
TIF2	translation initiation factor-2
TLR	toll-like receptor
TFIID	transcription factor II D
TLE	transducin-like enhancer protein 1
TNF	tumor necrosis factor
TRE	12- O-tetradecanoate-13-acetate-responsive element
Tris	trishydroxymethylaminomethane
UPR	unfolded protein response
XAP-2	hepatitis B X-associated protein-2
XBP-1	X-box protein-1

INTRODUCTION

I. TCDD

A. Sources of TCDD

TCDD is a common byproduct and trace contaminant in a wide variety of chemical reactions. It has been and continues to be generated worldwide by a number of industrial processes such as paper pulp bleaching and metal smelting, waste incineration and wood and coal burning and is released into the atmosphere during natural processes, such as forest fires (Thornton et al., 1996; Travis and Hattemer-Frey, 1991). Some TCDD is generated as a byproduct in the course of manufacturing of phenoxyacetic acid herbicides, 2, 4 –dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and may be found as a trace contaminant in products containing these compounds. Whereas the agricultural use of 2,4,5-T in United States was banned by the US Environment Protection Agency in 1979 due to concerns of human reproductive toxicity (Smith, 1979), 2, 4-D continues to be widely used today in the agricultural settings in United States and worldwide, and may pose a risk of TCDD exposure.

Once TCDD is released into the environment, it persists in soil and aquatic sediments, resulting in animal exposure and bioaccumulation. Thus, significant amounts of TCDD may accumulate in tissues of animals grown for human consumption. In addition to natural accumulation in the food chain, TCDD has been recently detected in ball clay that was commonly added to chicken and catfish feed, resulting in abnormally high TCDD levels in these animal products marketed for general consumption. Identification of high TCDD levels in chickens recently led to a ban on ball clay additives use in animal feed by US Food and Drug Administration (Hayward et al., 1999). Today,

consumption of TCDD-contaminated food is the main route by which the general population is exposed to this compound, with highest concentration of TCDD detected in fish, meat and dairy products (Travis and Hattemer-Frey, 1991). In summary, TCDD remains an eminent health concern due to its high environmental persistence and presence in food products.

B. **Human exposure to TCDD**

It is believed that the general population is exposed to TCDD primarily through diet, however the majority of our knowledge regarding TCDD-related adverse effects in humans stems from observations in cohorts of individuals that were exposed to TCDD in occupational accidents. Whereas dietary exposure implies exposure to low TCDD doses over a long period of time, accidental exposures to TCDD are usually acute and doses are higher. However, information gathered from the accidental TCDD exposure has been seminal to our understanding of the TCDD-mediated toxicity in humans. Some of the major documented instances of accidental human exposure to TCDD are discussed below.

a. **Nitro plant accident**

One of the first major occupational exposure events involving TCDD occurred on March 9, 1949 at the Monsanto Company's chemical plant in Nitro, West Virginia. The accident occurred in the process of trichlorophenol manufacturing. Pressure within the reaction vessel exceeded the safety limits and the safety valve opened to release pressurized TCDD-contaminated trichlorophenol inside the plant building (Gough, 1985).

A number of workers who were either present at the time of the accident or took part in the cleanup of the contaminated facility were exposed to TCDD. This accident was later recognized as a major event in occupational and environmental health, and led to a number of seminal studies (Gough, 1985; Hay and Silbergeld, 1986; Senger, 1991; Zack and Suskind, 1980).

b. **Times Beach accident**

Another major accident involving TCDD occurred in the early 1970s at Times Beach, Missouri, where dioxin-contaminated waste was accidentally used for dirt road spraying in order to reduce dust formation. The Northeast Pharmaceutical and Chemical Corporation (NEPACCO) located in Verona, Missouri, was manufacturing hexachlorophene from trichlorophenol contaminated with 3 to 5 ppm of dioxin. A sub-contractor, Russell M.Bliss, was hired by NEPACCO to dispose of the oily residue from the bottom of process vessels so-called "still-bottoms". Waste oil was occasionally sprayed on dirt roads and dirt surfaces to reduce the generation of dust. In 1971, Bliss sprayed Shenandoah Stables and Jefferson Stables with "still-bottoms oil" leading to horses death and children sickness. These events prompted an investigation by the Center for Disease Control and Prevention, which has identified dioxin-contaminated trichlorophenol in both arenas. Further joint investigation by the Center for Disease Control and the Environment Protection Agency revealed that miles of unpaved roads in the town of Times Beach, Missouri that were extensively sprayed by Bliss between 1972 and 1976 contained dioxin at concentrations as high as 300 ppb (I.O.M., 1993). Extensive effort has been made since to eliminate TCDD and to establish safe TCDD

levels in the soil at Times Beach's industrial and residential areas (Gough, 1991; Paustenbach et al., 1992).

c. Seveso accident

In Europe, the most notorious chemical incident involving TCDD occurred in Italy in 1976. A chemical manufacturing plant located near the town of Seveso (ICMESA) produced trichlorophenol as an intermediate compound for the production of hexachlorophene. As was revealed later, the trichlorophenol produced by the Seveso plant was contaminated with TCDD. In July 1976, an explosion of a reaction vessel containing trichlorophenol produced a release of a chemical mixture into the air, which spread approximately half pound of TCDD over several square kilometers inhabited by almost 40,000 people (Pesatori et al., 2003). The chemical fallout resulted in death of small animals and birds. Residents were evacuated from the zones proximal to the plant and strict measures were taken in order to limit human exposure to the released chemicals in the zones designated as less contaminated (Pesatori et al., 2003). The only certain effect of human exposure to TCDD at Seveso was chloracne. Increased mortality from cardiovascular disease, diabetes, and cancer of gastrointestinal, lymphatic and hematopoetic systems were also reported, but the results were not conclusive due to small sample size and confounding factors (Bertazzi et al., 1998; Reggiani, 1978).

d. Operation Ranch Hand

Whereas the majority of human cases of acute exposure to TCDD occurred as a result of industrial accidents, TCDD received the highest notoriety in United States due to

use of the defoliant Agent Orange in the course of Vietnam War. From 1962 to 1971, a military operation called Ranch Hand was executed in Vietnam. The goals of this operation were the defoliation of trees and plants to improve observation and the destruction of enemy crops. During that period, nearly 19 million gallons of herbicides were sprayed, of which at least 11 million gallons were agent orange, a 1:1 mixture of 2, 4-D and 2, 4, 5-T, used for defoliation of a wide array of broadleaf plant species (I.O.M., 1993). It was later determined that 2,4,5-T, a component in the Agent Orange was contaminated with TCDD at levels up to 50 ppm (I.O.M., 1993). Due to emerging experimental evidence of birth defects in animals and the rising concern for human health, the US Department of Defense temporarily suspended the use of 2, 4, 5-T, and therefore Agent Orange in military operations in April 1970, and the Operation Ranch Hand was eventually terminated October 31, 1971 (I.O.M., 1993). It is believed today that the majority of Vietnam veterans were under low risk of exposure to Agent Orange (Young et al., 2004). However, in a subgroup of Operation Ranch Hand veterans who were involved in handling and spraying of Agent Orange, high serum levels of TCDD and a number of associated toxicities have been detected. Namely, among veterans of Operation Ranch Hand with the highest serum TCDD levels, there was an increased incidence of peripheral neuropathies, hepatic abnormalities and impairment of cognitive functions (Barrett et al., 2001; Michalek et al., 2001). These and other incidents raised concerns over the health outcomes of human exposure to TCDD, and deemed the research of the TCDD toxicity in animal models and their correlation with human toxicity necessary.

C. Signs of TCDD toxicity in animals and humans

A wide array of toxic effects has been associated with TCDD exposure in humans and in animal models. In animal models, TCDD adverse effects include carcinogenicity, reproductive and developmental toxicity, hepatotoxicity, nephrotoxicity, endocrine toxicity and immunotoxicity. In humans, however, the only effects unequivocally proven to result from TCDD exposure are chloracne and alterations in lipid metabolism (Panteleyev and Bickers, 2006; Sweeney and Mocarelli, 2000). Furthermore, TCDD is categorized as Class I carcinogen (*i.e.*, " a known human carcinogen") by the International Agency for Research on Cancer based on human epidemiological studies and mechanistic studies in animals, but this classification remains controversial due to weak evidence of TCDD carcinogenicity in humans (Cole et al., 2003). A generalized profile of TCDD toxicity that takes into account the most prominent effects observed in individual species and effects shared by multiple species is presented below.

a. Chloracne and hyperkeratosis

Chloracne and hyperkeratosis are among the most consistent effect associated with acute human exposure to TCDD. In fact, chloracne was one of a few symptoms that were shown to be proportional to the serum concentrations of TCDD in the exposed population (Sweeney and Mocarelli, 2000). Although the mechanism of TCDD-mediated chloracne eruption is not clearly understood, impaired vitamin A metabolism in the skin (Coenraads et al., 1994) and TCDD-induced activation of skin stem cells (Panteleyev and Bickers, 2006) have been proposed as possible mechanisms for the TCDD-induced chloracne formation. Rabbits, monkeys, cows and hairless mice also exhibit chloracne

(Andersen et al., 1994; Knutson and Poland, 1982; Puhvel et al., 1982). *In vitro* studies utilizing mouse and human keratinocytes have shown that the TCDD-induced hyperkeratosis was dose-related (Greenlee et al., 1985a; Knutson and Poland, 1980), providing additional evidence for the dependence of chloracne eruption on TCDD.

b. **Wasting syndrome**

The most common effect of TCDD and related compounds in rodents is wasting syndrome – a starvation-like effect leading to weight loss and lethality. Symptoms of wasting syndrome are reduction in adipose tissue mass, hypertriglyceridemia and redistribution of fatty acids (Brewster and Matsumura, 1988; Chapman and Schiller, 1985; Gasiewicz and Neal, 1979). Animals affected by wasting syndrome gradually develop hypoglycemia, which has been attributed to the TCDD-mediated inhibition of phosphoenol pyruvate carbokinase (Stahl et al., 1993; Weber et al., 1995), and eventually die.

c. **Hepatotoxicity**

Liver is another target organ of TCDD. Hepatic effects in rodents and humans range from CYP450 enzymes induction, hepatomegaly, fatty change and bile duct hyperplasia to impaired liver function. In rats and mice, the appearance and severity of TCDD-induced hepatotoxicity depends on the expression of AHR (Birnbaum et al., 1990; Shen et al., 1991). In addition TCDD exposure is also associated with reduction in bilary excretion (Yang et al., 1977) and accumulation of porphyrins in the liver, kidney and spleen (Goldstein et al., 1982). Induction of liver enzymes is the most extensively

studied effect of TCDD. The induction of mixed-function oxidase and the aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin-O-deethylase (EROD) as markers of CYP1A1 induction are considered the most sensitive biochemical responses to TCDD, and have been used to determine the relative potency of dioxin-like compounds in relation to TCDD (Poland and Glover, 1973; Safe, 1990). Induction of liver enzymes is species-dependent. For example, in guinea pig, species most sensitive to mortality by TCDD, the induction of AHH activity is slight, even at lethal doses (Neal et al., 1979). By contrast, in Syrian Golden hamsters induction of cytochrome P450 enzymes was observed at doses significantly lower than doses required to produce tissue damage and lethality (Gasiewicz et al., 1986). In mice, cytochrome P450 enzyme induction segregated with the Ah locus. Namely, in C57BL/6J mice (AHR-responsive) TCDD was ten-fold more potent than in DBA/2 mice (AHR-nonresponsive) (Nebert, 1989; Poland and Knutson, 1982). A similar relationship has been observed in C57BL/6L mice congenic at the Ah locus (Birnbaum et al., 1990).

d. **Vitamin A storage by the liver**

According to several reports, another biological function of the liver, vitamin A storage, is compromised by TCDD exposure. Vitamin A storage is sensitive to TCDD even at low doses in a variety of species. TCDD is capable of decreasing the vitamin A storage in the liver of rats, guinea pigs and mice (Brouwer et al., 1989; Hakansson and Hanberg, 1989; Hakansson et al., 1991; Kay et al., 1997; Thunberg et al., 1979). The proposed mechanism for impaired vitamin A storage in the liver is inhibition of the storage of vitamin A in the livers stellate cells (Hakansson and Hanberg, 1989). A single

dose of TCDD administered to a female Sprague-Dawley (SD) rat decreased vitamin A levels in liver, as well as in lung, intestines, and adrenal gland, while increased vitamin A concentrations in serum, kidneys and urine. Concomitantly, the free fraction of serum retinol binding protein was increased by 150% (Brouwer et al., 1989). The dysregulation of vitamin A by TCDD has been proposed to contribute to a number of TCDD-induced toxicities. For example, the enhanced vitamin A metabolism has been linked to cleft palate (Birnbaum et al., 1989) and liver tumor promotion (Bock and Kohle, 2005) by TCDD in mice.

e. **Impairment of thyroid function**

Chronic and subchronic exposures to TCDD may impair thyroid functions. Dose-dependent reductions of plasma thyroxine levels have been observed in TCDD-exposed animals. Decrease in plasma thyroxine levels was detected in SD rats (Van Birgelen et al., 1995) and in adult great blue heron (Janz and Bellward, 1996). The disbalance of thyroid hormones was associated with the TCDD-induced induction of hepatic UDPGT isozymes (Lucier et al., 1973; Lucier et al., 1986). The induced UDPGT isozymes conjugate thyroxine, leading to deactivation and elimination of this thyroid hormone (Bastomsky, 1977; Henry and Gasiewicz, 1987).

f. **Diabetes**

Recent epidemiological studies suggested that increased diabetes risk exists in human populations exposed to low levels of TCDD and TCDD-like compounds (Everett et al., 2007; Fujiyoshi et al., 2006; Pesatori et al., 2003). Several molecular entities have

been linked to this toxicity of TCDD. Epidemiological study in Operation Ranch Hand veterans reported that the induction in the mRNA levels of glucose transporter GLUT4 and the inflammatory mediator nuclear factor kappa B (NF-κB) correlated with the development of diabetes (Fujiyoshi et al., 2006). The involvement of AHR in glucose metabolism is evidenced by the fact that AHR-knockout mice had altered insulin regulation and glucose tolerance tests (Thackaberry et al., 2003), providing additional evidence for the putative involvement of TCDD in diabetes etiology.

g. Cross-talk between TCDD and sex steroids

Activity of sex steroids is affected by TCDD exposure. Specifically, estrogen seems to contribute to the capacity of TCDD to induce tumors. Long-term TCDD exposure bioassays demonstrated liver tumors in female, but not male rats (Kociba et al., 1978). This tumorogenic effect can be effectively prevented by removing ovaries from female rats before exposure to TCDD (Lucier et al., 1991). The metabolism of testosterone is disrupted by TCDD as well. Namely, the activity of testicular 16-α-testosterone hydroxylase, 6-β-hydroxytestosterone and 7α-hydroxytestosterone, was decreased in SD rats post single peritoneal dose of TCDD (Mittler et al., 1984). In addition, decreased levels of testosterone and dihydrotestosterone were detected in serum of male SD rats following TCDD exposure (Moore et al., 1985). Modulation of the hepatic microsomal testosterone hydroxylases has been also observed in young male Wistar rats (Keys et al., 1985).

h. **Developmental toxicity**

In general, developmental effects of TCDD were found to be dependent on regimen, dose and gestational period throughout which the experimental species at question were exposed to TCDD. For the majority of species, developing fetus is more sensitive to TCDD as compared with an adult, *i.e.*, toxic effects were observed at lower doses.

In fish, TCDD is most toxic at the early stages of life. For example, rainbow trout, and zebrafish embryos and fry sac showed increased sensitivity to TCDD compared to adult animals (Prasch et al., 2003; Zabel et al., 1995). TCDD toxicity in fish is manifested in edema, hemorrhages, arrested growth and development, and ultimately death. These effects are believed to be mediated through AHR, which is assumed to be present in the early life stages of fish (Binder and Lech, 1984; Binder and Stegeman, 1983).

Among birds, chicken has been the most sensitive species for developmental toxicity of TCDD. Exposure of fertilized chicken eggs to TCDD resulted in pericardial and subcutaneous edema, liver lesions, inhibition of lymphoid development in thymus and Bursa of Fabricius, beak deformities, cardiovascular malformations and mortality (Cheung et al., 1981; Nikolaidis, 1990; Wood et al., 2002). Compared to chicken, responses to TCDD varied in other species. For example, in ring-necked pheasants injection of TCDD to the embryo resulted in embryo mortality, but other signs of toxicity observed in chickens, such as cardiac malformations, did not occur (Nosek et al., 1992).

Mammalian embryos and fetuses are also more sensitive to TCDD than adult animals, as has been shown for hamsters, rats and macaque monkeys (Moran et al., 2004;

Olson et al., 1980a; Olson et al., 1980b; Sparschu et al., 1971). Gestational exposure to TCDD in mammals typically results in thymic hypoplasia, subcutaneous edema, decreased fetal growth and perinatal mortality. In the mouse, hydronephrosis is the most sensitive effect of prenatal toxicity, followed by cleft palate formation and thymic atrophy, subcutaneous edema and mortality (Abbott et al., 1987a; Abbott et al., 1987b; Courtney, 1976; Courtney and Moore, 1971; Neubert and Dillmann, 1972). Similarly, in the rat, TCDD prenatal toxicity is manifested by internal hemorrhage, subcutaneous edema, decreased fetal growth, and mortality (Brouwer et al., 1995; Sparschu et al., 1971)

i. **Carcinogenicity**

TCDD is linked to a number of cancers in animals and humans. Evidence for cancer in human studies has been presented for Seveso accident, (Bertazzi et al., 2001; Pesatori et al., 2003), Operation Ranch Hand (Akhtar et al., 2004) and additional TCDD human exposure accidents (Johnson, 1991; Kogevinas, 2000). Long term TCDD exposure studies in rodents demonstrate that TCDD is carcinogenic for mice and rats (Kociba et al., 1978). The observed effects included tumors of nasal turbinates and hard palate, lung, thyroid, liver, and thymus. In addition, carcinogenicity following long term TCDD exposure has been reported in hamsters, which developed facial skin carcinoma (Rao et al., 1988).

The mechanism of TCDD carcinogenic effects is not completely understood, but it is clear that TCDD is not a direct genotoxic agent, as it does not form DNA adducts (Randerath et al., 1988; Turteltaub et al., 1990). Furthermore, TCDD is negative in the

Salmonella/Ames test in the presence or absence of mixed-function oxidase activating system (Shu et al., 1987; Wassom et al., 1977). Consequently, TCDD acts as promoter rather than as an initiator in the two-stage liver and skin tumor promotion models (Lucier et al., 1991; Pitot et al., 1980; Poland and Knutson, 1982). In rat liver, TCDD promotion of diethylnitrosamine-initiated tumors was dependent on ovarian hormones, and occurred to a lower extent in ovarectomized females as compared to intact females (Graham et al., 1988; Lucier et al., 1991). Unexpectedly, some *in vitro* studies suggest that TCDD acts as an antiestrogen in rat uterus (Safe et al., 1991) and human breast cancer cells (Narasimhan et al., 1991), suggesting that the dependence of the TCDD-induced tumors on estrogen in rat is tissue-specific.

By comparison, in some instances exposure to TCDD was associated with decreased incidence of cancer. For example, carcinogenic changes in mammary glands were detected with lower frequency in rats following a two-year TCDD exposure, as compared with the non-exposed control rats (Kociba et al., 1979; Kociba et al., 1978). Similarly, follow-up study of cancer occurrence performed on individuals exposed to TCDD during the Seveso accident revealed decreased incidence of breast and endometrial cancers in women residing in the TCDD-contaminated zones (Bertazzi et al., 1993). Whereas the exact explanations for these instances of reduced cancer incidence remain to be elucidated, recently published report demonstrates the ability of TCDD to promote degradation of a number of steroid receptors, among them the estrogen receptor (Ohtake et al., 2007). In their studies, Ohtake and colleagues have demonstrated that TCDD-dependent degradation of estrogen receptor α and some androgen receptors is mediated through the formation of steroid receptor complex with an atypical ligase E3,

targeting the steroid receptor molecule for degradation via the ubiquitin pathway. Hence, the reduced incidence of breast and endometrial cancers, which are often estrogen-receptor-dependent, may be in part due to the TCDD-induced degradation of the estrogen receptors.

The International Agency for Research on Cancer lists TCDD as class I human carcinogen since 1997 (McGregor et al., 1998). This assessment is based on substantial evidence in animal models, limited human evidence obtained from epidemiological studies in four industrial cohorts, and supportive mechanistic evidence. The epidemiological human evidence was that TCDD increased the incidence of all cancers combined in four cohorts of individuals exposed to TCDD in industrial settings. The judgment of the mechanistic evidence was based on the fact that many of the carcinogenic effects of TCDD in rodents were AHR-dependent, that AHR is evolutionary conserved among species, and that the concentration of TCDD in heavily exposed human population were similar to the concentrations sufficient to produce tumors in rat two-stage carcinogenic models (Steenland et al., 2004). However, this classification has been disputed based on the fact that TCDD exhibited a modest risk to increase the incidence of a number of human cancers, but did not pose a significant risk to development of any particular cancer type (Cole et al., 2003). Overall, existing evidence suggests that the carcinogenic risk of TCDD exposure may have been overstated. In the future, better understanding of the biologic mechanisms underlying the TCDD-mediated tumorogenicity will be necessary to better assess the risks of exposure to this compound.

j. **Immunotoxicity**

The ability of TCDD to induce toxicity in the immune system has been established by evidence derived from studies in animal species, including rodents, guinea pigs, rabbits, and marmosets (Holsapple et al., 1991a; Kerkvliet, 1995; Neubert et al., 1993; Ross et al., 1997; Vos and van Loveren, 1995). Human studies in TCDD-exposed populations have indicated numerous abnormalities of the immune system, but the results were inconclusive and the recent follow-up studies have not identified consistent relationships between TCDD exposure and immune system abnormalities (Baccarelli et al., 2002; Michalek et al., 1999), possibly due to confounding variables present and small sample sizes. Nevertheless, the immune system is thought to be one of the systems most sensitive to TCDD toxicity (Holsapple et al., 1991b; Kerkvliet, 1984; Vos et al., 1973). The immunosuppressive effects of TCDD include involution of lymphoid organs, especially of the thymus, suppressed cytotoxic T cell responses to antigens, suppressed immunoglobulin production, and in some cases, paradoxical induction of immune mediators (Funseth and Ilback, 1992; Neff-LaFord et al., 2003) .

One of the first reports linking TCDD exposure to immune toxicities in multiple animal species has shown that host resistance to pathogens was compromised by TCDD (Vos et al., 1973). Specifically, the delayed type hypersensitivity (DTH) response to tuberculin in guinea pigs and the graft versus host response (GVH) in mice were suppressed by TCDD. In addition, host resistance to influenza A virus (Neff-LaFord et al., 2003), and to nematode parasite *Trichinella Spiralis* (Luebke et al., 2002), and cytotoxic T lymphocyte response (Kerkvliet et al., 2002) in mice were found to be suppressed by TCDD. The cellular and mechanistic aspects of the TCDD-mediated

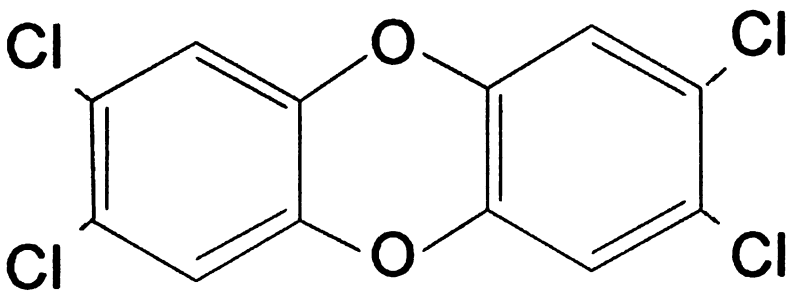
immunotoxicity are discussed in detail in section II C.

D. Molecular mechanisms of TCDD action

a. AHR is a receptor for TCDD and other structurally related compounds

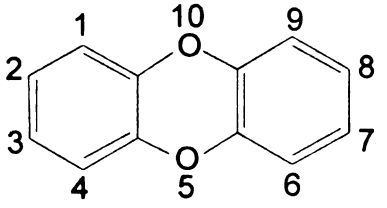
TCDD (Fig.1) is the most toxic congener in the class of structurally related halogenated aromatic hydrocarbons (HAH), a family of polycyclic organic compounds that includes halogenated dibenzodioxins, biphenyls and dibenzofurans (Fig. 2). Many of the HAH congeners act as ligands for the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor. A large number of physiologic and toxic responses to TCDD that have been characterized to date are mediated through a specific interaction of TCDD with AHR. The existence of the AHR was first demonstrated in the cytosolic fraction of C57BL/6J mouse liver (Poland and Knutson, 1982). The AHR was first identified as a receptor for TCDD in the C57BL/6J mouse liver fractions based on its ability to bind TCDD in a saturable and reversible manner, and with high affinity (*i.e.*, in the nanomolar range).

Notably, the activation of AHR by TCDD and other HAH is known to induce the expression of liver cytochrom P450 metabolizing enzymes, such as CYP1A1, CYP1B1, and CYP1B2. Therefore, the induction of CYP1A1 has been widely employed as a biomarker of exposure to TCDD and structurally related compounds.

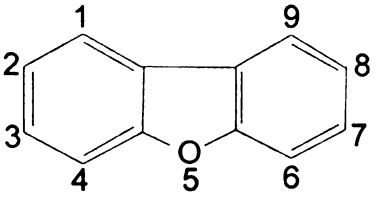


2,3,7,8-Tetrachlorodibenzo-para-dioxin

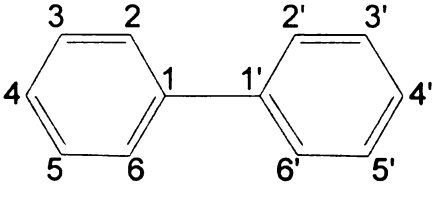
Figure 1. Chemical structure of the 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin. TCDD is the most toxic of all HAH congeners. The high affinity of this compound for AHR is conferred by its planar shape and substitution of the four *para* positions with chlorine atoms.



Dibenzo-para-dioxin



Dibenzofuran



Biphenyl

Fig. 2. General chemical structures of dibenzo-*p*-dioxin, dibenzofuran and biphenyl congeners. Fluorine, bromine or chlorine substitution at most of the *ortho* and *para* positions in these molecules yields toxic congeners.

A structure activity relationship (SAR) has been established for HAH, which relates the HAH compound toxicity to its binding affinity for AHR, and can be measured by the induction of CYP4501A1 gene (Poland and Glover, 1973, Safe, 1990).

b. SAR for HAH congeners that are ligands for AHR

Competition binding studies with HAH congeners revealed that multiple aromatic hydrocarbons can specifically bind to AHR, however TCDD had the highest binding affinity of all dioxin congeners. Ligands with the highest binding affinity for the AHR were planar and contained halogen atoms in at least three out of four *para* positions (2,3,7,8 for dioxin congeners). Conversely, halogenation of the *ortho* positions (1,4,6,9 for dioxin congeners) in addition to the *para* positions reduced the congener's affinity for the AHR, due to the distortion of the molecule's single plain configuration (Poland and Knutson, 1982).

Furthermore, SAR studies for TCDD congeners revealed that for coplanar dioxins AHR-binding affinity correlated with the congener potency. In general, the rank order of potency for dioxin compounds to elicit a broad spectrum of physiologic and toxic responses has been demonstrated to be similar to their rank order binding affinity to TCDD (Knutson and Poland, 1982; Safe, 1986). However, it has been recognized that some AHR ligands are weaker agonists than others, and some act as antagonists, suggesting that not all AHR ligands have the same degree of efficacy (Henry et al., 1999; Hestermann et al., 2000).

The involvement of the AHR in the TCDD toxicity was corroborated by studies in inbred strains of mice known to express AHR alleles that possess either high (C57BL/6J)

or low (DBA/2J) affinity for TCDD. For example, the TCDD-responsive mouse strain, C57BL/6J, showed signs of toxicity at about 10-times lower concentrations of TCDD when compared with the TCDD-resistant DBA/2J strain, (Chapman and Schiller, 1985; Lusska et al., 1991). In C57BL/6J mice congenic for the Ah locus, 8-24 times higher TCDD concentrations were required to elicit toxic responses in the AHRd/d (resistant) strain when compared with the AHRb/b (sensitive) strain (Birnbaum et al., 1990). Furthermore, the AHR null-allele mice are resistant to the TCDD-mediated hepatic enzyme induction and toxic effects of very high doses of TCDD (Fernandez-Salguero et al., 1996).

c. AHR structure and function

Structural analysis of AHR revealed that it belongs to of basic helix-loop-helix (bHLH) class of transcription factors. In the AHR, bHLH domain is located towards the N-terminal end of the protein. Based on the analysis of structurally related proteins it has been determined that whereas the basic region mediates DNA binding, the helix-loop-helix domain is necessary for protein-protein interactions. An additional domain involved in protein-protein interactions in bHLH transcription factors is the periodictyl/AHR nuclear translocator/single-minded ((PER-ARNT-SIM), (PAS)) domain (Huang et al., 1993). In AHR, the PAS domain consists of two adjunct regions designated PAS-A and PAS-B. In the absence of an agonist, the PAS-B associates with one of the two 90-kDA heat shock protein (hsp90) subunits, allowing the other hsp90 to bind the bHLH region of AHR (Coumailleau et al., 1995; Fukunaga et al., 1995; Whitelaw et al., 1993b). TCDD has been shown to bind to a ligand-binding pocket

located near the PAS-B region, the conformation of which is maintained by hsp90. Dimerization between AHR and ARNT is mediated through their bHLH regions, but is further stabilized by PAS-PAS interactions (Fukunaga et al., 1995; Reisz-Porszasz et al., 1994). The carboxyl terminus of AHR protein contains a transcription activation domain comprised of acidic and glutamine-rich regions that are capable of interaction with the transcriptional machinery proteins, such as the TATA-binding protein, and with co-activators such as steroid receptor co-activator (SRC-1a) and translation-initiation factor 2 (TIF2) to advance transcription (Kumar et al., 2001; Watt et al., 2005).

Interestingly, the human form of AHR protein is homologous with that of other mammalian species (Hahn, 1998), but compared with mouse and rat AHR, the human AHR has a several-fold lower affinity for TCDD (Ema et al., 1994), and appears to be less sensitive in eliciting a response. For example, CYP1A1 induction in human embryonic palatal cells by TCDD is approximately 200 times lower than in cultured mouse palatal cells (Abbott et al., 1999). Furthermore, AHR receptor concentration and characteristics in human population vary considerably (Roberts et al., 1986; Roberts et al., 1991), making interspecies comparisons challenging.

d. **AHRR structure and function**

The AHR repressor (AHRR) protein was identified as a factor capable of repressing the activity of AHR (Haarmann-Stemmann and Abel, 2006; Mimura et al., 1999). The mechanism whereby AHRR is repressing AHR transcriptional activity involves competition with AHR for dimerization with ARNT and for subsequent DRE binding (Mimura et al., 1999). The expression of AHRR is induced, in part, by activated

AHR-ARNT complex binding to DRE in AHRR promoter creating a negative feedback loop for AHR transcriptional activity (Mimura et al., 1999). Interestingly, the expression of AHRR in SD rat liver was shown to be induced by TCDD treatment, but not by AHR ligands 3-methylcholanthrene and β-naphtoflavone (Brauze et al., 2006), suggesting that the induction of AHRR may be TCDD-specific.

Like AHR, AHRR is a member of the bHLH family. Considerable sequence similarity exists between AHR and AHRR bHLH domain, and PAS-A domain, however the other domains present in AHR to date have not been identified in AHRR (Haarmann-Stemmann and Abel, 2006; Mimura et al., 1999). AHRR localizes to the nucleus where it constitutively binds to ARNT (Mimura et al., 1999). Furthermore, the heterodimerization of AHRR with ARNT can not be induced by HAH compounds (Mimura et al., 1999). AHRR has been implicated in the etiology of breast cancer, where it has been proposed to suppress tumor growth by a mechanism involving competition with AHR for DRE binding (Kanno et al., 2006), and may play a role in the mechanism of other TCDD toxicities. Furthermore, AHRR polymorphisms in human populations have been implicated in the development of endometriosis (Tsuchiya et al., 2005). The levels of AHRR expression in an organism is highly dependent on species, tissue type and stage of development, giving rise to the notion that AHRR may mediate tissue-specific AHR responses. However, although literature published to date provides evidence for attenuation of AHR-mediated responses by AHRR *in vitro* (Haarmann-Stemmann and Abel, 2006; Kanno et al., 2006; Mimura et al., 1999), very little is known about the role AHRR plays in AHR-mediated responses *in vivo*.

e. **ARNT structure and function**

ARNT is also a member of the bHLH family. Similarly to AHRR, ARNT appears to be a nuclear protein. ARNT has been detected in the nuclear, but not cytosolic compartment of uninduced hepatoma cells, and TCDD exposure of cells produced no change in its intracellular distribution (Pollenz et al., 1994). Interestingly, ARNT protein neither binds TCDD nor associates with DREs in the absence of ligand-bound AHR (Whitelaw et al., 1993a). On other hand, ARNT interaction with AHR is necessary for AHR-DRE binding and transcriptional activity. AHR and ARNT interact in the heterodimeric complex through their respective bHLH domains. This notion is supported by the fact that deletion of bHLH domain of ARNT abrogates its functional interactions with the ligand-bound AHR (Whitelaw et al., 1993a).

The structure of ARNT protein is similar to the structure of AHR. ARNT contains the bHLH domain and PAS region comprised of PAS-A and PAS-B subdomains at the N-terminal. However, the carboxyl terminal transactivation domain in ARNT is shorter as compared with AHR, and the relative contribution of ARNT domain to transcriptional activation depends on availability of cell specific co-activators (Corton et al., 1996). Multiple ARNT isoforms have been detected in several species (Drutel et al., 1996; Hirose et al., 1996; Pollenz, 1996). In mice and rats ARNT1 and ARNT2 are 83% homologous in sequence and are capable of heterodimerization with AHR and specific recognition of DREs *in vitro* (Hirose et al., 1996). However, the distribution of these two proteins differs, as ARNT1 is widely expressed in a variety of tissues, whereas ARNT2 is detected primarily in adult brain and kidney (Drutel et al., 1996; Hirose et al., 1996). By comparison with rodents, multiple homologous forms of AHR and ARNT have been recently identified in zebrafish (AHR1, AHR2, ARNT1, ARNT2) and a bird species

termed common cormorant (ARNT1, ARNT2) (Carney et al., 2006) (Lee et al., 2007). Furthermore, the developmental toxicity of TCDD in zebrafish was shown to be mediated by AHR1 and ARNT2, but not by AHR2 or ARNT1 proteins (Antkiewicz et al., 2006), suggesting that a distinct isoform of ARNT may mediate the toxicity of TCDD in other species as well. However, the respective roles of ARNT1 and ARNT2 in contributing to the variety of TCDD-elicited responses in rodents or humans have not been fully elucidated.

In addition to interacting with AHR, ARNT1 acts as a dimerization partner for a number of other transcription factors, such as SIM, hypoxia inducible factor HIF1α, the endothelial PAS protein, and several additional proteins that have not yet been characterized (Ema et al., 1997; Hogenesch et al., 1997; Probst et al., 1997). In addition, ARNT plays an essential role in development (Kozak et al., 1997; Maltepe et al., 1997). The AHRR has been shown to repress AHR activity by sequestering ARNT (Mimura et al., 1999). Therefore, ARNT is an important protein with diverse functions in development, hypoxia and other cellular processes, including the mediation of AHR transcriptional activity.

f. **Activation of the AHR pathway**

In the absence of ligand, AHR resides in the cytosolic compartment of the cell bound to a complex of cytosolic proteins (Fig.3). The cytosolic AHR-binding complex contains two subunits of hsp90 (Denis et al., 1988; Perdew, 1988), an immunophillin-like protein termed AHR-associated protein-9/AHR inducible protein/hepatitis B

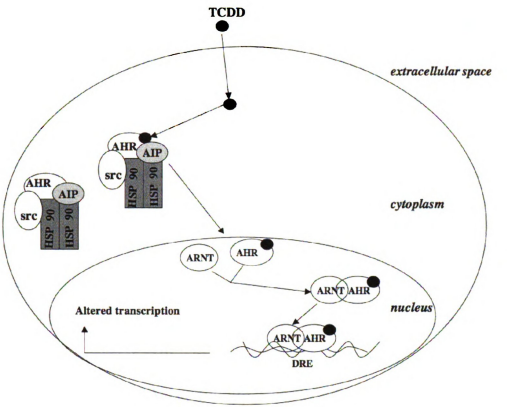


Figure 3. Schematic representation of the AHR-ARNT signaling pathway. TCDD freely penetrates cell membrane due to its lipophilic nature. In the cytosol, TCDD associates with its receptor, the AHR, leading to AHR dissociation from the cytosolic protein complex and translocation to the nucleus. In the nucleus, TCDD forms dimers with its partner, ARNT. The resulting AHR-ARNT dimer functions as a transcription factor by binding to its cognate motifs, DREs. AHR-ARNT binding to DRE is believed to be responsible for modulation of a large number of genes by TCDD.

X-associated protein-2 (ARA9/AIP/XAP-2), (Carver et al., 1998; Ma and Whitlock, 1997; Meyer et al., 1998), a 23 kDa protein (p23) (Kazlauskas et al., 1999), and additional proteins that have not been fully elucidated. The hsp90 protein is an abundant cellular chaperone commonly involved in regulation of folding and stability of steroid receptors.

Similarly, hsp90 subunits were shown to be involved in the regulation of AHR nuclear translocation and DNA binding (Phelan et al., 1998). The immunophillin proteins are often present in the hsp90 chaperone cytosolic complexes, where they play an important role in protein trafficking. In the AHR cytosolic complex, the immunophillin-like protein ARA9/AIP/XAP-2 has been shown to enhance the transcriptional activity of AHR-ARNT complex (Ma and Whitlock, 1997; Meyer et al., 1998). In addition, the ARA9 protein, also termed AIP/XAP-2, was shown to increase the number of AHR molecules in the cytosol (LaPres et al., 2000) and to stabilize the AHR interaction with the cytosolic complex (Bell and Poland, 2000). Another protein associated with the cytosolic complex, p23, was to associate with hsp90 and the ligand-free form of AHR and to increase the ligand responsiveness of AHR *in vitro* (Kazlauskas et al., 1999). According to one report, c-src kinase also participates in the complex (Enan and Matsumura, 1996).

Upon ligand binding, AHR dissociates form the cytosolic complex and translocates to the nucleus. It has not been fully elucidated yet whether or not the additional factors of the AHR cytosolic complex are capable of dissociating upon AHR ligand binding, and initiating additional signaling pathways. To this end, evidence exists for c-src dissociation and activation upon AHR dissociation form the complex. The

dissociated c-src has been shown to initiate a protein phosphorylation pathway (Enan and Matsumura, 1996), providing a possible explanation for the increase in phosphorylation associated with TCDD treatment (Carlson and Perdew, 2002).

g. **Initiation of the AHR-ARNT transcriptional activity**

As depicted in figure 3, binding of TCDD to AHR in the cytosol of the cell leads to AHR dissociation from the cytosolic protein complex and translocation to the nucleus (Pollenz et al., 1994). In the nucleus AHR heterodimerizes with ARNT. The AHR-ARNT heterodimer acts as a transcription factor, *i.e.*, it may up- or downregulate the transcription of susceptible genes in DNA-binding-dependent manner. The AHR-ARNT heterodimer specifically recognizes and binds dioxin response elements (DREs) located in promoter or enhancer regions of susceptible genes (Hankinson, 1995). The core nucleotide sequence for DRE has been identified as 5'-TNGCGTG-3'(Yao and Denison, 1992), the four-base sequence of which, 5'-GCGTG-3' is required for the receptor heterodimer to bind DNA *in vitro* (Yao and Denison, 1992). Furthermore, it has been determined that ARNT always binds to the 3'half site-GTG, whereas AHR binding affinity to the adjacent DNA may depend on the sequence. Interestingly, whereas ARNT can dimerize with proteins other that AHR to act as a transcription factor, AHR has not been shown to dimerize with any proteins except ARNT. Furthermore, the AHR-ARNT heterodimerization is necessary in order for AHR to bind DRE sequences (Whitelaw et al., 1993a), thus the heterodimerization with ARNT is necessary for the AHR transcriptional activity. Therefore, the specificity of AHR-ARNT transcription complex for DREs is conferred, to a large part, by AHR, whereas the abundance of ARNT in the

nucleus may modulate the transcriptional activity of the AHR-ARNT complex.

Existing evidence suggests that ARNT is not involved in the AHR translocation to the nucleus, but is involved in the translocation of AHR from the nucleus to the cytosol, as in ARNT-deficient cells AHR accumulates in the nuclear compartment (Hankinson, 1995). The AHR-ARNT heterodimer is formed through interaction of bHLH domains of the two proteins. The heterodimer can selectively bind to DRE motifs in the regulatory regions of susceptible genes, resulting in altered gene transcription. This mechanism is responsible for the induction of liver CYP450 enzymes, and is prototyped by the induction of CYP1A1 gene in mice and rats. Notably, the induction of metabolizing enzymes *per se* is a physiologic, rather than toxic response to TCDD, but it has been postulated that similar mechanisms mediate many of TCDD-induced toxicities. Because, the induction of CYP1A1 is a rapid and sensitive response to chemicals acting as AHR ligands, it has been widely used as a measure of exposure to TCDD congeners.

The activity of the AHR-ARNT complex at DRE site is twofold. First, the AHR-ARNT complex recruits transcriptional co-activators, such as specificity protein-1 (SP-1) and SRC-1 (Beischlag et al., 2002; Kumar and Perdew, 1999) through its transactivation domain. Second, the AHR-ARNT complex interacts with the basic transcriptional machinery, including TATA box-binding protein (TBP), transcription factor II D (TFIID), and RNA polymerase II (Tian et al., 2003; Watt et al., 2005). These AHR functions appear to be dependent on specific AHR tyrosine residues, phosphorylation of which is required for AHR binding activity (Minsavage et al., 2004).

In case of CYP1A1, CYP1B1 and many other genes, the engagement of DRE site by AHR-ARNT complex upregulates transcription. However, for some other genes DRE

engagement results in transcriptional repression. DRE motifs that mediate transcriptional repression have been identified in a number of β-estradiol-regulated genes (Duan et al., 1999; Safe et al., 2000). It has been proposed that AHR-ARNT binding to DRE will repress transcription if the binding occurs at overlapping DNA site that also binds another transcription factor. For example, overlapping NF-κB/DRE site has been identified in the IgH 3'α enhancer. The interaction of AHR and NF-κB at the overlapping binding site have been proposed to contribute to the repression of the 3'α IgH enhancer, associated with TCDD exposure (Sulentic et al., 2004a; Sulentic et al., 2004b).

h. AHR-ARNT-DRE pathway-independent effects of TCDD

Although many of the toxic and physiologic responses to TCDD reported to date are mediated through the classical AHR-ARNT-DRE pathway, some responses appear to be DRE-independent. These responses can be further subdivided into AHR-dependent, and AHR-independent. Despite the fact that the functional implications of the DRE-independent effects are not fully understood, they may play an important role in the realization of the TCDD-mediated toxicity.

(i). AHR-dependent effects of TCDD, for which DRE involvement has not been established

A number of AHR interactions with important cellular factors have been reported. Specifically, AHR has been shown to physically interact with retinobalstoma protein (Rb) (Andersen and Barton, 1998; Elferink et al., 2001; Puga et al., 2000b; Puga et al., 2000c; Strobeck et al., 2000). The Rb tumor suppressor protein inhibits cell cycle progression at G1 stage and promotes differentiation. Direct AHR-Rb interaction has been shown to

contribute to the cell cycle arrest at G1 stage (Ge and Elferink, 1998), in agreement with the documented ability of TCDD-bound AHR agonist to arrest cell cycle (Andrysik et al., 2007; Elferink et al., 2001; Levine-Fridman et al., 2004; Trapani et al., 2003). On the mechanistic level, AHR-Rb interaction has been shown to result in the repression of E2F-mediated transcription (Puga et al., 2000c). This effect was mediated, in part, by AHR preventing the interaction of stimulatory p300 protein with E2F-dependent promoters (Marlowe et al., 2004). In addition, AHR-Rb interaction resulted in upregulation of p27Kip1, a cyclin-dependent kinase 2 repressor (Strobeck et al., 2000). Overall, the interaction of AHR with Rb activated a number of molecular mechanisms leading to cell cycle arrest in TCDD-treated cells.

Another protein, which has been reported to interact with AHR, is NF-κB. AHR-NF-κB interaction was shown to result in mutual repression between these two transcription factors (Tian et al., 1999). Transcription factor NF-κB plays an important role in immune cell activation, differentiation and responses to cytokines, therefore the interaction of AHR and NF-κB may explain, in part, the immunosuppressive effects of TCDD. Notably, other mechanisms that were not shown to involve direct NF-κB interaction with AHR play a role in the expression and function of NF-κB in TCDD-treated cells. For example, TCDD has been shown to induce the DNA-binding activity of NF-κB p50/p50 complex, which has inhibitory properties (Puga et al., 2000a). This response was speculated to be mediated by oxidative stress that TCDD treatment exerts. In addition, TCDD has been shown to increase the expression of I κ B and I κ B kinases within the hepatic microsomes (Bruno et al., 2002), which may lead to inhibition the NF-κB transcriptional activity and an increase in NF-κB degradation. Furthermore,

interaction of AHR-ARNT complex and NF-κB at DRE-like sites in the IgH gene 3'α enhancer was implicated in the repression of IgH by TCDD (Sulentic et al., 2004a). In addition, there appears to be a considerable cross-regulation of AHR with other inducible transcription factors, including receptors to estrogen, thyroid hormone, and retinoic acid (Duan et al., 1999; Hogenesch et al., 1997; Murphy et al., 2007).

(ii). **Putative AHR-independent effects of TCDD**

For a number of observed effects of TCDD, AHR involvement has not been established. Among these effects are alterations in hepatic plasma cell membrane (Matsumura et al., 1984) and increase in activated protein kinase C (PKC), (Bombick et al., 1985), increases in the activities of nuclear protein kinases (Ashida et al., 2000) and activation of extracellular signal-activated kinase, p38, and other mitogen-activated protein kinase pathways (Park et al., 2005; Tan et al., 2002). In addition, TCDD has been shown to mediate increases in the expression of c-fos and c-jun genes and subsequent increase in the DNA-binding activity of transcription factor AP-1 (Hoffer et al., 1996; Puga et al., 1992). By comparison, TCDD induced the DNA-binidng activity of transcription factor NF-κB in BCL-1 cells, a murine B cell line devoid of AHR (Sulentic et al., 2000). Furthermore, TCDD resulted in sustained elevation in the intracellular calcium levels in hepatoma cell lines with high as well as low AHR expression levels (Puga et al., 1997), and in murine lymphocytes (Karras et al., 1996). Moreover, TCDD has been shown to induce apoptosis in AHR-deficient EL-4 cells (Park et al., 2003) and induce morphological changes in the reproductive tissue of a soft shell clam *Mya arenaria*, in AHR-independent manner (Butler et al., 2004).

One possible explanation for these effects is that although they were not directly induced by AHR, they may represent secondary, tertiary or even more remote consequences of AHR activation. In addition, a wide range of TCDD concentrations and treatment regiments were employed in the studies above, and high amounts of TCDD may have produced toxic effects by AHR-independent mechanisms, which would not have been observed at lower amounts. Therefore, the above results should be interpreted with caution.

In spite of the caveats, several observations argue that AHR-independent effects of TCDD may be relevant for the TCDD toxicity. First, the majority of the above effects were detected shortly after TCDD exposure, in agreement with the hypothesis that these effects do not involve changes in gene expression. Second, corroborating the assertion that these effects are AHR and/or DRE-independent, a number of the aforementioned changes occurred in cells or tissues not expressing AHR. Therefore, although additional evidence is needed to unequivocally determine that the aforementioned TCDD effects are AHR-independent, we believe that some aspects of TCDD toxicity occur via AHR-independent mechanisms.

II. TCDD interference with the immune system

A. Structure and function of the immune system

a. Immune organs and cells

The immune system is a complex organization of organs and circulating cells. All organs of the immune system are classified as either primary or secondary immune organs. The primary lymphoid organs are responsible for generation of the various cell types of the immune system. Development of immune cells occurs in the yolk sack and the liver during the fetal life, and in bone marrow and thymus after birth. Secondary immune system organs include the spleen and the lymph nodes distributed throughout the body. Within this category, tonsils, adenoids and Peyer's patches play an important role in maintaining the immunity of mucosal surfaces of respiratory, digestive and reproductive systems. The cellular arm of the immune system includes macrophages, neutrophils, eosinophils, mast cells, and lymphocytes, specifically T and B cells. All immune cell types develop from the pluripotent stem cells and then circulate throughout the extracellular fluid. B cells reach maturity within the bone marrow, whereas T cells migrate to the thymus to undergo maturation. Activation and terminal differentiation of mature lymphocytes usually occurs in the secondary lymphoid organs, namely the lymph nodes, the spleen, and the mucosa-associated lymphoid tissues (Delves and Roitt, 2000a; Delves and Roitt, 2000b). Secondary lymphoid organs are vital for the activation of T and B cells, since they provide the environment in which lymphocytes can interact with each other and with the antigen presenting cells.

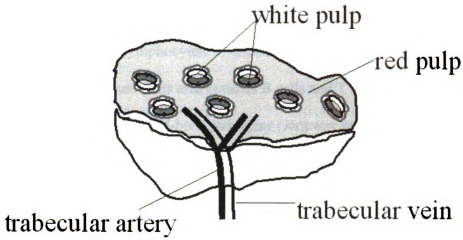
b. **Structure of lymphoid organs – lymph node and spleen**

Lymph node consists of an outermost cortex and an inner medulla. The cortex is further divided to the outer cortex, consisting primarily of B cells, and a deep inner cortex comprised primarily of T cells and dendritic cells. The outer cortex contains lymphoid follicles (FO), in which germinal center reactions occur upon B and T cell activation. In the medulla, macrophages and terminally differentiated plasma cells are grouped in string-like formations known as medullary cords. The lymph streams through the lymph node from the afferent lymphatic tract, located at the cortical side of lymph node, to the efferent lymphatic tract located in the medulla. Naïve lymphocytes enter the lymph node from the bloodstream through specialized postcapillary venules and exit by the efferent lymphatics in the medulla. Upon infection, antigen presenting cells, most commonly dendritic cell and macrophages that have encountered the antigen in one of the body cavities, carry the processed antigen to the local lymph nodes. They enter the lymph node by afferent lymphatics and present the antigen to naïve lymphocytes, thus initiating T and B cell activation (Janeway et al., 2003).

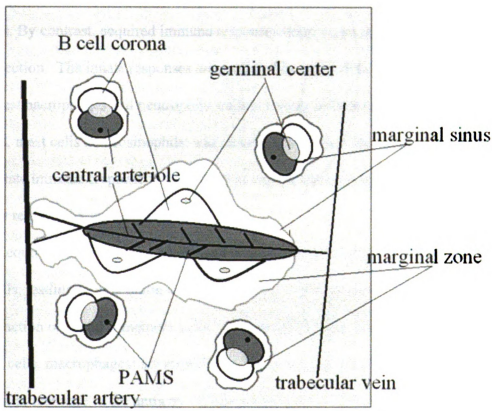
The organization of the lymphoid tissue in the spleen is somewhat resembling that of the lymph node, with the exception that antigen enters spleen from the blood rather than from the lymph (Fig. 4). The spleen consists of red pulp, where defective or old red blood cells are being destroyed, intercepted with areas of lymphoid white pulp. Blood flow, carrying lymphocytes and antigens from the bloodstream enters each white pulp area from the trabecular artery and flows through the central arteriole and the marginal sinus before draining into the trabecular vein. The periartheriolar marginal shealth (PAMS), comprised of T cells, surrounds the central artheriole.

Figure 4. Structure of the lymphoid tissue in the spleen A) The lymphoid white pulp areas are distributed within the red pulp tissue of the spleen. Blood enters the spleen through the trabecular artery and leaves through the trabecular vein. B) Longitudinal and transverse cross-section view of the lymphoid tissue in the spleen. Each white pulp area consists of B cell corona, germinal center and PAMS, all surrounded by the marginal zone. Blood from the trabecular artery flows into the central arteriole and then to the marginal sinus and drains into the trabecular vein (Janeway et al., 2003).

A


white pulp
red pulp
trabecular artery
trabecular vein

B


B cell corona
germinal center
marginal sinus
central arteriole
marginal zone
PAMS
trabecular artery
trabecular vein

Between PAMS and the marginal sinus lies the B cell zone, also termed B cell corona. Germinal centers are formed between the PAMS and B cell corona, allowing for interaction between T and B lymphocytes. Lymphocyte-containing marginal zone (MZ) surrounds each marginal sinus. This area consists B cells and T helper (Th) cells involved in rapid responses to blood-born pathogens (Janeway et al., 2003).

c. **Innate and acquired immunity**

There are two fundamentally different types of responses to invading pathogens. The innate immune response typically occurs to the same extent however many times the pathogen is encountered. The exception to that rule is the phenomenon of tolerance, such as tolerance to LPS, when successive exposures to LPS elicit progressively weaker innate responses. By contrast, acquired immune responses improve on repeated exposure to a given infection. The innate responses are mediated by phagocytic cells, such as monocytes/macrophages and neutrophils; cells releasing inflammatory mediators, such as basophils, mast cells and eosinophils; and natural killer cells. The molecular component of the innate immune response is comprised of complement, acute-phase proteins, and cytokines released by immune cells.

Acquired responses involve specific recognition of an antigen by a subset of T and B cells, leading to generation of T and B clones with high specificity to the antigen, and production of T and B memory cells. Antigen-presenting cells, (*i.e.*, B cells, dendritic cells, macrophages) are required to display the antigen to T cells and collaborate with them in response to the antigen. In addition, B cells secrete immunoglobulins, the antigen-specific antibodies responsible for eliminating extracellular pathogens. Th cells

help to activate B cells to produce immunoglobulins, whereas cytotoxic T cells can eliminate virally infected cells (Delves and Roitt, 2000a; Delves and Roitt, 2000b).

d. B cell activation and terminal differentiation

The humoral arm of acquired immunity is mediated primarily by B cells. Upon encountering an antigen, resting mature B cells can terminally differentiate into immunoglobulin-secreting plasma cells, or into memory cells. The initial two steps that resting B cell undergoes after recognizing an antigen are activation and clonal expansion, termed so because the activated B cell undergoes multiple cycles of divisions. Clonally expanded antigen-specific B cells then differentiate into one of two terminal phenotypes: they either become plasmablasts, the antigen-specific dividing cells that secrete immunoglobulins and eventually terminally differentiate into plasma cells; or antigen-specific B cells that terminally differentiate into memory cells (Adopted from Kallies and Nutt, 2007), (Fig. 5). Plasma cells are a specialized cell type capable of massive immunoglobulin secretion. Plasma cells are not dividing and short-lived. They are distributed throughout the blood circulation and some migrate to the bone marrow. Bone marrow plasma cells receive survival signals from the bone marrow stromal cells, which may extend bone marrow plasma cell survival to month or even years. Memory cells do not secrete immunoglobulins, but they retain specificity to the activating antigen and rapidly differentiate to plasmablasts and plasma cells in case of secondary exposure to the same antigen. The memory cells reside in bone marrow, where, like the long-lived plasma cells, they receive survival signals from bone marrow stromal cells, which allow memory cells to survive for month or years.

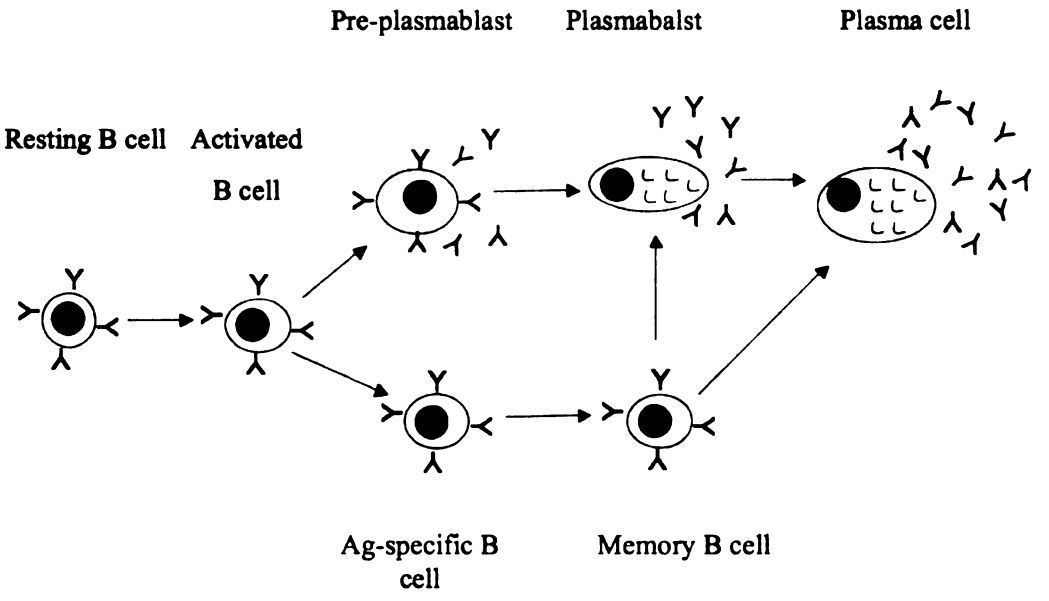


Figure 5. Stages of terminal B cell differentiation. Upon activation, a resting B cell follows one of the two distinct differentiation pathways, both of which ultimately lead to the formation of plasma cell. One pathway proceeds through pre-plasmablast to plasmablast, to plasma cell developmental stage. The other pathway generates memory cells, which also differentiate into plasma cells following a recurring encounter with the specific antigen (Kallies and Nutt, 2007).

e. Functional differences among B cell lineages in terminal differentiation

For classification purposes, naïve mature B cells in the mouse are commonly divided into two lineages, B1 and B2. B1 cells reside mainly in the peritoneal and pleural cavities, whereas B2 cells are commonly located in the secondary lymphoid organs. Furthermore, each of the B1 and B2 lineages can be subdivided into two subsets based on the surface markers these cells express. Thus, B1 cells subdivide into B1a ($B220^{low}IgM^{high}CD11b^{+}CD5^{+}$) and B1b ($B220^{low}IgM^{high}CD11b^{+}CD5^{-}$). The B2 lineage subdivides into FO B cells ($B220^{+}CD23^{high}CD21^{low}$), (Song and Cerny, 2003), and MZ B cells ($B220^{+}CD23^{low}CD21^{high}$) (Martin and Kearney, 2000). Upon primary infection by a pathogen, B1 cells, as well as MZ cells can rapidly become activated by a thymus-independent mechanism and differentiate into plasma cells (Berland and Wortis, 2002; Cariappa and Pillai, 2002; Fagarasan and Honjo, 2000; Martin and Kearney, 2000). These plasma cells usually have a life span of several days and secrete a low affinity immunoglobulin, IgM. Therefore, these short-lived IgM-secreting plasma cells are capable of providing an early humoral response to invading pathogens, particularly to bacteria (English et al., 1976). On the other hand, FO B cells do not develop into plasma cells directly. Instead, FO B cells form germinal centers where they undergo a complex set of reactions, which lasts for several days. The reactions occurring in the germinal centers require B cell interaction with an antigen-specific activated Th cells and an activated follicular dendritic cell. Furthermore, B cell activation requires stimulatory cytokines that are generated by activated Th cells, activated antigen presenting cells and additional cell types.

f. **Germinal center reactions**

In the beginning of germinal center formation, antigen specifically binds to Ig expressed on the surface of a B cell, which functions as the B cell receptor (BCR). Many of the additional signals required for B cell activation are delivered through B cell - Th cell interaction, whereby both B cells and Th cells become activated (van Seventer et al., 1991). During this interaction, signals are transferred between the two cell types through cell surface proteins and by secreted cytokines. The first stage of Th-B cell interaction is termed Th cell surveillance (Noelle and Snow, 1991). During this stage, lymphocyte function-associated antigen-1 (LFA-1) adhesion molecule on the Th cell interacts with intercellular adhesion molecule-1 (ICAM-1) on the B cell, whereas the Th-derived CD4 molecule binds to the monomorphic MHC class II domains on the B cell. This initial interaction is antigen non-specific, and its affinity is weak (Hodgkin et al., 1990; Noelle and Snow, 1991; Springer, 1990). The surveillance phase helps Th cell to locate those B cells expressing the appropriate processed antigen. The next stage, the recognition phase, involves high affinity, antigen specific Th-B cell interaction. At this stage, the T cell receptor (TCR) is recognizing the processed antigen in the context of MHC class II molecule, causing a transient increase in the affinity of LFA-1 on the T cell to the ICAM-1 on the B cell (Dustin and Springer, 1989), leading to the upregulation of CD2 and CD28 molecules on the surface of Th cell. Subsequent binding of Th-derived CD2 and CD28 to B7 and LFA-3 on the B cell surface, helps to stabilize the Th-B cell interaction and enhance intracellular signaling in Th and B cells (Alberola-Ila et al., 1991; June et al., 1990; Linsley et al., 1990; Selvaraj et al., 1987). The next stage is the B

cell activation phase. During this stage, IL-4 and IL-5 released by the activated Th cell bind their cognate receptors on the B cell and prompt the resting B cell to move from stage G0 to G1 and to enter the cell cycle (Hodgkin et al., 1990; Noelle et al., 1991). The final stage of B cell activation is the cytokine-dependent proliferation of B cell. The production of cytokines by Th cell is initially mediated by TCR ligation of the processed antigen/MHC class II complex, and the subsequent CD28-B7 interaction increases cytokine release by Th cell (Thompson et al., 1989). The IL-4, which is released by the activated Th cell at this stage, mediates B cell clonal expansion by increasing the number of activated B cells and inducing the entry of dividing cells into the S phase of the cell cycle (Noelle and Snow, 1991) On other hand, IL-5, initiates IgM secretion and class switch to other Ig isotypes. Additional cytokines that are being released by Th cells or other cell types, such as IL-2 and interferon gamma (INF-γ), further advance the progress of B cell differentiation (Noelle and Snow, 1991), whereas IL-6 suppresses the terminal B cell differentiation until germinal center reactions are completed (Calame, 2006).

g. **Affinity maturation and class switch recombination**

First, activated B cells in the germinal center undergo a process termed affinity maturation. Affinity maturation is mediated by a succession of rapid proliferation cycles accompanied by hypermutation of the rearranged immunoglobulin variable region genes. Eventually the immunoglobulin molecule with high affinity to a specific antigen is expressed on B cell surface to serve as the BCR. B cells that fail to express the high affinity BCR are eliminated by apoptosis. An additional process occurring in germinal centers is isotype switch recombination. During switch recombination immunoglobulins

with isotypes other then IgM (*i.e.*, IgG of various subtypes, IgA, IgE, or IgD) are generated. The reactions at the germinal centers peak at 10-12 days after infection, and generate antigen-specific dividing plasmablasts, which eventually differentiate into non-dividing plasma cells and can generate high-affinity antibodies of secondary isotypes against the antigen (McHeyzer-Williams et al., 2001; Przylepa et al., 1998). Therefore, the germinal center reaction generates immunoglobulins that are more effective against the initiating pathogen as compared to the IgM generated by B1 and MZ cells. In addition, the germinal center reaction is responsible for generation of memory cells, allowing for a rapid secondary response to the antigen upon reoccurring infection (Hofer et al., 2006).

h. **LPS is a thymus-independent activator of the B cell lineage**

Naïve murine B cells are known to proliferate in response to LPS, an agonist to toll-like receptor-4 (TLR4). All B cell subsets express TLR-4, but its expression is significantly higher in B-1 and MZ cells as compared with FO cells (Genestier et al., 2007). Subsequently, the proliferation and immunoglobulin secretion in response to LPS is higher in B1 and MZ cells as compared with FO cells (Genestier et al., 2007). Notably, recent evidence suggests that germinal center reaction can be initiated not only by thymus-dependent antigens, but also by some thymus-independent antigens (Gaspal et al., 2006). Thymus-independent germinal center reactions require activated follicular dendritic cells and the engagement of B cell-derived CD40 receptor. However, these reactions do not require Th cells, as CD40 interaction with the T cell-derived CD40 ligand was not required for B cell activation under the aforementioned experimental

conditions (Gaspal et al., 2006). In sum, murine B cells in culture can be efficiently activated in TI manner by TLR4 agonist LPS. This activation will mimic the primary IgM response of B1 cells and MZ cells to bacterial antigens *in vitro*, but not FO responses, as these responses require formation of germinal centers in the spleen. CH12.LX cells are a murine B cell lymphoma that, upon LPS activation, responds in a manner that closely mimics B cell differentiation *in vitro*. Historically, CH12.LX have been utilized in our laboratory to study the mechanisms of B cell differentiation. Thus, the characteristics of CH12.LX utilized in the B cell differentiation modeling are of interest, and are discussed below.

i. CH12.LX as experimental model for terminal B cell differentiation

CH12.LX cells are a useful model of terminal B cell differentiation, because they exhibit many characteristics of the primary B cell. CH12.LX are a subclone of CH12 cells that were generated in the ascites fluid of B10-H2^{a}H4^{b}p/Wts mouse following repeated intraperitoneal injections of sheep red blood cells (SRBC) (Bishop and Haughton, 1986). *In vitro* cultured CH12.LX cells readily respond to SRBC stimulation, when co-cultured with T cells. This activation mechanism is MHC class II restricted, and involves TCR binding to I-E, but not I-A MHC class II haplotype (Bishop et al., 1988). Interestingly, CH12.LX cells are CD5^{+}. In conjunction with the origin of these cells from the peritoneal cavity, and their high surface expression of BCR (IgM), they resemble the B1a lineage, which is known to be readily activated by bacterial antigens to produce a rapid IgM response. Indeed, CH12.LX cells are readily activated in response to bacterial lipopolysaccharide (Sulentic et al., 1998; Sulentic et al., 2000). Previous studies from our

laboratory have shown that the IgM response in CH12.LX cells is strongly suppressed by TCDD treatment. Furthermore, CH12.LX cells express functional AHR, and the suppression of IgM response to LPS in these cells is AHR-dependent (Sulentic et al., 1998; Sulentic et al., 2000). This effect may be mediated, in part, by TCDD-dependent repression of IgH 3'α enhancer, which may contribute to the repression of IgH production. The repression of the IgH 3'α enhancer by TCDD was shown to be mediated, in part, by AHR acting as a transcriptional repressor in the 3'α enhancer DRE-like sites (Sulentic et al., 2004a; Sulentic et al., 2004b). In addition, in LPS-activated CH12.LX cells, TCDD has been shown to repress factors involved in B cell growth and differentiation, such as AP-1 (Suh et al., 2002) and p27kip1(Crawford et al., 2003). Therefore, the CH12.LX cell line is a crucial tool in our studies aiming to characterize the molecular events contributing to the dysregulation of B cell differentiation.

B. TCDD effects in innate and acquired immunity

a. Innate immunity

TCDD has been demonstrated to adversely affect both innate and acquired immune responses. Intriguingly, whereas the acquired immune response is suppressed by TCDD, several components of the innate immune response are enhanced following TCDD treatment. For example, exposure to TCDD caused increase in inflammatory mediators interferon gamma (INF-γ), interleukin-1 (IL-1) and tumor necrosis factor (TNF). In addition, TCDD treatment was shown to increase the numbers of neutrophils and natural killer (NK) cells, but decreased the numbers of macrophages (Choi et al., 2003; Fan et al., 1997; Funseth and Ilback, 1992; Mantovani et al., 1980; Neff-LaFord et

al., 2003; Warren et al., 2000). Despite the fact that levels of the aforementioned inflammatory mediators are enhanced by TCDD treatment, innate inflammatory responses and in particular neutrophil function did not appear to be enhanced following TCDD treatment (Ackermann et al., 1989; Vorderstrasse and Lawrence, 2006). Therefore, it has been proposed that the enhanced level of inflammatory mediators and elevated neutrophil and NK numbers are compensatory mechanisms deeming to offset the immunosuppressive effects of TCDD (Choi et al., 2003; Funseth and Ilback, 1992).

b. **Acquired immunity**

(i). **Cell-mediated responses**

Studies have shown that both cell-mediated and humoral immune responses are suppressed by TCDD (Holsapple et al., 1991b). For example, TCDD has been shown to suppress the T-cell mediated GVH disease and the generation of cytotoxic T lymphocytes. Nevertheless, direct addition of TCDD to mixed lymphocyte cultures *in vitro* had little effect on T cell proliferation and differentiation (Oughton et al., 1995), suggesting that the T cell is not a direct target of TCDD. However, the involvement of T cells in the TCDD-induced suppression of GVH response *in vivo* has been recently demonstrated. Furthermore, the suppression of this response by TCDD was shown to be dependent on the expression of functional AHR by both $CD4^+$ and $CD8^+$ T cells (Kerkvliet et al., 2002).

The role that thymic involution plays in the suppression of cell-mediated immune responses by TCDD is of interest, because thymic involution is a common and very sensitive outcome of TCDD exposure in rodents, as it occurs even following single or

low dose exposure to TCDD (reviewed in Holsapple et al., 1991b). The mechanism for TCDD-induced thymic involution involves altered thymocyte maturation and differentiation, an effect observed both *in vivo* and *in vitro* studies (Gehrs and Smialowicz, 1997; Greenlee et al., 1985b; Holladay et al., 1991). Furthermore, the involution is mediated, in part, by TCDD-mediated induction of premature terminal differentiation of the thymic epithelial cells (Greenlee et al., 1985b) and by damage to prethymic T cell precursor stem cells in both bone marrow and liver (Silverstone et al., 1994).

Evidence demonstrates that thymic involution contributes to TCDD-induced immunosuppression in animals exposed prenatally, as thymus plays an important role in thymocyte maturation at the early stages of life. However, in adult animals thymus plays only a minor role in cell-mediated immune responses (Colombi et al., 1994), thus thymic involution in adult animals may only modestly contribute to the TCDD-associated immunosuppression. This notion is supported by the fact that adult thymectomy prior to TCDD treatment did not affect the TCDD-induced suppression of the humoral response to SRBC (Tucker et al., 1986)

(ii). **Humoral responses**

The suppression of humoral immune responses by TCDD in mice was established by several plaque-forming cell (PFC) studies performed by different laboratories. All studies have consistently demonstrated suppression of humoral responses to thymus-dependent as well as thymus-independent antigens following TCDD treatment (Holsapple et al., 1986a; Smialowicz et al., 1996; Tucker et al., 1986; Vecchi et al.,

1980). Furthermore, the B cell is the only immune cell type, whose immune function is compromised by direct addition of TCDD (Kramer et al., 1987; Luster et al., 1988). *Ex vivo* studies were performed in order to identify the sensitive cell types in TCDD-induced suppression of anti-SRBC response in B6C3F1 mice (Dooley and Holsapple, 1988). Splenocytes from vehicle- and TCDD-treated mice were fractionated into T cells and macrophages (T group) or B cells and macrophages (B group), sensitized *in vitro* with T-dependent antigen (SRBC), or T-independent antigen, dinitrophenol-Ficoll (DNP-Ficoll), and reconstituted. The PFC response to SRBC or DNP-Ficoll was measured in presence or absence of TCDD. The identification of TCDD-sensitive cellular targets was based on the fact that antigens used in these studies require a different cellular cooperativity to elicit an antibody response. Reconstitutions of T and B cell populations were performed following TCDD or vehicle treatment *in vitro*. Statistically significant and similar in magnitude suppression of PFC response was observed in the reconstitution of TCDD-treated B group with TCDD-treated T group, and in reconstitution of TCDD-treated B group with vehicle treated T group. Only slight suppression of PFC response was observed in the reconstitution of vehicle treated B group with TCDD treated T group. Further investigation of the role of T cells in the TCDD-induced suppression of antibody response focused on T helper and on T suppressor cellular populations (Dooley et al., 1990). TCDD treatment of neither T cell population caused a suppression of antibody response to either SRBC or DNP-Ficoll antigens. Therefore, the B cell was identified as a primary target in TCDD-induced suppression of *ex vivo* antibody response. An earlier study showed that TCDD suppressed *in vitro* antibody responses to the above-mentioned antigens in a concentration-dependent manner (Holsapple et al., 1986a). Further studies

have suggested that TCDD inhibits terminal differentiation of B cells by alteration of an early activation event (Holsapple et al., 1986a; Holsapple et al., 1991a; Karras and Holsapple, 1994; Luster et al., 1988). The mechanisms underlying inhibition of B cell activation by TCDD might involve induction of tyrosine kinase activity and increased phosphorylation by TCDD (Kramer et al., 1987; Lucier et al., 1991), as well as inhibition of calcium-dependent B cell activation by TCDD (Karras and Holsapple, 1994; Karras et al., 1996).

Moreover, the TCDD-associated immunotoxicity has been shown to segregate with the Ah locus (Vecchi et al., 1983). In mice different alleles encode AHR variants that differ in their affinity for AHR (Poland and Glover, 1990). As a result, mice strains expressing b/b- or d/d AHR variants have been shown to have high or low sensitivity to TCDD, respectively (Birnbaum et al., 1990). One of the first studies to report this effect compared humoral antibody production in response to TCDD in mice strains that differed in their AHR expression. Humoral antibody response was suppressed by TCDD in the "TCDD-sensitive" mice strains, C57BL/6 and C3H/HeN that express the higher affinity (b/b) AHR, at much lower concentration as compared with the "TCDD-resistant" DBA/2 and AKR mice, which express the lower affinity (d/d) AHR. Furthermore, the severity of TCDD-induced immunosuppression in these mice strains correlated with the level of aryl hydrocarbon hydroxylase activity, corroborating the involvement of AHR in the suppression of humoral immune response (Vecchi et al., 1983). The involvement of AHR in the suppression of the humoral immune responses was further corroborated by studies performed in mice congenic for the Ah locus (Kerkvliet et al., 1990; Silkworth et al., 1993). B6 mice used in these studies were homozygous for either (d/d) or (b/b)

variant of the AHR gene. As expected, humoral response to SRBC antigen was more sensitive to suppression by TCDD in AHR (d/d) mice as compared to AHR (b/b) mice (Kerkvliet et al., 1990; Silkworth et al., 1993). An additional line of evidence implicating AHR in the suppression of humoral immune responses stems from SAR studies demonstrating order of rank potency for HAH compounds with different affinity for AHR (Harper et al., 1995a; Harper et al., 1995b).

c. Evidence for AHR-independent mechanisms of the suppression of humoral immune responses by TCDD

Although the vast majority of published reports support the involvement of AHR in the suppression of immunoglobulin response, some *in vitro* studies suggest that the TCDD-induced suppression of humoral immune response may be mediated, in part, by AHR-independent mechanisms. To this end, one study comparing the suppression of PFC response by TCDD on splenocytes isolated from B6C3F1 mice and DBA/2 reported that comparable concentrations of TCDD were sufficient to suppress plaque cell formation by B6C3F1 and DBA/2 splenocytes, suggesting that this effect was independent on the Ah locus (Holsapple et al., 1986a). Similarly, no difference in suppression was observed when comparing the antibody response suppression by TCDD in congenic AHR-homozygous (b/b) and AHR-heterozygous (b/d) mice (Holsapple et al., 1986a). In addition, in one study and 2,7- dichlorodibenzo-*p*-dioxin, a congener devoid of AHR affinity was demonstrated to produce TCDD suppression of antibody response in female B6C3F1 mice (Holsapple et al., 1986b). Another study performed direct comparisons between the potency of HAH that differ in their affinity to AHR *in vivo* to

induce suppression of antibody response to SRBC in *in vitro* cultures of B6 and DBA/2 splenocytes, expression the "sensitive" and the "resistant" form of AHR, respectively (Davis et al., 1991). Surprisingly, results from this study indicated that all of the congeners were equipotent and produced a similar concentration-dependent suppression of the *in vitro* antibody response in splenocytes from either B6 or DBA/2 mice. The authors of the study concluded that some mechanism mediating the suppression of humoral response by TCDD *in vitro* are independent of AHR. Since the studies where the suppression of SRBC response was found to be AHR-independent are overweighed by the body of evidence suggesting AHR-dependence of this response, and because some of the HAH concentrations used *in vitro* studies were relatively high, reports of AHR-independent suppression of humoral responses should be interpreted with caution. Overall, scientific evidence accumulated to date strongly suggests that the suppression of humoral immune responses by TCDD is AHR-dependent.

d. AHR involvement in the suppression of IgM response in LPS-activated CH12.LX cells

IgM response to LPS in CH12.LX B cells is suppressed by TCDD (Sulentic et al., 1998) through mechanism involving AHR (Sulentic et al., 2000). One of the proposed molecular mechanisms of humoral immune response suppression in CH12.LX cells involves activation of the AHR-ARNT transcriptional pathway. The transcriptional regulation of the IgM heavy chain subunit is mediated, in part, by a heavy chain enhancer sequence. Heavy chain enhancer is located 3' to the α heavy chain-coding region and consists of hs1, 2 hs3 and hs4 domains (Madisen and Groudine, 1994; Saleque et al.,

1997). Putative DRE sites have been identified within the hs1,2 and hs4 domains of the 3'α enhancer (Sulentic et al., 2000). TCDD treatment suppressed the activity of reporter gene construct containing the entire 3'α enhancer, in concordance with the suppression of the IgM heavy chain transcription. However, the activity of the reporter gene construct driven by the hs4 domain alone was induced by TCDD treatment, and little TCDD effect has been reported for the hs1,2-driven reporter gene construct (Sulentic et al., 2004b). In addition, mutation of the DRE-like site in the hs4 domain abolished the induction of the reporter gene construct by TCDD (Sulentic et al, 2004a). Interestingly, the hs4 DRE-like site overlapped with an NF-κB response element, and both LPS and TCDD induced AHR and NF-κB binding at the site of interest. Based on these findings, it has been proposed that the AHR-NF-κB interaction at the hs4 DRE-like site is contributing to the suppression of 3'α enhancer activity by TCDD (Sulentic et al., 2004a, Sulentic et al 2004b). In summary, the DNA-binding activity at the DRE-like site in the hs4 domain of the 3'α IgH enhancer appears to be involved in the dysregulation of the 3'α IgH activity by TCDD, but its role in the suppression of IgH gene by TCDD remains to be determined.

III. **Signaling cascades governing the terminal B cell differentiation**

As stated earlier, mature naïve B cells become activated upon encountering specific antigens. Activated B cells clonally expand and then embark on the path to differentiation. The critical feature of resting mature B cells is their ability to become activated in response to antigenic stimuli. This ability is granted by expression of surface receptors mediating the transmission of the activation signals. Activation signals are transmitted through a number of signaling surface molecules, such as BCR components surface Ig and Igα; B cell receptor-associated kinase BLNK, B cell co-receptor components CD19, CD21, CD81; and MHC class II molecules, which present the processed antigen to Ag-specific T cells. Upon activation, mature B cells undergo a proliferative burst termed clonal expansion. The clonal expansion is mediated, in part, through induction of the transcription factor c-myc, which is an important activator of cell cycle in B cells (Calame, 2001). Consequently, c-myc is readily expressed in the mature B cells, but is silenced in the plasma cells, which are non-dividing.

In contrast to resting mature B cells, terminally differentiated plasma cells are a highly specialized cell type committed to secreting large quantities of Ag-specific immunoglobulins, but insensitive to antigenic stimulation. Therefore, plasma cells produce very large amounts of the secreted Ig form, and drastically increase those organelles involved in protein folding and secretion, such as golgi, ER and mitochondria. The secretory capacity in plasma cells is mediated, in part, by the transcription factor XBP-1 (Shaffer et al., 2004). In addition, plasma cells strongly downregulate the molecular components of the activation pathway such as MHC class II and BLINK, and BRC signaling components CD19, CD21 and CD45 (Shaffer et al., 2002; Silacci et al.,

1994)

The molecular changes in B cell differentiation are governed by an orchestrated multistep process, in which signals are passed along from one factor to another, ultimately changing the phenotype of resting mature B cell into plasma cell. Whereas the initial activation steps may vary between thymus-dependent and thymus-independent B cell activation, the downstream signaling cascades hinge on a number of common critical regulators, which are discussed in detail in the next chapter.

A. **Critical transcriptional regulators in B cell differentiation**

Blimp-1 has been named the "master regulator" of B cell differentiation due to the ability of ectopically expressed Blimp-1 to induce differentiation in resting mature B cells. Although recent reports have demonstrated that modest Ig secretion can occur prior to the upregulation of Blimp-1 (Kallies et al., 2007), Blimp-1 is absolutely required for the terminal B cell differentiation into plasma cell (Kallies and Nutt, 2007). Blimp-1 is a transcriptional repressor targeting a large number of downstream factors. Among B cell targets are Oct-2, a positive regulator of the B cell activation pathway components Igα, CD19, and BLNK; c-myc; a critical positive regulator of cell cycle, and CIITA, which acts as a strong positive regulator of MHC class II (Shaffer et al., 2002). Thus, Blimp-1 upregulation contributes to the suppression of genes responsible for B cell activation and cell cycle in activated B cells.

Importantly, Blimp-1 acts as a repressor of Pax5, a dual nature activator/repressor transcription factor that positively regulates the expression of CD19, Igα, BLNK, CIITA and indirectly, MHC class II, but negatively regulates the expression of XBP-1,

immunoglobulin joining chain (IgJ), IgH, and possibly Igκ light chain (Schebesta et al., 2007). Activation-induced upregulation of Blimp-1 leads to repression of Pax5 and further contributes to the repression of B cell surface proteins and signaling molecules involved in B cell activation (Lin et al., 2002). By contrast, the expression of Ig genes, and XBP-1 is upregulated as the Pax5-mediated repression of these genes is relieved by Blimp-1. Although the factors leading to XBP-1 induction following B cell activation are not fully understood, experimental evidence clearly indicates that the removal of Pax5 repression contributes to the upregulation of XBP-1 (Lin et al., 2002). In summary, the key transcriptional regulators of B cell differentiation Blimp-1, Pax5 and XBP-1 regulate a wide spectrum of genes that drive the molecular program to transform the mature B cell into a plasma cell. A detailed overview of structure, mechanism of activity and expression patterns for each one of these transcription factors is provided in the next chapter.

a. **B lymphocyte-induced maturation protein-1**

Blimp-1, B lymphocyte-induced maturation protein 1, is encoded by prdm1 (positive regulatory domain 1 binding factor 1) gene. Blimp-1 analogs have been identified in human (Huang, 1994), mouse (Turner et al., 1994), *Xenopus* (de Souza et al., 1999), sea urchin (Wang et al., 1996), *D. melanogaster* and *C. elegans* (Tunyaplin et al., 2000) genomes. Initially Blimp-1 was identified in B lymphocytes (Huang, 1994; Turner et al., 1994), where it was named "the master regulator" of terminal differentiation (Turner et al., 1994). Later Blimp-1 was shown to regulate differentiation in granulocytes and macrophages (Chang et al., 2000), and recently – in T cells, as Blimp-1

is responsible for homeostasis in the terminally differentiated T cell (Fink, 2006; Jameson, 2006; Kallies et al., 2006; Kallies and Nutt, 2007; Martins et al., 2006). Moreover, Blimp-1 is involved in embryonic development in zebrafish (Baxendale et al., 2004; Lee and Roy, 2006), and mice, where it is necessary for germ cell and sebaceous gland development (Horsley et al., 2006; Ohinata et al., 2005), and where the unconditional Blimp-1 knockout results in lethality (Davis, 2007).

The organization of the mouse Blimp-1 gene has been determined (Tunyaplin et al., 2000; Turner et al., 1994). The Blimp-1 open reading frame in the mouse is 865 amino acids long and is comprised of eight exons, encoding five Krupfel-type zink finger motifs and proline-rich and acidic regions characteristic of transcription factors. Blimp-1 is transcribed from a TATA-less promoter and has multiple initiation sites. Multiple Blimp-1 isoforms exist, however the three major Blimp-1 isoforms result from the use of alternative polyadenylation sites and do not encode different proteins (Tunyaplin et al., 2000). The mechanism whereby Blimp-1 represses transcription of target genes involves Blimp-1 binding to specific motifs located in the regulatory regions of target genes. The DNA-binding specificity of Blimp-1 is conferred by zink-finger motifs. Blimp-1 acts as a transcriptional repressor by binding to specific motifs located in the promoter regions of susceptible genes. A 12-bases long consensus binding site for Blimp-1 has been identified as GTAGTGAAAGTG (Gyory et al., 2003). DNA-binding site-dependent repression by Blimp-1 has been reported for Pax-5 (Lin et al., 2002) , c-myc (Lin et al., 1997) and CIITA promoters (Piskurich et al., 2000). Moreover, a proline rich (PR) region N-terminal to the zink finger motif has been shown to mediate the Blimp-1 repressor activity by association with histone deacetylases (Gyory et al., 2003; Gyory et

al., 2004; Yu et al., 2000) and groucho family proteins (Ren et al., 1999; Yu et al., 2000).

Recruitment of HDAC to DNA appears to alter nucleosome structure in a local region and inhibit transcription. Specifically, HDAC is removing acetyl groups from lysines on histone tails, possibly exposing the positive charge on lysines and making them less accessible for the transcription machinery (Yu et al., 2000). Mutation analysis of Blimp-1 domains required for the HDAC recruitment to the c-myc promoter, revealed that multiple Blimp-1 domains, including the N-terminal acidic region, were involved in the recruitment of HDAC (Yu et al., 2000). Furthermore, Blimp-1 was able to associate with HDAC1 and HDAC2 and to deacetylate the histone H3 bound to the c-myc promoter (Yu et al., 2000). Groucho family is a large family of proteins that do not bind DNA directly, but rather are recruited to the DNA template by repressor proteins to serve as co-repressors (Chen and Courey, 2000). Groucho co-repressors hGRg, TLE1 and TLE2 associate with Blimp-1 through Blimp-1 N-terminal domain and are involved in the repression of INF-β gene by Blimp-1 (Ren et al., 1999). In addition, Blimp-1 has been shown to recruit methyl transferases to susceptible gene promoters to silence transcription in lineages other than B cell (Ancelin et al., 2006; Gyory et al., 2004). Specifically, the repression of INF-β promoter by Blimp-1 in the osteosarcoma cell line U2OS involved the recruitment of the histone 3 lysine methyltransferase G9a (Gyory et al., 2004), whereas the repression of mouse germ-cell lineage by Blimp-1 involved the recruitment of Prmt5 , an arginine-specific histone methyltransferase (Ancelin et al., 2006). In the nuclei of mouse embryonic primordial germ cells, the presence of the Blimp-1- Prmt5 complex was high on embryonic day 8.5, but not on day 11.5. In correlation with this observation, Blimp-1-Prmt5 putative target gene, Dhx38, was

upregulated on embryonic day 11.5 (Ancelin et al., 2006). Although to date Blimp-1 interaction with methyltransferases has not been reported in the B cell lineage, they may be putatively involved in the repression of B cell-specific genes by Blimp-1.

Expression of Blimp-1 transcripts can be rapidly induced in splenic primary B cells or B cell cultures by either LPS treatment or IL-2 and IL-5 co-treatment, but not by IL-4 + anti-CD40 or IL-4 + anti-μ F' (ab) 2 combination (Knodel et al., 2001; Randall et al., 1998; Schliephake and Schimpl, 1996; Soro et al., 1999). It has been shown that IL-4 and either CD40 or anti-μ F' (ab) 2 co-stimulation promotes the memory cell phenotype (Knodel et al., 2001) and diverts B cell differentiation from the plasma cell track. By contrast, Blimp-1 over-expression can rescue the plasma cell phenotype in CD40 or anti-mu F'(ab)2-stimulated B cells (Angelin-Duclos et al., 2000; Knodel et al., 2001; Schliephake and Schimpl, 1996). The fact that in the B cell lineage Blimp-1 has been detected in plasma cells (Shaffer et al., 2002), but not in memory cells, further supports the notion that Blimp-1 expression commits B cells to the plasma cell, rather than memory cell phenotype. The kinetics of Blimp-1 mRNA induction has been reported to have a biphasic pattern in B cells as well as in monocyte and granulocyte cells (Chang et al., 2000; Turner et al., 1994). Effects of Blimp-1 expression include terminal B cell differentiation, suppression of B cell proliferation and apoptosis.

The role of Blimp-1 in terminal B cell differentiation involves activation of genes promoting antibody production, while repressing the genes associated with the resting mature B cell phenotype (Sciammas and Davis, 2004; Sciammas and Davis, 2005; Shaffer et al., 2002; Shapiro-Shelef et al., 2005). Among well-characterized Blimp-1 target genes are c-myc, Pax-5 and CIITA, for which direct regulation by Blimp-1 has

been established (Lin et al., 2002; Lin et al., 1997; Piskurich et al., 2000).

Recently, two DNA microarray studies were performed aiming to identify genes that are regulated by Blimp-1 during the terminal B cell differentiation. One study transduced Blimp-1 into human B cell lines that do not endogenously express Blimp-1. Cell lines used in this study were: WI-L2, an EBV^+ mature lymphoblastoid cell line; SUDHL4, an EBV^- B cell-like diffuse large B cell lymphoma (DLBCL) cell line; BJAB, an EBV^- mature B lymphoma cell line; and RAJI, an EBV^+ Burkitt's lymphoma cell line (Shaffer et al., 2002). Another study employed murine cell lines (M12, CH12.LX, MOPC315J and Phoenix-e lymphoblastoid cell lines that arose spontaneously) that were transduced with Blimp-1, as well as a murine B cell line, BCL-1, treated with IL-2 + IL-5 to induce Blimp-1 expression. In addition, primary murine B cells were however treated with LPS or anti IgM F (ab)'2 before transfection with Blimp-1 vector (Sciammas and Davis, 2004). In both studies, the microarray analyses aimed to identify genes, whose expression is controlled by Blimp-1. Although large differences existed between the findings of the two studies, both studies identified a broad array of genes involved in cell proliferation and growth, immunoglobulins secretion, B cell lineage commitment and apoptosis as Blimp-1 targets (Sciammas and Davis, 2004; Shaffer et al., 2002).

Whether the regulation of these genes by Blimp-1 is direct or indirect, *i.e.* occurs through intermediate regulatory proteins that are Blimp-1 direct targets, was not established, since a large portion of all genes identified as Blimp-1 targets are transcription factors, each of which regulates its own target set of genes (Shaffer et al., 2002). Blimp-1 targets associated with proliferation and growth included the previously identified Blimp-1 target c-myc, and c-myc downstream targets RCL1, ODC, LDH-A,

and DHFR. In addition, Blimp-1 also repressed E2F-1, and its targets c-myc, DHFR, PCNA and CDC2. Additional genes repressed by Blimp-1 are the genes required for replication and mitosis, such as PCNA, DNA polymerase δ and MCM2. Furthermore, Blimp-1 induced the expression of proapoptotic genes GADD45 and GADD153. The fact that Blimp-1 is repressing proliferation signals and promotes apoptosis is consistent with the paradigm of terminal B cell differentiation in that plasma cells do not divide, and are prone to apoptosis unless they receive specific survival signals (Minges Wols et al., 2002). A set of genes involved in immunoglobulin secretion was induced by Blimp-1, including IgJ, XBP-1 and hsp70. The induction of these genes by Blimp-1 is likely occurring through a repression of an intermediate regulator. For example, the repression of IgJ and XBP-1 is mediated, in part, through the repression by Blimp-1 of Pax5 (Lin et al., 2002). Pax5, in turn, is an established repressor of IgJ and XBP-1 (Reimold et al., 1996; Rinkenberger et al., 1996). Cells responsible for B cell lineage and function were also repressed by Blimp-1. Among genes downregulated by Blimp-1 were the genes encoding BCR signaling components such as CD79A, BLNK, Btk, PKC-β, lyn, syk, BRDG-1, CD45, CD19, CD21, CD22 (Shaffer et al., 2002). This finding is consistent with the fact that BCR signaling inhibits differentiation of mature B cells into plasma cells and involves the repression of Blimp-1, whereas Blimp-1 overexpression can restore the B cell differentiation process (Knodel et al., 2001).

(i). **The role of activation protein-1 in Blimp-1 regulation**

Factors mediating the expression of Blimp-1 during terminal B cell differentiation are poorly understood. However, the induction of Blimp-1 was shown to involve the

transcription factor activating protein-1 (AP-1). AP-1 is a dimer commonly comprised of fos, jun, or related proteins (Karin et al., 1997). AP-1 dimers recognize core motifs TGACTCA (Novak et al., 1990), termed 12- O-tetradecanoate-13-acetate-responsive elements (TRE). Mitogens, such as LPS, were shown to induce the AP-1 component c-fos expression, through activation of mitogen-activated protein kinase (MAPK) pathways. Specifically, extracellular signal-regulated kinase (ERK) (Gille et al., 1992; Marais et al., 1993), jun-amino terminal kinases (JNK) (Treisman, 1992) and p38 (Raingeaud et al., 1995) were shown to contribute to c-fos induction. Similarly to c-fos, the expression of c-jun is induced by JNK and p38 MAPK pathways (Derijard et al., 1994; Hibi et al., 1993; Raingeaud et al., 1995)

AP-1 activity is known to positively regulate a large number of genes involved in cell growth, proliferation, and specialized responses such as inflammation. AP-1 motifs have been identified in the human and mouse Blimp-1 promoters (Ohkubo et al., 2005; Vasanwala et al., 2002). Furthermore, exogenously expressed c-fos was able to induce Blimp-1 and promote B cell differentiation in cultured B cells from c-fos-transgenic mice (Ohkubo et al., 2005), demonstrating that AP-1 is capable to induce the expression of Blimp-1. Furthermore, the expression and activity of AP-1 was shown to be induced by TCDD treatment in liver cells (Ashida et al., 2000; Hoffer et al., 1996; Puga et al., 2000a; Puga et al., 1992). By contrast, in LPS-activated B cells c-jun expression and AP-1 DNA-binding activity were shown to be reduced in the presence of TCDD (Suh et al., 2002).

b. **Paired box gene 5**

Pax5 is a product of paired box gene 5, and is also termed B cell specific activator protein (BSAP). In addition to Blimp-1, cytosine methylation is involved in regulating the expression of Pax5 promoter during B cell terminal differentiation. Regulatory regions of many genes contain sequences with high density of cytosines located 5' to guanines, termed CpG islands. Covalent addition of a methyl group to cytosine bases in CpG islands is a ubiquitous gene silencing mechanism regulating cell growth, proliferation and differentiation (Tate and Bird, 1993). Transcriptional regulation by CpG cytosine methylation is common for Pax family genes. In addition to Pax5 gene silencing, regulation through cytosine methylation has been described for Pax3 and Pax7 (Danbara et al., 2002; Kay et al., 1997).

In the Pax5 gene, silencing is necessary for the progression of terminal B cell differentiation. Recently, CpG motifs have been identified in the first two hundred bp. 5' of the Pax5 gene transcription start site (Danbara et al., 2002). The causal relationship between the CpG methylation in Pax5 promoter and Pax5 gene silencing is supported by several lines of evidence. First, the CpG sites were found to be methylated in myeloma cell lines (FO and Sp-2/0), representing the terminally differentiated B cells, in which Pax5 was not expressed, but remained unmethylated in pre-B (38B9) and mature (P2K-3) B cell lines, in which Pax was detected at the mRNA level. Furthermore, Pax5 was re-expressed in terminally differentiated cell lines following 5-aza-2'-deoxycytidine treatment, which demethylated the CpG sites in the Pax5 promoter. Finally, Pax5 re-expression correlated with the renewed expression of the Pax5 transcriptional target genes mb-1 and CD19. Additional evidence supporting the role for Pax5 promoter

methylation comes from cancer studies examining the link between the abnormal Pax5 expression and uncontrolled cell proliferation (Palmisano et al., 2003). In this study, tumor cells were examined for the methylation status of Pax5 gene 5'CpG region in relation to Pax5 expression. A strong correlation was found between the Pax5 5'CpG region methylation and Pax5 gene transcriptional silencing. As in Danbara and co-workers study, 5-aza-2'-deoxycytidine treatment led to Pax5 re-expression. Furthermore, Pax5 promoter methylation correlated with silencing of CD19, in agreement with Danbara et al. studies. Taken together, these studies point to the importance of Pax5 5'CpG methylation in the regulation of Pax5 expression.

Among the nine mammalian members of paired box family of genes, only Pax5 is expressed in the hematopoetic lineage (Cobaleda et al., 2007). Pax5 is a transcription factor that can act as either activator or repressor of transcription, depending on the context of co-activators or co-repressors (Cobaleda et al., 2007). The entire Pax5 open reading frame is comprised of 10 exons. In the mouse, several Pax5 isoforms have been detected, in which exon 2 is spliced out, or exons 6-10 have been replaced by a novel alternative sequence of unknown function (Zwollo et al., 1997). Additional isoforms have been recently identified in malignant B cells of human origin (Borson et al., 2006; Oppezzo et al., 2005; Robichaud et al., 2004). Differential expression of these new Pax5 isoforms has been implicated in the etiology of multiple myeloma (Borson et al., 2006), chronic lympholytic leukemia (Oppezzo et al., 2005) and B cell lymphoma (Robichaud et al., 2004). Pax5 gene consists of N-terminal DNA-binding motif, homeodomain homology region, octamer sequence and a C-terminal transactivation domain (Zwollo et al., 1997). In the mouse Pax5 isoforms lacking functionally important domains can be

differentially expressed by normal B cells in the process of B cell maturation (Lowen et al., 2001; Zwollo et al., 1997), whereas the presence of Pax5 splice variants may increase or decrease the repressive activity of the full length Pax5 isoform (Lowen et al., 2001).

Additional Pax5 isoforms can be generated by transcription from an alternative promoter that has been identified within the first intron of Pax5 gene (Busslinger et al., 1996; Morrison et al., 1998). These alternative isoforms have been identified in B cell malignancies, where the translocation of strong positive regulatory motifs upstream to the alternative Pax5 promoter can allow the expression of an alternative first exon, termed exon 1B, at the 5'end of Pax5 mRNA template. Such is the case in non-Hodgkin's lymphoma termed marginal zone lymphoma (MZL). In MZL, the immunoglobulin switch Smicro promoter has translocated upstream to exon 1B of Pax5 gene, resulting in an inappropriate expression of Pax5 (Morrison et al., 1998). However, the exon 1B-comprised Pax5 isoform appears to be functional, as it could effectively repress the expression of IgJ gene in MZL (Morrison et al., 1998). Although the analysis of the 5'-flanking region of the alternative exon 1B in human Pax5 gene revealed no consensus TATA box, binding motifs for 3 CAT boxes, SP-1 box, 1E box and additional transcription factors were identified (Liu et al., 2002), suggesting that the downstream TATA-less Pax5 promoter is capable to drive transcription of the Pax5 gene.

Pax5 interaction with DNA is mediated by Pax5 paired domain, a conserved Pax family motif. This DNA-binding domain located in the N-terminal region of Pax5 protein and is comprised of two subdomains (Czerny et al., 1993). Each of the aforementioned subdomains can specifically bind to a cognate half-site in the Pax5 recognition sequence, located in adjacent major grooves of DNA helix. Therefore, each

half-site contributes independently to the overall affinity of a given binding site (Garvie et al., 2001). The transcriptional activity of Pax5 at other regulatory elements is determined by interaction of distinct partner proteins with central and C-terminal protein interaction motifs of Pax5. The homeodomain region of Pax5 associates with the TATA-binding protein of the basal transcription machinery (Eberhard and Busslinger, 1999), while a potent C-terminal transactivation domain (Dorfler and Busslinger, 1996) is capable of interacting with histone deacetylases (HAT), such as coactivator CBP (Emelyanov et al., 2002) or a SAGA complex (Barlev et al., 2003). In contrast, co-repressors of the Groucho protein family, which are a part of a larger histone deacetylase complex, are able to convert Pax5 from a transcriptional activator to a repressor by binding to a conserved octapeptide motif of Pax5 (Eberhard et al., 2000).

Pax5 is required for commitment to the B cell fate, as it downregulates the Notch pathway that is required for T cell lineage commitment (Souabni et al., 2002). During B cell development, Pax5 plays a critical role in the V-D-J rearrangement of heavy chain locus and in the expression of light chain locus (Fuxa et al., 2004; Rolink et al., 1999). In addition, throughout B cell development, Pax5 positively regulates a panel of genes involved in maintaining B cell identity, including CD19 (Kozmik et al., 1992), Igα (also termed mb1 or CD79), BLNK and CIITA (Horcher et al., 2001). By contrast, throughout the maturation process and in mature B cells, but not in plasma cells, Pax5 is negatively regulating genes associated with the plasma cell phenotype, including IgJ, IgH and XBP-1 (Reimold et al., 1996; Rinkenberger et al., 1996; Singh and Birshtein, 1993). Transcription factor XBP-1, which is repressed by Pax5, is strictly required for plasma cell development and antibody secretion (Reimold et al., 2001), whereas IgJ gene, also

repressed by Pax5, is necessary for IgM and IgA secretion. However, whereas repression of Pax5 is required for plasma cell function, the absence of Pax5 is not sufficient to drive plasma cell differentiation (Horcher et al., 2001), suggesting that additional factors are involved in this process.

Recent studies employing conditional inactivation (*in vivo*) or conventional deletion (*in vitro*) of the Pax5 gene further demonstrate the importance of Pax5 in the late stage of B cell differentiation (Horcher et al., 2001; Nera et al., 2006). Upon Pax5 inactivation in mature mouse B cells, many of the B cell surface molecules that are involved in B cell activation and signal transduction, such as CD19, CD21, CD40, BLNK, CIITA, CD79A were downregulated (Horcher et al., 2001). Furthermore, Pax5 deficient mature B cells fail to respond to LPS and are unable to differentiate into germinal center B cells (Horcher et al., 2001). Taken together, these findings demonstrate that functional Pax5 is required for the proper mature B cell activation. Conventional deletion of Pax5 in chicken DT40 B cells results in a phenotype similar to the Pax5-deleted mouse cells in that Pax (-/-) DT40 cells are resistant to activation through BCR cross-linking (Nera et al., 2006). Furthermore, Pax5 (-/-) DT40 cells exhibit high secretion of IgM and high expression of Blimp-1 and XBP-1, which is characteristic of plasma cells (Nera et al., 2006). In addition, the inappropriate lineage genes that are silenced by Pax5 at the onset of terminal B cell differentiation require continuous repression by Pax5 to remain silent. To this end, conditional deletion of Pax5 in mature B cells led to re-expression of genes such as CD28 and CD22, which were re-expressed again in plasma cells, and which are required for plasma cell function (Horcher et al., 2001). Therefore, the removal of repression by Pax5 in the end of terminal B cell

differentiation contributes to initiation and maintenance of the plasma cell transcriptional program.

c. The role of X-box protein-1 in B cell differentiation

Transcriptional activator x-box binding protein 1 (XBP-1) has been initially identified as a transcription factor binding to a conserved DNA motif termed "x" box in the MHC class II gene promoter (Liou et al., 1990; Ono et al., 1991). XBP- 1 is a basic region leucine zipper transcription factor of CREB/ATF (cAMP response element binding protein/activating transcription factor) family. XBP-1 is ubiquitously expressed in all adult tissues. During embryonic development, particularly high XBP-1 levels were detected in the developing exocrine glands, osteoblasts and chondroblasts, suggesting the importance of XBP-1 for the development of secretory and skeletal systems. Another system in which XBP-1 plays a critical role during embryonic development is the liver, as XBP-1 deficient embryos die *in utero* from liver hypoplasia due to compromised hepatocyte development (Reimold et al., 2000).

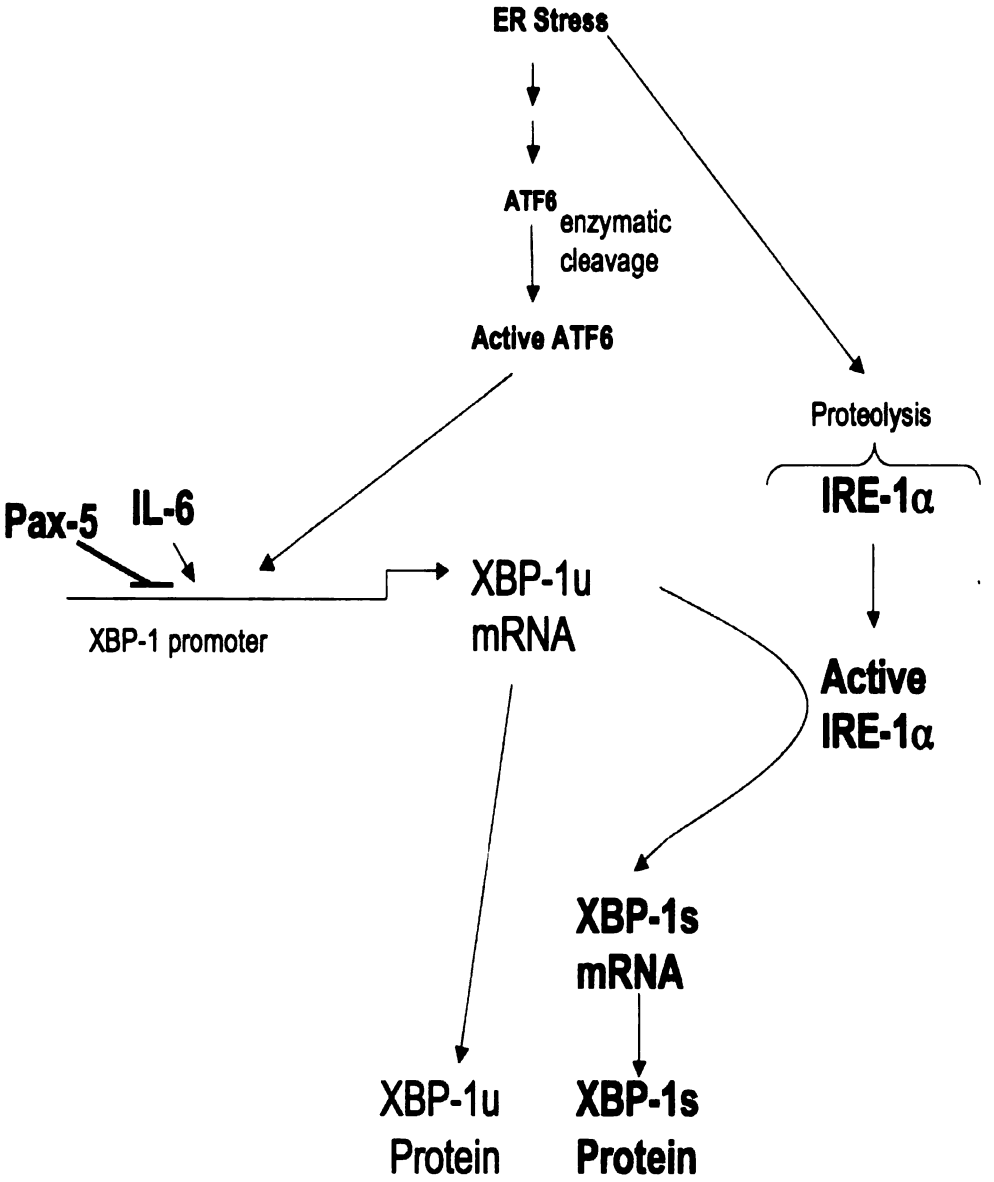
The role of XBP-1 during terminal B cell differentiation was assessed by RAG-2 (recombination-activating gene-2)-deficient blastocyst complementation system (Chen et al., 1993). In this system, RAG(-/-) lymphoid chimeras defective in the generation of plasma cells and immunoglobulin secretion were also defective in XBP-1. Purified B cells from XBP-1/RAG(-/-) chimeric mice stimulated with LPS *in vitro* produced lower levels of secreted IgM than wild-type control B cells. Re-expression of XBP-1 in XBP-1/RAG(-/-) B cells was able to rescue IgM secretion. Furthermore, XBP-1/RAG (-/-) mice had severely diminished numbers of plasma cells as compared to control mice, and were unresponsive to either T-independent (TNP-Ficoll) or T-dependent (TNP-chicken γ-

globulin) antigens *in vivo*. In addition, XBP-1/RAG (-/-) chimaeras transferred into host severe combined immunodeficiency (SCID) mice failed to generate an immunoglobulin response to polyoma virus. Taken together, these findings demonstrate that XBP-1 is absolutely required for the terminal B cell differentiation and immunoglobulin secretion (Reimold et al., 2001). Additional studies confirmed the requirement for XBP-1 for the terminal B cell differentiation and immunoglobulins response *in vitro*, employing isolated murine primary B cells and B cell line BCL-1 (Iwakoshi et al., 2003). In agreement with previous studies (Reimold et al., 2001), Iwakoshi and co-workers have shown that the overexpression of XBP-1 induces IgM secretion in BCL-1 cells. Furthermore, these studies confirmed the earlier finding (Reimold et al., 2001) that transfection of XBP-1 into XBP-1-deficient B cells can restore immunoglobulin secretion (Iwakoshi et al., 2003)

The mechanism whereby XBP-1 mediates immunoglobulin responses in terminally differentiated B cells is closely related to the unfolded protein response (UPR) pathway in yeast, *Caenorhabditis elegans* worms and mammalian secretory cells (Calfon et al., 2002; Foti et al., 1999), (Fig. 6). UPR allows cells to respond to ER stress initiated due to accumulation of large quantities of unfolded proteins in the lumen of ER. UPR pathway is initiated from the ER membrane and is thought to monitor the load of generated proteins destined to secretion. This effect is achieved by upregulating protein folding and degradation pathways in the ER and inhibiting protein synthesis (Rutkowski and Kaufman, 2004). Upregulation of protein folding is achieved, in part, by induction of ER resident molecular chaperones and folding enzymes that promote protein folding and assembly. Increased expression of ER chaperones is mediated to the large extent by two

ER-membrane bound transcription factors, ATF6α and β. These ATF6 proteins undergo ER-stress induced proteolytic cleavage to generate soluble basic leucine-zipper transcription factors (Haze et al., 2001; Haze et al., 1999). Activated ATF6α and β transcription factors then target the *cis*-acting ERSE (ER stress response elements) in the promoters of several genes encoding ER chaperones and folding enzymes (Yoshida et al., 2000; Yoshida et al., 2001b), thus enhancing the folding capacity of the ER. Since one of the ERSE targets is XBP-1, the transcription of XBP-1 is induced, in part by UPR (Yoshida et al., 2001a). XBP-1 mRNA undergoes site-specific cleavage by IRE-1α, an ER transmembrane kinase/endonuclease that is activated in the UPR (Calfon et al., 2002; Yoshida et al., 2001a). Activated IRE-1α splices a-26nt-long sequence out of XBP-1 mRNA resulting in a translational frame shift encoding a longer form of XBP-1 protein, XBP-1s. XBP-1s is a more potent transcriptional activator compared with the unspliced form of XBP-1, XBP-1u (Calfon et al., 2002), thus splicing by IRE-1α is necessary for the complete activation of transcription factor XBP-1. Studies employing primary mouse B cells and the BCL-1 B cell line determined that the immunoglobulin response is dependent on the UPR (Iwakoshi et al., 2003). Several lines of evidence were presented in support of this conclusion. First, it has been shown that XBP-1 splicing by IRE-1α occurs during terminal B cell differentiation, and correlates with the induction of UPR markers Grp94 and Grp78. Furthermore, it has been established that the IRE-1α-mediated splicing of XBP-1 is dependent on the IgM heavy chain production (Iwakoshi et al., 2003). In another study ectopic expression of a dominant-negative form of ATF6α in differentiating splenic B cells significantly diminished IgM secretion (Gunn et al., 2004). Therefore, UPR is an integral part of terminal B cell differentiation program.

Figure 6. XBP-1 expression and activation pathways. Unfolded protein response is initiated at the ER membrane by a stressful event, such as accumulation of large quantities of unprocessed proteins in the ER. As a result, members of the ATF6 transcription factor family are expressed and activated by enzymatic cleavage. Activated ATF6 proteins contribute to the induction of XBP-1 transcription, yielding XBP-1u mRNA. Another arm of ER stress produces massive proteolysis, which promotes the activation of IRE-1α. Activated IRE-1α cleaves the XBP-1u mRNA to XBP-1s isoform. Whereas both XBP-1u and XBP-1s are being translated to protein, generation of XBP-1s is crucial for the IgM secretion process. In addition to transcriptional regulation by ATF6, IL-6 has been shown to induce, and Pax5 to suppress XBP-1 promoter activity.


ER Stress
ATF6
enzymatic cleavage
Active ATF6
Proteolysis
IRE-1α
Pax-5
IL-6
XBP-1 promoter
XBP-1u mRNA
Active IRE-1α
XBP-1s mRNA
XBP-1u Protein
XBP-1s Protein

In addition to ATF6, several other factors contribute to the induction of XBP-1 transcription in activated B cells. One of the factors that play an important role in XBP-1 regulation during terminal B cell differentiation is Pax5. Transient expression studies have shown a strong downregulation of the XBP-1 promoter by Pax5 in mature B cells. Pax5 has been shown to repress XBP-1 transcription through XBP-1 promoter binding (Reimold et al., 1996). Removal of Pax5 repression may account for the high-level expression of XBP-1 in plasma cells, a developmental stage where Pax5 is no longer expressed (Reimold et al., 1996). Notably, the induction of XBP-1 does not occur in the absence of the induction of Blimp-1, an upstream repressor of Pax5 (Shaffer et al., 2004). This finding supports of the notion that Blimp-1 dependent repression of Pax5 is required for XBP-1 expression in terminally differentiating B cells.

Another factor implicated in XBP-1 regulation is IL-6. Initial studies in human multiple myeloma cells have shown that XBP-1 is strongly induced following IL-6 treatment (Wen et al., 1999). Furthermore, IL-6 can drive resting primary B cells to the differentiated plasma cell stage (Hirano and Kishimoto, 1989). In addition, IL-6 is known to promote proliferation in malignant plasma cells (Wen et al., 1999). These findings led to the suggestion that IL-6 is a positive regulator of XBP-1. In extension of these result, later studies have shown that spliced XBP-1 can induce IL-6 secretion in activated mouse splenic B cells (Iwakoshi et al., 2003). Taken together, these studies suggest the existence of a positive feedback loop between XBP-1 and plasma cell growth factor IL-6.

The roles of activated XBP-1 in terminal B cell differentiation include the expansion of the secretory apparatus and other organelles, and increases in protein

synthesis (Shaffer et al., 2004). Gene expression studies were performed in a human mature B cell line, Raji, transduced with retrovirus encoding XBP-1s or XBP-1u. Genes that were differentially induced by XBP-1s, but not XBP-1u included genes involved in translocation of proteins across the ER membrane (srp54, srpr, ssr3, ssr4, rpn1, tram1, spc22/23), ER protein folding (erp70, ppib, grp58, fkbp11, dnaJb9, hspa5), protein glycosylation (gcs1, ddost, dad1), and vesicle trafficking (sec23B, sec24C, os-9, golgB1, mcfd2) (Shaffer et al., 2004). In addition, an increase in cell size and ER content were observed in cells transduced with XBP-1s constructs, whereas RNA-interference to XBP-1s in a melanoma cell line was able to diminish the increase in cell size, suggesting that XBP-1s is mediating the increase in cell size. In addition, XBP-1s-transduced cells increased the size of their mitochondria and ribosomes, suggesting that in addition to protein processing, metabolic capacity is positively regulated by XBP-1 during terminal B cell differentiation (Shaffer et al., 2004).

B. Surface markers of B cell differentiation

a. Syndecan-1

Syndecan-1, also known as CD138, is a surface adhesion molecule expressed on epithelia and B cells. The backbone of syndecan-1, a proteoglycan, is bearing heparan sulfate chains, which are capable to bind soluble and insoluble effector molecules. These interactions promote syndecan-1 binding to extracellular matrix and the adjacent cells (Sanderson and Borset, 2002). The expression of murine syndecan-1 is mediated, in part, by transcription factors Hox and MyoD (Bernfield et al., 1993). In fibroblasts and keratinocytes, the growth factors fibroblast growth factor-2 (FGF-2), epithelial growth

factor (EGF) and keratinocyte growth factor (KGF) were also involved in the activation of syndecan-1 promoter (Jaakkola and Jalkanen, 1999).

Within the B cell lineage, syndecan-1 expression has been detected on pre-B and plasma cells, but not on mature B cells (Sanderson et al., 1989), making syndecan-1 a valuable phenotypic marker of terminal B cell differentiation (Angelin-Duclos et al., 2000; Kopper and Sebestyen, 2000; Shapiro-Shelef et al., 2005; Turner et al., 1994). The expression of syndecan-1 was found to correlate with the expression of Blimp-1 (Turner et al., 1994) but it has not been elucidated whether Blimp-1 is directly involved in the induction of syndecan-1 transcription in differentiating B cells.

b. **Major histocompatibility complex class II**

The major histocompatibility complex class II molecule is a cell surface protein expressed on all APC, including the mature B cells, but not on plasma cells. This molecule consists of α1, α2, β1 and β2 glycoprotein chains held together by disulfide bonds and anchored into the cell plasma membrane. In human and mouse, MHC class II gene is encoded by several loci. In human, the loci are termed human leukocyte antigen (HLA)-DO, HLA-DR and HLA-DP, and in the mouse I-A and I-E. MHC class II is crucial for antigen presentation by the B cells to interacting $CD4^+$ Th cells, and mounting T-cell dependent immune responses (see section II.B.c. for details).

The critical role for MHC class II in initiating immune responses against bacterial infections was demonstrated by the fact that MHC class II-deficient mice succumbed to the infection, in contrast with their wild-type counterparts (Ladel et al., 1995; Morrison et al., 1995).Similarly, in humans defects in MHC class II expression lead to a severe

immunodeficiency (Mach et al., 1996). Conversely, overexpression of MHC class II was implicated in T cell hyper-responsiveness (Bottazzo et al., 1986; Wraith et al., 1989). The expression of MHC class II is modulated by a number of genes, but the evidence for the involvement of most of these genes in MHC class II regulation has been inconclusive. One of the genes identified in this group is MHC class II transcriptional activator (CIITA) (Mach et al., 1996). Notably, the CIITA transcriptional activity in MHC class II promoter was INFγ-inducible (Silacci et al., 1994). CIITA plays a crucial role in the modulation of MHC class II expression, as evidenced by a positive quantitative correlation between MHC class II and CIITA expression and the fact that CIITA expression precedes the induction of MHC class II by several hours (Mach et al., 1996). Furthermore, studies in CIITA-deficient mutants and co-transfections of CIITA (Benoist and Mathis, 1990; Glimcher and Kara, 1992) and DRA-CAT cassette (Silacci et al., 1994) corroborated the assertion that CIITA is a positive regulator of the proximal MHC class II promoter.

Importantly, Blimp-1 has been shown to repress MHC class II expression in terminally differentiating B cells by repressing the expression of CIITA (Piskurich et al., 2000). In addition, deletion of Blilmp-1 direct target gene Pax5 correlated with downregulation of MHC class II expression (Horcher et al., 2001), which may suggest that Pax5 contributes to the maintenance of MHC class II expression in mature B cells.

c. Cluster of differentiation 19

Cell surface protein cluster of differentiation 19 is a component of B cell co-receptor, also comprised of CD21 and CD81 protein (Barrington et al., 2005; Roberts and

Snow, 1999; Shoham et al., 2003). CD19 is expressed on B cells and on a minor population of dendritic cells (Baban et al., 2005; Bjorck and Kincade, 1998). Within the B cell lineage, CD19 is expressed in mature B cells, but not in plasma cells (de Rie et al., 1989; Kozmik et al., 1992).

Numerous studies demonstrated the involvement of CD19 in B cell activation and differentiation (de Rie et al., 1989; Engel et al., 1995; Sato et al., 1995). The CD19 molecule is comprised of one extracellular and one cytosolic domain (Zhou et al., 1991). When the extracellular portion of the B cell co-receptor is engaged in BCR interaction, a conformational change occurs in the cytosolic portion of the CD19 protein, leading to phosphorylation of cytosines on the CD19 cytoplasmic tail (Carter et al., 1997). This action initiates Src family protein tyrosine kinase activation, (Fujimoto et al., 2000) which is in addition to signaling through the BCR cytosolic residue. As a result, the B cell activation threshold can be reduced up to 100-fold (Roberts and Snow, 1999). Due to its ability to lower the B cell activation threshold, CD19 is considered the leverage factor modulating B cell activation.

The expression of CD19 diminishes as B cell differentiation progresses. This effect is due, in part, to the suppression of B cell differentiation repressor Pax5, which is a strong positive regulator of the CD19 gene. Pax5 recognizes cognate recognition motifs in the murine and human CD19 promoters and acts to induce CD19 expression in a DNA-motif-binding manner (Kozmik et al., 1992). In fact, confirmation of CD19 expression was used by a number of studies as an indirect indication of Pax5 functionality (Danbara et al., 2002; Palmisano et al., 2003). Therefore, the differential expression of CD19 in the course of terminal B cell differentiation is a useful tool to

assess the progress of B cell differentiation.

MATERIALS AND METHODS

I. Chemicals

TCDD, in 100% dimethylsulfoxide (DMSO), was purchased from AccuStandard (New Haven, CT). DMSO and LPS were purchased from Sigma (St. Louis, MO).

II. Cell line

CH12.LX B cell line was derived from the murine B cell lymphoma, CH12, which arose in B10.H-2^{a}H-4^{b}p/Wts mice (B10.A X B10.129) and has been previously characterized (Bishop and Haughton, 1986). CH12.LX cells were maintained in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 13.5 mM HEPES, 23.8 mM sodium bicarbonate, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Cells (1 x 10^{5}/ml) were activated with 5 μg/ml LPS, and treated with 0.01% DMSO or TCDD for indicated times.

III. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was performed on CH12.LX cells cultured at density 1.0 x 10^{5} cell/ml for 72 h in LPS (5 μg/ml)-containing medium in the presence of TCDD (10 nM) or the vehicle (0.01% DMSO). Untreated cells (Naïve) or TCDD alone treated cells served as negative as well as comparative control. The supernatants were harvested at 24, 48 and 72 h and analyzed by sandwich ELISA as previously described (Sulentic et al., 1998). Briefly, a 96-well microtiter plate (Immulon

4, Dynex Technologies Inc., Chantilly, VA) was pre-coated with anti-mouse Ig capture antibody (Sigma-Aldrich, St. Louis, MO) and 100 μl supernatant or mouse IgM κ light chain (standard) were added in each well. Plates were incubated at 37°C for 1.5h and washed twice with 0.05% Tween-20 PBS and three times with H_2O. A horseradish peroxidase (HRP)-linked anti-mouse IgM detection antibody was added to the plate, incubated for 1.5h at 37°C, and the plates were washed as stated above. ABTS substrate (Roche Molecular Biochemicals, Indianapolis, IN) was added for kinetic colorimetric detection, which was performed on a-405-nm wavelength over a 1-h period using an EL808 automated microplate reader (Bio-Tek, Winooski, VT). The concentration of total IgM in the supernatants was calculated using a standard curve of known IgM κ concentrations.

IV. **Flow cytometry**

Cells were harvested from culture at the indicated times by centrifugation at 300 x g for 10 min at 4°C, washed twice in ice-cold 1X HBSS and stained using BD Cytofix /Cytoperm kit (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. Briefly, cells were incubated with 1 μg/10^6 cells of purified rat anti-mouse CD16/CD32 monoclonal antibody (BD Pharmingen, San Diego, CA) for 15 min at 4°C to prevent non-specific binding, then stained with surface marker detection antibody (anti-FITC-conjugated mouse anti-mouse I-A^P, APC-conjugated rat anti-mouse CD19, or PE-conjugated anti-mouse syndecan-1) or a respective isotype control (BD Pharmingen, San Diego, CA). To exclude non-viable cells, 2 μl of 7-amino-actinomycin D (7-AAD) solution (Sigma-Aldrich, St. Louis, MO) containing 1 mg 7-AAD, 50 μl methanol, 950 μl

HBSS were added simultaneously with detection antibodies to the cells in 50 µl of staining buffer. The cells were then fixed, washed and maintained in staining buffer containing 10 mM actinomycin D (Sigma-Aldrich, St. Louis, MO) to prevent 7-AAD leakage from fixed cells. For the detection of nuclear Pax5 protein, cells were fixed and permeabilized prior to staining with FITC-conjugated anti-Pax5 antibody, or isotype control (Santa Cruz Biotechnologies, Santa Cruz, CA). Fluorescence detection was performed using a BD FACSCalibur flow cytometer (BD Biosciences, CA) on 10,000 viable cells per sample. Data analysis was performed using BD CellQuest Pro Software (BD Biosciences, CA).

V. **Real Time RT-PCR**

Total RNA was isolated from naïve or LPS-activated cells using a SV Total RNA Isolation kit (Promega, Madison, WI). To synthesize cDNA, 1,000 ng/sample of total RNA was incubated with 600 ng random primer (Invitrogen, Carlsbad, CA) in 10 µl endonuclease-free water at 70°C for 10 min, cooled on ice for 10 min and reverse transcribed in 20 µl 1X First Strand Synthesis buffer (Invitrogen, Carlsbad, CA), containing 0.2 mM dNTPs, 10 mM DTT, and 200 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction mixture was incubated at 42°C for 60 min and the reaction was stopped by incubation at 75°C for 15 min. Real time PCR detection was performed using TaqMan primers and probes (Table 1). The PCR cycling conditions were as follows: initial denaturation and enzyme activation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Detection of PCR transcripts was performed on a PE Applied Biosystems PRISM 7900HT Sequence Detection System

(Foster City, CA). The absolute copy number for each of the Pax5 amplicons in naïve CH12.LX cells was calculated by standard curve method. The relative levels of Pax5 amplicons during the 72 h time course were calculated by the comparative threshold method using 18S ribosomal subunit expression in order to control for differences in loading.

VI. **RT-PCR**

Total RNA was isolated from naïve or LPS-activated cells using a SV Total RNA Isolation kit (Promega, Madison, WI). To synthesize cDNA, 1,000 ng/sample of total RNA was incubated with 600 ng random primer (Invitrogen, Carlsbad, CA) in 10 µl endonuclease-free water at 70°C for 10 min, cooled on ice for 10 min and reverse transcribed in 20 µl 1X First Strand Synthesis buffer (Invitrogen, Carlsbad, CA), containing 0.2 mM dNTPs, 10 mM DTT, and 200 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction mixture was incubated at 42°C for 60 min and the reaction was stopped by incubation at 75°C for 15 min. RT-PCR was performed using SYBR Green PCR Core Reagents (PE Applied Biosystems). The PCR cycling conditions were as follows: initial denaturation and enzyme activation for 10 min at 95°C followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Primers for the detection of alternative spliced XBP-1 isoforms (XBP-1s/u), (Table 3), were designed to span the 26-nucleotide splice site in XBP-1 mRNA (Applied Biosystems, Foster City, CA). Detection of XBP-1 spliced and unspliced forms was achieved by resolution of PCR products on a 4% agarose gel.

VII. **Electrophoretic mobility shift assay**

A. **Isolation of nuclear AHR protein.** CH12.LX cells were incubated with 0.01% DMSO or 30 nM TCDD in DMSO for 1 h at 37°C. Cells were harvested by centrifugation at 300g for 10 min, washed once with 1X PBS and incubated on ice in lysis buffer (10 mM HEPES, 3 mM $MgCl_2$) for 15 min, and centrifuged as above. All buffers below contained protease inhibitor cocktail (Roche, Boehringer Mannheim, Germany). One milliliter of MDH buffer (3 mM $MgCl_2$, 1 mM dithiotretiol (DTT), 25 mM HEPES) was added to the cell pellet and homogenized with tight-fitting pestle. Nuclei were pelleted by centrifugation at 1,000g for 5 min, washed twice with MDHK buffer (3 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES, 100 mM KCl) and then resuspended in 100 µl of HEDGK buffer (25 mM HEPES, 1 mM EDTA, 1 mM DTT, 10% glycerol, 400 mM KCl), incubated on ice with agitation for 40 min, and centrifuged at 14,000g for 15 min. The supernatant was aliquoted and stored at -80°C before use in the electrophoretic mobility shift assay (EMSA). Protein concentrations were determined using the bicinchonic acid protein determination assay (Sigma, St Louis, MO).

B. **Isolation of nuclear AP-1 and Blimp-1 proteins.** CH12.LX cells treated with LPS, TCDD or both were harvested at the indicated times by centrifugation at 300g for 10 min, washed once with 1X PBS and lysed in lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$). Nuclei were pelleted by centrifugation at 6,700g for 10 min, and the pellet was lysed in a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA, and 10% glycerol), which contained 1 mM DTT and protease inhibitors cocktail (Roche Boehringer Mannheim, Germany), for 15 min on ice. Samples were then

centrifuged at 17,500g for 15 min and the supernatant was retained. Protein determinations were performed using the bicinchoninic acid assay (Sigma, St. Louis, MO).

C. **Analysis of DRE and TRE recognition motifs**

Putative DRE and TRE motifs in the Blimp-1 and Pax5 promoters were identified by sequence analysis using MacVector software (MacVector Inc., San Diego, CA). In addition, position weight matrix program with qualifying score of 0.75 or higher was used to confirm the presence of the identified DRE sites (Sun et al., 2004). Consensus TRE oligonucleotide (Faubert and Kaminski, 2000) containing the core sequence TGACTCA (Novak et al., 1990), and high affinity DRE oligonucleotide, DRE3, containing the core sequence GCGTG (Denison and Yao, 1991) were used as positive control probes. For AHR EMSA, 20 μg nuclear protein was placed in 20 μl AHR-binding buffer (25 mM HEPES (pH=7.5), 1 mM EDTA, 2 mM DTT, 10% glycerol, 110 mM KCl). For AP-1 and Blimp-1 EMSA, 11 μg and 20 μg nuclear protein, respectively, were placed in 20 μl of binding buffer (30 mM HEPES, 1.5 mM $MgCL_2$, 0.3 mM EDTA, 100 mM NaCl, 10% glycerol, 0.05% Nonidet P-40, 1 mM DTT). Each binding buffer contained protease inhibitor cocktail (Roche). Samples were then incubated with 1.0 μg poly dI-dC (Roche Boehringer Mannheim, Germany) at room temperature for 15 min. Double-stranded ^{32}P-labeled probes (Table 2) were added and incubated at room temperature for another 30 min. The binding of protein to DNA was resolved by a 4.0% nondenaturing polyacrylamide gel electrophoresis (PAGE). The gel was then dried on 3-mm filter paper (Bio-Rad, Hercules, CA), and autoradiographed.

VIII. **Assessment of the 5'CpG methylation levels in Pax5 promoter**

Total genomic DNA was isolated from naïve or treated CH12.LX cells after the 72 h culture period by GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, St. Louis, MO). Our studies focused on the methylation status of CpG sites located within the first 200 bp of the Pax5 promoter region (AF148961). Treatment-related differences in Pax5 promoter CpG sites' methylation were assessed by restriction studies using methylation-sensitive enzymes BstUI, BssHII and HpaII (New England Biolabs, Ipswich, MA), for 16 h as instructed by the manufacturer. Naïve CH12.LX cells treated with a CpG methylase, M.SssI, (New England Biolabs, Ipswich, MA), were used as a positive control for methylation. Genomic DNA subjected to the restriction treatment was amplified by PCR using primers spanning the restriction sites (Table 3). PCR reactions were performed in 12.5 µl each containing 100 ng DNA template, 1µM of each primer, 6.25 µl 2 x FailSafe mix G (Epicentre Biotechnologies, Madison, WI) and 0.75 U native *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The PCR product was then resolved on a 2% agarose gel, visualized by ethidium bromide, and optical density for each transcript was assessed by autoradiography.

IX. **Western blotting**

CH12.LX cells (1 x 10^5cells/ml) were cultured for indicated times in the presence of LPS (5 μg/ml) and TCDD (10 nM) and/or vehicle (0.01% DMSO). Cells were harvested and lysed in HEG buffer (25 mM HEPES, 2mM EDTA and 10% glycerol) containing protease inhibitors (complete mini tablets, Roche Molecular Biochemicals), sonicated three times for 5 s, and centrifuged at 10,000 x g for 20 min at 4°C. Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Twenty-five μg lysate was loaded per lane on denaturing 10% SDS-PAGE (Life Science Products Inc., Denver, CO), resolved by electrophoresis and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). Blots were blocked in blocking buffer containing 4% low-fat dry milk, 1% BSA in 0.1% Tween-20 TBS) for 1-2 h at room temperature. Immunochemical staining was performed as previously described (Williams et al., 1996). Detection was performed using primary rabbit anti-monkey XBP-1 antibody (Santa Cruz Biotechnology, CA.) and secondary HRP-linked anti-rabbit antibody (Pierce Biotechnology, Rockford, IL). For loading control, blots were stripped and re-probed for β-actin using primary donkey anti-mouse β-actin antibody (Sigma-Aldrich) and secondary anti-donkey antibody (Pierce Biotechnology, Rockford, IL). Stripping was performed by submerging the membranes in stripping buffer containing: 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris (pH=6.7), for 30 min at 37°C. The protein blots were then washed, blocked and reprobed as stated above. Optical density for XBP-1 and β-actin was measured by densitometry using a model 700 imaging system (Bio-Rad).

X. **Statistical Analysis of Data- Mean ± S.E.** was determined for each treatment group of a given PCR or ELISA experiment. Statistical differences between groups in each experiment were determined by two way ANOVA followed by Bonferroni post-hoc test.

XI. **Primer and probe sequences**

Table 1. TaqMan RT-PCR primers and probes 5' to 3'

AMPLICON	
Pax5, I	
Forward primer	CCC GAC TCC TCG GAC CAT
Reverse primer	AGT GGC CGT CCA TTC ACA AAA
Probe	CTC CTC CAT GTC CTG TCC TG
Pax5, II	
Forward primer	CGA CTC CTC GGA CCA TCA G
Reverse primer	CTT CCT GTC TCA TAA TAC C
Probe	CAA TCA CCC CCG GCT TGA
Pax5, III	
Forward primer	CGA CAC CAA CAA ACG CAA GA
Reverse primer	CCG GAA GTG AGT GGC CAT T
Probe	ACT CCT GAA TAC CTT CAT CCC
Pax5, IV	
Forward primer	GCA TCC CCA CCC GGA AT
Reverse primer	CCT TCT GCG AGG GTT CCA
Probe	CAC CTA GCA GGG TCT GTG
Blimp-1	
Forward primer	CTTTGGACTCTTACTCAACTGTACAAGCT
Reverse primer	CAGTCTCTGCCAGTCCTTGAAA
Probe	CCCAAGTCTAGCTCCGGCTCCGTG
CD19	
Forward primer	GGG AGC AGT TTG AAT CAG AGC CCG GAG GAG
Reverse primer	CCA CAG GAC AGC CAA AGT GT
Probe	CCA CAG TGA GAT CTT G
XBP-total	
Forward primer	GAG CCC GGA GGA GAA AGC
Reverse primer	TCT GCG CTG CTA CTC TGT TTT
Probe	CTG CGG AGG AAA CTG

Table 2. EMSA oligonucleotide probes 5' to 3'.

PROBE	TOP STRAND	BOTTOM STRAND
TRE consensus	GATCCGGCTGACTCATCAGTA	CTACTGATGAGTCAGCCGGAT
TRE-47	GCTGGTAGGAGTGAATCAGACCGT	ACTGACGGTCTGATTCACTCCTAC
TRE-1060	ACTTCATTGTATGACTAAGTTGGT	TGATACCAACTTAGTCATACAATG
TRE-1610	CATAGTGGTGCTGACTCAGCATCG	TAACCGATGCCTGAGTCAGCACCAC
DRE3	GATCTGGCTCTTCTCACGCAACTCCG	GATCCGGAGTTGCGTGAGAAGAGCCA
DRE -506	GACGGACGGGTCGCACGGTCTGCCCG	GACGGGCAGACCGTGCGACCCGTCCG
DRE -75	CCAGGTGCGGCCACGCCCCCATCGCG	GCCGCGATGGGGGCGTGGCCGCACCT
DRE -107	GCCCTGAACCCCACGCTGCACGGCTG	CCCAGCCGTGCAGCGTGGGGTTCAGG

Table 3. Semi-quantitative PCR probes 5' to 3'

AMPLICON	FORWARD PRIMER	REVERSE PRIMER
XBP-1s/u	ACACGCTTGGGAATGGACAC	CCATGGGAAGATGTTCTGGG
Pax5 promoter	GGGTGAATCTGAGGATGCTG	GTGCAAGAGGCCCAGAGAG

EXPERIMENTAL RESULTS

I. TCDD-mediated suppression of the IgM humoral immune response and differentiation in LPS-activated CH12.LX cells

A. TCDD-mediated suppression of the IgM response to LPS in CH12.LX cells

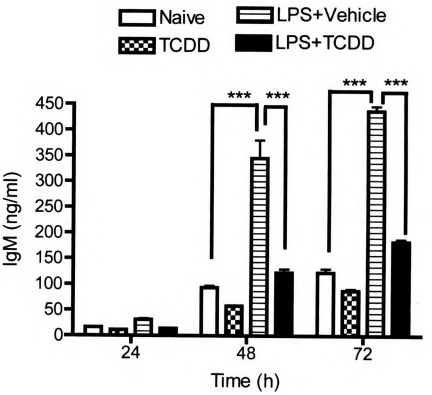
The suppression of humoral immune responses is a well-established effect of TCDD (Holsapple et al., 1986a; Luster et al., 1988; Sulentic et al., 1998; Sulentic et al., 2000; Tucker et al., 1986). A typical kinetic profile of the IgM suppression by TCDD in our experimental model, CH12.LX cells, is depicted in figure 7A. CH12.LX cells were activated by LPS in the presence and absence of TCDD during the 72 h time course. In the absence of activation stimulus, the CH12.LX cells secreted small amounts of IgM, which was detected at 24 h and continued to accumulate in the supernatant until 72 h. LPS activation strongly induced IgM secretion, which was significantly different from the time-matched naïve control at 48 and 72 h. By contrast, TCDD treatment significantly suppressed the secretion of IgM at 48 and 72 h, when compared to the LPS-activated time-matched control CH12.LX cells. Notably, TCDD treatment had little effect on CH12.LX cells viability (Fig. 7B).

B. TCDD attenuated cell surface MHC class II down-regulation in LPS-activated CH12.LX cells

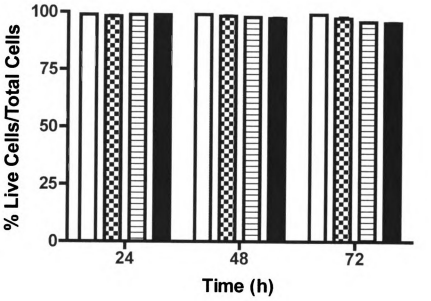
To evaluate the extent of terminal differentiation in LPS-activated CH12.LX cells following TCDD treatment, the levels of surface MHC class II were monitored by flow

Figure 7. Kinetics of the TCDD-mediated IgM response suppression in LPS-activated CH12.LX cells. LPS-activated (5 μg/ml) CH12.LX cells were cultured for 72 h in the presence of TCDD (10 nM) and/or vehicle (0.01% DMSO). Naïve and TCDD treated cells in the absence of LPS activation were used as a negative control. A) Supernatants were harvested at the indicated times and assayed for IgM concentration by sandwich ELISA. The supernatant IgM concentrations are represented on the *y*-axis as ng/ml. B) Cell viability was determined by flow cytometry using the 7-actinomyacin D exclusion method. The percentage of viable cells is represented on the *y*-axis. Results (mean ± S.E.) are representative of a triplicate determination in each group from at least three separate experiments. Statistical significance was determined by a two way ANOVA followed by Bonferroni post-hoc test. *** Denotes $p<0.01$

A


Naive
LPS+Vehicle
TCDD
LPS+TCDD
***
***
***
***
450
400
350
300
250
200
150
100
50
0
IgM (ng/ml)
24
48
72
Time (h)

B


100
75
50
25
0
% Live Cells/Total Cells
24
48
72
Time (h)

cytometry. LPS-activation induced a down-regulation of MHC class II on the CH12.LX cell surface between 24 and 72 h of culture when compared to the time matched naïve control (Fig. 8), which is consistent with the B cell differentiation. Conversely, TCDD treatment attenuated the LPS-induced down-regulation of MHC class II at 24 and 48 h when compared to the time-matched LPS control.

C. TCDD modestly suppressed cell surface syndecan-1 expression in LPS-activated CH12.LX cells

In light of the marked suppression of IgM secretion in LPS-activated CH12.LX cells in the presence of TCDD, cell surface expression of plasma cell marker, syndecan-1, was examined by flow cytometry (Fig. 9). In LPS-activated CH12.LX cells, a modest increase in syndecan-1 expression was detected at 48 and 72 h, as compared with the time-matched naïve control. TCDD treatment of the LPS-activated CH12.LX cells produced a modest, but detectable decrease in syndecan-1 cell surface expression, which was prominent at 72 h post LPS-activation.

D. TCDD alters the levels of CD19 in LPS-activated CH12.LX cells.

As a marker for assessing B cell differentiation, we examined the expression of CD19 in LPS-activated CH12.LX cells in the presence or absence of TCDD. During the 72 h time course, flow cytometric analysis revealed a continuous downregulation of CD19 surface expression in the LPS-activated group as compared with the time-matched naïve control between 24 and 72 h, in concordance with the progression of terminal B cell differentiation (Fig. 10). Following TCDD treatment, LPS-activated CH12.LX cells

Figure 8. Flow cytometric analysis of cell surface expression of MHC class II protein in LPS-activated CH12.LX cells. Naïve or LPS (5μg/ml)-activated CH12.LX cells were treated with TCDD (10 nM) and/or vehicle (0.01% DMSO). Cells were harvested at 24, 48 and 72 h, incubated with anti-MHC class II ($I\text{-}A^P$)-FITC or isotype control antibodies and analyzed by flow cytometry. Results are representative of triplicate determinations in each treatment group from three separate experiments.

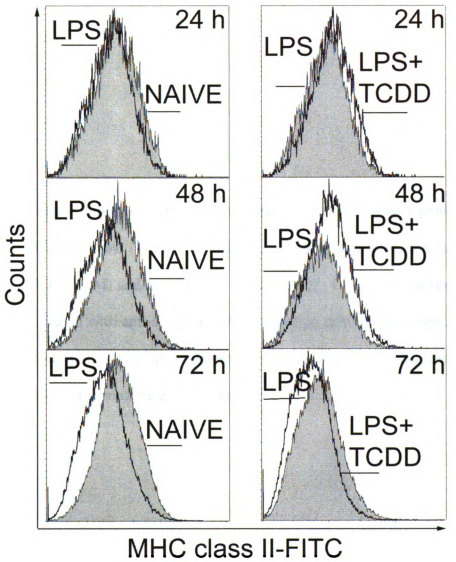

LPS
24 h
NAIVE
LPS
24 h
LPS+
TCDD
LPS
48 h
NAIVE
LPS
48 h
LPS+
TCDD
LPS
72 h
NAIVE
LPS
72 h
LPS+
TCDD
Counts
MHC class II-FITC

Figure 9. Flow cytometric analysis of cell surface expression of syndecan-1 protein in LPS-activated CH12.LX cells. Naïve or LPS (5μg/ml)-activated CH12.LX cells were treated with TCDD (10 nM) and/or vehicle (0.01% DMSO). Cells were harvested at 24, 48 and 72 h, incubated with anti-syndecan-1-PE or isotype control antibodies and analyzed by flow cytometry. Results are representative of triplicate determinations in each treatment group from three separate experiments.

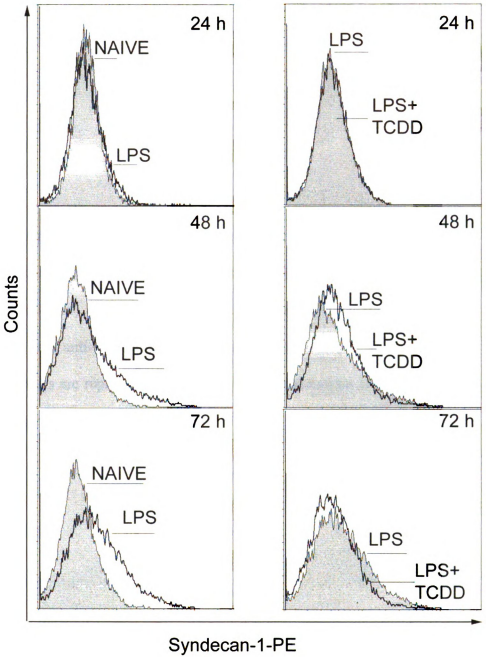
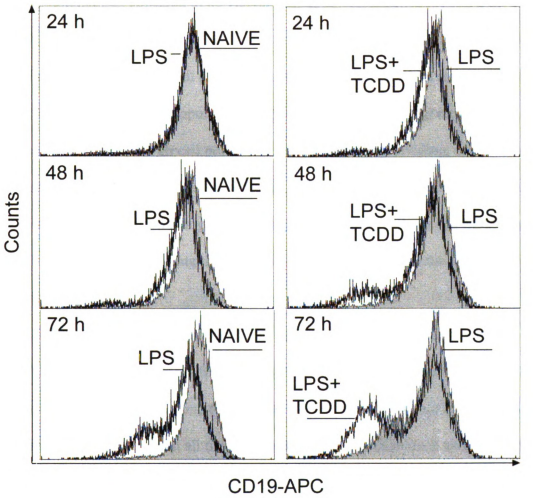

24 h
NAIVE
LPS
24 h
LPS
LPS+
TCDD
48 h
NAIVE
LPS
48 h
LPS
LPS+
TCDD
72 h
NAIVE
LPS
72 h
LPS
LPS+
TCDD
Counts
Syndecan-1-PE

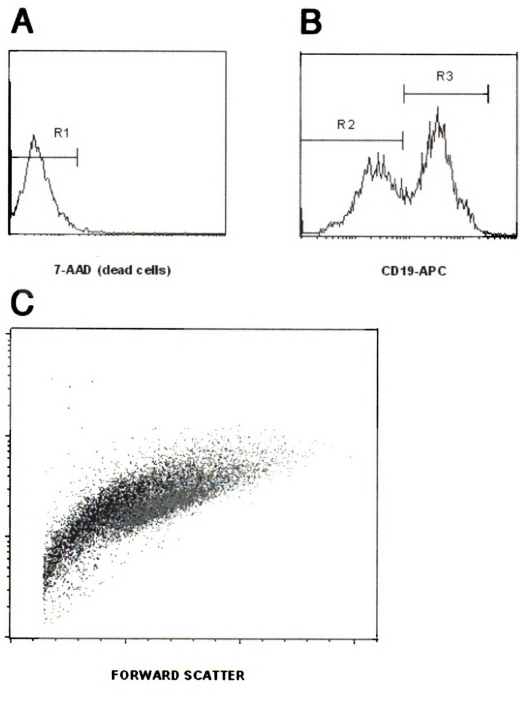
Figure 10. Flow cytometric analysis of cell surface expression of CD19 in LPS-activated CH12.LX cells. Naïve or LPS (5 μg/ml)-activated CH12.LX cells were treated with TCDD (10 nM) or vehicle (0.01% DMSO). Cells were harvested at 24, 48 and 72 h, incubated with anti-CD19-APC or isotype control antibodies and analyzed by flow cytometry. Results are representative of triplicate determinations in each treatment group from three separate experiments.


24 h
NAIVE
LPS
24 h
LPS+
TCDD
LPS
48 h
NAIVE
LPS
48 h
LPS+
TCDD
LPS
72 h
NAIVE
LPS
72 h
LPS
LPS+
TCDD
Counts
CD19-APC

exhibited an additional decrease in CD19 surface expression as compared with the time-matched LPS-activated control group. Notably, by 72 h two cell populations were detected based on the levels of CD19 surface expression. Since 7-AAD staining was performed to exclude non-viable cells (Fig. 11A), both CD19-high and CD19-low-expressing populations appeared to be viable. Gating on viable cells only (R1), the entire CH12.LX population at 72 h was plotted (Fig. 11B), and gates were set for the CD19-low (R2) and the CD19-high (R3) cell populations. As indicated by the increase in side scatter (Fig 11C), the CD19-low cell population (black) exhibited increased granularity as compared with the CD19-high cell population (gray), indicating dividing, and possibly non-differentiating CH12.LX cells. Importantly, TCDD treatment produced a decrease in CD19 surface expression following LPS activation of both CD19-high and CD19-low CH12.LX populations, suggesting that this effect was independent of the B cell differentiation state.

To further address the effect of TCDD on CD19 expression in LPS-activated CH12.LX cells, the mRNA levels for CD19 were analyzed by real time PCR during the 72 h time course (Fig. 12). A trend towards CD19 mRNA down-regulation following LPS-activation was observed at all times, with statistical significance detected at 48 h. Conversely, TCDD treatment of LPS-activated CH12.LX cells showed a trend towards attenuation of the down-regulation of CD19 as compared with the time-matched LPS-activated control at 48 and 72 h. The mRNA levels of CD19 in LPS activated CH12.LX cells in absence of TCDD were concordant with the CD19 protein surface expression at 24 h, 48 and 72 h, and were consistent with the notion that LPS-activation results in diminished CD19 expression. In addition, although TCDD treatment of LPS-activated

Figure 11. Two CD19-expressing cell populations were detected in LPS-activated TCDD-treated CH12.LX cells at 72 h. LPS (5 μg/ml)-activated CH12.LX cells were treated with TCDD (10 nM), harvested at 72 h, incubated with anti-CD19-APC or isotype control antibodies and analyzed by flow cytometry. A) Exclusion of non-viable cells was performed by staining with 7-AAD. Only the 7-AAD-negative cell population was analyzed for CD19 expression. B) CH12.LX cells were discriminated based on CD19 expression as CD19-high (gate R3) and CD19-low (gate R2). C) The CD19-low cells (gate R2) are denoted in black, whereas the CD19 high cells (gate R3) are denoted in gray.


A
R1
7-AAD (dead cells)
B
R3
R2
CD19-APC
C
FORWARD SCATTER

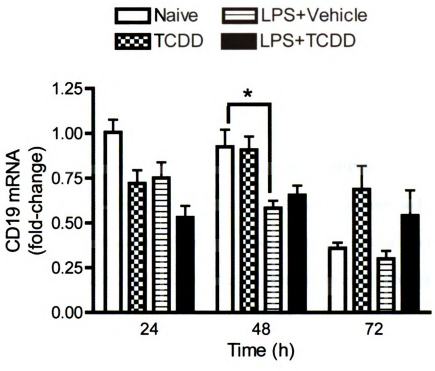


Figure 12. Effects of TCDD on CD19 mRNA levels in CH12.LX cells. Naïve or LPS (5 μg/ml)-activated CH12.LX cells were treated with 10 nM TCDD and/or vehicle (0.01% DMSO). Cells were harvested at the indicated times post LPS-activation and quantitative RT-PCR was performed to determine the total levels of CD19 transcripts. Results were normalized to 18S ribosomal subunit amplification, which was used as a loading control. The fold-change in CD19 transcripts relative to naïve sample at 24 h, which was arbitrarily given the value of 1, is represented on the *y*-axis. Results represent the mean ± S.E. of triplicate determinations in each treatment group from at least three separate experiments. Statistical significance is denoted as * $p<0.05$.

CH12.LX cells decreased the CD19 surface expression, the CD19 mRNA levels remained elevated. Therefore, the CD19 mRNA expression is in agreement with our hypothesis postulating that the TCDD impairment of the terminal B cell differentiation program will result in attenuation of the suppression of CD19 in LPS-activated CH12.LX cells.

II. TCDD decreased the expression and activation of XBP-1.

XBP-1 is a critical factor involved in immunoglobulin assembly and secretion. In addition to suppression of immunoglobulin production and attenuation of a number of B cell differentiation markers, TCDD affected the expression and activation of XBP-1, as discussed below.

A. TCDD decreased the total mRNA levels of XBP-1 in LPS-activated CH12.LX cells

The effects of TCDD on XBP-1, which is essential for IgM secretion by B cells (Reimold et al., 2001) were examined. First, the levels of total XBP-1 mRNA in CH12.LX cells following LPS-activation and/or TCDD treatment were assessed by real time PCR. As expected, XBP-1 was induced in the LPS-activated cells between 24 and 72 h compared to the time-matched naïve control (Fig. 13A). TCDD treatment down-regulated the LPS-induced XBP-1 mRNA levels at 48 and 72 h when compared to LPS alone.

B. TCDD decreased the mRNA levels of the activated form of XBP-1, XBP-1s

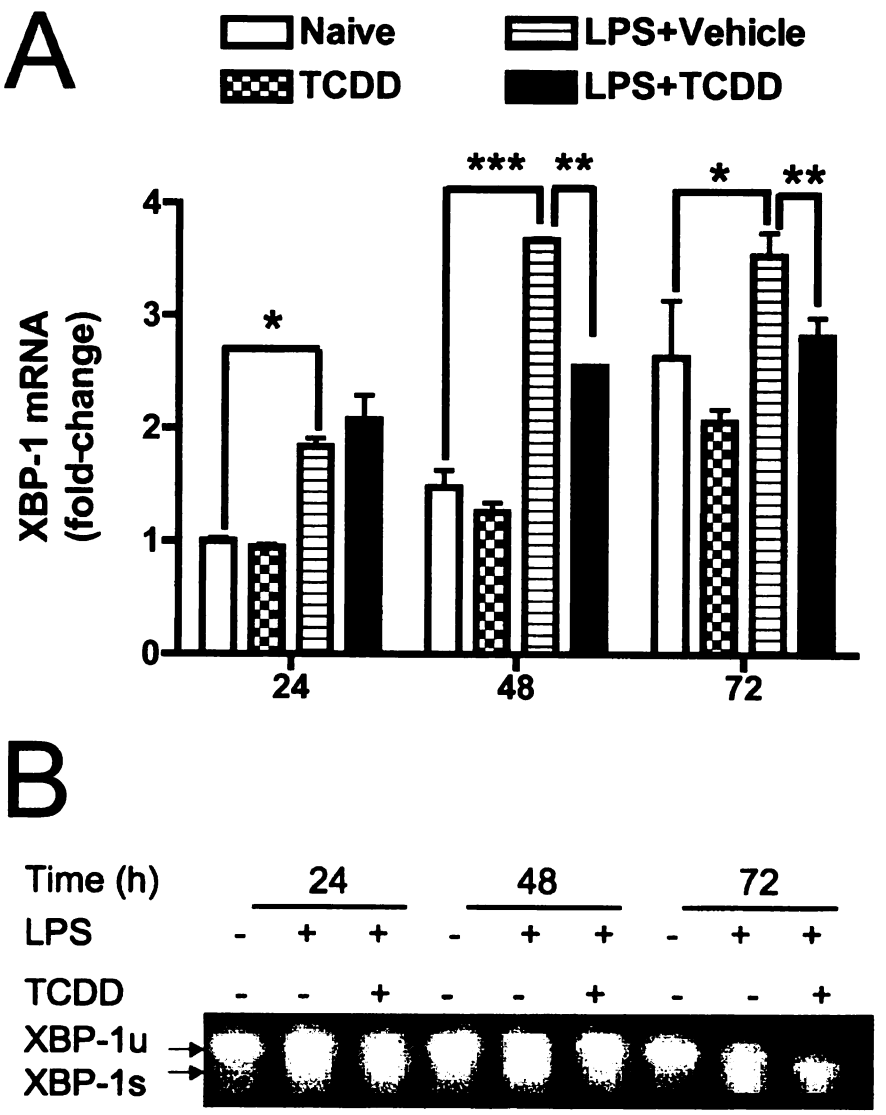
The following experiments were designed to determine whether or not LPS and/or

TCDD treatment affects the post-transcriptional modification of XBP-1. Transcriptional activity of XBP-1 is known to be enhanced by a splicing event, which removes a 26-nt-long fragment from XBP-1 mRNA, resulting in an open reading frame shift and yielding a longer XBP-1 protein that is more potent as a transcription factor (Calfon et al., 2002). Amplification of XBP-1 mRNA from CH12.LX cells activated with LPS in the presence or absence of TCDD by PCR primers that span the splice region detected two amplicon bands: a 171 bp amplicon representing the unspliced (XBP-1u) and a 145 bp amplicon representing the active spliced form of XBP-1 (XBP-1s) (Fig. 13B). Double bands were observed following LPS treatment at 24, 48 and 72 h indicating that both splice variants were present. By contrast, TCDD treatment significantly reduced the amount of XBP-1s isoform at 48 and 72 h.

C. TCDD decreased the protein levels of the activated form of XBP-1, XBP-1s

To determine whether the TCDD-mediated changes in XBP-1 mRNA expression and modification persist at the protein level, XBP-1 protein expression by was assessed by Western blotting. Due to a shift in the XBP-1 open reading frame, XBP-1s isoform yields a fully functional transcription factor of larger molecular weight (54 kDa), whereas the XBP-1u isoform is of lesser molecular weight (33 kDa) and likely does not contribute to the immunoglobulin secretion (Calfon et al., 2002). The expression of XBP-1s and XBP-1u was determined by Western blotting using an anti-XBP-1 antibody capable to detect XBP-1s and XBP-u, and anti-β-actin antibody as a loading control (Fig. 14). The detected immunoreactive bands were discriminated based on molecular weight. The

Figure 13. Effects of TCDD on XBP-1 mRNA levels in CH12.LX cells. A) Naïve or LPS (5 μg/ml)-activated CH12.LX cells were treated with TCDD (10 nM) and/or vehicle (0.01% DMSO). Cells were harvested at the indicated times post LPS-activation and quantitative RT-PCR was performed to determine the total levels of XBP-1 transcripts. The determinations of XBP-1 transcripts were normalized to 18S mRNA, which was used as a loading control. The fold-change in XBP-1 mRNA levels relative to naïve samples at 24 h, which was arbitrarily given the value of 1, is represented on the *y*-axis. Results represent the mean ± S.E. of triplicate determinations in each treatment group from at least three separate experiments. Statistical significance is denoted as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. B) Cells were harvested at the indicated times and amplified by RT-PCR using primers spanning a splicing site, and resulting XBP-1 amplicons were resolved on 4% agarose gel. Results are representative of at least three separate experiments.


A
Naive
LPS+Vehicle
TCDD
LPS+TCDD
XBP-1 mRNA (fold-change)
0
1
2
3
4
*
***
**
*
**
24
48
72
B
Time (h)
24
48
72
LPS
- + + - + + - + +
TCDD
- - + - - + - - +
XBP-1u
XBP-1s

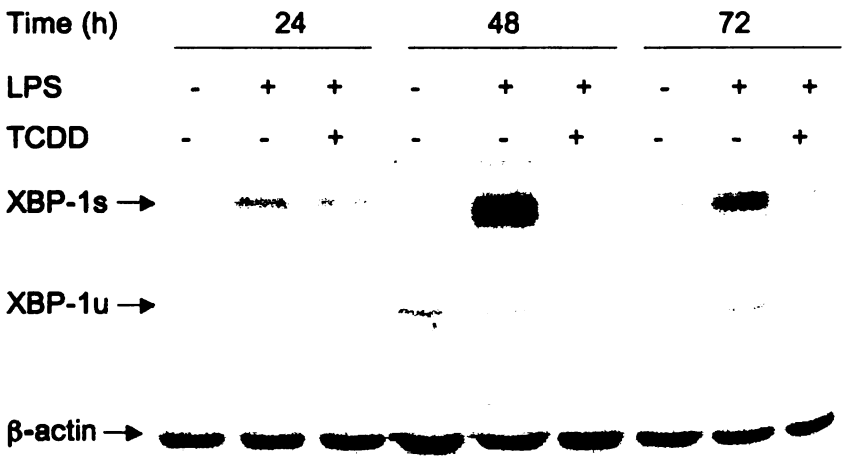


Figure 14. TCDD-mediated suppression of the XBP-1 protein in LPS-activated CH12.LX cells. Naive or LPS-activated CH12.LX cells treated with TCDD (10 nM) and/or vehicle (0.01% DMSO) were harvested at the indicated times post LPS-activation and whole cell lysates were isolated. Proteins (25 μg/lane) were resolved on a 10% SDS-PAGE and probed with an anti-XBP-1 antibody. For loading control, blots were stripped and re-probed with anti-β-actin antibody. Results are representative of three separate experiments.

expression of the two XBP-1 isoforms was induced by LPS-activation of CH12.LX cells at 24, 48 and 72 h (Fig. 14). In LPS-activated CH12.LX cells, the expression of the XBP-1s isoform was dramatically higher as compared with XBP-1u, with the largest difference detected at 48 h. Interestingly, the suppression of XBP-1s by TCDD was stronger on the protein level than on the mRNA level, possibly indicating that TCDD treatment increased the degradation rate or decreased the translation rate of XBP-1s protein. Importantly, TCDD treatment suppressed the expression of XBP-1u and XBP-1s at all time points. This effect was most prominent in the active XBP-1 form, XBP-1s, in concordance with the suppression of the IgM response by TCDD.

III. TCDD dysregulated transcription factors crucial for the terminal B cell differentiation program

A. TCDD-induced changes in transcription factor Pax5

a. TCDD attenuated the LPS-induced down-regulation of Pax5 protein in LPS-activated CH12.LX cells.

In light of the suppressed IgH, Igκ and IgJ expression (Yoo et al., 2004), suppression of the IgM response, and reduction in the expression of XBP-1 observed in LPS-activated CH12.LX cells in the presence of TCDD, studies were undertaken to characterize the effect of TCDD on Pax5, a transcriptional modulator of IgH, Igκ, IgJ and XBP-1 (Neurath et al., 1994; Reimold et al., 1996; Singh and Birshtein, 1993; Tian et al., 1997). Expression of the Pax5 protein was characterized in CH12.LX cells by flow cytometric analysis, facilitating the evaluation of individual cells in contrast to prior Western blotting studies (Yoo et al., 2004) examining large numbers of pooled cells

(Fig. 15). LPS-activated CH12.LX exhibited a gradual reduction in Pax5 protein between 24 and 72 h. By contrast, in the CH12.LX cells that were LPS-activated in the presence of TCDD, Pax5 protein levels remained abnormally elevated compared to the time-matched LPS-activated control. This result correlates with the observed temporal profile of Pax5 mRNA expression in LPS-activated CH12.LX cells in the presence or absence of TCDD.

b. Characterization of Pax5 isoforms in CH12.LX cells

Since TCDD attenuated the suppression of Pax5 at the protein level in LPS-activated CH12.LX cells, a detailed analysis of Pax5 mRNA levels in LPS-activated CH12.LX cells in the presence or absence of TCDD was performed. Pax5 transcripts are known to undergo alternative splicing of exons 2 and/or 6-10, resulting in removal of functionally important domains (Fig 16A), (Zwollo et al., 1997). A hypothesis that post-transcriptional modifications of Pax5 following LPS and/or TCDD treatment could differentially regulate the expression of the full-length Pax5 isoform, Pax5a was tested. PCR primers and probe sets were designed to differentially spliced regions in Pax5 mRNA to determine whether the aforementioned splice events occur in CH12.LX cells (Fig.16B). In naïve CH12.LX cells four distinct Pax5 amplicons, I, II, III and IV, representative of at least three Pax5 isoforms, were detected (Fig. 16C). Furthermore, the expression of amplicons I and III was approximately one hundred-fold greater than the expression of amplicons II and IV, demonstrating that Pax5a is the major isoform expressed in resting CH12.LX cells (Fig. 16C).

Figure 15. Flow cytometric analysis of Pax5 in LPS-activated CH12.LX cells. Naïve or LPS (5 μg/ml)-activated CH12.LX cells were treated with TCDD (10 nM) and/or vehicle (0.01% DMSO). Cells were harvested at 24, 48 and 72 h, incubated with anti-Pax5-FITC or isotype control antibodies and analyzed by flow cytometry. Results are representative of triplicate determinations in each treatment group from three separate experiments.

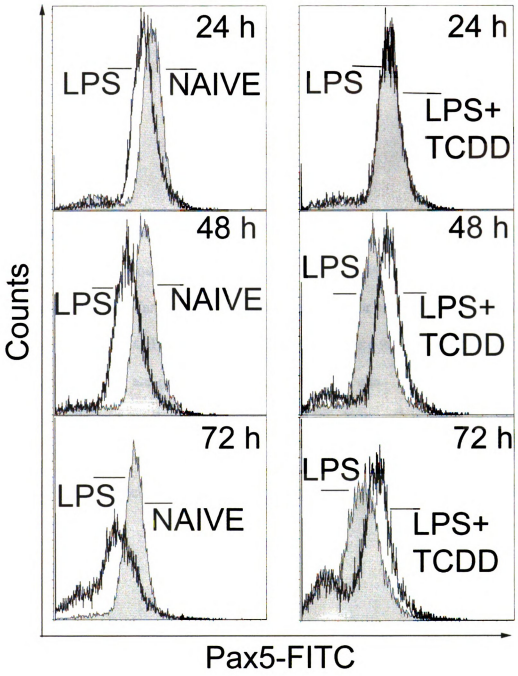
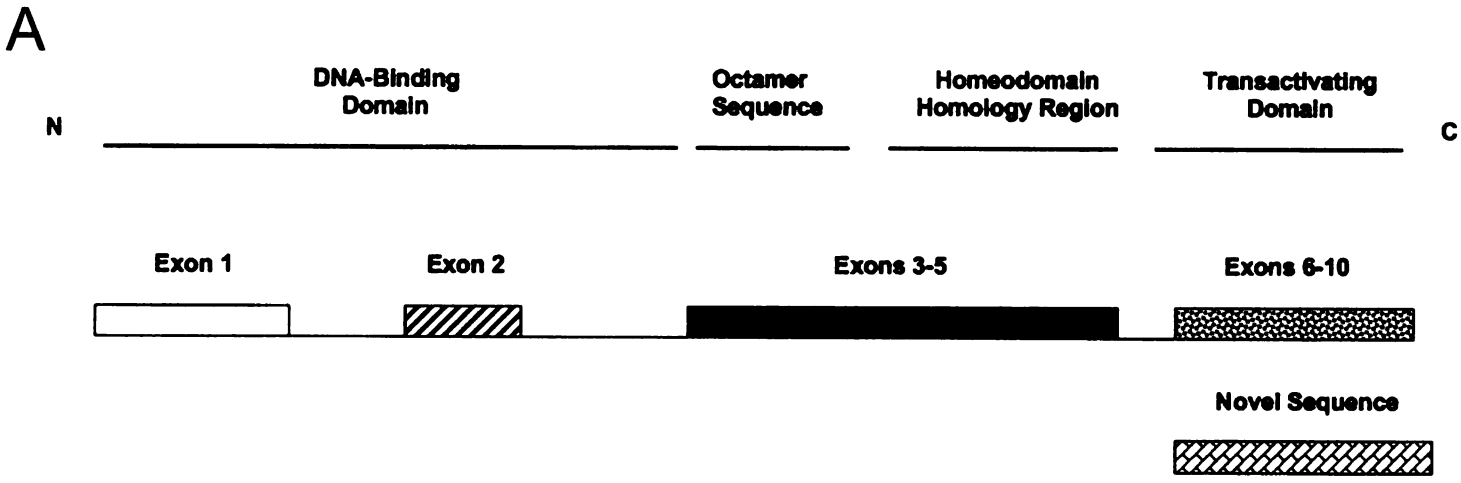
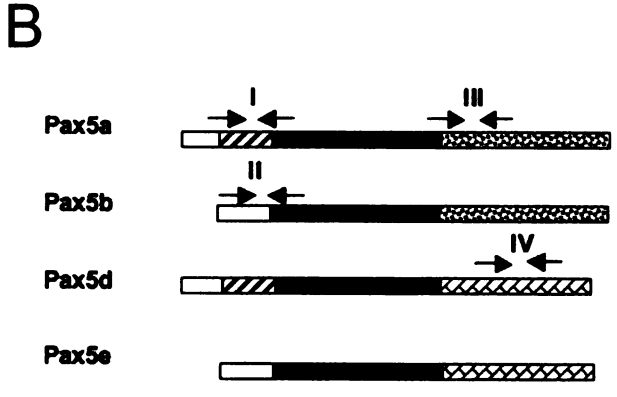
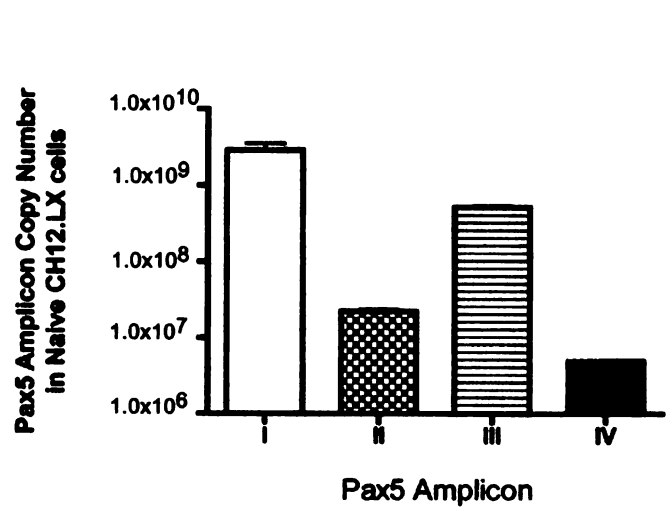

24 h
LPS
NAIVE
24 h
LPS
LPS+
TCDD
48 h
LPS
NAIVE
48 h
LPS
LPS+
TCDD
72 h
LPS
NAIVE
72 h
LPS
LPS+
TCDD
Counts
Pax5-FITC

Fig. 16. Identification of Pax5 isoforms in CH12.LX cells. A) Structure of Pax5 isoforms that have been previously characterized in B cells (adopted from Zwollo et al., 1997). B) Location of TaqMan primers and probes utilized for the detection of Pax5 amplicons I, II, III and IV in CH12.LX cells. C) Four Pax5 splice variants, identified by amplicons I, II, III and IV, are present in resting CH12.LX cells. Total mRNA from naïve CH12.LX cells was analyzed by quantitative RT-PCR. Copy number was determined for each amplicon using amplicon standard curves. Results represent the mean ± S.E. of triplicate determinations in each treatment group from two separate experiments.


A
N
DNA-Binding Domain
Octamer Sequence
Homeodomain Homology Region
Transactivating Domain
C
Exon 1
Exon 2
Exons 3-5
Exons 6-10
Novel Sequence


B
Pax5a
Pax5b
Pax5d
Pax5e
I
II
III
IV


C
Pax5 Amplicon Copy Number in Naive CH12.LX cells
1.0x10^10
1.0x10^9
1.0x10^8
1.0x10^7
1.0x10^6
I
II
III
IV
Pax5 Amplicon

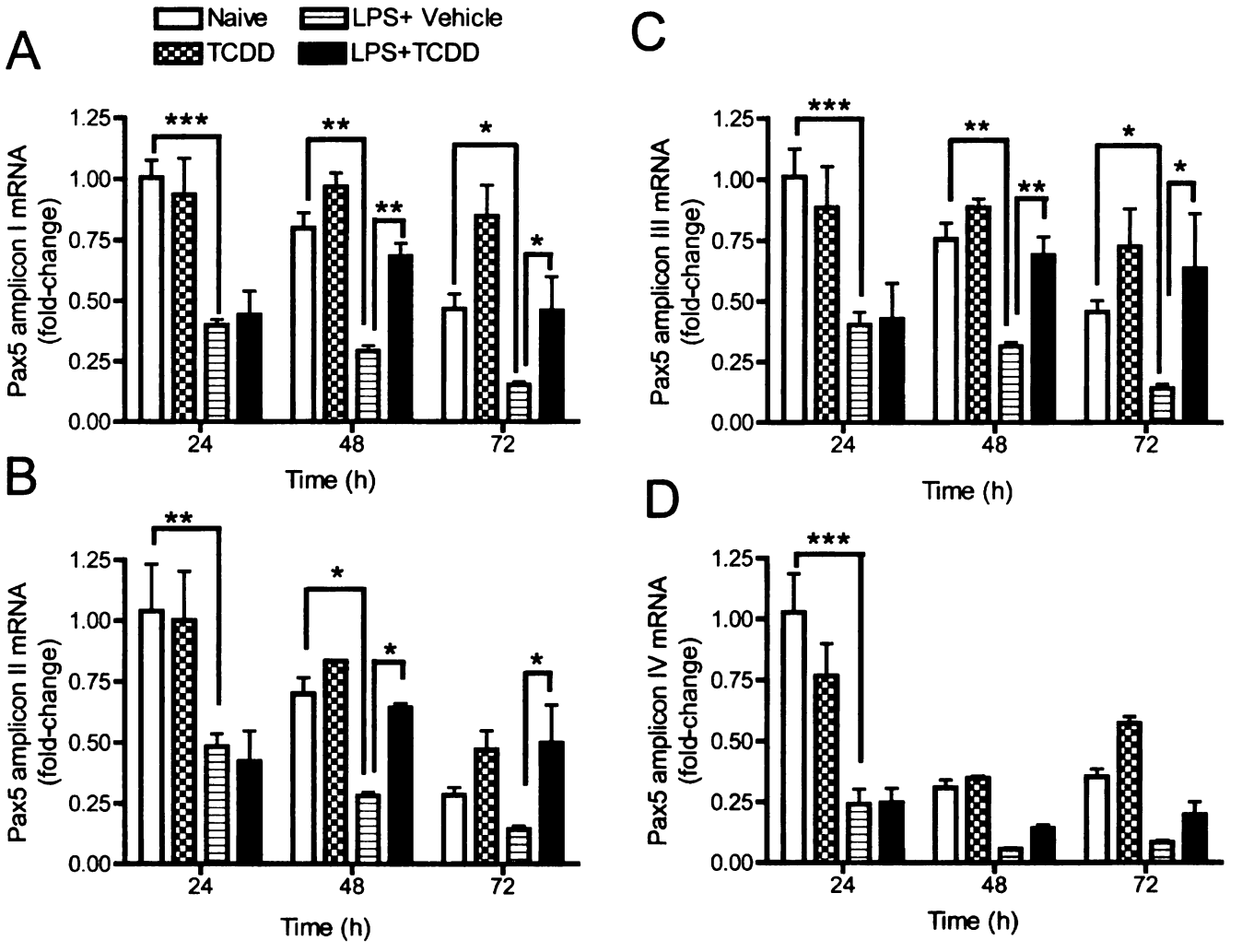
As determined by quantitative PCR, the levels of amplicons I, II and III were downregulated by LPS between 24 and 72 h (Fig. 17). In contrast, in the presence of 10 nM TCDD, a concentration that produces approximately 90% suppression of the LPS-induced IgM response with no effect on CH12.LX cell viability (Yoo et al., 2004), amplicons I, II and III were abnormally elevated at 48 and 72 h when compared with the time-matched LPS-activated control. Amplicon IV, representative of the Pax5 novel sequence, was suppressed by LPS-activation at 24 h, but the effect of LPS at 48 and 72 h and the effect of TCDD between 24 and 72 h were not significant. Collectively, the synchronous temporal profile of expression for Pax5 amplicons I, II, III and IV ruled out post-transcriptional regulation of the Pax5 gene by alternative splicing at exon 2 or exons 6-10 as a result of LPS-activation or TCDD treatment, but suggested a common mode of dysregulation of the Pax5 isoforms by TCDD at the transcriptional level.

c. Regulation of Pax5 promoter in the presence of TCDD

(i). Identification of a DRE-like site in the Pax5 promoter.

To test the hypothesis that the synchronous attenuation of down-regulation of the four Pax5 isoforms by TCDD is mediated through DRE binding within the Pax5 promoter, the first 1,000 bp of Pax5 promoter were examined for DRE-like sites. A motif search of the Pax5 promoter region identified one DRE-like site possessing the core DRE (GCGTG) sequence (Swanson et al., 1993) in a 3' to 5' orientation, located 506 bp upstream to the putative Pax5 transcription start site (DRE-506). A radiolabeled double stranded oligonucleotide probe containing the DRE3 sequence from the murine CYP1A1 promoter (Denison and Yao, 1991), a well characterized high affinity AHR-ARNT binding motif,

Figure 17. Levels of Pax5 transcripts in LPS-activated CH12.LX cells are altered by TCDD treatment. CH12.LX cells were activated with LPS (5 μg/ml) in the presence of TCDD (10 nM) and/or vehicle (0.01% DMSO), harvested at the indicated times post LPS-activation and analyzed for the levels of Pax5 amplicons. 18S ribosomal subunit amplification was used as a loading control. The fold-change in Pax5 transcript levels relative to untreated samples at 24 h, which was arbitrarily given the value of 1, is represented on the *y*-axis. Results represent the mean ± S.E. of triplicate determinations in each treatment group from at least three separate experiments. Statistical significance was determined using a two way ANOVA and Bonferroni post-hoc test. Statistical significance is denoted as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

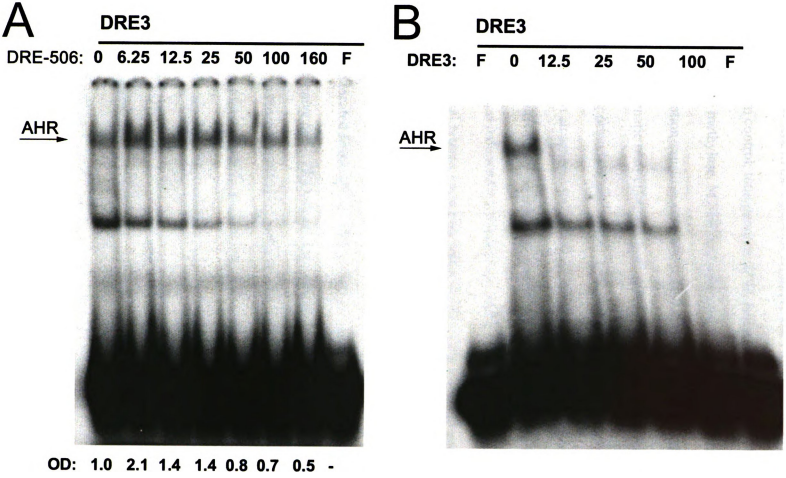

A
Naive
LPS+ Vehicle
TCDD
LPS+TCDD
Pax5 amplicon I mRNA (fold-change)
1.25
1.00
0.75
0.50
0.25
0.00
***
**
*
**
*
24
48
72
Time (h)
B
Pax5 amplicon II mRNA (fold-change)
**
*
*
*
C
Pax5 amplicon III mRNA (fold-change)
D
Pax5 amplicon IV mRNA (fold-change)

was used as a positive control for assessing AHR DNA-binding activity in EMSA analyses. The DRE-506 probe produced no detectable gel shift band (data not shown). A consequent EMSA competition study showed that excess cold DRE-506 competitor displaced the DRE3 probe from binding to the AHR protein in a molar excess-dependent manner (Fig 18A), however, in order to compete for DRE3, a large molar excess of cold DRE-506 competitor was required. By comparison, only a modest excess of cold DRE3 used as competitor was sufficient to completely abrogate the DRE3 probe binding to AHR (Fig. 18B), suggesting no specific protein binding to DRE-506.

(ii). **TCDD alters the extent of cytosine methylation at the HpaII-sensitive restriction site in the Pax5 promoter.**

Methylation of the 5'CpG motifs is an important mechanism of gene transcription regulation during development and differentiation stages in many cell types. During the process of terminal B cell differentiation, methylation of specific 5'CpG sites in the Pax5 promoter contributes to silencing of the Pax5 gene (Danbara et al., 2002). Because an attenuation of Pax5 suppression has been detected in LPS-activated CH12.LX cells treated with TCDD, we aimed to determine whether changes in the level of methylation at the critical 5'CpG sites in Pax5 promoter are contributing to this effect. For this purpose, LPS-activated (5 μg/ml) CH12.LX cells were maintained in culture for 72 h in presence or absence of TCDD. Naïve or TCDD-treated CH12.LX cells were used as control. Total genomic DNA isolated from each of the experimental groups was subjected to restriction by each one of the three methylation-sensitive restriction enzymes

Figure 18. Characterization of a DRE-like site in the Pax5 promoter by competition gel shift assay. CH12.LX cells were incubated with TCDD (30 nM) for 1 h at 37 °C and nuclear protein extracts were isolated. Nuclear protein (20 μg) and the ^{32}P-labeled DRE3 binding nucleotide were incubated with the indicated molar excess of A) cold DRE-506 or B) cold DRE3 for 30 min and resolved on a 4% nondenaturing PAGE gel, dried on 3-mm filter paper, and analyzed by autoradiography. Binding of AHR to the ^{32}P-labeled DRE3 was quantified by densitometry. The adjusted volumes (OD x area) for all samples are expressed as fold-change from AHR binding in the absence of cold competitor. Results are representative of three separate experiments. F denotes free probe.


A
DRE3
DRE-506: 0 6.25 12.5 25 50 100 160 F
AHR
OD: 1.0 2.1 1.4 1.4 0.8 0.7 0.5 -
B
DRE3
DRE3: F 0 12.5 25 50 100 F
AHR

(BstUI, BssHII and HpaII), which recognize and restrict the unmethylated, but not the methylated sequences present in the CpG-rich region of the Pax5 promoter (Fig. 19). For positive methylation control, total genomic DNA isolated from naïve CH12.LX cells was treated with a CpG methylase, M.SssI, and subjected to restriction with the aforementioned methylation-sensitive enzymes alongside other experimental groups.

Following enzymatic restriction, the CpG-rich region of the Pax5 promoter was amplified by PCR using primers spanning the restriction sites, and the obtained PCR product was resolved on an agarose gel. No CpG methylation has been detected at the BssHII- and BstUI-sensitive sites, as demonstrated by the lack of detectable PCR product (Fig. 20). This effect was independent of treatment, and no PCR product was detected in either of the experimental groups, including naïve, TCDD, LPS + vehicle and LPS + TCDD. By contrast, restriction by the HpaII demonstrated notable differences between experimental groups (Fig. 21). Specifically, CpG methylation has been detected at the HpaII-sensitive site located from -104 to -109 respective to the Pax5 transcription start site (AF148961), in naïve and TCDD alone treated cells (Fig. 21A). LPS-activation of the CH12.LX cells reduced the extent of methylation at the HpaII-sensitive sites, as evidenced by reduced levels of PCR product resolved on the agarose gel (Fig. 21B). Finally, LPS+TCDD treatment restored the extent of CpG methylation to the levels observed in the naïve cells. The reduction of methylation at the HpaII-sensitive sites has been observed repeatedly in four separate experiments, and the difference between the mean values in LPS and LPS+TCDD groups was statistically significant at the $p<0.05$ level, (Fig. 21C).

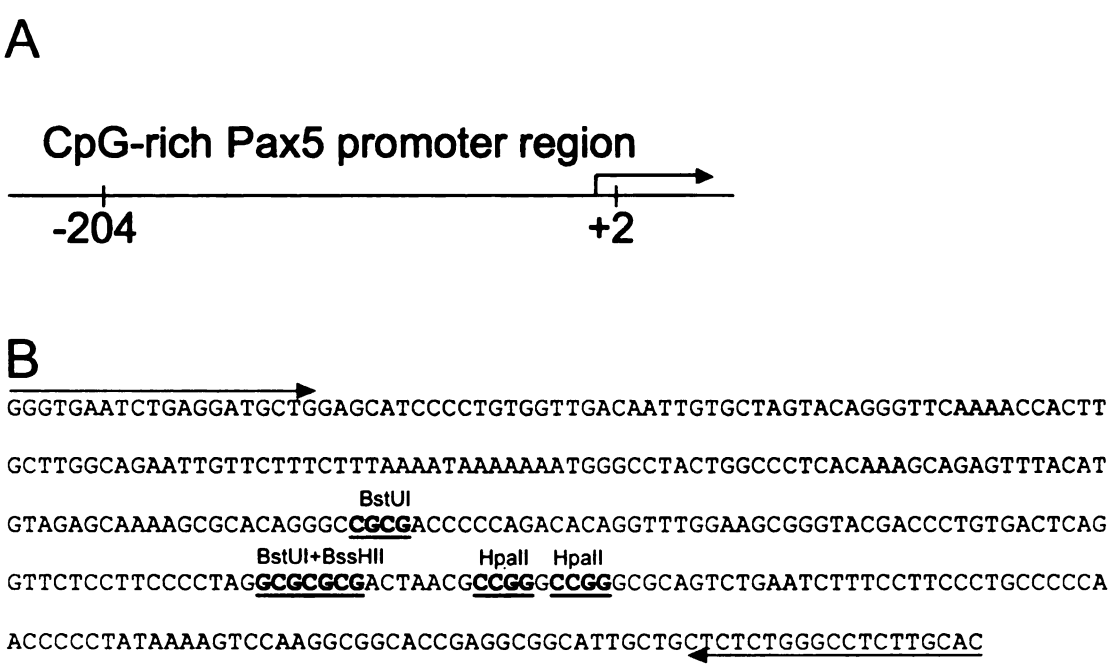


Figure 19. Location of methylation-sensitive enzymatic restriction sites within the Pax5 promoter. A) Schematic representation of the CpG-rich region in Pax5 promoter. B) The sequence of the Pax5 promoter region that was amplified by PCR to assess the restriction of the non-methylated CpG sites. The restriction sites for BstUI, BssHII and HpaII methylation-sensitive enzymes are bolded and underlined. Locations of PCR primers are indicated by arrows.

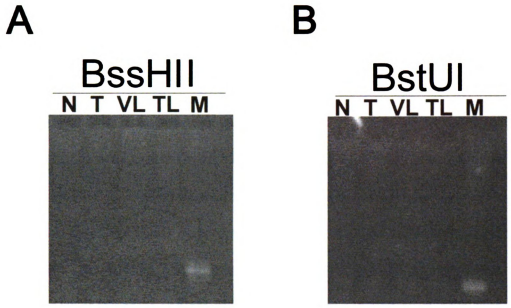
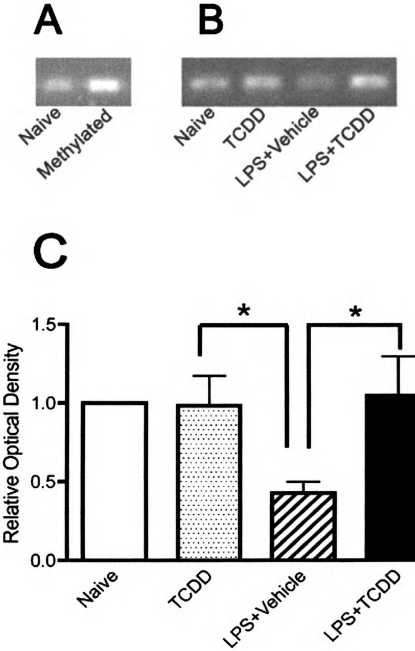


Figure 20. Analysis of BssHII- and BstUI-sensitive sites within the Pax5 promoter in naïve or LPS-activated CH12.LX cells treated with TCDD. CH12.LX cells were activated with LPS (5μg/ml) in the presence of TCDD (10 nM) and/or vehicle (0.01% DMSO) and harvested at 72 h post LPS-activation. Naïve and TCDD (10 nM) alone treated groups were used as comparative controls. Total genomic DNA was extracted for each experimental group, restricted by A) BssHII or B) BstUI, and amplified by PCR using primers spanning the restriction sites. Naïve CH12.LX cells subjected to M.SssI methylation were used as methylation positive control. PCR products were resolved on a 2% agarose gel. N-naïve, T-TCDD, VL-Vehicle+LPS, TL-TCDD+LPS, M-positive methylation control.

Figure 21. Analysis of HpaII-sensitive sites within the Pax5 promoter in naïve or LPS-activated CH12.LX cells treated with TCDD. A) Genomic DNA from naïve or M.SssI methylase-treated CH12.LX cells was restricted by HpaII methylation-sensitive enzyme and amplified by PCR. The PCR product was then resolved on a 2% agarose gel and autoradiographed. B) CH12.LX cells were activated with LPS (5 μg/ml) in the presence of TCDD (10 nM) and/or vehicle (0.01% DMSO) and harvested at 72 h post LPS-activation. Naïve and TCDD (10 nM) alone groups were used as methylation comparative control. Total genomic DNA for each experimental group was restricted by HpaII and amplified by PCR using primers spanning the restriction site. PCR products were resolved on a 2% agarose gel. C) Relative optical intensity for each experimental group in (B) was determined by autoradiography. The ratio of relative optical intensity of the treatment groups to the naive group, which was arbitrarily given the value of 1, is represented on the *y*-axis. Results reflect the mean $\pm$ S.E. of single determinations in each treatment group from four separate experiments. Statistical significance was determined using a two way ANOVA and Bonferroni post-hoc test; * denotes $p<0.05$.

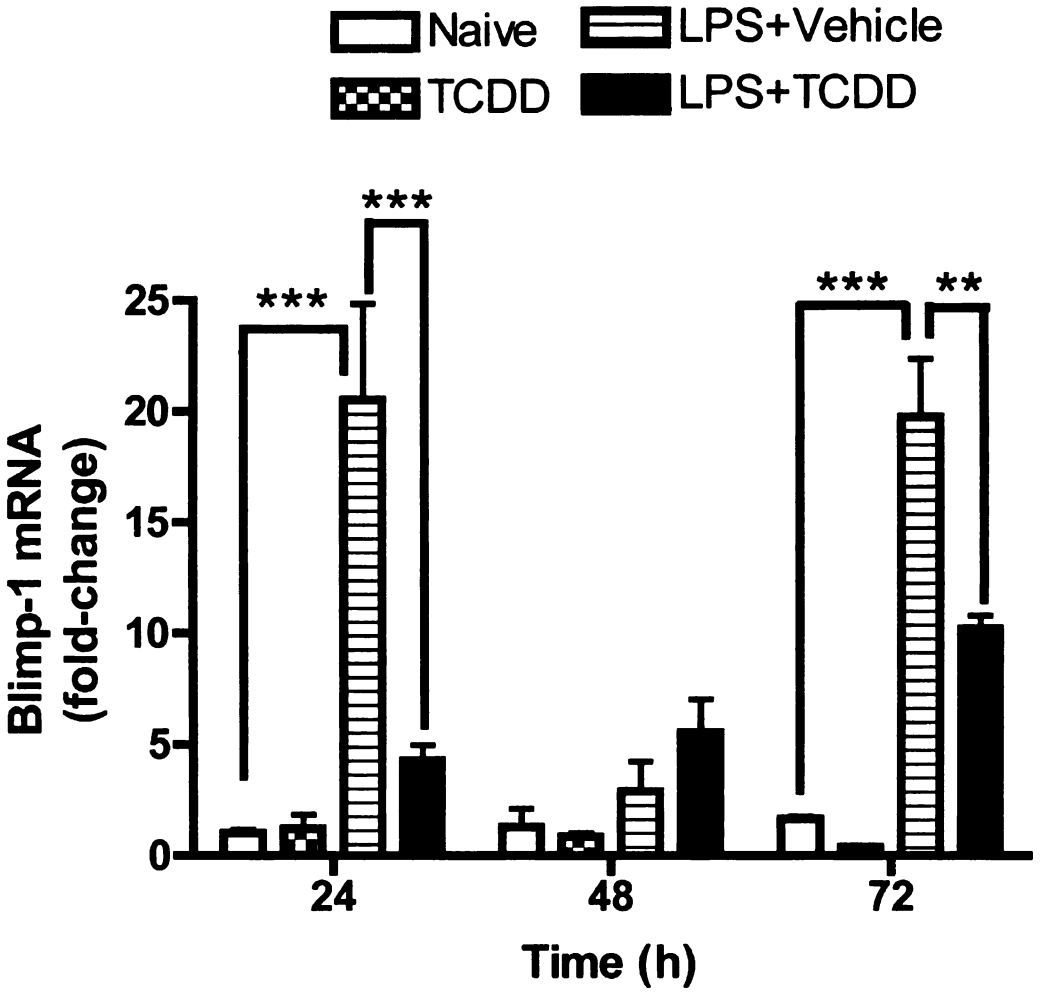

A
B
Naive
Methylated
Naive
TCDD
LPS+Vehicle
LPS+TCDD
C
Relative Optical Density
1.5
1.0
0.5
0.0
*
*
Naive
TCDD
LPS+Vehicle
LPS+TCDD

B. **Dysregulation of Blimp-1 by TCDD**

a. **TCDD altered Blimp-1 mRNA levels in LPS-activated CH12.LX cells**

Blimp-1 is a transcriptional repressor, whose expression is induced in differentiating B cells. Repression of Pax5 by Blimp-1 is a necessary step in the B cell differentiation program, and is mediated by Blimp-1 binding to its cognate motif located within the Pax5 promoter (Lin et al., 2002). In LPS-activated CH12.LX cells treated with TCDD, Blimp-1 DNA-binding activity within the Pax5 promoter was suppressed (Schneider et al., in preparation). In addition, previous studies implicated the dysregulation of Pax5 in the TCDD-induced suppression of the IgM response (Yoo et al., 2004) and B cell differentiation, linking Blimp-1 to the dysregulation of B cell differentiation by TCDD. In addition, Blimp-1 is involved in regulation of a number of genes modulated during B cell differentiation, for which direct regulation by Pax5 has not been established, such as MHC class II (Piskurich et al., 2000), whose downregulation is attenuated by TCDD in LPS-activated CH12.LX cells. Therefore, the effects of TCDD on Blimp-1 were investigated. The mRNA levels of Blimp-1 were monitored over a 72 h period in LPS-activated CH12.LX cells (Fig. 22). Blimp-1 mRNA levels were strongly induced by LPS activation at 24 and 72 h, as compared to the time-matched naïve (*i.e.*, untreated) controls. In contrast, TCDD treatment of CH12.LX cells at 24 and 72 h resulted in a marked decrease in Blimp-1 mRNA levels compared to the time-matched LPS-activated controls (Fig 22). Blimp-1 mRNA levels were strikingly low in LPS as well as in LPS plus TCDD treated cells at 48 h, when compared to their respective treatment groups at 24 and 72 h.

Figure 22. Blimp-1 mRNA levels are suppressed by TCDD in LPS-activated CH12.LX cells. CH12.LX cells were activated with LPS (5 μg/ml) in the presence of TCDD (10 nM) and/or vehicle (0.01% DMSO), harvested at the indicated times post LPS-activation and analyzed for the levels of Blimp-1 mRNA. Samples were normalized per 18S ribosomal subunit amplification, which was used as loading control. The fold-change in Blimp-1 mRNA levels relative to naïve sample at 24 h, which was arbitrarily given the value of 1, is represented on the *y*-axis. Results represent the mean ± S.E. of triplicate determinations in each treatment group from at least three separate experiments. Statistical significance was determined using a two way ANOVA and Bonferroni post-hoc test and is denoted as ** $p<0.01$, *** $p<0.001$.


Naive
LPS+Vehicle
TCDD
LPS+TCDD
***
***
***
**
25
20
15
10
5
0
Blimp-1 mRNA
(fold-change)
24
48
72
Time (h)

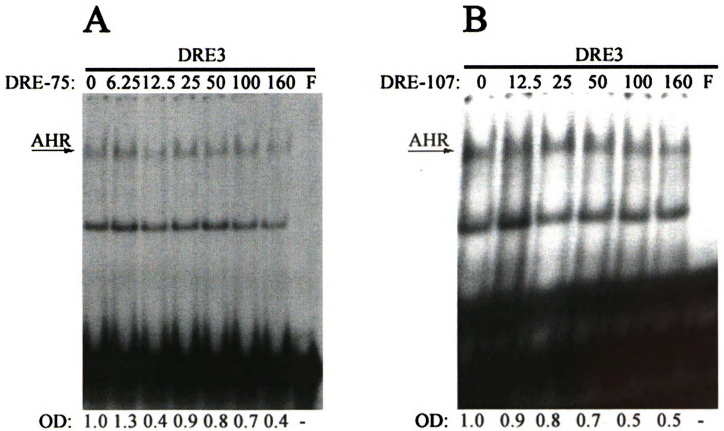
Repeatedly, a biphasic profile of Blimp-1 mRNA levels post LPS activation was observed in our studies, a phenomenon which has been previously described by others (Turner et al., 1994).

b. Studies of DNA-binding activity within the Blimp-1 promoter in the presence of TCDD.

(i). DRE-like motifs identified within the Blimp-1promoter.

A detailed analysis of the first 2,000 bp of Blimp-1 promoter has identified two putative DRE motifs, based on sequence similarity with the consensus core DRE (GCGTG) and the composition of regions flanking the core (Yao and Denison, 1992). These motifs are located –75 and –107 bp upstream from the Blimp-1 gene and are designated here DRE-75 and DRE-107, respectively (Table 2). A radiolabeled double-stranded oligonucleotide probe containing the DRE3 sequence from the murine CYP1A1 promoter (Denison and Yao, 1991), which is a well-characterized high affinity AHR-ARNT binding motif, was used as a positive control for assessing AHR DNA-binding activity in EMSA analyses. Using nuclear proteins isolated from TCDD-treated CH12.LX cells, no TCDD-inducible specific DNA-binding activity was detected to either DRE-75 or DRE-107 (data not shown). Co-incubation of a previously characterized radiolabeled positive control DRE3 motif with increasing amounts of cold DRE-75 or DRE-107 reduced gel retardation by the radiolabeled DRE3 in a molar excess-dependent manner (Fig. 23A, 23B). However, 50% reduction in gel retardation by radiolabeled DRE3 required at least 100-fold excess of cold DRE-75 or DRE-107 competitor.

Figure 23. Identification of DRE-like motifs within the Blimp-1 promoter. Two DRE-like sites within the Blimp-1 promoter were characterized by competition EMSA. CH12.LX cells were incubated with TCDD (30 nM) for 1 h at 37°C and nuclear protein extracts were isolated. Nuclear protein (20 μg/lane) and the ^{32}P-labeled DRE3 nucleotide were incubated with the indicated molar excess of A) cold DRE-75 or B) cold DRE-107 for 30 minutes and resolved on a 4% non-denaturing PAGE gel, dried on 3-mm filter paper and analyzed by autoradiography. Binding of AHR to the ^{32}P-labeled DRE3 was quantified by densitometry. The adjusted volumes (OD x area) for all samples are expressed as fold-change from AHR binding in the absence of cold competitor. Results are representative of three separate experiments. F denotes free probe.


A
DRE3
DRE-75: 0 6.25 12.5 25 50 100 160 F
AHR
OD: 1.0 1.3 0.4 0.9 0.8 0.7 0.4 -
B
DRE3
DRE-107: 0 12.5 25 50 100 160 F
AHR
OD: 1.0 0.9 0.8 0.7 0.5 0.5 -

This finding is in contrast with the competition EMSA performed for radiolabeled and cold DRE3 probes, where 12.5-fold molar excess of cold DRE3 was sufficient to completely abrogate the radiolabeled DRE3 gel shift band (Fig. 18), suggesting negligible or no specific protein binding to DRE-75 or DRE-107.

(ii). **Characterization of TRE-like motifs within the Blimp-1 promoter**

Since no TCDD-inducible specific protein DNA-binding activity was detected to DRE-75 and DRE-107, the role of additional transcriptional regulators of Blimp-1 was investigated. The ability of AP-1 transcription factors to induce Blimp-1 transcription has been recently reported (Ohkubo et al., 2005). We identified three TRE-like motifs within the first 2,000 bp of Blimp-1 promoter. These motifs are located -47, -1060 and -1610 bp upstream from the Blimp-1 transcription start site and are designated here TRE-47, TRE-1060 and TRE-1610, respectively (Fig. 24A). Notably, TRE-1610 corresponds to the AP-1-binding motif identified by Ohkubo and co-workers (Ohkubo et al., 2005). EMSAs were performed using radiolabeled probes possessing TRE-47, TRE-1060 and TRE-1610 (Fig. 24B). A consensus TRE motif (Faubert and Kaminski, 2000) containing the core TGACTCA (Novak et al., 1990) was used as a positive, as well as comparative, control for AP-1 DNA-binding activity. Gel retardation bands indicative of background AP-1 DNA-binding activity were detected in naïve cells incubated with the TRE consensus, TRE-47, TRE-1060 and TRE-1610 probes (Fig. 24B). The DNA-protein complexes containing theTRE-like probes migrated slightly further than the TRE consensus-containing complex, indicating that different AP-1 proteins might be participating in the complexes formed by TRE-like probes. The AP-1 DNA-binding activity was strongly induced 2 h after LPS treatment to the consensus TRE, TRE-1060

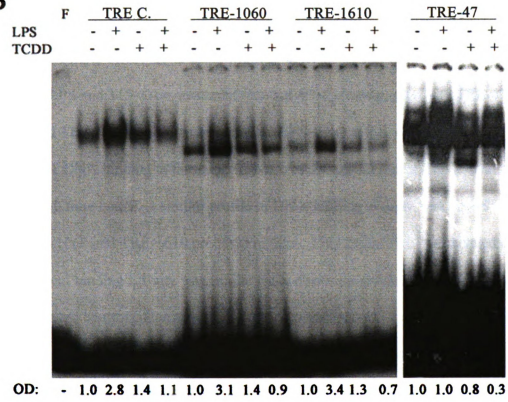
Figure 24. Identification of three TRE-like motifs within the Blimp-1 promoter.

A) Schematic of the three TRE-like motifs identified within the Blimp-1 promoter. B) CH12.LX cells were activated with LPS (5 μg/ml) in the presence of TCDD (10 nM) and/or vehicle (0.01% DMSO) and harvested 2 h post LPS-activation. Nuclear protein (20 μg/lane) and the ^{32}P-labeled TRE consensus motif (TRE C.) or putative TRE-like motifs from Blimp-1 promoter (TRE-47, TRE-1016, TRE-1610) were incubated for 30 min and resolved on a 4% nondenaturing PAGE gel, dried on 3-mm filter paper and analyzed by autoradiography. Protein binding to the ^{32}P-labeled TRE motifs was quantified by densitometry. The adjusted volumes (OD x area) for all samples are expressed as fold-change from naïve control. Results are representative of three separate experiments. F denotes free probe.

A


Blimp-1
-1610
-1060
-47
+1

B


F
TRE C.
TRE-1060
TRE-1610
TRE-47
LPS
TCDD
OD: - 1.0 2.8 1.4 1.1 1.0 3.1 1.4 0.9 1.0 3.4 1.3 0.7 1.0 1.0 0.8 0.3

and TRE-1610 probes, but not to TRE-47 probe. TCDD treatment of LPS-activated cells resulted in reduced protein binding to all four probes when compared to LPS alone (Fig 24B). Interestingly, TCDD treatment of non-activated CH12.LX cells at 2 h resulted in a very modest induction of protein binding to probes TRE-1060 and TRE-1610, but not TRE-47, when compared to the time matched naïve control (Fig. 24B). The induction of AP-1 DNA-binding activity by TCDD alone was short-lived and was not observed at 24, 48 and 72 h (Fig. 25).

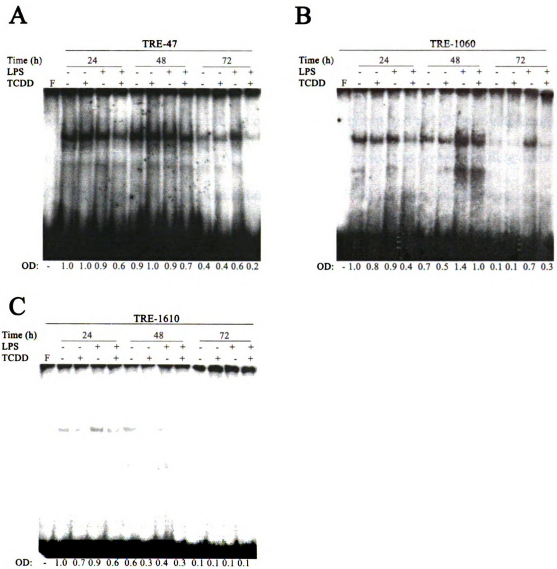
(iii). **TCDD alters AP-1 DNA-binding activity within the Blimp-1 promoter**

Since LPS and TCDD co-treatment reduced AP-1 binding activity to TRE-47, TRE-1060 and TRE-1610 probes as compared to LPS alone at 2 h, we examined the profile of AP-1 DNA binding to these motifs between 24 and 72 h post LPS activation. During the 72 h time course, a similar profile of DNA-binding activity was observed for TRE-47, TRE-1060 and TRE-1610 probes (Fig. 25A, 25B, 25C). In LPS-activated cells, the DNA-protein binding activity was similar to, or above the levels observed in time-matched untreated controls. Conversely, in LPS-activated and TCDD treated cells, protein binding to TRE-47, TRE-1060 and TRE-1610 probes was reduced when compared with CH12.LX cells activated with LPS in the absence of TCDD as well as untreated time-matched control group.

(iv). **The suppression of AP-1 DNA-binding activity in Blimp-1 promoter is dependent on TCDD concentration**

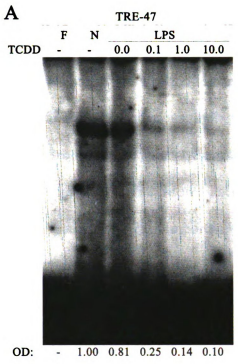
To establish a causal relationship between TCDD treatment and the suppression

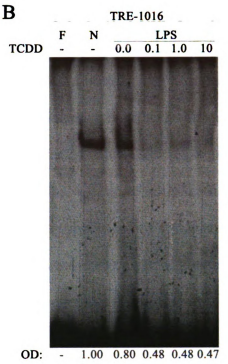
Figure 25. TCDD alters the kinetics of AP-1 binding in the Blimp-1 promoter. CH12.LX cells were activated with LPS (5 μg/ml) in the presence of TCDD (10 nM) and/or vehicle (0.01% DMSO) and harvested at the indicated times post LPS-activation. Nuclear protein (20 μg/lane) and each ^{32}P-labeled TRE-like motif was incubated for 30 min and resolved on a 4% nondenaturing PAGE gel, dried on 3-mm filter paper and analyzed by autoradiography. Protein binding to the ^{32}P-labeled A) TRE-47, B) TRE-1016, or C) TRE-1610 motifs from the Blimp-1 promoter was quantified by densitometry. The adjusted volumes (OD x area) for all samples are expressed as fold-change from naïve cells at 24 h. Results are representative of three separate experiments. F denotes free probe.

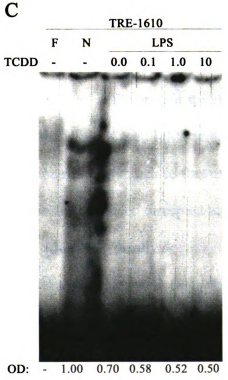

A
TRE-47
Time (h)
24
48
72
LPS
TCDD
F
OD: - 1.0 1.0 0.9 0.6 0.9 1.0 0.9 0.7 0.4 0.4 0.6 0.2
B
TRE-1060
Time (h)
24
48
72
LPS
TCDD
F
OD: - 1.0 0.8 0.9 0.4 0.7 0.5 1.4 1.0 0.1 0.1 0.7 0.3
C
TRE-1610
Time (h)
24
48
72
LPS
TCDD
F
OD: - 1.0 0.7 0.9 0.6 0.6 0.3 0.4 0.3 0.1 0.1 0.1 0.1

of AP-1 binding activity in the Blimp-1 promoter, LPS-activated CH12.LX cells were treated with TCDD over a broad concentration range (0.1 to 10 nM) employing the TRE-47, TRE-1060 and TRE-1610 motifs (Fig. 26). The TRE-47 containing ^{32}P-labelled probe was incubated with nuclear CH12.LX extracts collected 72 h after LPS-activation in presence of increasing concentrations of TCDD. As compared to LPS-activated non-treated cells, TCDD concentrations of 0.1 to 10 nM decreased the protein binding activity to TRE-47 in a concentration-dependent manner (Fig. 26A). Similar results were observed in EMSAs employing the TRE-1016 and TRE-1610 probes (Fig 26B, 26C), indicating a concentration-dependent reduction in AP-1 DNA-binding activity within the Blimp-1 promoter in TCDD-treated CH12.LX cells and providing additional evidence that the suppression of AP-1 DNA-binding activity within the Blimp-1 promoter is mediated by TCDD.

Figure 26. The suppression of AP-1 binding in the Blimp-1 promoter is dependent on TCDD concentration. CH12.LX cells were activated with LPS (5 μg/ml) in the presence of the indicated concentrations of TCDD (nM) and/or vehicle (0.01% DMSO) and harvested 72 h post LPS-activation. Nuclear protein (20 μg/lane) and the ^{32}P-labeled motifs TRE-47, TRE-1060 or TRE-1610 were incubated for 30 min and resolved on a 4% nondenaturing PAGE gel, dried on 3-mm filter paper and analyzed by autoradiography. Protein binding to the ^{32}P-labeled motifs TRE-47, TRE-1060 and TRE-1610 was quantified by densitometry. The adjusted volumes (OD x area) for all samples are expressed as fold-change from naïve (*i.e.*, untreated) cells (N). Results are representative of at least two separate experiments. F denotes free probe.


A
TRE-47
F N LPS
TCDD - - 0.0 0.1 1.0 10.0
OD: - 1.00 0.81 0.25 0.14 0.10


B
TRE-1016
F N LPS
TCDD - - 0.0 0.1 1.0 10
OD: - 1.00 0.80 0.48 0.48 0.47


C
TRE-1610
F N LPS
TCDD - - 0.0 0.1 1.0 10
OD: - 1.00 0.70 0.58 0.52 0.50

SUMMARY AND DISCUSSION

Suppression of the humoral immune responses is a well-established toxic outcome of exposure to TCDD. Numerous findings supporting this notion have been obtained *in vivo* and *in vitro* in murine splenocytes and B cell lines treated with TCDD (Holsapple et al., 1986a; Luster et al., 1988; Morris and Holsapple, 1991; Sulentic et al., 1998; Tucker et al., 1986; Vos et al., 1973). Importantly, only a modest suppression of B cell proliferation has been detected at TCDD doses that produced marked suppression of the Ig response, suggesting that Ig suppression by TCDD was not due to delayed proliferation (Holsapple et al., 1986a; Luster et al., 1988).

I. Effects of TCDD on surface markers of B cell differentiation

In agreement with the previous observations, in the experimental model of B cell differentiation used in the present studies, CH12.LX, TCDD treatment markedly suppressed the LPS-induced IgM secretion. In addition, changes in the B cell differentiation markers indicated that the differentiation process in LPS-activated CH12.LX cells was indeed disrupted by TCDD treatment. Specifically, MHC class II expression, which is typically downregulated during terminal B cell differentiation, remained elevated in LPS-activated CH12.LX cells treated with TCDD. Expression of syndecan-1, a surface proteoglycan involved in cell-to-cell interactions and a marker of plasma cell phenotype, was modestly induced by LPS. TCDD treatment appeared to reduce the syndecan-1 surface expression on LPS-activated CH12.LX cells, although to modest extent. These results were interpreted as follows: LPS treatment was able to

drive a fraction of CH12.LX cells toward plasma cell phenotype, which resulted in syndecan-1 expression. Even provided the modest induction of syndecan-1 expression by LPS, suppression of syndecan-1 was detected following TCDD treatment, pointing to the suppression of terminal differentiation in LPS-activated CH12.LX cells. As expected, surface expression of CD19, which is typically downregulated in the course of terminal B cell differentiation, was diminished on LPS-activated CH12.LX cells. TCDD treatment led to a trend towards de-repression of the CD19 mRNA levels, but not the CD19 protein levels. Thus, the surface expression of CD19 did not follow the proposed hypothesis. CD19 protein trafficking and stability, as well as additional factors may have contributed to the diminished CD19 surface expression in LPS-activated and TCDD treated CH12.LX cells. However, the trend towards de-repression of CD19 at the mRNA level favors our hypothesis that the repression of CD19 in LPS-activated CH12.LX cells is attenuated by TCDD treatment. Taken together, our results demonstrated attenuation of plasma cell phenotype by TCDD in LPS-activated CH12.LX cells.

II. Effects of TCDD on the crucial regulator of IgM secretion, XBP-1s

Another key factor associated with terminal B cell differentiation and the humoral immune response is XBP-1. Present studies demonstrated XBP-1 induction in LPS-activated CH12.LX cells, as evidenced by an increase in total XBP-1 and its active form XBP-1s. This effect was observed on mRNA and protein levels, in support of the notion that the molecular mechanism of immunoglobulin secretion is attenuated by TCDD treatment. These findings are in agreement with the TCDD-associated suppression of humoral immune responses, and with our hypothesis linking the Ig suppression by TCDD

to the impaired B cell differentiation.

III. **Effects of TCDD on transcriptional mediators of B cell differentiation**

Provided that in LPS-activated CH12.LX cells TCDD treatment produced marked IgM suppression, changes in differentiation surface markers and suppression of the key secretory factor XBP-1s, possible dysregulation of the transcriptional mechanism of B cell differentiation by TCDD has been investigated. To date, two transcription factors have been identified as critical components of the B cell differentiation pathway: Blimp-1 and Pax5.

We have previously shown that Pax5 DNA-binding activity, protein, and total mRNA levels were abnormally elevated in the presence of TCDD in LPS-activated CH12.LX cells (Yoo et al., 2004). This effect was concordant with the dysregulation of downstream target genes that are differentially expressed in plasma cells as compared to mature B cells, such as IgH, Igκ, IgJ, (Yoo et al., 2004), XBP-1, MHC class II, syndecan-1 and CD19. The attenuation of Pax5 repression by TCDD in LPS-activated CH12.LX cells has been observed at the mRNA and protein level. Four Pax5 transcripts have been identified, which are representative of at least three distinct Pax5 isoforms. Differential expression of Pax5 isoforms has been shown to affect Pax5 function during B cell development (Lowen et al., 2001; Zwollo et al., 1997), leading us to hypothesize that the dysregulation of Pax5 function by TCDD is mediated, in part, by a similar mechanism. However, in LPS-activated CH12.LX cells treated with TCDD the kinetics of mRNA expression for all Pax5 amplicons were strikingly similar, suggesting that differential Pax5 isoforms expression is not contributing to the dysregulation of Pax5 function by

TCDD. Notably the most abundant isoform detected in CH12.LX cells was the full-length Pax5 isoform, Pax5a, to which the majority of regulatory effects by Pax5 have been attributed. The fact that Pax5a was de-repressed by TCDD at the transcriptional level, lead to investigation of factors involved in the regulation of Pax5 promoter. One DRE-like motif has been identified in the first 1,000 bp of Pax5 promoter, termed here DRE-506. However, no inducible protein binding to this sequence was detected by EMSA, and competition EMSA with radiolabeled DRE3 from CYP1A1 promoter required a large excess of cold DRE-506, suggesting no specific protein binding to DRE-506. Therefore, we examined other mechanisms that may have contributed to the attenuation of Pax5 suppression by TCDD. Examination of CpG-rich region of the Pax5 promoter, which have been previously shown to contribute to Pax5 silencing in plasma cells through CpG methylation (Danbara et al., 2002), revealed one region that was differentially methylated in naïve and LPS-activated CH12.LX cells in the presence or absence of TCDD. This region contained two proximal sites prone to restriction by methylation-sensitive HpaII enzyme. The HpaII-sensitive region was methylated in naïve CH12.LX cells, but exhibited reduced methylation levels in LPS-activated CH12.LX cells. Notably, the background methylation levels at the HpaII-sensitive region were restored in LPS-activated CH12.LX cells in the presence of TCDD.

Interestingly, in our experimental system the reduced CpG methylation of the HpaII-sensitive site in the Pax5 promoter correlated with Pax5 de-repression, whereas typically reduction in CpG methylation in regulatory region of a gene leads to reduced gene expression. However, in some instances decreased methylation promotes gene silencing. Such is the case for Igf2 gene, which is silenced when the ICR regulatory

region is demethylated (Reik and Murrell, 2000). In addition, methylation of a specific intron region on the maternal copy of the mouse insulin-like growth factor type 2 receptor leads to the expression of the maternal copy of this gene (Stoger et al., 1993). In light of our results, it is plausible to speculate that methylation of the HpaII-sensitive site in the Pax5 promoter is contributing to the expression of Pax5 gene. However, the exact mechanism for this effect remains to be elucidated.

In light of the fact that Pax5 is dysregulated by TCDD at the transcriptional level, we examined the signaling events that precede Pax5 suppression during B cell differentiation, and probed for TCDD-mediated effects. The plasma cell commitment factor Blimp-1 is considered to be a critical regulator of the Pax5 gene (Angelin-Duclos et al., 2000; Lin et al., 2002), and the earliest differentiation factor known, whose activation is required for the terminal B cell differentiation (Kallies and Nutt, 2007). Our results indicate that Blimp-1 DNA-binding activity in the Pax5 promoter was suppressed by TCDD at all times up to 72 h as compared to the time-matched LPS-activated control. The repression of Blimp-1 DNA-binding activity by TCDD corresponded to the previously reported abnormal elevation in Pax5 mRNA, protein and DNA-binding activity in LPS-activated CH12.LX cells (Yoo et al., 2004), and attested to the involvement of Blimp-1 in the TCDD-induced attenuation of Pax5 repression. Blimp-1 mRNA levels were induced by LPS activation of CH12.LX cells as compared to the time-matched naïve control at 24 and 72, but not 48 h. The absence of Blimp-1 mRNA induction at 48 h was detected consistently throughout series of experiments, and although the explanation to this effect is not known, a similar pattern of Blimp-1 mRNA induction has been reported in BCL-1 cell line (Turner et al., 1994), suggesting that it is a

common pattern of Blimp-1 induction *in vitro*. Importantly, the suppression of Blimp-1 mRNA levels by TCDD was robust at 24 and 72 h, demonstrating the ability of TCDD to interfere with the LPS-mediated induction of Blimp-1 mRNA. TCDD effects on Blimp-1 expression at 24 and 72 h were concordant at the mRNA level and the DNA-binding activity level. This result suggests that although additional factors, such as Blimp-1 protein stability, may affect the Blimp-1 DNA-binding activity in the presence of TCDD, Blimp-1 transcriptional repression at 24 and 72 h may be involved in the suppression of Blimp-1 DNA-binding activity by TCDD.

IV. **Effects of TCDD on AP-1 DNA-binding activity in the Blimp-1 promoter**

In light of Blimp-1 mRNA suppression by TCDD, we proceeded to examine the signaling events involved in the dysregulation of Blimp-1 transcription in the presence of TCDD. Transcriptional repression through inhibitory DRE motifs has been previously reported (Safe et al., 2000), and we hypothesized that AHR binding to DREs in Blimp-1 promoter could contribute to the attenuation of Blimp-1 induction in the presence of TCDD. However, the two DRE-like motifs that were identified within the first 2,000 bp of Blimp-1 promoter exhibited no specific inducible protein binding affinity, suggesting that additional factors are involved in the dysregulation of Blimp-1 transcription in the presence of TCDD.

Interestingly, previous studies from this laboratory have reported that the TCDD-mediated suppression of c-jun led to suppression of AP-1 DNA-binding activity in LPS-activated CH12.LX cells (Suh et al., 2002). Furthermore, AP-1-binding motifs are involved in the induction of human and mouse Blimp-1 genes (Ohkubo et al., 2005;

Vasanwala et al., 2002). EMSA assays revealed that AP-1 binding to the consensus AP-1 motif (Novak et al., 1990) and to motifs TRE-1060 and TRE-1610 (but not TRE-47) was induced at 2 h post LPS-activation. Importantly, AP-1 binding to all motifs was suppressed following LPS and TCDD co-treatment. By comparison, TCDD alone resulted in a transient modest induction of AP-1 binding as compared to the time matched naïve control at 2 h. The observed modest induction of AP-1 binding by TCDD alone is in agreement with the previously reported results obtained in cultured hepatoma cells treated with TCDD (Puga et al., 1992), suggesting that the induction of AP-1 binding by TCDD is independent of cell type. The low magnitude of AP-1 binding induction by TCDD in our studies as compared to Puga et al. may stem from the differences in experimental models (B lymphocytes compared to hepatocytes), and possibly from differences in the kinetics of AP-1 DNA-binding activity. By contrast, LPS and TCDD co-treatment resulted in suppression of AP-1 binding to TRE-47, TRE-1060 and TRE-1610 motifs at 24, 48 and 72 h post treatment as compared to LPS alone. The TCDD-mediated reduction in the AP-1 DNA-binding activity may be a result of reduction in the levels of AP-1 complex, reduced accessibility of TRE-like elements within in Blimp-1 promoter, or additional factors. Previous studies in LPS-activated CH12.LX cells have revealed that the levels of the AP-1 component c-jun were reduced by TCDD treatment at 48 and 72h. Therefore, the reduction of c-jun availability may be responsible, in part, for the reduced AP-1 binding activity at 48 h and later times. However, additional factors must be involved in the suppression of AP-1 DNA-binding activity at the earlier times *(i.e.,* 2, and 24h) Overall, the reduction in AP-1 DNA-binding activity in LPS-activated CH12.LX cells treated with TCDD was dependent on the TCDD concentration, as

demonstrated by EMSA. Importantly, the range of TCDD concentrations we have used to demonstrate TCDD concentration-dependent suppression of the AP-1 DNA-binding activity (*i.e.*, 0.1nM to 10 nM) have been previously shown to effectively suppress the IgM response in LPS-activated CH12.LX cells (Yoo et al., 2004, Sulentic et al., 1998). This finding confirms the potency of TCDD treatment to suppress the AP-1 DNA-binding activity within the Blimp-1 promoter, which correlates with the potency with which TCDD suppresses the IgM response in LPS-activated CH12.LX cells.

V. **Concluding remarks**

In continuation of previous studies of TCDD-mediated suppression of the humoral immune response, present findings demonstrate TCDD-mediated impairment of a number of molecular entities involved in B cell differentiation (Table 4). Specifically, our results indicate changes at different stages of the terminal B cell differentiation pathway, from the functional outcome - the suppression of the IgM response to a very early effect - suppression of AP-1 DNA-binding activity observed at 2 h post TCDD treatment. Collectively, our studies suggest that the TCDD-mediated suppression of the humoral immune response in B cells is produced, in part, by dysregulation of the well-orchestrated transcriptional cascade mediating the terminal B cell differentiation (Fig. 26). In addition, our studies suggest that TCDD treatment is interfering with a critical early event required for the proper differentiation of B cells into plasma cells at the level of, or preceding the AP-1 activation by LPS. The overall contribution of the current studies to the field of immunotoxiology is in demonstrating that the early non-genomic

Table 4. Summary of differentiation-related changes detected in LPS-activated CH12.LX cells during the 72 h culture period

FACTOR	FORM	NAIVE	LPS	LPS+TCDD
IgM	Protein	++	+++++	++
IgH	mRNA	++	+++++	++
Igκ	mRNA	++	+++++	++
IgJ	mRNA	++	+++++	++
CD-19	Protein	++++	+++	++
	mRNA	++++	+++	++++
MHC-II	Protein	+++	++	+++
Syndecan-1	Protein	+	++	+
sXBP-1	Protein	+	++++	+
	mRNA	+++	++++	+++
uXBP-1	Protein	+	++	+
	mRNA	+++	+++	+++
Pax5	DNA-binding activity*	++	+	++
	Protein	++++	++	+++
	mRNA	++	+	++
Blimp-1	DNA-binding activity**	+	++	+
	mRNA	+	++++	++
AP-1	DNA-binding activity	+	+++	+
	c-jun protein***	+	++	+
	c-jun mRNA***	+	++	+

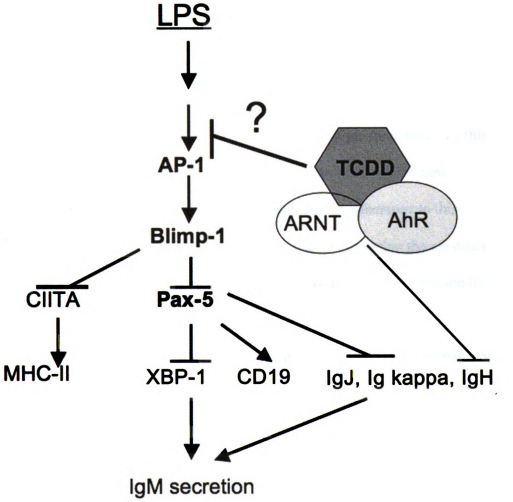
* - (Yoo et al., 2004)
** - (Schneider et al., in preparation)
*** - (Suh et al., 2002)

AHR-mediated mechanisms, as opposed to activation of the classical AHR-ARNT genomic pathway, may be responsible, in part, for the suppression of B cell differentiation by TCDD.

VI. **Significance and relevance**

Current studies were based on the existing body of knowledge gathered in two distinct scientific fields: one examining the molecular events of terminal B cell differentiation and another focusing on the mechanisms of suppression of humoral immune responses by TCDD. Findings of the former field have unequivocally established that the B cell may only assume its effector function (*i.e.* immunoglobulin secretion) if it terminally differentiates into a plasma cell. Evidence accumulated by the later field provided the knowledge that many, but not all immunosuppressive effects of TCDD are mediated through the specific receptor for TCDD, the AHR. To date, only a few molecular TCDD targets putatively involved in the suppression of the humoral immune responses have been elucidated. Furthermore, few of the proposed molecular mechanisms for the suppression of humoral immune responses in B cells by TCDD, among them the dysregulation of suppressor of cytokine signaling 2 (SOCS2) and the suppression of IgH 3'α enhancer, suggested mediation through the AHR-DRE genomic pathway (Boverhof et al., 2004; Sulentic et al., 2004a; Sulentic et al., 2004b). For many other TCDD effects observed on the molecular level, such as dysregulation of the activity of p27kip1, PLC and PKC, interaction with NF-κB and Rb proteins, and upregulation of the intracellular Ca^{++} levels by TCDD, DRE involvement has not been established

Figure 27. Proposed scheme for TCDD involvement in the impairment of B cell differentiation program. B cell differentiation is a multistep process. A B cell activation event modeled in our experimental system by LPS treatment of cultured CH12.LX cells initiates an early signaling cascade leading to activation of AP-1 and induction of Blimp-1. Blimp-1 represses a large number of genes, among them transcription factor Pax5 (directly), and B cell surface molecules MHC class II and CD19 (indirectly). Repression of Pax5 by Blimp-1 allows for the expression of XBP-1, IgH, IgJ, Igκ and ultimately, the IgM secretion. TCDD treatment resulted in dysregulation of each one of the aforementioned factors, suggesting that an early B cell activation event is responsible for the suppression of B cell differentiation and IgM response by TCDD.


LPS
?
AP-1
TCDD
ARNT
AhR
Blimp-1
CIITA
Pax-5
MHC-II
XBP-1
CD19
IgJ, Ig kappa, IgH
IgM secretion

(Crawford et al., 2003; Elferink et al., 2001; Ge and Elferink, 1998; Karras and Holsapple, 1994; Kramer et al., 1987; Tian et al., 2002). Furthermore, much of the existing evidence is fragmented, *i.e.*, even when evidence of a molecular entity being altered by TCDD treatment exists, it often does not explain how this change is linked to the functional deficiency in the IgM secretion.

Based on the recent advances in our understanding of B cell differentiation, a hypothesis was formed, stating that the molecular program of B cell differentiation is disrupted by TCDD treatment, resulting in a number of phenotypic outcomes. To this end, suppression of the IgM production in response to antigen has been the best-documented outcome, but other outcomes may exist as well. In adherence to this hypothesis, the primary objective of the current studies was to determine the existence and the magnitude of alterations in the molecular program of B cell differentiation by TCDD.

First, it has been investigated whether phenotypic plasma cell characteristics other than IgM secretion are altered by TCDD. Using the B cell differentiation surface markers MHC class II, syndecan-1 and CD19 as an indication of impaired terminal differentiation, it has been confirmed that multiple alterations to the differentiated B cell phenotype are conferred by TCDD treatment of LPS-activated B cells. Therefore, we proceeded to examine the molecular factors involved in the regulation of the phenotypic elements that were found to be altered by TCDD in our model.

A crucial late B cell differentiation effector protein is the transcription factor XBP-1. Current studies demonstrate that the steady state XBP-1s protein levels are suppressed following TCDD treatment. Furthermore, the suppressed XBP-1s protein

levels correlated with the reduction in XBP-1s mRNA and total XBP-1 mRNA levels. The observation of the reduction in XBP-1 mRNA provides evidence for both the impaired UPR (possibly due to suppressed IgH, Igκ and/or IgJ expression) and impaired XBP-1 transcription. The reduction in total XBP-1 mRNA levels clearly attests to the repression of XBP-1 transcription and implicates the transcriptional XBP-1 regulators as potential targets of TCDD.

Interestingly, each one of the two major known regulators of B cell differentiation, Blimp-1 and Pax5, has been shown to contribute to physiological XBP-1 upregulation in the course of the terminal B cell differentiation (Lin et al., 2002; Reimold et al., 1996). Recent studies employing Pax5 and Blimp-1-deficient systems have demonstrated that the differentiation program critically depends on the proper function of these two regulators (Kallies and Nutt, 2007; Nera et al., 2006). Namely, Pax5 has to be repressed by Blimp-1 (Lin et al., 2002), whereas Blimp-1 has to be induced by early B cell activation signals (Soro et al., 1999). Although Pax5 and Blimp-1 are critically important for B cell differentiation, their behavior in the TCDD-treated environment has not been previously elucidated. Our laboratory was the first to report that Pax5 is abnormally elevated following TCDD treatment (Yoo et al., 2004). Furthermore, we have shown that the attenuation of Pax5 repression occurs at the transcriptional level, which suggest the involvement of Blimp-1. Indeed, Blimp-1 DNA-binding activity (Schneider et al., submitted) and expression in LPS-activated CH12.LX cells are suppressed by TCDD, which may contribute to the attenuation of Pax5 repression, and is consistent with our results showing that TCDD treatment results in an abnormally elevated Pax5.

Due to the observation that Blimp-1 is repressed at the transcriptional level following TCDD treatment, we sought to identify the molecular effectors involved in Blimp-1 transcription regulation that may have been targeted by TCDD. AP-1 was considered a plausible candidate, being a factor that positively regulates the Blimp-1 promoter (Ohkubo et al., 2005; Vasanwala et al., 2002), and whose activity has been previously reported to be altered by TCDD in a hepatoma and a B cell lymphoma (Puga et al., 2000a; Suh et al., 2002). Although TCDD treatment in the absence of stimulation produced an upregulation in AP-1 DNA-binding activity in the hepatoma (Puga et al., 2000a), in LPS-activated B cells TCDD repressed AP-1 (Suh et al., 2002). The significance of these findings is that although AP-1 repression in LPS-activated CH12.LX cells has been reported previously, we were able to link this effect to a functional endpoint in B cell differentiation – the induction of Blimp-1. As present studies demonstrate, the suppression of AP-1 DNA-binding activity by TCDD within the Blimp-1 promoter may contribute to the repression of Blimp-1 activity, and ultimately, to the suppression of the IgM response, which for many years served as a hallmark of TCDD toxicity in B cells.

As stated above, the classical molecular mechanism for biochemical and toxic effects of TCDD is the genomic pathway, mediated by the AHR-ARNT heterodimer binding to DRE elements located in regulatory regions of susceptible genes. Apart from mediating CYP450 isozyme induction by TCDD, this mechanism has been implicated in a wide range of TCDD toxicities, including in the immune system. Furthermore, TCDD suppression of the IgM responses in B cells has been shown to depend on a functional AHR, and was proposed to be mediated, in part, by DRE-like motifs located in the IgH

3'α enhancer. Conversely, some molecular effects of TCDD exposure, such as intracellular Ca^{++} elevation, or PKC activation were shown to be AHR-independent, and there are a number of reported TCDD effects that were mediated by AHR, but do not appear to involve the genomic DRE pathway, such as AHR interaction with NF-κB or Rb proteins. Although the suppression of the IgM response in CH12.LX cells was shown to depend on a functional AHR, it has not been determined whether it was mediated by genomic mechanisms alone. Our studies of transcription factor-DNA-binding activity demonstrated no specific protein binding to the DRE-like motif DRE-506 within the Pax5 promoter, and DRE-like motifs DRE-75 and DRE-107 within the Blimp-1 promoter, suggesting that additional transcriptional mechanisms are involved in the dysregulation of Blimp-1 and Pax5 by TCDD.

Taken together, these findings provide evidence for impairment of the B cell differentiation program by TCDD. The contribution of this work to the field is in establishing a functional link between the dysregulation by TCDD of the B cell differentiation program, and the suppression of IgM response. Furthermore, this work provides the necessary justification for further investigation into the molecular mechanisms whereby TCDD impairs expression and transcriptional activity of Blimp, Pax5 and XBP-1, because proper and timely function of each one of these factors is crucial for generation of the IgM response. In light of the current findings, we propose that the impairment of Ig secretion should not be approached as a stand-alone toxic outcome of TCDD exposure. Rather, we suggest that Ig secretion be viewed as one among many outcomes of terminal B cell differentiation that are impaired by TCDD. Furthermore, as our results and existing literature reports imply, mechanisms other than

the classical AHR pathway may be involved in the immunosuppressive effects of TCDD in general, and the suppression of the humoral immune response in B cells in particular. We envision that such broad perspective will facilitate new approaches to the question of TCDD involvement in the complex array of molecular events that are necessary for proper execution of terminal B cell differentiation, and will expand our understanding of the TCDD-induced toxicity in B cells.

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