

Developmental Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin:

Induced and Exacerbated Autoimmunity in Adulthood

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(ABSTRACT)

Developmental 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) exposure can permanently alter immune system ontogeny, resulting in the dysregulation of a number of vital immune pathways. We hypothesized that developmental exposure to TCDD may also impair the establishment of self-tolerance, resulting in an increased risk of autoimmunity. For example, we observed that a single prenatal TCDD exposure given to non-autoimmune-prone high affinity aryl hydrocarbon receptor (*AhR*) C57BL/6 mice resulted in an immune complex-mediated autoimmune disease during the adult stage. Further using a similar TCDD exposure protocol, autoimmune-prone low affinity *AhR* SNF₁ mice exhibited acceleration and exacerbation of lupus-like nephritis in adulthood. Examination of these mice showed that perinatal TCDD exposure adversely affected both primary immune organs of the adaptive immune system. In the thymic compartment, prenatal TCDD affected thymocyte cellularity, differentiation and maturation as well as central tolerance as indicated by high levels of autoreactive V β TCR T cells in the periphery. Prenatal TCDD also altered bone marrow B lymphopoiesis and B cell maturation and differentiation in the spleen. Functionally, these B cell changes resulted in high

serum autoantibodies titers to dsDNA, ssDNA and cardiolipin suggesting a loss in central B cell tolerance. The functional assessment of T cells, via cytokine production showed that prenatal TCDD mice altered Th1/Th2 levels. As a result, significant changes were detected in the kidney characterized by increased immune complex deposition in the glomeruli, lymphocytic infiltration and general pathologic changes. This would suggest that multiple immune pathways are affected by prenatal TCDD and work either independently or synergistically to display immune-mediated disease during aging. Importantly, this study has also shown that the sex of an individual appears to influence both the type of immune pathways affected by TCDD as well as the progression and severity of the autoimmunity. In summary, these studies clearly demonstrate that postnatal immune system impairment due to prenatal TCDD exposure is not limited to immunosuppression but also can include inappropriate immune activation manifested as a hypersensitivity that can lead to the onset of autoimmune disease.

This dissertation is dedicated to my mother and father,
to whom I owe everything

In the memory of my father

To my mother, who has been a source of encouragement and inspiration to me
throughout my life

In the memory of my sister

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CHAPTER 1. LITERATURE REVIEW

Dioxin

The chemical 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), also referred to as "dioxin", is an environmental contaminant that is classified as the most potent of toxic halogenated aromatic hydrocarbons (HAHs). All HAHs produce similar patterns of toxicity and appear to have a common mechanism of action, although they differ in potency (Poland and Knutson, 1982; Safe, 1986). TCDD has a triple-ring structure that consists of two benzene rings connected by a third oxygenated ring with a pair of oxygen atoms. TCDD can be formed as an unintentional by-product through a variety of chemical reactions and combustion processes. According to recent U.S. Environmental Protection Agency (US EPA) data, the major sources of dioxins include coal fired utilities, municipal waste incinerators, metal smelting, diesel trucks, land application of sewage sludge, burning treated wood and trash burn barrels. Dioxin was also a contaminant in the herbicide Agent Orange, which was used as a defoliant by United States forces during the Vietnam War (Holsapple et al. 1991; Schechter et al. 2003).

The continued concern with TCDD is due to its widespread distribution, persistence as an environmental contaminant, bio-accumulation within the food chain (with food from animal origin being the major source), and its broad toxicity. TCDD is chemically stable and this property makes it very resistant to biodegradation. Moreover, TCDD is a lipophilic compound that tends to deposit in adipose tissue. Dioxins are absorbed primarily through the consumption of diet rich

in fat, although occupational and accidental exposures have been reported. In humans, the highly chlorinated dioxins are stored in fatty tissues and are neither readily metabolized nor excreted. Because of its relative resistance to metabolism, TCDD persists in the body, with a half-life in humans ranging from 7 to 10 years while highly chlorinated dioxins (4-8 chlorine atoms) persist in the body from 7.8 to 132 years (Pirkle et al. 1989; Michalek et al. 1996; Michalek & Tripathi 1999; Geyer et al. 2002). Therefore, dioxin tends to accumulate in human tissues over time, raising concern that repeated exposures, even small exposures may eventually reach dangerous levels, and evoke adverse health effects. Perinatal exposure of human infants is of concern because pregnant and nursing women can pass dioxins to their babies both trans-placentally and lactationally (Wang et al. 2004).

TCDD is associated with multiple biochemical and biological toxic effects *in vivo* and *in vitro* (Safe 1995). TCDD elicits a wide range of biological effects, including alterations in metabolic pathways, immunological changes and immunotoxicity, wasting syndrome, chloracne, hepatotoxicity, gastric lesions, carcinogenesis, reproductive toxicity, teratogenicity and embryo toxicity (Poland and Knutson, 1982; Maronpot et al., 1986; Safe, 1986; Birnbaum, 1994). At the biochemical level, TCDD can modulate endocrine response, enzyme activities, mRNA and protein levels, as well as alter the phosphorylation and glycosylation states of proteins (Safe 1995; Safe et al. 2000; Kim et al. 2008).

Dioxin and Aryl Hydrocarbon Receptor

The biological and toxic manifestations of dioxins are mediated primarily by the Aryl hydrocarbon receptor (*AhR*) (Poland & Knutson 1982; Safe 1990; Hogaboam et al. 2008). The resistance of *AhR*-deficient (knockout) mice to the enzyme inductive and toxic manifestations of very high doses of TCDD supports the role of this protein in mediating TCDD's effects (Fernandez-Salguero et al. 1996; Schmidt & Bradfield 1996; Tijet et al. 2006; Sarioglu et al. 2008).

Through the generation of a large gene database, dioxin responsive genes have been identified. However, only a few of these genes have been reported to have promoter regions targeted by *AhR*. This would suggest that not all genes modulated after dioxin exposure have dioxin responsive elements (DREs) in their promoter regions (Sun et al. 2004; Majora et al. 2005; Frericks et al. 2006). Therefore, it has been hypothesized that many of the genes regulated by the *AhR* /ligand complex are mediated indirectly by secondary signaling cascades.

AhR -deficient (-/-) mice displayed slowed growth, a spectrum of hepatic defects, enlarged spleens, increased neonatal mortality rate, inflammation of several major organ systems and a severely depressed immune system suggesting fundamental involvement of the *AhR* in normal growth, development, differentiation and organ functions. These mice at the same time are resistant to dioxin-induced toxicity (Fernandez-Salguero et al. 1995; Schmidt & Bradfield 1996). However, few reports have addressed the changes in gene expression by the absence of the *AhR* in relation to cells or organs known to be sensitive targets of *AhR* activity, such as thymus. These limited studies have shown that in the absence of the *AhR*, normal

gene expression is affected indicating that constitutive expression of genes can also be dependent on the *AhR* (Karyala et al. 2004; Frericks et al. 2006; Tijet et al. 2006).

Dioxin-dependent gene expression regulation has shown a high degree of cell specificity in response to the *AhR* activation. For example, major immune subpopulations of CD4, CD8 and dendritic cells exhibited completely different effects and gene expression profiles for dioxin-dependent gene regulation. Surprisingly, there is no overlap at all, for example, all cell types initiate a unique molecular signaling pathway leading to regulation of a distinct set of genes after *AhR* over-activation by TCDD (Majora et al. 2005; Frericks et al. 2006). More striking, the same population with similar cell differentiation stages, such as thymic CD4-CD8-cells, can display different effects and *AhR*-mediated gene expression profiles in the fetus and the adult in response to TCDD treatment (Frericks et al. 2006). Thus, the idea of a TCDD-specific signature across various organs or many specific cell types or even within closely related cell subsets of a single organ is not plausible, suggesting special care is required in the interpretation of toxic data for risk assessment relative to TCDD. The underlying causes for this highly cell-specific response outcome of *AhR* signaling suggest the involvement of other factors including, a direct interaction of the *AhR* with cellular proteins, such as the retinoblastoma protein (Elferink et al. 2001), transcriptional co-factors (Hankinson 2005), promoter accessibility modulation such as histone deacetylation or DNA methylation (Nakajima et al. 2003), secondary signaling mechanisms involving *AhR* chaperoning proteins, or genes that are primary targets of the *AhR*, such as the *AhR*

repressor and pathways for *AhR* action that do not involve *AhR* nuclear translocator (Arnt).

TCDD enters the responsive cell and binds with high affinity to the cytosolic unbound *AhR*, which exists as a multiprotein complex, containing two molecules of the chaperone protein, a heat shock protein of 90 kDa (hsp90), the X-associated protein 2 (XAP2), *AhR*-activated 9 (ARA9) and a recently identified 23-kDa co-chaperone protein referred to as prostaglandin E synthase 3 (p23) (Meyer et al. 1998; Kazlauskas et al. 1999; Cox & Miller 2004). Once the ligand has bound to *AhR*, hsp90 is released and the *AhR* is presumed to undergo a conformational change that exposes a nuclear localization sequence(s), resulting in translocation of the complex into the nucleus (Hord and Perdew, 1994; Pollenz et al., 1994). Release of the ligand:*AhR* from this complex and its subsequent dimerization with a related nuclear protein called *AhR* nuclear translocator (Arnt) converts the *AhR* into its high affinity DNA binding form (Probst et al. 1993; Hankinson 1995). After orchestrating the appropriate transcriptional co-factors, this heterodimer then binds to a specific DNA sequence (5'-GCGTG-3') which is in the core binding motif of dioxin response element (DRE)(5'-T/GNGCGTGA/CG/CA-3') (Shen & Whitlock 1992; Lusska et al. 1993; Schmidt & Bradfield 1996; Hankinson 2005). The DRE recognition sequence has been found within enhancer elements upstream of the promoter region of various genes, including cytochrome P4501A1 (CYP1A1), cytokines and growth factors (Denison et al. 1988; Phelan et al. 1998; Whitlock 1999). The net result of TCDD-induced *AhR* signaling pathway is direct and indirect altered transcription of

genes involved in critical cellular process, such as cell differentiation, cell proliferation and cell death.

The *AhR* protein contains several domains critical for function and is classified as a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors (Burbach et al. 1992; Fukunaga et al. 1995). An analysis of this and other bHLH proteins indicates that the basic region mediates DNA binding, whereas the helix-loop-helix domain is necessary for dimerization with other proteins. Two other receptor regions function in dimerization. These are designated as "PAS" regions. The PAS B region also contains part of the ligand-binding domain of the receptor and a domain necessary for interaction with hsp90 (Coumailleau et al. 1995). The capability for DNA-binding requires that the receptor interact with another factor (such as the Arnt protein). Thus, the active form of the receptor is heteromeric. Endogenous ligands for Ah receptor include tryptophan derivatives, carotinoids and arachidonic acid metabolites. *AhR* binds and mediates the response of TCDD and other aromatic hydrocarbons. The *Ah* genetic locus that controls this receptor polymorphism also controls the biological responses to TCDD and other ligands.

Arnt is a member of basic helix-loop-helix, Per-Arnt-Sim family of heterodimeric transcription factors, which include *AhR* hypoxia-inducible factor-1 α (HIF-1 α), and *Drosophila* single-minded protein (SIM) (Hoffman et al. 1991; Burbach et al. 1992; Tomita et al. 2003). To function, Arnt undergoes dimerization with other basic helix-loop-helix, Per-Arnt-Sim members such as *AhR*, HIF-1 α , and SIM. Arnt also has PAS domains that function in transcriptional activation (Schmidt & Bradfield 1996;

Rowlands & Gustafsson 1997; Whitlock 1999). Arnt protein does not bind TCDD. Ah receptor recognition sites on DNA (DRE) require the presence of both proteins (ligand-AhR and Arnt) to generate a specific DNA-binding and to activate the expression of a dioxin-responsive reporter gene. Heteromorphy formation might contribute to the diversity of responses that specify dioxin action. However, Arnt is the only protein that has been demonstrated to be a functional partner for the Ah receptor in terms of conferring specificity of DNA binding and transactivation.

Consequences of Developmental Immunotoxicant Exposure

Establishment of the vertebrate immune system is initiated by the formation of pluripotent hematopoietic stem cells (HSC) in intraembryonic splanchnopleura mesenchyme early in embryonic/fetal life. This is followed by a sequential series of temporal and well coordinated developmental events that continue through the early period after birth (Dieterlen-Lievre 1975; Le Douarin et al. 1984; Good 1995; Medvinsky & Dzierzak 1996). Perturbation or abrogation of this developmental sequence of events is a vulnerable event that can lead to heritable immune dysfunctions.

Studies in laboratory rodents have shown that exposure to various types of immunotoxicants (e.g., environmental chemicals, drugs, and ionizing radiation) during perinatal ontogeny of the immune system can lead to persistent, and in some cases dramatic, effects on postnatal immune competence, suggesting that developmental exposure to immunotoxic chemicals may be a more sensitive

window for the immune system than exposure during adult life (Holladay 1999; Holladay & Smialowicz 2000). For example, the insecticide chlordane has been shown to cause persistent immune alterations in BALB/c mice, including a significant depression of cell-mediated immunity still present 100 days after birth and a significant enhancement in the survival of the offspring to influenza virus infection (Spyker-Cranmer et al. 1982; Barnett et al. 1985). Mice exposed to chlordane during fetal life also display alterations in bone marrow colony formation as well as long-term depression of both delayed-type hypersensitivity and mixed lymphocyte reactivity (Barnett et al. 1985; Barnett et al. 1990). However, adult mice exposed to chlordane at equal dose levels were resistant to these immune alterations compared to perinatal exposure. Similar results have been reported in mice exposed during development to benzo[*a*]pyrene (a polycyclic aromatic hydrocarbon (PAH))(Urso & Gengozian 1984).

Low-level prenatal exposure to certain HAHs, particularly dioxins, also gives rise to severe, long-lasting immunologic defects in rodents (Poland & Knutson 1982; Dencker et al. 1985; Fine et al. 1989). A review by Holladay (1999) provided a list of additional diverse agents that produce developmental immunotoxicity in rodents which include PAHs other than B[*a*]P such as 7,12 dimethylbenz[*a*]anthracene, 3-methylcholanthrene; pesticides other than chlordane such as hexachlorocyclohexane and DDT; polycyclic halogenated hydrocarbons such as TCDD; heavy metals such as cadmium and mercury; hormonal substances such as diethylstilbestrol (DES), testosterone, and cortisone; mycotoxins (most notably T toxin); and therapeutic agents such as acyclovir and cyclophosphamide. Several of

these agents target the fetal thymus, producing fetal thymic atrophy as well as altered differentiation of fetal T-lymphocyte precursor cells (thymocytes). Such chemical insult on thymocyte maturation, during critical periods of self tolerance may have detrimental consequences on immune function in postnatal life, including possible expression of autoimmunity (Holladay & Smialowicz 2000; Fisher et al. 2005). These reports provide compelling evidence that rodents prenatally exposed to immunotoxic compounds will display altered fetal immune development, causing severe and sustained postnatal immunosuppression into adulthood. Such defects in the development of the immune system may lead to adverse health consequences manifested by cancer, hypersensitivity or increased incidence of microbial infections which may be life threatening (Holladay & Smialowicz 2000). To this end, TCDD has been the one of the most studied of the environmental contaminants.

Consequences of Developmental TCDD Exposure

Prenatal exposure to TCDD targets various vital components of both the humoral and cell mediated immune system leading to a broad range of immune deficiencies. Further, perinatal TCDD exposure has been associated with what appears to be lifelong immunosuppression. Numerous studies in laboratory animals have shown that perinatal exposure to TCDD can result in immunosuppression lasting into adulthood (Faith & Moore 1977; Thomas & Hindsill 1979; Gehrs & Smialowicz 1997; Gehrs & Smialowicz 1999; Hogaboam et al. 2008). This would suggest a permanent impairment in the developing immune system, which could lead to an

increased susceptibility to infections, cancer, and autoimmunity in adulthood. For example, perinatal TCDD exposure impairs host resistance against infectious agents or syngeneic tumor cells (Faith & Moore 1977; Luster et al. 1979). In addition, perinatal TCDD exposure elicits suppression of cell-mediated immune functions, including T-cell mitogen responses (Luster et al. 1979), skin graft rejection times, graft-versus-host reactivity (Vos et al. 1974), delayed hypersensitivity (Faith & Moore 1977; Blaylock et al. 1992; Gehrs & Smialowicz 1997; Gehrs & Smialowicz 1999), and cytotoxic T-cell responsiveness (Holladay et al. 1991). These studies strongly suggest that prenatal exposure to TCDD permanently alters the developing immune system, leading to deficiencies in postnatal immunity.

Effects of TCDD on T Cell Development

A number of past studies seem to infer that the prenatal effects are more selective toward cell-mediated immunity as compared to humoral or innate immunity (Vos et al. 1974; Faith & Moore 1977; Luster et al. 1980). For instance, perinatal TCDD exposure causes thymic atrophy and alters thymocyte differentiation and maturation as indicated by changes in proportion of thymocyte subpopulations in both mice and rats (Faith & Moore 1977; Holladay et al. 1991; Blaylock et al. 1992; Gehrs et al. 1997; Gehrs & Smialowicz 1999; Camacho et al. 2004), an effect that is accompanied by depressed T cell responses (Gehrs et al. 1997; Gehrs & Smialowicz 1999). For example, developmental exposure to TCDD between gestation day (gd) 6 and gd 14 results in offspring with diminished cytotoxic T lymphocytes (CTL) activity against allogeneic tumor cells (Holladay et al.

1991). Suppression of delayed type hypersensitivity and contact hypersensitivity responses were observed in rats exposed to TCDD *in utero* on gd 14 (Gehrs & Smialowicz 1999; Walker et al. 2004). Functional defects in the immune response to influenza virus infection have also been reported, and include suppressed expansion and differentiation of T cells and decreased antibody responses (Lawrence & Vorderstrasse 2004; Vorderstrasse et al. 2006). Conversely, these diminished responses in infected offspring from TCDD-treated dams were accompanied by enhanced innate immunity as demonstrated by an increased number of neutrophils and higher pulmonary interferon gamma (IFN- γ) levels. This would also suggest that the consequences of developmental exposure to TCDD are not limited to immunosuppression, as some immune responses are inappropriately enhanced. In support of this idea, offspring exposed to TCDD via lactation showed decreased host resistance while serum IFN- γ levels were increased after infection with *Listeria monocytogenes* (Sugita-Konishi et al. 2003).

In addition to thymic atrophy, developmental exposure to TCDD suppresses lymphocyte responses and enhances aspects of inflammation. However, unlike thymic atrophy, alterations in leukocyte function appear to persist into adulthood (Luster et al. 1980; Gehrs & Smialowicz 1997; Walker et al. 2004). These T cell effects might be linked to the observations that perinatal TCDD exposure alters terminal deoxynucleotidyl transferase-synthesizing thymic precursor stem cells in the fetal liver and bone marrow, but not thymus (Fine et al. 1990). In addition, *in vivo* TCDD exposure alters the fetal thymus by inhibiting thymocyte maturation and reducing expression of thymic MHC class II molecules (De Waal et al. 1992).

Additionally, there is sufficient evidence suggesting that TCDD may directly target thymocytes by either preventing the differentiation of CD4⁺CD8⁺ T cells and the transition phase from CD4⁻CD8⁺CD24⁺ to CD4⁺CD8⁺ (Fine et al. 1989; Kerkvliet & Brauner 1990; Lundberg et al. 1990; Holladay et al. 1991; Blaylock et al. 1992; Gehrs et al. 1997; Gehrs & Smialowicz 1997), by reducing entrance into cell cycle of intrathymic progenitor cells especially in the CD4⁻CD8⁻CD3⁻ (Laiosa et al. 2003), by affecting thymic stroma (Nagarkatti et al. 1984; Greenlee et al. 1985; De Waal et al. 1992; Kremer et al. 1995; Camacho et al. 2005), by inducing thymic apoptosis (Rhile et al. 1996; Kamath et al. 1997; Zeytun et al. 2002; Camacho et al. 2004), by deviating the emigration pattern of thymic T cell subpopulations specifically double negative (DN) CD4⁻CD8⁻ cells (Majora et al. 2005; Temchura et al. 2005; Frericks et al. 2006), or by altering the transcription of genes involved in critical cellular process in various thymic T cell subpopulations (Hundeiker et al. 1999; Esser et al. 2004; Frericks et al. 2006). Still, other studies have shown TCDD alters the expression of a large array of thymus genes (Zeytun et al. 2002). Therefore, it is reasonable to propose that TCDD may be influencing multiple immune mechanisms or pathways simultaneously to produce thymic atrophy.

The primary function of the thymus is the stringent generation of a T cell repertoire capable of MHC-restricted antigen recognition while purging T cells that are potentially autoreactive through the process of negative selection (Sebzda et al. 1999; Palmer 2003; Siggs et al. 2006). TCDD appears to alter the dynamics of thymic selection in part through dysregulation of microenvironment chemokines, adhesion molecules, coreceptors, and MHC expression and costimulatory molecules

important in thymic selection (Esser et al. 2004; Fisher et al. 2004; Vogel et al. 2007). These observations led to the suggestion that TCDD may alter critical selective developmental steps through which differentiating or maturing thymocytes expressing autoreactive T cell receptors (TCRs) are deleted via lympho-stromal communication. Failure in this thymic selection checkpoint can lead to autoimmune disease where negative selection is inefficient or positive selection is blocked.

The elimination of strongly self-reactive T cells has long been considered the exclusive function of negative selection or clonal deletion in thymus (Siggs et al. 2006). Presently, the affinity/avidity model of thymic selection is widely accepted. Based on this model, lympho–stromal communications, a bilateral coordination or crosstalk, between the rearranged TCRs expressed on traveling CD4⁺CD8⁺ double-positive (DP) thymocytes and MHC/self-peptide complexes expressed by architectural stromal cells or hematopoietic cells, induce TCR-dependent signaling in DP thymocytes. Following TCR recognition of peptide–MHC ligands at low-avidity interactions, DP thymocytes are induced to receive signals for survival and further differentiation into single-positive (SP) thymocytes. This process, referred to as positive selection, enriches T cells that are potentially reactive to foreign antigens, but not to self antigens, presented by self-MHC molecules. Most (approximately 95%) DP thymocytes, after V(D)J gene rearrangement, express a TCR that is not capable of interacting with MHC/self-peptide complexes expressed by stromal cells, such as cortical thymic epithelial cell (cTECs), and dendritic cells in the cortex.

These thymocytes undergo apoptosis because they are not rescued by TCR-mediated survival signals (Singer 2002; Palmer 2003).

Thymocytes bearing TCRs capable of communicating with MHC/ self-peptide complexes are either positively or negatively selected. It is generally believed that low-avidity interactions of TCR on DP thymocytes with MHC/self-peptide complexes on thymic antigen-presenting cells rescue approximately 5% of thymocytes from death by neglect (positive selection) and further differentiation into single-positive (SP) thymocytes (Sprent & Kosaka 1993; Jameson et al. 1995; Hogquist 2001; Sprent & Kishimoto 2002). In contrast, high-avidity interactions elicit signals that lead to the deletion of potentially autoreactive thymocytes. Specifically, MHC class I and class II molecules act as thymic self-antigen-presenting molecules in a process whereby thymocytes expressing TCRs with high affinity to self-antigen are eliminated (negative selection) (Palmer 2003).

Most of the studies have focused on the interactions involved in positive and negative selection and specifically, how signals mediated through the TCR are able to sort both survival and apoptosis signals. Current models of T cell selection suggest that the affinity/avidity of TCR toward MHC/self peptide complex is critical for T cell fate determination during selection by affecting the strength and duration of the interaction between T cells and various antigen-presenting cells (Dave et al. 1999). In particular, the interactions of high affinity TCR for MHC/self-peptide complex are more transient than the interactions between low-affinity TCR and MHC/self-peptide. Under the duration model, high-affinity TCR interactions result in transient but strong activation of extracellular signal-regulated kinases (ERKs)

intracellular signaling pathway via TCR, leading to negative selection, whereas the TCR signaling mediated by low-affinity interactions is sustained and results in weak activation of ERK intracellular signaling pathway, leading to positive selection. Whereas ERK has been shown to be critical for positive selection, as has calcineurin, which activates the transcription factor nuclear factor of activated T-cells (NFAT) downstream of calcium elevation, two other types of mitogen-activated protein (MAP) kinases, c-Jun N-terminal kinases (JNK) and p38 MAP kinase are important in negative selection, thus, illustrating one striking difference between the two signals (Mariathasan et al. 2000; Mariathasan et al. 2001).

The kinetic interaction and strength between T cells undergoing selection and APCs is dependent on a variety of factors in addition to the affinity and avidity of TCR for MHC/self-peptide. Data have shown that thymic selection outcome is dictated by the level of TCR expression on thymocytes, as well as by the nature of the ligand and/or its presentation by different subsets of APCs (Ashton-Rickardt et al. 1994; Sebzda et al. 1994; Dave et al. 1999). In addition, other experiments provide compelling evidence that co-stimulatory molecules have a prominent role during negative selection. For example, studies have shown that CD30 is involved in negative selection as demonstrated by impaired negative selection in CD30^{-/-} mice, whereas negative selection was enhanced in CD30 overexpressing mice (Amakawa et al. 1996; Chiarle et al. 1999; DeYoung et al. 2000). Similarly, mice deficient in tumor necrosis factor family member LIGHT show reduced negative selection, whereas overexpression of LIGHT enhances negative selection (Wang et al. 2001; Granger & Rickert 2003; Palmer 2003; Wang & Fu 2003). These observations

demonstrate that altered expression of specific molecules involved in thymic selection apparatus can modulate the outcome of the selective process. Interestingly, the full effects of perinatal TCDD exposure on co-stimulatory molecules and signaling pathways have yet to be investigated.

TCDD Exposure Impact on Central Tolerance

Several studies have established a connection between prenatal chemical exposure and enhanced or induced postnatal autoimmunity with HAHs such as TCDD (Holladay & Smialowicz 2000). For example, the therapeutic immunosuppressant cyclosporin A (CsA) induces a T-cell-mediated autoimmunity in rodents by altering the thymic development process via a mechanism similar to TCDD (Holladay & Smialowicz 2000). TCDD produces a significant dysregulation in fetal thymocyte development, as demonstrated by decreased percentage of double-positive cells and increased percentage of both double-negative cells and immature TCR⁺ CD8^{lo} thymocytes, a pattern similar to the maturational dysregulation produced in fetal thymic organ culture exposed to the therapeutic immunosuppressive drug CsA (Holladay & Smialowicz 2000). Due to CsA inability to cross the placenta, the *in vitro* fetal thymic organ culture was used instead of *in vivo* exposure to study the effect of CsA on developing T cells.

Fetal mouse exposure to TCDD or *ex vivo* exposure of fetal mouse thymi to CsA showed a similar pattern of effects on thymocytes. The demonstration that CsA produces autoimmune disease in rodents mediated in part by altering the lympho-stromal communications between the rearranged TCRs expressed on traveling

thymocytes and MHC/self-peptide complexes expressed by architectural stromal cells or hematopoietic cells during negative selection of autoreactive T cells, may be an effect related to down regulation of thymic MHC class II molecules. TCDD produces an inhibition of fetal thymocyte differentiation very similar to that seen in fetal thymic organ cultures exposed to CsA and also interferes with expression of thymic MHC class II molecules (De Waal et al. 1992; Vorderstrasse et al. 2003). It has also been shown that similar patterns of inhibited thymic T cell differentiation occur spontaneously in various autoimmune mice models such as MRL-lpr/lpr mice (Kakkanaiah et al. 1990), in TCDD-treated mice (Blaylock et al. 1992), and in mice treated *in vivo* with monoclonal antibodies to MHC class I and class II molecules (Kruisbeek et al. 1985), suggesting the importance of these MHC molecules in thymocyte differentiation and maturation. TCDD has also been shown to decrease expression of an MHC class I gene ($Q1^b$) in a mouse hepatoma cell line (Dong et al. 1997). This study revealed that MHC $Q1^b$ cDNA encoded for the $\alpha 3$ domain and transmembrane domain of the $Q1^b$ class I protein, implying that the MHC gene product could interact with $\beta 2$ -microglobulin, a component of MHC class I molecules. These authors therefore suggested that TCDD might impair the antigen presentation process in the liver as well as the thymus by targeting the MHC $Q1^b$ molecule expression, thus altering the CD8 thymocyte selection process (Dong et al. 1997). Another study has shown that exposure to TCDD increases the expression of MHC class II molecules on splenic Dendritic cells (DC) in the absence of antigen suggesting that TCDD modulates the processing and presentation of antigen in thymic DC, thus altering the CD4 thymocyte selection process (Vorderstrasse et al.

2003). In autoimmune-prone (low affinity *AhR*) SNF_1 mice, resistant males were rendered susceptible to lupus glomerulonephritis early in life by a mid-gestation dose of 10 $\mu\text{g}/\text{kg}$ TCDD treatment (Silverstone 1998).

TCDD's ability to alter T cell maturation and differentiation may negatively impact central tolerance leading to autoimmunity. Using the popliteal lymph node assay in male rats, the investigators concluded that TCDD has the potential to induce or exacerbate autoimmune-like reactions (Descotes 1992; Fan et al. 1995). Targeting of thymic parenchyma and thymic stromal cells by TCDD, led to suggestions that TCDD may have the potential to alter critical, selective and dynamic developmental steps through which thymocytes expressing autoreactive T-cell receptors (TCRs) are deleted via lympho-stromal communication (negative selection) (De Waal et al. 1992; Schuurman et al. 1992). In another study, thymic epithelial distribution of major histocompatibility complex (MHC) class II molecules was altered in TCDD-treated mice, an effect that was hypothesized as having potential for autoreactive thymocytes to escape deletion into the periphery and increase extrathymic T cell differentiation (Hanawa et al. 1993; De Heer et al. 1994). Although this extrathymic pathway is limited in normal mice, it becomes a predominant pathway in mice with autoimmune diseases, athymic mice, and aged mice (Hanawa et al. 1993).

In mice, extrathymic T cells were activated in association with thymic atrophy, expressed high levels of LFA-1 and IL-2R beta-chains, and contained a significant proportion of double negative CD4⁻CD8⁻ T cells. Investigators have

identified several T cells carrying T cell receptor (TCR)-variable regions associated with self-reactivity in TCDD-treated animals. The TCR-variable, (V β) chains are usually deleted in the thymus by reaction with self-MHC and minor lymphocyte stimulatory antigens (Okuyama et al. 1992; Hanawa et al. 1993) and have been associated with autoimmunity in some experimental mouse models such as lupus nephritis and Sjogren's syndrome (Rocha et al. 1992; Suttmuller et al. 1998; Yamamoto et al. 1998). In one study, spleens from nephritic SNF₁ animals showed an expansion of several forbidden autoreactive T helper cell clones expressing TCR V beta genes including V β 17a⁺ TCR (Adams et al. 1990). Other studies have shown that both TCDD and estradiol induce extrathymic T-cell differentiation in the liver of young adult mice, and that such extrathymic cells expressed elevated levels of V β ⁺ TCR (Okuyama et al. 1992; Silverstone et al. 1994). Further, the increase in V β autoreactive T cells has been proposed as a mechanism by which TCDD may promote autoimmunity by inducing extrathymic T-cell differentiation in the liver (Okuyama et al. 1992; Silverstone et al. 1994).

TCDD Effects on the Endocrine System

TCDD has attracted a great deal of attention as a prototypical endocrine disruptor capable directly or indirectly modulating multiple endocrine signaling pathways (Birnbaum 1995; Safe & Wormke 2003). Perinatal TCDD exposure has been shown to suppress the development of the male reproductive system (Mably et al. 1992; Mably et al. 1992; Gray et al. 1997; Ohsako et al. 2001), to induce sexual

dimorphism in the rat brain (Zareba et al. 2002), and also cause damage to the ovaries (Chaffin et al. 1996; Chaffin et al. 1997; Heimler et al. 1998; Chaffin et al. 2000; Davis et al. 2000). The similarity in observations between TCDD-induced toxicities and thyroid dysfunction initiated investigations into the effects of TCDD on thyroid hormone (T₃ and T₄) levels (Bastomsky 1977; Rozman et al. 1985; Henry & Gasiewicz 1987). These results showed decreased circulating T₄ in animals treated with TCDD mediated by the induction of glucuronyl transferase activity and subsequent increased formation and excretion of T₄ glucuronides (Sewall et al. 1995; Wu et al. 1995).

TCDD also affects steroidogenesis *in vitro* and in laboratory rodents (Mebus & Piper 1986; Mebus et al. 1987; Kleeman et al. 1990; Moore et al. 1991). TCDD may also contribute to the decrease in spermatogenesis in male rats and the ability to conceive and carry a pregnancy to term in female rats exposed *in utero* (Peterson et al. 1993). In juvenile, pubertal and postpubertal male rats exposed *in utero* and *via* lactation to TCDD, plasma androgen levels tended to be elevated while testosterone levels were decreased (Roman et al. 1995; Gray et al. 1997; Faqi et al. 1998). Studies in laboratory animals and cells in culture show that TCDD and other *AhR* agonists downregulate the epidermal growth factor receptor (Madhukar et al. 1984; Hudson et al. 1985; Madhukar et al. 1988; Sewall et al. 1993), decrease follicle stimulating hormone and luteinizing hormone receptors mRNAs in cultured granulosa cells (Hirakawa et al. 2000; Hirakawa et al. 2000), and decrease insulin-like growth factor I (IGF-I)-induced binding of the IGF-I receptor (Liu et al. 1992).

Aryl Hydrocarbon Receptor-Estrogen Receptor α Cross-Talk

Investigators have studied *AhR*-estrogen receptor (ER) cross-talk both *in vivo* and *in vitro*, and showed that estrogen treatment enhances TCDD-induced cytochrome P450 1A1 (CYP1A1) expression in the livers of ovariectomized rats, suggesting that ER signaling can modulate *AhR* signaling (Sarkar et al. 2000). Studies have also shown that estrogen receptor α (ER- α) acts as a positive modulator in the regulation of TCDD-inducible genes and is necessary for the transactivation of genes mediated through the DRE in both the human breast carcinoma cell line MCF-7 and human endometrial epithelial carcinoma cell line RL95-2 (Jana et al. 1999; Jana et al. 2000). However, many studies have demonstrated the existence of inhibitory *AhR*- ER cross-talk in rodent uteri and mammary glands, as well as in human breast cancer cells. In human mammary cell lines and ER-positive human breast cancer cells, TCDD inhibits 17 β -estradiol (E2)-dependent cell proliferation (Biegel & Safe 1990) and prevents the secretion of E2-induced proteins, such as tissue plasminogen activator and cathepsin-D (Gierthy et al. 1987).

There is substantial evidence that TCDD does not interact directly with the ER or the progesterone receptor (PR) (Romkes & Safe 1988), so that the antiestrogenic effects of TCDD cannot be explained by direct interaction with these receptors, but must involve modulation of estrogen-induced gene transcription (Kharat & Saatcioglu 1996). Recently, Ohtake et al. (2003) showed that the agonist-activated *AhR*/ARNT heterodimer directly associated with estrogen receptors ER- α and ER- β , with the concomitant recruitment of unliganded ER and

activation of the p300 coactivator, resulting in activation of the transcription of estrogen-responsive genes (Ohtake et al. 2003). They also showed that the activation of *AhR* by dioxins impaired the expression of estrogen-responsive genes when E2 was present. It was suggested that ligand-activated *AhR* signaling varies depending on the concentration of estrogen bound to the ERs. Based on those observations, it could also be speculated that the interaction between TCDD and ER signaling affects expression of a variety of genes. Investigators identified 32 TCDD-responsive genes out of a total of 165 estrogen-responsive including the gene *IGFBP5*, which is involved in fetal bone development (Tanaka et al. 2007). These results suggest that TCDD affects the expression levels of a series of estrogen-responsive genes (Tanaka et al. 2007).

TCDD can induce estrogenic action or inhibit estrogen-induced effects in various tissues because of *AhR*-ER cross-talk (Safe & Wormke 2003; Tanaka et al. 2007). Estrogenic agents produce effects on B cell development in the bone marrow similar to those caused by TCDD. Studies have confirmed that an elevation in estrogen is responsible for the reduced number of B cell precursors in the bone marrow. Further, examination of this phenomenon showed that estradiol impaired progression from the pro-B cell stage to early pre-B cell stage (Medina & Kincade 1994; Medina et al. 2001). In addition to an estradiol-induced decrease in B cell lymphopoiesis, estradiol also modulates the later stages of B cell development. BALB/c mice treated with 17 β -estradiol time-release pellets display a marked reduction in the percentage of transitional type 1 and transitional type 2 B cells (Grimaldi et al. 2001). Studies have demonstrated that E2 treatment of BALB/c mice

transgenic for the heavy chain of a pathogenic anti-DNA Ab induces a lupus-like phenotype with expansion of anti-DNA B cells, elevation of anti-DNA Ab titers, and glomerular immunoglobulin deposition (Bynoe et al. 2000; Grimaldi et al. 2002). Thus, autoreactive B cells that arise in the naïve repertoire are rescued and activated in estradiol-treated mice (Bynoe et al. 2000).

Mouse models of SLE, including B/W F1 and MRL/lpr mice, develop autoimmune disease spontaneously. As with human SLE, female B/W F1 mice exhibit an earlier onset of disease and mortality than male littermates. Studies demonstrated that oophorectomized female B/W F1 mice displayed an increased lifespan similar to male mice, whereas castrated mice exhibited disease kinetics similar to intact female B/W F1 mice (Roubinian et al. 1978; Roubinian et al. 1979). Treatment of B/W F1 or MRL/lpr mice with estrogen also accelerates the onset of disease and mortality (Roubinian et al. 1979; Carlsten et al. 1989; Carlsten et al. 1990; Carlsten et al. 1992).

B-Cell Maturation and Development

B cells are generated from pluripotent hematopoietic stem cells (HSC) in the liver during mid-to-late fetal development and in the bone marrow after birth. HSC differentiate into common lymphoid progenitors (CLP), which then become committed to the B lineage. This lineage choice is reinforced by the absence of notch signaling, which promotes T cell development. Commitment to the B lineage is controlled by sequential expression within the cell of the transcription factors E2A, EBF and Pax5, which direct the expression of B-lineage-specific gene products such

as Ig α , Ig β and the surrogate light κ -chain subunits $\lambda 5$ and VpreB, and RAG-1 and RAG-2 (DeFranco 2007). Progress through the B cell development program is defined in two ways: by the status of Ig gene rearrangement and by the cell surface expression of proteins characteristic of various stages of B cell development. The general scheme of early B-cell development starts with a series of well-characterized molecular steps involving the assembly of gene segments encoding the immunoglobulin molecule. This is followed by heavy chain D-J rearrangements joined by V to yield progenitor B cells and finally by light chain V-J rearrangements to generate B cells expressing complete cell-surface Ig (Alt et al. 1984; Hardy et al. 1991; Hardy & Hayakawa 1991).

B lymphopoiesis can be identified in the fetal mouse liver by gd 12-13 (Hayakawa et al. 1994). Pre-B cells can be found in the fetal liver of mice by gd 14, and B lymphocytes, which are for the most part restricted to IgM expression, can be found by gd 16-17. The basic molecular pathways involved in B-cell lymphopoiesis that have been demonstrated in both the murine fetal liver and neonatal spleen in the mouse, appear identical to those occurring throughout life in adult bone marrow. However, neonatal mouse B lymphopoiesis has no terminal deoxynucleotidyl transferase (TdT). In addition to the absence of TdT, fetal precursors have also been shown to lack precursor lymphocyte-specific regulatory light chain (PLRLC), a lymphocyte-restricted myosin L chain, and to show distinctive delayed expression of MHC class II. One study suggested that the rationale for these fetal differences compared to the adult B lymphopoiesis during development may be related to differences in susceptibility to tolerance (Hayakawa et al. 1994).

Analogous to the function of thymus, as a primary lymphoid organ, bone marrow is responsible for the generation of a B-cell repertoire capable of recognizing a diverse and broad array of antigens critical for survival. However, the diversity of antigenic recognition must be stringent to avoid autoreactivity. B cell autoreactivity is dictated by the specificity of the antibody or immunoglobulin molecule. The immunoglobulin molecule consists of two identical heavy chain and two identical light chain molecules linked by disulfide bonds. The variable regions of the heavy chain and light chain form the antigen binding site. The heavy chain variable region is encoded by gene segments termed the V, D and J genes (Early et al. 1980; Sakano et al. 1980) and the light chain is encoded by V and J genes (Bernard & Gough 1980). The random recombination of these variable gene segments, coupled with the random association of heavy and light chain molecules gives rise to a diverse repertoire of different antibody molecules. Based on this mechanism, non-autoreactive as well as autoreactive B cells are spontaneously generated during B cell repertoire development. When the immunoglobulin is expressed as a secreted molecule, it executes effector functions by the constant region, which vary for the μ , γ , α and ϵ constant region heavy domains. When expressed as a cell surface molecule, it forms the major component of the B cell receptor (BCR), which also includes the molecules $Ig\alpha$ and $Ig\beta$ (CD79). The cytoplasmic tails of the $Ig\alpha$ and $Ig\beta$ transduce signals critical for BCR signaling events involved B cell development and activation (Clark et al. 1994). The BCR plays a critical role in the negative selection of autoreactive B cells (Healy & Goodnow 1998; Sandel & Monroe 1999).

In the adult, the early stages of B cell development occur in the bone marrow (Hardy et al. 1991). Pluripotent stem cells give rise to the first recognizable B cell stage, which is the pro-B cell stage expressing CD45R/B220. Heavy chain gene rearrangement, which is mediated by proteins encoded by the recombination activation genes RAG-1 and RAG-2, occurs at the pro-B cell stage (Oettinger et al. 1990; Li et al. 1993). Expression of the heavy chain molecule coupled with surrogate light chain is required to signal, via BCR, to continue maturation of pro-B cells into pre-B cells. The surrogate light chain acts as a screen for those heavy chains with V regions that will associate well with light chain to form a functional B cell receptor. During the late pre-B cell stage, light chain gene rearrangement takes place, which is also mediated by RAG-1 and RAG-2 (Li et al. 1993). Those pre-B cells with unfavorable heavy chain structures do not undergo pre-B cell clonal expansion and fail to develop further. Upon successful rearrangement of the light chain molecule, pre-B cells differentiate into immature B cells. Surface expression of the newly rearranged IgM molecule occurs at the immature B cell stage of development. Immature B cells migrate to the spleen to continue the differentiation and maturation process. Before becoming fully competent mature B cells in the spleen, the newly arrived immature B cells develop into transitional B cells. The most recognized immature transitional B cells are called transitional type 1 B cells (T1) (Carsetti et al. 1995; Loder et al. 1999) (**Figure 1**).

In the spleen, the new the bone marrow-derived transitional type 1 B cells give rise to the more mature transitional type 2 B cells. Mature B cells develop directly from the transitional type 2 populations or possibly from cells described as

transitional type 3 B cells (Allman et al. 2001). B cells that mature to immunocompetence become either follicular or marginal zone cells or B1 cells (originate from fetal liver precursor). Whether a cell differentiates into a follicular or marginal zone B cell or B1 cells appears to be determined by the strength of the BCR-mediated signal (Niiri & Clark 2002). Each subset predominates in distinct anatomical locations and differ in their phenotype (Sagaert & De Wolf-Peeters 2003) (**Figure 2**).

Exploitation of the BCR to generate very strong signals, as seen in CD22-deficient mice, favors the development of the nonconventional hyperresponsive B1 B cell population (which arises through mechanisms distinct from the conventional B cell subsets) (O'Keefe et al. 1996). A modest decrease in signaling strength, as observed in PKC deficient mice, favors the development of follicular B cells (Leitges et al. 1996). Follicular B cells, which are located in B cell follicles, require antigen and helper T cells for activation. Finally, a strongly diminished BCR signal, which can be achieved by a deficiency in Btk (Cariappa et al. 2001), favors the development of marginal zone B cells. Thus, a general paradigm model has emerged, with some exceptions, in which a strong BCR signal favors B1 cells, a strong-to-intermediate signal favors follicular B cells, and a weak signal favors marginal zone B cells (Leitges et al. 1996; O'Keefe et al. 1996; Cariappa et al. 2001). When follicular B cells are activated, they become germinal center cells and undergo heavy chain class switching and somatic mutation, resulting in plasma cells and memory B cells.

Establishing B cell Tolerance

Purging autoreactive B cells that are spontaneously generated following the random rearrangement and association of heavy and light chains is necessary to ensure self-tolerance. There are a number of “checkpoints” at which autoreactive B cells are eliminated or neutralized, thus, ensuring B cell tolerance. The mechanisms that regulate tolerance induction of autoreactive B cells have been extensively studied in mice that express transgene-encoded autoantibodies. These occur both during B lymphopoiesis in the bone marrow (central tolerance) and after antigen interaction in the spleen (peripheral tolerance). Several mechanisms have been shown to be involved in generating a repertoire of non-autoreactive immunocompetent cells including receptor editing, clonal deletion and anergy. Receptor editing has been characterized as the first mechanism to tolerize autoreactive cells occurring in the bone marrow at the early immature B cell stage following crosslinking of the BCR by self-antigen (Gay et al. 1993; Tiegs et al. 1993).

In the presence of strong BCR signaling, self-reactive immature B cells in the bone marrow arrest maturation and undergo receptor editing, replacing the light chain of a self-reactive antibody by a new light chain, or less commonly the heavy chain by a new heavy chain, thus replacing the autoreactive specificity with a non-autoreactive one. This rescue is accompanied with the re-expression of RAG-1 and RAG-2 (Tiegs et al. 1993; Yu et al. 1999). There is evidence that the early immature B cell stages IgM^{lo}/IgD^{lo} are susceptible to receptor editing to achieve tolerization, whereas the more developed IgM^{hi}/IgD^{lo} B cells are regulated by antigen-induced apoptosis (clonal deletion) (Melamed et al. 1998), suggesting that tolerization in B

cells is developmentally regulated and the stage of B cell development determines how B cells are tolerized. Clonal deletion is thought to occur when receptor editing fails, and is mediated by apoptosis of autoreactive B cells (Sandel & Monroe 1999). If the signaling strength of the BCR by self-antigen is weak or insufficient to trigger apoptosis, autoreactive B cells may leave the bone marrow via the blood and enter the next stage of maturation in the spleen.

Self-reactive immature B cells that escape the correct receptor editing or negative selection (apoptosis) in the bone marrow may be subject to peripheral tolerance in the spleen. Transitional B cells in the spleen that react to self-antigen undergo clonal deletion or anergy but not receptor editing (in a state of non-responsiveness) (Goodnow et al. 1988; Carsetti et al. 1995; Sandel & Monroe 1999). This would suggest that the mechanism of tolerance induction of immature B cells is determined, not only by developmental stage, but by the environment in which immature B cells encounter antigen (Sandel & Monroe 1999). Anergic B cells respond poorly to antigen and T cell help, and display decreased expression of IgM. They will, nevertheless, respond normally to stimulation with T cell-derived mediators such as CD40 ligand and IL-4, suggesting that they retained the capacity to respond under pro-inflammatory conditions and to secrete antibodies (Cooke et al. 1994). Whether transitional B cells undergo receptor editing has yet to be determined. During an immune response, self-reactive B cells can emerge through hypermutation of immunoglobulin genes in B cells proliferating within germinal centers. Thus, tolerization mechanisms must also be active in the germinal center. Studies have shown that newly formed self-reactive B cells within the germinal

center are rapidly eliminated by apoptosis (clonally deleted) (Han et al. 1995; Pulendran et al. 1995; Shokat & Goodnow 1995; Kuo et al. 1999).

B Cells and Autoimmunity

Disruption in normal B cell selection or function can lead to autoimmunity, in which B cell participation traditionally has been viewed as limited to that of antibody producer. However, it has recently been suggested that B cells contribute to the autoimmunity in many different ways. They can function as potent antigen-presenting cells (APCs), or may regulate T cells and dendritic cells (DCs) through the production of cytokines or regulatory mediators (Fujimoto & Sato 2007). B cells tend to have a major role in the pathogenesis of immune-mediated glomerulonephritis (GN) in SLE mouse models and SLE-like autoimmune disease. In these models, antibodies may bind directly to glomerular antigens or form immune complexes, which deposit in glomeruli initiating inflammation. The increased presence of autoantibodies in SLE-like model (SNF₁) suggests that one of the major abnormalities underlying disease development is a breakdown in the mechanisms which maintain B cell tolerance after TCDD exposure. Thus, failure to purge autoreactive cells during B cell lymphopoiesis, abnormal exposure of self-antigen and dysregulation of B cell activation thresholds have all been implicated in the pathogenesis of SLE and other autoimmune models (Clatworthy & Smith 2007).

Mechanisms for B Cell Tolerance Breakdown

A. Failure to Purge Autoreactive B Cells

A.1 The Role of Bcl-2 Family

Programmed cell death (apoptosis) is a mechanism used to enforce central and peripheral tolerance by purging autoreactive B and T cells. Dysregulation in the pathways of programmed cell death may well lead to the survival of autoreactive cells and to autoimmune disease. The proto-oncogene *bcl-2* protects cells from apoptosis (Vaux et al. 1988). Transgenic overexpression of Bcl-2 in B cells prevents apoptosis, obstructs peripheral self-tolerance (negative selection) and can predispose to autoantibody production and immune-mediated glomerulonephritis (Strasser et al. 1991; Hande et al. 1998). Immature autoreactive B cells in the bone marrow escaped central deletion when the anti-apoptotic molecule Bcl-x_L is upregulated (Fang et al. 1998). Overexpression of Bcl-2 in several transgenic mouse models prevents apoptosis and enhances survival of autoreactive B cells that arise in the bone marrow (Hartley et al. 1993; Fulcher & Basten 1997; Janani et al. 1998) and of B cells that attain autoreactivity following somatic hypermutation in germinal centers (Hande et al. 1998). In immature B cells, overexpression of Bcl-2 enhances receptor editing of autoreactive B cells that would normally be eliminated by apoptosis (Lang et al. 1997). In Bcl-2 transgenic BALB/c mice, autoreactive germinal center B cells overexpressing Bcl-2 escape deletion and mature into plasma cells but are not sustained within the memory compartment (Hande et al. 1998; Kuo et al. 1999).

Normally, B cells at the immature and transitional stages are most susceptible to BCR-induced cell death (Sater et al. 1998). However, it has been reported that E2 treatment of primary B cells impaired anti-IgM-mediated cell death of transitional B cells. These data suggest that E2 can alter negative selection in naive immature B cells. An expansion of bone marrow progenitor B cells in E2-treated mice has been reported (Medina et al. 2000; Grimaldi et al. 2002). Interestingly, studies have shown that perinatal TCDD exposure modulates the Bcl-2 gene family in the rat offspring in a sex dependent manner (Chang et al. 2005). In adult mice, TCDD alters Bcl-2 family member's gene expression in thymocytes (Fisher et al. 2004). Whether TCDD exposure mediates this mechanism, of altering Bcl-2 family member's expression in autoreactive B cells that escape negative selection has yet to be fully investigated.

A.2 The Role of Fas-FasL

CD95 (Fas) is a member of the nerve growth factor/tumor necrosis factor receptor superfamily and is a widely expressed glycosylated cell-surface molecule of relative molecular mass 45,000–52,000 (335 amino-acid residues) (Huang et al. 1996; Krammer 2000). Fas can trigger a signal transduction cascade leading to apoptosis via interaction with CD95L (FasL) (Ju et al. 1995; Siegel et al. 2000). MRL mice homozygous for the *lpr* or *gdl* mutation (leading to non-functional Fas or FasL proteins, respectively) have a disrupted homeostasis of the T and B cells, increased proliferation of polyclonal T and B cells, and on certain genetic backgrounds develop autoantibodies, hepatosplenomegaly, lymphadenopathy and immune complex-

mediated glomerulonephritis (Bossu et al. 1993; Russell & Wang 1993; Poppema et al. 2004). The role of perinatal TCDD exposure in altering thymic Fas-FasL interaction has been established, however its role in modulating B lymphopoiesis has yet to be investigated (Kamath et al. 1999; Camacho et al. 2005). Furthermore, mice heterozygous (PTEN+/-) for PTEN, a phosphatase involved in apoptosis, have impaired Fas response and develop a lethal polyclonal autoimmune disorder similar to those observed in Fas-deficient mutants (Di Cristofano et al. 1999; Cheng et al. 2008). Recently, it was shown that Pten is an essential mediator of BCR-induced cell death during the negative selection in immature B cells (Cheng et al. 2008).

A.3 The Role of FcγRIIb in Apoptosis

It has been proposed that FcγRIIb plays a role in central B cell tolerance and peripheral tolerance. Antigens can be retained in the form of immune complexes bound to follicular dendritic cells (FDCs) in the germinal center, and hence, B cell stimulation can occur either through FcγRIIb independently of BCR coligation to directly mediate an apoptotic response or through both FcγRIIb and the BCR blocking apoptotic pathway (Pearse et al. 1999). Therefore, a malfunction of FcγRIIb might allow autoreactive B cells to escape deletion (negative selection). Recent studies suggest that FcγRIIb provides a distal peripheral checkpoint by limiting the accumulation of autoreactive plasma cells, thereby maintaining the peripheral tolerance (Fukuyama et al. 2005).

In addition to its contributing role in maintaining peripheral tolerance, FcγRIIb may also contribute to central B cell tolerance. At the pre-B cell stage of

development, the inhibitory FcγRIIB is expressed in the absence of a functional BCR, suggesting an inhibitory function independent of BCR expression and antigen specificity. Thus, FcγRIIb ligation can induce apoptosis, inhibit migration and inhibit B cell production in the bone marrow (Brauweiler & Cambier 2004). However, if the pre-B cell receptor is cross-linked, then FcγRIIb can negatively regulate apoptosis (Kato et al. 2002). FcγRIIb is also important in modulating plasma cell survival since it is expressed on plasma cells and controls their persistence in the bone marrow. Crosslinking FcγRIIb can induce apoptosis of plasma cells. Plasma cells from lupus-prone mice, including NZB and MRL, do not express FcγRIIb and are thus resistant to apoptosis via this pathway. Thus, failure of FcγRIIb-mediated apoptosis could contribute to plasma cell accumulation and thus, the production of autoantibody (Xiang et al. 2007). Surprisingly, nothing currently published has established a link between perinatal TCDD exposure and FcγRIIb expression on immune cells.

B. Defect in Apoptotic Cell Disposal

B.1 Clearance of Apoptotic Cells Modulates Immune Responses

Efficient apoptotic cell clearance is critical for maintenance of self-tolerance. Abnormally high exposure to self-antigen is one of the mechanisms implicated in SLE and other autoimmune diseases. Therefore, either an increased production or dysfunctional clearance of apoptotic cells might lead to a breakdown of tolerance and autoimmune responses.

Under normal conditions, apoptotic cells are efficiently removed by professional phagocytes to prevent significant inflammatory response (Savill et al. 2002). This rapid removal process prevents the exposure of potential autoantigens from apoptotic "bodies", which might then trigger a pro-inflammatory immune response and generate autoantibodies to nuclear components. Apoptotic cells exposed with a number of "eat me" signals, like phosphatidylserine, to assist in phagocytosis are recognized by receptors on phagocytic cells, including the phosphatidylserine receptor, CD14, the C1q receptor, the vitronectin receptor and the MER receptor (Scott et al. 2001; Savill et al. 2002). The uptake of apoptotic cells involves a wide variety of phagocytoreceptors and soluble bridging molecules which bind these cells in response to "eat me" signals (Savill et al. 2002). Recognition of tag molecules such as C1q, C-reactive protein, serum amyloid P protein (SAP), pentraxin-3, thrombospondin and prothrombinon apoptotic cells by corresponding receptors on phagocytic cells can facilitate uptake by phagocytes (D'Agnillo et al. 2003).

B.2 The Role of the Complement

Phosphatidylserine is one of the C1q ligands on apoptotic cells, and C1q-PS interaction takes place at early stages of apoptosis (Paidassi et al. 2008). Deficiencies in the classical pathway of the complement system have been implicated in the etiology and pathogenesis of SLE. C1q-deficient mice show high levels of ANAs and increased numbers of apoptotic bodies within glomeruli,

suggesting that C1q deficiency causes autoimmunity by impairment of the clearance of apoptotic cells (Botto et al. 1998; Liu et al. 2004). This idea is supported by the observation that most patients with C1q deficiency do develop a lupus-like disease (Slingsby et al. 1996). Deficiency of C4 (Chen et al. 2000), SAP (Paul & Carroll 1999) and MER (Cohen et al. 2002) in mice results in an antinuclear autoimmune response and a glomerulonephritis. Prothrombin binds to apoptotic cells and appears to be a target of lupus anticoagulant antibodies (D'Agnillo et al. 2003).

A few studies have shown that the complement system might be a potential target of TCDD acute exposure. These studies have shown that complement component C3 levels in female mouse serum were decreased following *in vivo* acute or subchronic TCDD exposure (White et al. 1986; Lin & White 1993). However, there still is a significant gap in literature addressing the effects of TCDD perinatal exposure on the complement system.

To summarize, there is sufficient evidence to suggest that the display of nuclear antigens on the cell surface during apoptosis may facilitate the activation of autoreactive B cells and the presentation of these antigens to autoreactive T cells. Thus, impaired apoptotic cell clearance, in part via a deficiency in complement, may contribute to the breakdown of tolerance to nuclear antigens.

C. Altering B Cell Signaling Threshold

C.1 Signaling via BCR Apparatus

BCR apparatus signaling is implicated in both self- tolerance induction and B cell activation threshold. BCR signaling strength is modulated by the synchronized activity of stimulatory and inhibitory receptor and coreceptor molecules. Stimulatory receptors contain immunoreceptor tyrosine-based activation motifs (ITAMs) and inhibitory receptors contain tyrosine-based inhibitory motifs (ITIMs). Antigen engagement by the BCR triggers a signaling cascade, which results in the phosphorylation of the ITAMs within the cytoplasmic tail of the accessory molecules $Ig\alpha$ and $Ig\beta$, which is a critical upstream event for B cell activation. BCRs with phosphorylated ITAMs result in the activation of Src family kinases such as Syk and Btk that are critical for downstream signal transduction events.

BCR signaling strength is also modulated by several membrane positive and negative coreceptors. The most important of these response regulators include CD19/CD21 complex, CD22, CD72, and $Fc\gamma RIIb$. CD19 and CD22 do not merely regulate BCR signals independently, but they have their own regulatory system (Sato et al. 1997; Fujimoto & Sato 2007). When BCR binds with C3d-coupled antigens, the CD19/CD21 complex is congregated with the BCR and undergoes phosphorylation (Dempsey et al. 1996). CD19 serves as a positive response regulator by recruiting signaling molecules necessary for a Src-family protein tyrosine kinase (PTK) activation amplification loop that controls basal signaling thresholds and amplifies Src-family PTK activity following BCR ligation, as well as

maintaining phosphatidylinositol (PI) 3-kinase and Vav stimulation(Fujimoto et al. 2000). By contrast, CD22, CD72, and FcγRIIb act as negative response regulators by downregulating BCR signaling through the recruitment of tyrosine phosphatases including SHP-1 and/or SHIP on phosphorylated ITIMs within the cytoplasmic tail (Cyster & Goodnow 1997; Nitschke & Tsubata 2004). CD22 is an inhibitory regulator of BCR signaling (Carter et al. 1991; O'Rourke et al. 1998). Phosphorylation of the ITIMs within the cytoplasmic tail of CD22 allows association with the SHP-1 phosphatase (Otipoby et al. 2001). SHP-1 inhibits BCR-mediated signals by dephosphorylating molecules in the BCR signaling pathway (Doody et al. 1995).

When antigen interacts with the BCR, the net effects of the signaling loop system mediated by both positive and negative regulatory molecules modulate the threshold of activation for the B cell. Autoimmunity can occur as a consequence of disruption in the balance between these inputs. A reduction of negative feedback signals due to malfunction of negative regulatory molecules such as FcγRIIb, CD22 and PD-1 or an excess of activatory signaling due to abnormalities of CD19/21 system can both lead to a breakdown in tolerance. Thus, altering the expression/function of these signaling components in mice can lead to autoimmune phenotypes.

C.2 The Role of FcγRIIb in BCR Signaling

In addition to its role in apoptosis, FcγRIIb plays an important role in regulating BCR signals. FcγRIIb contains an immunoreceptor inhibitory motif (ITIM) within its cytoplasmic tail, a hallmark of this inhibitory protein family, which when phosphorylated at its tyrosine residue, triggers a negative feedback signal (Takai 2002; Nimmerjahn & Ravetch 2006). FcγRIIb is the only Fc receptor present on B cells, which can co-ligate with the BCR during immune complex binding, to trigger two ITIM-dependent signaling pathways that inhibit cell activation and proliferation. There is compelling evidence to suggest that defective FcγRIIb inhibition is important in the pathogenesis of SLE. Autoimmune-prone mouse strains such as NZB, NOD, BXSB, and MRL express reduced levels of this receptor on macrophages and activated B cells, which has been attributed to a deletion in the FcγRIIb promoter gene (Jiang et al. 2000; Xiu et al. 2002).

FcγRIIb-deficient mice have augmented humoral responses (immunoglobulin levels) following immunization with both thymus-dependent and independent antigens (Takai et al. 1996) and showed an enhanced potential to generate a number of antibody- or immune complex-dependent inducible models of autoimmunity, including collagen-induced arthritis (Kagari et al. 2003), Goodpasture's syndrome (Nakamura et al. 2000) and immune complex-mediated alveolitis (Clynes et al. 1999). In addition, 29/B6/FcγRIIb^{-/-} strain mice derived by being back-crossed for 12 generations onto a C57BL/6 background, but not BALB/c background, develop proteinuria, hypergammaglobulinaemia, autoanti- nuclear antibodies (such as anti-chromatin and anti-dsDNA) and an immune complex-

mediated autoimmune disease resembling SLE in a strain-dependent fashion (Bolland & Ravetch 2000). In the same study, animals reconstituted with RIIB-deficient bone marrow developed glomerulonephritis after the transfer without requiring the presence of RIIB^{-/-} myeloid cells in the recipient, suggesting that the disease is fully transferable and dependent on B cells. This idea is supported by the increased survival of NZB, BXSB and FcγRIIb-deficient mice accompanied by a reduction in antibodies to dsDNA and chromatin, and renal immune complex deposition following normalization of bone marrow FcγRIIb expression by retroviral transduction of bone marrow cells compared with mice reconstituted with control transfected bone marrow (McGaha et al. 2005). Furthermore, mice heterozygous for FcγRIIb deficiency express only a modest reduction in receptor, but have an increased susceptibility to autoimmunity (Bolland et al. 2002). Thus, even minor modulation of FcγRIIB expression or signaling may have a fundamental effect on maintenance of self-tolerance. More recently, a polymorphism in the human FcγRIIB promoter that is linked to lupus has defective inhibitory function. This polymorphism leads to decreased transcription and surface expression of FcγRIIB, which is associated with B cell hyperactivity of human lupus patients (Blank et al. 2005; Kono et al. 2005).

C.3 The Role of CD22

CD22 is a B cell-specific 130–140 kDa protein transmembrane molecule generally categorized as negative regulator (O'Keefe et al. 1996). CD22-deficient

mice show B cell hyper-responsiveness, which correlates with the development of various autoantibodies, including anti-cardiolipin Ab, anti-dsDNA and anti-myeloperoxidase Ab (O'Keefe et al. 1999). Mice deficient in other molecules involved in the CD22 negative feedback pathway, such as Lyn and SHP-1, also display chronically activated B cells and autoimmune disease (Hibbs et al. 1995). Conversely, overexpression can improve survival of a specific subset of autoreactive B cells (Cyster & Goodnow 1995).

CD22 has also been reported to be both a positive and negative regulator of BCR-mediated signal transduction that is necessary for B cell development and activation (Sato et al. 1998). However, since CD22 is involved in downregulating signals mediated through the BCR, it is likely that increased expression of CD22 would impair tolerization of either high-affinity autoreactive naïve B cells, causing them to behave as indifferent or ignorant B cells, or antigen-activated germinal center B cells. Signaling transduction studies suggest that CD19 expression enhances CD22 phosphorylation and that CD22 expression inhibits CD19 phosphorylation (Fujimoto et al. 2000). Thus, CD19 and CD22 launch a loop that reciprocally regulates the function of each to modulate cellular phosphorylation and signaling transduction (Fujimoto & Sato 2007). Interestingly, a genetic variant of *cd22* has been shown to be associated with SLE in a study of Japanese patients suggesting that CD22 could be considered a candidate for the susceptibility genes to autoimmune diseases (Hatta et al. 1999).

C.4 The Role of PD-1

Programmed cell death gene-1 (PDCD1 or PD-1) is a 55-kDa transmembrane protein of the immunoglobulin superfamily containing an ITIM in its cytoplasmic domain. *PD-1*-deficient B6 mice spontaneously developed typical lupus-like glomerulonephritis and destructive arthritis (Nishimura et al. 1999). PD-1 is expressed strongly in the peripheral lymphocytes of both T and B lineages, thus being classified as an activation molecule (Agata et al. 1996). A polymorphism in PDCD1 has also been reported in humans.

An intronic single-nucleotide polymorphism (SNP) in PDCD1 is associated with the development of SLE in ethnic groups such as Europeans and Mexicans. The SNP alters a binding site for the runt-related transcription factor 1 (RUNX1) located in an intronic enhancer. Disruption of RUNX1 binding by the SNP could lead to abnormal regulation of PDCD1 expression, thus contributing to a dysregulation in self-tolerance and leading to chronic lymphocyte hyperactivity. This disruption of RUNX1 could serve as a possible mechanism to facilitate the development of SLE in humans (Prokunina et al. 2002). Thus, disruption in inhibitory signal of B cell activation can lead to B cell hyperactivity and autoimmunity.

C.5 The Role of CD19

CD19 is a 95-kDa immunoglobulin (Ig) superfamily member expressed solely on B cells in a complex with CD21 (complement receptor 2), CD81 and Leu 13 (Smith &

Fearon 2000). CD19 regulates the proliferation and activation signals delivered to the B cell by a variety of extracellular stimuli. Upon CD21 ligation, CD19 serves as a signal-transducing element of the CD19/CD21/CD81 complex to the BCR and results in a decrease in the activation threshold of B cells stimulated through surface IgM (Carter & Fearon 1992). Transgenic mouse studies have shown that overexpression of CD19 in mice results in elevated levels of anti-DNA Abs, rheumatoid factor and hyperactive B cells (Sato et al. 1996). In addition, in transgenic mice expressing a model autoantigen (soluble hen egg lysozyme, sHEL), B cells in sHEL/IgHEL double-transgenic mice are functionally anergic and do not produce autoantibodies. However, overexpression of CD19 in these mice results in the breakdown of peripheral tolerance and the production of anti-HEL antibodies (Inaoki et al. 1997). Further, CD19 density on blood B cells was significantly (~20%) higher in patients with systemic sclerosis compared with normal individuals (Sato et al. 2000). Thus, changes in the activation threshold on B cells even by modest changes in the expression or function of regulatory molecules such as CD19 may contribute to the breakdown of tolerance and the development of autoimmune disease.

Paired box 5 (Pax5), a transcription factor essential for B-cell lymphopoiesis, was downregulated by TCDD, resulting in impaired B cell differentiation and immunoglobulin gene expression in the murine CH12.LX B cell line (Yoo et al. 2004; Schneider et al. 2008). The *AhR* complex recognizes a DNA binding site for Pax5 in the promoter region of the human CD19 gene suggesting *AhR* activation interferes with Pax5-stimulated CD19 gene transcription (Masten & Shiverick 1995).

C.6 The Role of BAFF Family

The B cell survival factor BAFF (BAFF, also known as BLyS, TALL-1 and THANK), a member of the TNF family, is expressed on the surface of monocytes, dendritic cells, neutrophils, stromal cells and activated T cells, and in the serum as a biologically active homotrimer (Moore et al. 1999; Pers et al. 2005). Mice lacking BAFF are profoundly deficient in B cells, whereas BAFF transgenic mice have increased B cell numbers and develop a lupus-like syndrome. In addition, circulating BAFF is more abundant in NZB/W-F1 and MRL-lpr/lpr mice during the onset and progression of SLE (Gross et al. 2000). Increased levels of BAFF have also been found in patients with lupus and appear to be associated with SLE manifestations (Pers et al. 2005). Thus, levels of BAFF must be tightly regulated to maintain B cell survival without triggering autoimmunity.

B cells express three different BAFF receptors, transmembrane activator and calcium modulator ligand interactor (TACI), B cell maturation antigen (BCMA) and BAFF receptor (BAFF-R) at various times during their differentiation. BCMA is expressed on transitional type 1 (T1) cells and on plasma cells, whereas TACI and BAFF-R are expressed on transitional type 2/3 and mature B cells (Schneider 2005). BAFF-R is upregulated by BCR ligation on mature B cells and is expressed on resting memory B cells. BAFF-R mediates most BAFF-dependent functions in the naive B cell population, whereas BCMA is needed for the optimal generation of long-lived plasma cells (Schneider 2005). TACI has mixed positive and negative B cell regulatory functions; TACI-deficient mice have increased B cell numbers and develop an autoimmune phenotype. TACI and BCMA also bind APRIL (a

proliferation-inducing ligand), a molecule homologous to BAFF, which is not necessary for normal B cell development but induces B cell proliferation, class switching and survival (Schneider 2005).

BAFF prolongs B cell survival by regulating the expression of Bcl-2 gene family members. Attenuation of apoptosis by BAFF correlates with changes in the ratios between Bcl-2 family proteins in favor of cell survival, predominantly by reducing the pro-apoptotic Bak and increasing its pro-survival partners, Bcl-2 and Bcl-xL (He et al. 2004). In either resting or CD40L-activated B cells, the NF-kappaB transcription factors RelB and p50 are specifically activated, suggesting that they may mediate BAFF signals for B cell survival. BAFF might enhance signaling through the BCR by upregulating expression of the BCR co-receptors CD21 and CD19 and by potentiating BCR-mediated phosphorylation of CD19.

In non-Hodgkin's lymphoma (NHL) neutralization of endogenous BAFF and APRIL by soluble TACI and BCMA decoy receptors attenuates the survival of NHL B cells, decreases activation of the prosurvival transcription factor nuclear factor (NF)-kappaB, down-regulates the antiapoptotic proteins Bcl-2 and Bcl-x(L), and up-regulates the proapoptotic protein Bax (He et al. 2004). BAFF acts on primary splenic B cells autonomously, and directly cooperates with CD40 ligand (CD40L) in B cell activation *in vitro* by protecting replicating B cells from apoptosis (He et al. 2004). Studies have shown that BAFF- and APRIL-promoted viability of cycling lymphoblasts was associated with sustained expression of cyclooxygenase2 (COX-2), the rate-limiting enzyme for Prostaglandin E₂ (PGE₂) synthesis, within replicating cells, suggesting that a BAFF/APRIL -- TACI -- COX-2 -- (PGE₂) -- myeloid

cell leukemia-1 (Mcl-1) pathway reduces activation-related, mitochondrial apoptosis in replicating human B2 cell clones (Mongini et al. 2006). APRIL and BAFF were potent survival factors for exogenous cytokine-dependent myeloma. These factors activated NF-kappaB, phosphatidylinositol-3 (PI-3) kinase/AKT, and mitogen-activated protein kinase (MAPK) kinase pathways and induced a strong up-regulation of the Mcl-1 and Bcl-2 antiapoptotic proteins in myeloma cells. A recent study has demonstrated that TCDD induces the expression of BAFF in macrophages mediated by the *AhR*/ RelB complex (Vogel et al. 2007).

Marginal Zone B Cells and Autoimmunity

Marginal zone (MZ) B cells reside predominantly in the extra-follicular regions in the spleen and human tonsils. MZ B cells require antigen, but not cognate interactions with helper T cells, for activation and are defined by a pattern of surface marker expression (sIgM^{high} sIgD^{low} CD23^{low}CD21/35^{high} AA4) (Srivastava et al. 2005). The most important functional feature of these MZ B cells is their very early participation in immune responses, specially to blood-borne pathogens, due in part to a lower threshold than recirculating or immature B cells for activation, proliferation and differentiation into antibody-secreting cells (Martin & Kearney 2002). This B-cell subset helps to link the chronological gap in immune responses between rapid innate immune response and delayed T-cell dependent adaptive antibody response to reach peak levels (Martin & Kearney 2002).

MZ B cells express high basal levels of B7.1 and B7.2, and further upregulate these costimulatory molecules upon BCR, CD40, or LPS stimulation (Oliver et al.

1999; Martin & Kearney 2002). These features make them potent antigen presenting cells (APCs), triggering a much more vigorous proliferation of alloreactive T cells than follicular (FO) B cells at early immune response (Oliver et al. 1999). MZ B cells also respond to T-dependent (TD) antigens by generating small and poorly organized germinal centers. Further, they contribute significantly to the early IgM response however, it is the FO B cells that contribute most of the isotype-switched antibodies seen later in an immune response. Thus, while MZ B cells are known to be crucial to the T-independent (TI) response *in vivo*, they might also participate in TD responses (Lopes-Carvalho et al. 2005).

Finally, even though MZ B cells do not appear to secrete natural antibodies, it could be dangerous if they are stimulated into rapid differentiation to plasma cells and undergo class switch and affinity maturation. The fact that the majority of MZ B cells do not normally participate in T-dependent responses (Lane et al. 1986; Liu et al. 1991) may be important, as pathogenic antibodies are usually highly somatically mutated (Shlomchik et al. 1987).

The expansion of MZ B cells in lupus-prone SNF₁ mice has been observed typically before the onset of pathogenic T cell proliferation. These expanded MZ B cells express high levels of costimulatory molecules such as B7.1, B7.2 and ICAM-1 (Wither et al. 2000). In another study, naturally occurring anti-DNA B cells have been found in the splenic CD1⁺ population, enriched in the MZ, and appear to be precursors for autoantibody producing plasma cells in murine SLE (Zeng et al. 2000). Further, B cells appear to be required for priming of pathogenic T cells, as B-

cell deficient MRL/lpr mice do not show an expansion of activated T cells in murine SLE models (Chan & Shlomchik 1998).

Recently, studies have shown that MZ B cells are more efficient and potent than FO B cells at priming naïve T cells. Since these cells are over-represented in lupus-prone strains, it has been proposed that MZ B cells help trigger the T-cell dependent disease (Lopes-Carvalho et al. 2005). Whether the high specificity and affinity, class switched, autoantibodies would then be generated by the interaction of these primed T cells with FO B cells or by provision of help to the MZ B cells themselves needs to be tested.

Initially, the high levels of somatically hypermutated antibodies in SLE-prone mice indicated that the pathogenic plasma cells would be derived from the germinal center reaction (Shlomchik et al. 1987; Shlomchik et al. 1990), where MZ B cells have not been shown normally reside. However, another study also showed that rheumatoid factor reactive B cells in MRL/lpr and B6/lpr mice were not found in the germinal center (Shlomchik et al. 1990). Instead, they were detected in the bridging channels at the red pulp T-cell zone border. Thus, high-affinity pathogenic antibodies may be the product of extra-follicular hypermutation in the red pulp, where MZ B cells normally migrate and secrete antibody upon activation by T-independent antigens. These pathogenic B cells were observed to be affiliated with CD11c⁺ dendritic cells, similar to MZ B cells responding to blood-borne pathogen (Balazs et al. 2002; William et al. 2002).

The observation that lupus-prone mouse strains show impaired disposal of apoptotic cells (Potter et al. 2003), has led to reports that strains with impaired

genes needed for housekeeping debris-clearance functions show lupus-like symptoms (Botto et al. 1998; Napirei et al. 2000). Based on this connection, investigators proposed that abnormal cell death and clearance in lupus-prone strains may thus lead to selective uptake of necrotic cells by MZ B cells, which expand and prime T cells, initiating autoimmune disease. B-cell response to type I IFN is also essential in the induction of murine SLE and over-production of this cytokine and may also be linked to the expansion of MZ B cells seen in *lpr* mice (Braun et al. 2003; Santiago-Raber et al. 2003).

High IFN- γ levels, accompanied by expression of several interferon-regulated genes has been reported in patients with active lupus (Bennett et al. 2003). Dendritic cells that scavenge dead host cells can lead to autoantibody production in both wildtype and lupus-prone mouse strains, however clinical autoimmunity develops in susceptible lupus-prone mice only (Bondanza et al. 2003). If these cells are activated *in vivo* by the cellular debris seen in SLE, they may present host antigens to MZ B cells in the context of high IFN- γ and BAFF secretion, as seen with bacterial infections (Balazs et al. 2002). IFN- γ is a potent inducer of BAFF expression (Litinskiy et al. 2002), and INF- γ thus may also contribute indirectly to an activated, MZ-like phenotype of B cells in murine lupus models as well as high antibody levels.

Studies have demonstrated that 17 β -estradiol (E2) treatment of non-autoimmune BALB/c mice transgenic for the heavy chain of a pathogenic anti-DNA antibody can break B cell tolerance and initiate a lupus-like phenotype with expansion of anti-DNA secreting B cells, elevation of anti-DNA Ab titers, and

glomerular immunoglobulin deposition (Spatz et al. 1997; Bynoe et al. 2000; Peeva et al. 2000). A sustained increase in E2 resulted in an altered distribution of B cell subsets, with a diminished transitional population and an expanded marginal zone B cell population. Furthermore, the B cells that spontaneously secreted anti-DNA antibodies displayed a marginal zone phenotype (Grimaldi et al. 2001). Thus, a sustained increase in E2 alters the splenic B cell maturation process, resulting in the survival, expansion, and activation of a population of autoreactive marginal zone B cells; implicating this B cell subset in lupus-like autoimmunity (Grimaldi et al. 2001).

B-1a B Cells and Autoimmunity

B-1a cells are distinguished from conventional B cells (B2) by their developmental origin, their surface marker expression and their function. They were initially identified as a B cell subset of fetal origin that expresses the pan-T cell surface glycoprotein, CD5 (Berland & Wortis 2002). B-1 cells are larger than B-2 cells, and are defined by a pattern of surface marker expression, B220(CD45)^{lo}, IgM^{hi}, IgD^{lo}, CD9⁺, CD43⁺, CD23^{lo}, as opposed to conventional circulating B-2 cells that are B220(CD45)^{hi}, IgM^{hi/lo}, IgD⁺, CD9⁻, CD43⁻, and CD23^{hi} (Berland & Wortis 2002; Won & Kearney 2002). The majority of B-1a cells are located in the peritoneal and pleural cavities. Compared to B2 cells, B-1a are long-lived, non-circulating, with reduced BCR diversity and affinity (Kantor et al. 1997).

B-1a cells are largely responsible for the production of circulating IgM referred to as natural antibodies. These low affinity antibodies are polyreactive and constitute as such a first line of defense against bacterial pathogens (Carroll &

Prodeus 1998). This polyreactivity also results in the recognition of autoantigens, which functions in the clearance of apoptosis products. The weak autoreactivity of the B-1a cells has been postulated to play a role in autoimmune pathogenesis. In addition, other characteristics, such as the production of high level of IL-10 and enhanced antigen presentation capacities have linked B-1a cells with autoimmunity (O'Garra et al. 1992; Mohan et al. 1998). High numbers of B-1 cells have been reported in patients with SLE, Sjogren's syndrome and rheumatoid arthritis, and numerous associations between expansion of this cell compartment and systemic autoimmunity have been found in murine models (Berland & Wortis 2002).

Effects of TCDD on B-Cell Development

Whereas the majority of studies assessing the immunotoxicity of TCDD have focused on cell-mediated immunity (T cells), several reports have shown that antibody-mediated immunity (B cells) also appears to be a target. Early work by Vecchi and colleagues (Vecchi et al. 1980) showed that acute treatment with TCDD depressed the primary B cell response in adult C57BL/6 mice. Subsequent studies have shown that the degree of suppression correlated with the presence of the sensitive *AhR* allele (i.e. *Ah^{bb}*) expressed in these cells (Luster et al. 1985; Tucker et al. 1986; Blank et al. 1987). Other investigators have focused on B-cell toxicity elicited by TCDD; the majority of these studies have examined the impact of TCDD on the functionality of mature B cells, with little work addressing possible effects on development and maturation. These studies demonstrated that TCDD reduced the levels of mRNA for lymphocyte stem cell-specific enzymes terminal

deoxynucleotidyl transferase (TdT) and recombination-activation gene-1 (RAG-1) in murine bone marrow (Fine et al. 1990; Frazier et al. 1994; Frazier et al. 1994; Silverstone et al. 1994). Although these studies focused on prothymocyte activity the majority of TdT⁺/RAG⁺ bone marrow cells are in fact of the B-cell lineage. Thus, it is plausible that TCDD does also alter B-cell development.

In one study, a single dose of TCDD decreased immature bone marrow B cells in male C57BL/6 mice up to 9 days post treatment (Thurmond & Gasiewicz 2000). Furthermore, the same lab showed that TCDD significantly increased the pre-pro-B population in cultures with the S17 stromal cell line (Wyman et al. 2002). This increase in B220⁺/CD24⁻/CD43⁺ cell numbers was seen in cultures of both wild-type and *AhR*^{-/-} primary bone marrow cells. These findings would appear to suggest that the maturing B220⁺ B cells are not the direct target for TCDD-induced bone marrow B-cell alterations. However, this study did not investigate the effects of TCDD on more mature B cells because the cell line system did not support the differentiation of B cells to the stage of IgM expression.

At the molecular level, a more recent study reported impaired down-regulation of Pax5, a repressor of B cell differentiation and concomitant suppression of the IgM response by TCDD in the murine CH12.LX B cell line (Yoo et al. 2004). This observation suggested the involvement of altered Pax5 regulation in the suppression of the primary IgM antibody response by TCDD. However, the lack of studies investigating altered B cell function after early TCDD exposure has been identified as an important data gap worthy of further examination (Holsapple et al. 2005).

Genetic Factor of SLE

In many mouse models of lupus, genetic differences play a role in susceptibility to autoimmunity. For example, mice with the *Ipr* mutation in the *fas* gene exhibit a lupus phenotype when expressed on the MRL background, but not when expressed on the C57BL/6 background (Warren et al. 1984). Studies were able to identify lupus susceptibility genes [*Sle1*, *Sle2*, and *Sle3* on chromosomes (chr) 1, 4, and 7, respectively] from the lupus-prone NZM2410 mouse on the C57BL/6 background (Morel et al. 1994; Morel et al. 2000). *Sle1* mediates the loss of tolerance to nuclear antigens; *Sle2* lowers the activation threshold of B cells, and *Sle3* mediates a dysregulation of CD4⁺T cells. However, these results indicate that the loss of tolerance to chromatin mediated by *Sle1* is essential for disease pathogenesis (Morel et al. 2000).

Detailed mapping of *Sle1* indicated that the *z* allele of *Sle1* and its component sublocus *Sle1b*, derived from the lupus-prone NZM2410/NZW strain of mice, are linked to a variety of lupus-related disease pathologic manifestations (Morel et al. 2000). *Sle1^z/Sle1b^z* impairs B cell anergy, receptor revision, and deletion. Located within this locus are genes encoding the *SLAM* costimulatory molecules. One member of the *SLAM* family, *Ly108*, is highly expressed in immature B cells and exists in two isoforms *Ly108.1* and *Ly108.2*. The *Ly108.2* isoform functions to censor immature B cells to deletion by altering the strength of B cell signaling after BCR cross-linking. The lupus-associated *Ly108.1* isoform fails to augment BCR signaling leading to the persistence of self-reactive B cells. Thus, *Ly108* may function as a

molecular rheostat, determining the stringency with which autoreactive B cells are censored during early development (Kumar et al. 2006).

In this study, we investigated perinatal TCDD exposure as a potential initiator or/and promoter for autoimmune disease development in the high *AhR* affinity mouse strain C57BL/6, and the low *AhR* affinity autoimmune-prone mouse strain NZB × SWR (SNF₁). The fact that TCDD is capable of directly and indirectly altering transcription of genes involved in critical cellular process, such as cell differentiation, cell proliferation and cell death defines it as a potential chemical agent for immune modulation. On the other hand, autoimmunity can be caused by several mechanisms involving pathways for humoral and cell mediated components, although the exact steps involved in these pathways have yet to be fully detailed. Still, the wealth of available evidence indicates that most of the biological and toxic effects of dioxins are mediated by mechanisms leading to impairment of T cell and B cell self-tolerance mainly during the ontogeny of the immune system.

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Figures

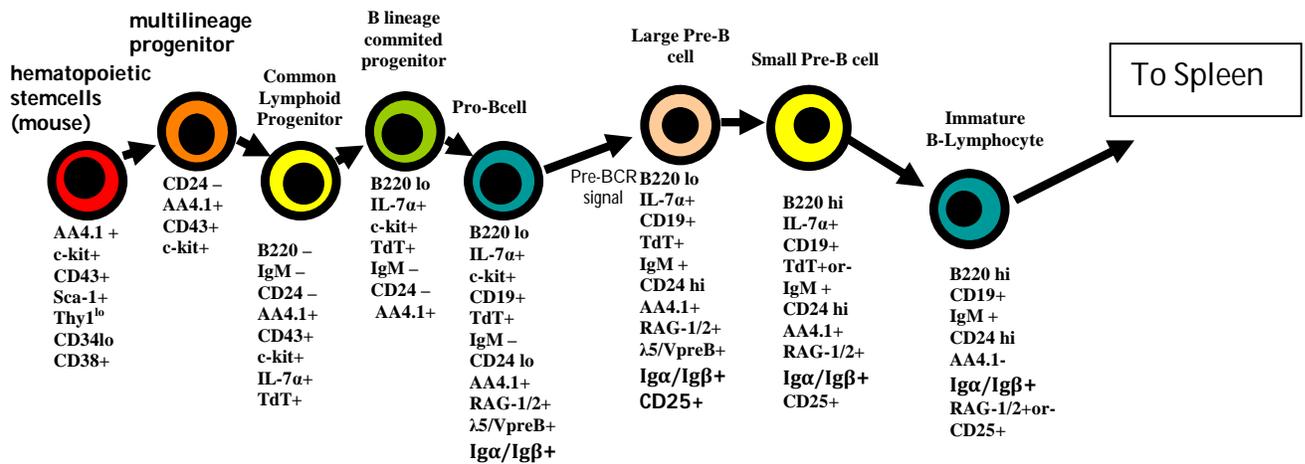


Figure 1. Diagram of B lineage development from hematopoietic stem cells (HSC) in mouse bone marrow through multilineage progenitor (MLP) and common lymphoid progenitor (CLP) to B cell committed stages showing cell surface phenotype.

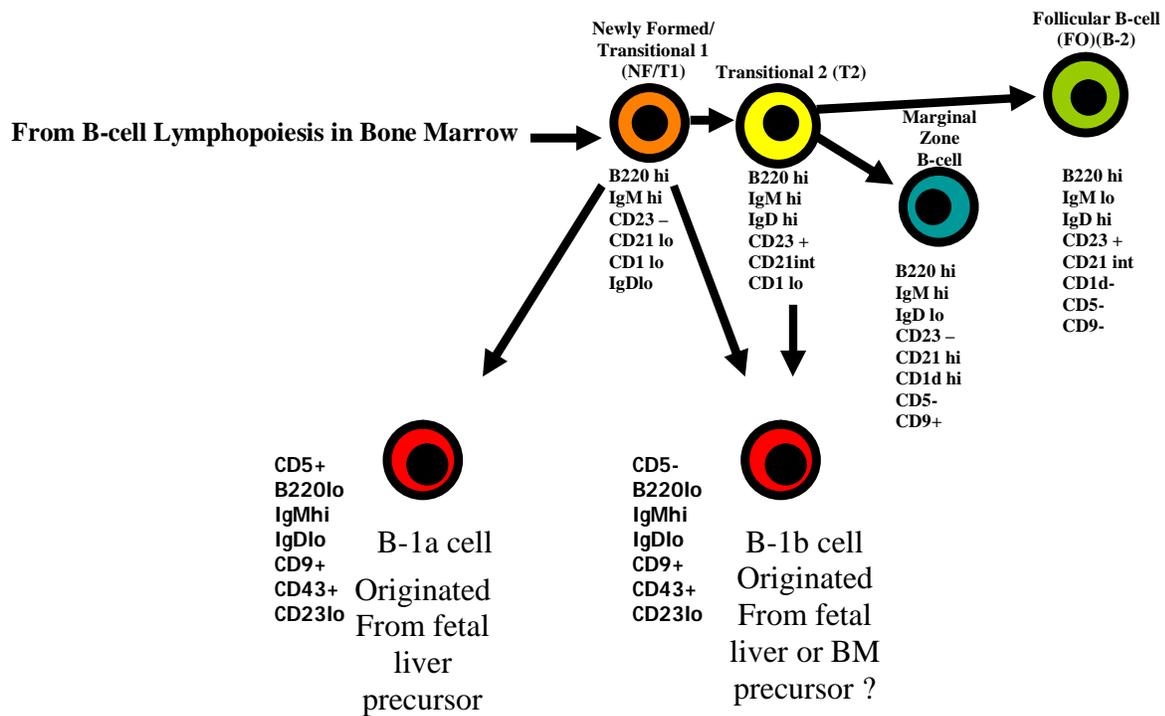


Figure 2. Surface markers that distinguish peripheral B cell populations in the murine spleen. Transitional stage 1 (T1) B cells differentiate into transitional stage 2 (T2), which may either differentiate into mature follicular (FO) B cells or marginal zone (MZ) B cells or B-1b. T1 B cells are also believed to differentiate into B-1a or B-1b.

CHAPTER 2. AN ENHANCED POSTNATAL AUTOIMMUNE PROFILE IN 24 WEEK-OLD C57BL/6 MICE DEVELOPMENTALLY EXPOSED TO TCDD

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Abstract

Developmental exposure of mice to the environmental contaminant and *AhR* agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), causes persistent postnatal suppression of T cell-mediated immune responses. The extent to which prenatal TCDD may induce or exacerbate postnatal autoimmune disease remains unknown. In the present study, time-pregnant high affinity *AhR* C57BL/6 mice received a single oral administration of 0, 2.5, or 5 $\mu\text{g}/\text{kg}$ TCDD on gestation day (gd) 12. Offspring of these mice (n=5/gender/treatment) were evaluated at 24 weeks-of-age and showed considerable immune dysregulation that was often gender-specific. Decreased thymic weight and percentages of CD4⁺CD8⁺ thymocytes, and increased CD4⁺CD8⁻ thymocytes, were present in the female but not male offspring. Males but not females showed decreased CD4⁻CD8⁺ T cells, and increased V β 3⁺ and V β 17a⁺ T cells, in the spleen. Males but not females also showed increased percentages of bone marrow CD24⁻B220⁺ B cell progenitors. Antibody titers to dsDNA, ssDNA and cardiolipin displayed increasing trends in both male and female mice, reaching significance for anti-dsDNA in both genders and for ssDNA in males at 5 $\mu\text{g}/\text{kg}$ TCDD. Immunofluorescent staining of IgG and C3 deposition in kidney glomeruli increased in both genders of prenatal TCDD-exposed mice, suggestive of early stages of autoimmune glomerulonephritis. Collectively, these results show that exposure to TCDD during immune system development causes persistent humoral immune dysregulation as well as altered cell mediated responses, and induces an adult profile of changes suggestive of increased risk for autoimmune disease.

Introduction

Several reports suggest TCDD may increase the risk of autoimmune responses. Similar patterns of inhibited thymocyte differentiation, as seen after TCDD, occur spontaneously in murine models of autoimmune disease (Kakkanaiah et al., 1990) and in mice treated *in vivo* with monoclonal antibodies to major histocompatibility complex (MHC) class I and class II molecules (Kruisbeek et al., 1985). These thymic MHC class I and class II antigens are required for normal thymocyte differentiation as well as for deletion of autoreactive cells (Blaylock et al., 2005). DeWall et al. (1992) observed reduced MHC class II antigen expression in the thymic epithelium of TCDD-treated mice; MHC class I antigen expression was not evaluated in these or subsequent studies. However, TCDD was found to down-regulate expression of an MHC class I gene (*Q1^b*) in a mouse hepatoma cell line (Dong et al., 1997). These authors demonstrated that MHC *Q1^b* cDNA encoded for the $\alpha 3$ domain and transmembrane domain of the *Q1^b* class I protein, implying that the MHC gene product could interact with $\beta 2$ -microglobulin. The authors therefore suggested that the MHC *Q1^b* molecule down-regulated by TCDD may function in antigen presentation, similar to MHC class I antigen in the thymus. These effects on MHC class I and II molecules by TCDD raise questions regarding ability of TCDD to impair autoreactive thymocyte deletion.

In addition to potentially increasing thymic release of autoreactive cells, TCDD increased extrathymic production of T cells, i.e., in compartments where negative selection does not occur or is less efficient than in thymus. For instance, Silverstone

et al. (1994) found that TCDD induced T cell differentiation in the liver of young adult mice. These extrathymic-derived T cells expressed elevated levels of CD4⁺ V β 17a and V β 3⁺ TCR. Such TCR variable β (V β) chains are usually deleted in the thymus by reaction with self-MHC and minor lymphocyte stimulatory antigens (Okuyama et al., 1992; Hanawa et al., 1993) and have been associated with autoimmunity in experimental mouse models (Rocha et al., 1992).

Exposure to immunotoxic compounds during the prenatal period of immune system establishment often results in more dramatic and persistent immunologic damage than does adult exposure (Holladay and Smialowicz, 2000; Dietert and Piepenbrink, 2006; Luebke et al., 2006). Using an autoimmune nephritis mouse model (SWR x NZB) and developmental exposure, Silverstone et al. (1998) observed significantly reduced time to postnatal onset of glomerulonephritis in the male SNF₁ mice offspring, after a single maternal exposure to 10 μ g/kg TCDD. T cells, which may be preferentially targeted by developmental TCDD (Vos et al. 1974; Faith & Moore 1977; Luster et al. 1980), do not appear to play a direct role in tissue damage in SNF₁ lupus nephritis. However cognate interaction between autoreactive T_h cells and autoreactive B cells may be involved in the proliferation of autoantibodies (Mohan et al., 1993). Sera of SNF₁ mice also contained high titers of IdLNF1 + IgG antibodies, which peaked at 22-24 weeks and coincided with an increase in the CD4 to CD8 ratio of IdLNF1-reactive T cells and IdLNF1 Ig (IgG + IgM) deposition in the kidneys (Uner et al., 1998). The presence of pathogenic autoantibody-inducing T_h cells specific for chromatin subparticles or histones has also been described in human patients with lupus as well as lupus-like SNF₁ mice (Fournel et al., 2003).

These data suggest the possibility that early onset of autoimmune nephritis in SNF₁ mice exposed to TCDD could result from a T cell lesion. However, postnatal day 3 thymectomy, which depletes CD4⁺CD25⁺ regulatory T cells and leads to multiple independent organ-specific autoimmune diseases, paradoxically protects SNF₁ mice from lupus-like glomerulonephritis and may suggest lack of T cell involvement (Bagavant et al., 2002).

In contrast to the unproven contributions by T cells, autoantibodies produced by B cells display strong polyreactive responses to DNA and glomerular substrate dominating the deposits in lupus kidneys (Xie et al., 2003). The possibility that prenatal TCDD may directly alter B cell activity to induce or exacerbate the autoimmune disease remains uninvestigated. In this regard, the lack of studies investigating B cell function following developmental TCDD exposure was recently identified as an important data gap worthy of further examination (Luster et al., 2003; Holsapple et al., 2005). The present studies therefore examined secondary lymphoid organ T cell phenotypes including V β TCR expression, and signs of altered B cell lymphopoiesis and autoantibody production, and immune complex deposition in kidneys, in high affinity *AhR* C57BL/6 mice after prenatal TCDD.

Methods

Mice and TCDD exposure. C57BL/6 mice were obtained from Charles River Laboratories (Portage, MI) at 4–5 weeks-of-age. Mice were acclimated to the animal care facility for at least 2 weeks prior to breeding. Briefly, 80 female C57BL/6 mice were bred overnight using one C57BL/6 male per two females. Mice were checked for the presence of a copulatory plug the following morning, which was designated as gestation day (gd) 0. Pregnant C57BL/6 mice were orally gavaged on gestation day 12 with 0, 2.5, 5 or 10 µg/kg TCDD dissolved in corn oil (n= 12 pregnant mice/treatment). The F₁ offspring remained with dams until weaning, then were separated by treatment and allowed to mature to 24 weeks-of-age. All animals were fed a commercial pelleted diet and provided water *ad libitum*, and were housed under controlled conditions of temperature (22 °C), humidity (40–60%), and lighting (12:12 light:dark cycle). Animal maintenance, care and use were at all times in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines at Virginia Tech, and approved prior to initiation of experiments.

Body weights and tissue collection. Mice were euthanized by cervical dislocation and weighed. The thymus, spleen, bone marrow and mesenteric, axillary and inguinal lymph nodes (LN) were immediately collected post-euthanasia under aseptic conditions, using dissection scissors and curved forceps. Mesenteric LN were used for histopathology, while axillary and inguinal LN were pooled for flow cytometry. Non-LN organs were weighed and then placed individually into pre-labeled sterile

Petri dishes (Corning, Corning, NY), containing 8 mL of RPMI-1640 culture medium (Mediatech, Herndon, VA). Dishes were placed on ice until tissue dissociation.

Cell dissociation and enrichment. Each organ was gently dissociated over a stainless steel sieve screen (Sigma, St. Louis, MO) using curved forceps. Cells were then pipetted through the sieve screen following dissociation to remove debris. Cells were washed in RPMI-1640 (Mediatech) for 10 min, 240 x g, and 23°C. The supernatant was then discarded and the cell pellet was resuspended in 8 mL of RPMI-1640. Spleen cells were then resuspended in 1 mL incomplete RPMI-1640. To each tube, 2 mL of ACK 0.83% ammonium chloride lysis buffer (pH 7.29) were added, to lyse red blood cells, and tubes were incubated for 5 min at 23°C. After lysis incubation, the cells were resuspended in 5 mL of incomplete RPMI-1640 (Mediatech) and washed twice (7 min, 290 x g and 7 °C). The splenic leukocyte-rich cells were then resuspended in 5 mL complete RPMI-1640 media containing 10% FBS (Sigma), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/mL penicillin (ICN), and 50 mg/mL of streptomycin (ICN), and maintained on ice. For bone marrow isolation, femurs were removed and the bone marrow cavities flushed with 2% FBS-PBS, washed once (7 min, 290 x g and 7 °C), resuspended in 1 mL incomplete RPMI-1640 media (Mediatech) and stored at 4 °C. Cells were adjusted to 5.0×10^6 cells/mL in complete media.

Cell enumeration. Cells were enumerated and size-analyzed using a Beckman Multisizer 3® Coulter cell counter (Fullerton, CA) according to the manufacturer's

protocol. Briefly, a 10 μ L aliquot of enriched cell suspension was transferred to a plastic counting-chamber containing 10 mL of PBS (Mediatech). The plastic chamber was capped, mixed by repeated gentle inversion, and counted.

Flow cytometric evaluation of cell-surface phenotypic markers. Cell suspensions ($5 \times 10^5/100 \mu$ L) from the thymus, spleen, lymph node and bone marrow were aliquoted into individual wells of a 96-well round-bottom tissue culture plate (Corning, NY). Monoclonal antibodies (mAbs) with phycoerythrin (PE) fluorescent labels were used according to manufacturer (BD Pharmingen; San Diego, CA) recommendation at a concentration of 0.2 μ g/ μ L; mAbs with fluorescein isothiocyanate (FITC) fluorescent labels were similarly used at the recommended concentration of 0.5 μ g/ μ L. Cells were stained as previously described (Gogal et al. 2001; Klein et al. 2006). Briefly, lymphocyte aliquots (5×10^5 cells/ 100 μ L) from thymus, spleen, lymph node and bone marrow were incubated with the following primary mAbs: PE-anti CD4, FITC-anti CD8, FITC-anti CD25, PE-anti CD45/B220 (Ebioscience, San Diego, CA); FITC-anti V β 3 TCR, FITC-anti V β 17a TCR, FITC-anti CD45/B220, PE-anti CD24 (HSA), FITC-anti CD1, PE-anti CD23 (BD Pharmingen). For double staining protocols, mAbs with different fluorescent labels were simultaneously added to the sample. For bone marrow, aliquots of 5×10^5 cells were pre-blocked with anti-Fc III/IIR (clone 2.4G2, Rat IgG2_b). Following staining, cells were washed and evaluated on a Coulter Epics XL flow cytometer (Coulter, Miami, FL). From each sample, 5000 events were collected and analyzed using the Immuno-4 software program. Dead

cells, clumps, and debris were excluded electronically by gating on forward (FSC) versus side scatter (SSC).

ELISA for autoantibodies to double-stranded DNA, single-stranded DNA and cardiolipin. To detect anti-dsDNA antibody, 96-well high-binding microtiter plates (Corning, Corning, NY) were coated with heat-denatured calf thymus DNA (100 mg/ml; Sigma). For anti-ssDNA antibody, DNA from calf thymus was used (10 mg/mL; Sigma), and for autoantibodies to cardiolipin a solution from bovine heart was used (10 mg/ml; Sigma). All pre-coated plates were incubated at 4°C overnight. The plates were washed three times with 300 µL PBS/0.05% Tween 20 (Mediatech), blocked with 1% BSA (Sigma) for 2 h at 23 °C, washed and then incubated with diluted serum samples to be tested (1/25). After 3 h at 23 °C, the serum-coated plates were washed three times with 300 µL of PBS-0.05% Tween 20. To each well, 0.2 mL of diluted alkaline phosphatase-conjugated anti-IgG antibody (1/3000) (Sigma) were added and the plates were incubated for 30 min at 23 °C. The plates were then washed three times with 300 µL of PBS-0.05% Tween 20. To each well, 0.2 mL prepared substrate for alkaline phosphatase conjugated secondary antibody (SIGMA FAST™ p-Nitrophenyl phosphate tablets) were added and allowed to develop for 45 min at 23 °C before adding 50 µL of 3 M NaOH stop solution (Sigma, St. Louis, MO). The absorbance (A_{405}) of the initial dilution was measured. Optical density (OD) readings represent the average from sera from each mouse performed in duplicate.

Histology of the kidneys, livers and lymph nodes. Kidneys, livers and mesenteric lymph nodes were collected at the time of euthanasia and immediately fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA) for 48 h, processed and embedded in a paraffin block. After embedding, a 5 µm section was cut from each tissue block, which was subsequently stained with hematoxylin and eosin (H&E, Richard-Allen Scientific, Kalamazoo, MI) using standard histologic methods. The prepared slides were then evaluated, with a light microscope, in a blinded-manner by a board certified veterinary pathologist (co-author PS).

Immunohistochemistry of the kidneys: C3 and IgG deposition. Kidney frozen sections were cut into 5 mm sections and stained with FITC conjugated antibodies. Briefly, tissue sections were thawed at room temperature and dried for 30 min. Slides were fixed in acetone for 10 min and then washed with PBS (Mediatech) three times for 3 min/wash. Goat anti-mouse IgG diluted 1:100 (Pierce, Rockford, IL) or goat anti-mouse C3 diluted 1:100 (Pierce, Rockford, IL) were incubated with tissues sections in humid chamber for 60 min at 23 °C. The sections were then rinsed three times for 5 min/wash with PBS (Mediatech). The slides were mounted using Vectashield™ mounting media (Vector Labs, Burlingame, CA) and then examined on an Olympus BX-60 fluorescence microscope (Center Valley, PA). The severity of glomerulonephritis and immune complex deposition was scored using a range from 0 to 3+, where 0 corresponded to a non-autoimmune healthy mouse and 3+ to the maximal alteration observed in the study. All slides were scored in a blinded manner by an experienced investigator (co-author CR).

Cytokine ELISA Assays. Splenocytes were plated into each well (1 mL in complete media; 5×10^6 cells per well) of a 24-well tissue-culture plate (Corning Cell Wells™, Corning, Corning, NY). Cells were co-cultured with 1 mL Con A (10 µg/mL, Sigma) and incubated at 37 °C, 5 % CO₂ for 48 h. The plates were centrifuged (7 °C, 250 x g, 7 min), and the supernatants were transferred to a sterile 12 x 75 mm cultured tubes (Fisher). Supernatants were stored at – 80 °C until use. The levels of interleukin 2 (IL-2), IL-4, IL-10, IL-12 and INF-γ were determined using ELISA kits (Ready-to-use; Ebioscience) according to the manufacturer's instructions.

Statistical analysis. Data were expressed as arithmetical mean ± SEM. The pregnant dam was maintained as the statistical unit in all cases such that each offspring analyzed represented a separate dam (one pup/gender/treatment). Analysis of variance (ANOVA) was used with Dunnett's *t*-test to establish significant differences among the same sex groups. Group size was 5 for all experiments ($n=5$). Results described as different in this report indicate significantly different at $p < 0.05$.

Results

Prenatal 10 µg/kg TCDD exposure resulted in postnatal mortality

The F₁ offspring of dams orally dosed with 0.0, 2.5, 5.0 or 10 µg/kg TCDD on gestational day 12 were monitored and allowed to mature to 24 weeks-of-age. By postnatal day 28, ninety-percent of the C57BL/6 pups born to the dams exposed to 10 µg/kg TCDD had succumbed to a wasting-like syndrome (Figure 1). This dose produced no overt toxicity in previous studies that utilized gd 18 data collection from pups derived by C-section (Holladay et al., 1991) or in pups cross-fostered to naïve dams (Blaylock et al., 1992), suggesting sufficient postnatal TCDD exposure occurred by lactation to induce the wasting syndrome. Alternately, the higher dose of TCDD may have diminished lactation due to impaired mammary gland differentiation (Vorderstrasse et al., 2004). The other treatment groups did not show any significant mortality. Therefore, the study was carried on from this point with the vehicle (0.0 µg/kg) and two treatment groups: 2.5 and 5 µg/kg TCDD.

Body and thymus weights

Body weight was reduced relative to controls in both 24-week-old male and female offspring of TCDD-treated dams, at the 2.5 µg/kg dose. Thymic weights showed a numerical trend toward decrease in males and significant decrease in females, in the 2.5 µg/kg and 5.0 µg/kg TCDD exposure groups. Thymic weight/body weight ratios were decreased significantly at 24 weeks, at both doses of TCDD, in females but not males. Similarly, mean thymic cellularity decreased

significantly at both doses of TCDD in females but not males. Splenic weight, splenic body weight ratio and cellularity yielded no significant changes across treatment groups (**Table 1**).

Thymic T cell differentiation

Female but not male offspring of dams dosed with 5.0 µg/kg TCDD exhibited marginal but significant thymic phenotypic changes at 24 weeks-of-age. The percentage of CD4⁺CD8⁺ double positive thymocytes was decreased and of CD4⁺CD8⁻ was increased in female mice exposed to 5.0 µg/kg TCDD on gd 12 (**Table 2**). Absolute numbers of cells in these phenotypes was decreased due to smaller thymuses in the female mice (data not shown).

T cell phenotypes in the spleen and lymph nodes

The 5.0 µg/kg gd 12 dose of TCDD caused a significant decrease in the percentage of CD4⁻CD8⁺ cells in the spleens of 24-week-old male, but not female, mice. The percentage of Vβ3⁺ TCR and Vβ17a⁺ TCR T cells showed a numeric (non-significant) dose-response trend toward increase in female mice. However, both of these Vβ phenotypes were significantly increased in the 5.0 µg/kg males (**Table 3**).

The 5.0 µg/kg gd 12 dose of TCDD caused a significant decrease in the percentage of the CD4⁺CD8⁻ cells and a significant increase in non-T cells (CD4⁻CD8⁻) in lymph nodes of female, but not male, mice. Total activated/regulatory T cells (CD4⁺CD25⁺) were not affected by prenatal TCDD (**Table 4**).

Phenotype of mature B cells in the spleen

The 5.0 µg/kg gd 12 dose of TCDD resulted in a significant decline in splenic CD24⁺B220⁺ B cells at 24 weeks, in both males and females. Staining of these cells with CD21 revealed a significant increase in cells expressing both the CD24-CD21⁺ and CD23-CD1⁺ in 5.0 µg/kg TCDD-exposed mice. This phenotype is consistent with the marginal zone (MZ) B cells suggesting an expansion of these cells (Grimaldi et al., 2001, Pillai et al., 2005). Additionally, CD138⁺ plasma cells increased by dose reaching significance in the 5.0 µg/kg TCDD-exposed male mice (**Table 5**).

B lymphoid progenitors in bone marrow

The 5.0 µg/kg gd 12 dose of TCDD caused significantly increased bone marrow-derived non B cell committed populations (CD24⁺B220⁻) in both genders and, in males, increased percentages of the earliest B cell progenitors (CD24⁻B220⁺). Immature and mature B cells (CD24⁺B220⁺) were decreased in both genders (**Table 6**).

Anti-dsDNA, anti-ssDNA, and anti-cardiolipin titers

Antibody titers to dsDNA, ssDNA and cardiolipin displayed increasing trends in both male and female mice, reaching significance for anti-dsDNA in both males and females at 5.0 µg/kg TCDD. A significant increase in ssDNA was present in 5.0 µg/kg male mice (**Figure 2**).

Deposition of anti-IgG and anti-C3 immune complexes in the kidney

Autoimmune glomerulonephritis was induced in male SNF₁ mice by prenatal TCDD exposure (Silverstone et al., 1998). Immunohistochemistry was therefore performed on kidneys from the present 24-week-old mice, and showed dose-related increased deposition of anti-IgG and anti-C3 immune complexes in mice that were exposed to TCDD during development. Visual and numeric increased mean immunofluorescence was clearly present in the 5.0 µg/kg TCDD group, and comparable, for both anti-IgG and anti-C3 probes (representative IgG deposition shown in **Figure 3**). The 2.5 µg/kg TCDD kidneys were more variable with some samples showing increased mean immunofluorescence and some comparable to control (not shown).

Lymph node and liver histopathology

Histological examination of the mesenteric lymph nodes from 2.5 and 5.0 µg/kg TCDD mice showed evidence of disruption of the architecture, including blurring of the usual distinct zones. In some mice the cell populations were regionally monomorphic suggestive of neoplasia. Moderately diminished follicular activity was seen in 5.0 µg/kg female mice and 2.5 µg/kg male mice (**Figure 4**).

Histological examination of the livers from 5.0 µg/kg TCDD-treated male group mice showed massive infiltration of lymphocytes around some central veins and increased number of small foci of necrosis with infiltration of inflammatory cells. The 2.5 µg/kg male group exhibited scattered lymphoid focus and a few small, scattered foci of necrosis with inflammation. Sections of livers from females in both

dosage groups had moderate vacuolation, moderate localized telangiectasis and moderate small foci of necrosis with inflammation. The liver sections from 2.5 µg/kg females exhibited scattered small foci of necrosis with no significant histologic changes compared to controls. In general, livers from females exhibited lesser pathological signs than the similar male dose group (**Figure 5**).

Th1/Th2 cytokine balance

Analysis of cytokine levels from supernatants of Con-A-stimulated splenic lymphocytes showed that the IFN- γ and IL-2 levels tended to increase non-significantly across treatment and gender of prenatal TCDD- exposed mice. IL-10 was significantly diminished in males dosed with prenatal TCDD, while females trended in the same direction. IL-12 was depressed in the females but not males at 5.0 mg/kg TCDD. Although not analyzed statistically in this study, IL-4 and IL-10 levels appeared to be markedly different between males and females, however were not affected by TCDD (**Figure 6**).

Discussion

The incidence of nephritis is low in autoimmune NZB mice, but when crossed with normal SWR mice, almost 100% of the female F₁ hybrids (SNF₁) develop glomerulonephritis that is fatal by about 8 months-of-age. Autoimmune disease progression in lupus-like SNF₁ mice as in the human version of the disease is critically dependent on T cell function, including accelerated antibody production subsequent to inappropriate activity of CD4⁺ T helper cells. The autoimmune nephritis is then characterized by IgG deposition in kidney glomeruli (Mohan et al., 1993).

Our laboratory and others have suggested that TCDD exposure during the sensitive period of immune system development may increase risk of postnatal autoimmune disease (reviewed by Holladay, 1999). In the single study of its kind, Silverstone et al. (1998) exposed autoimmune-predisposed (SWR x NZB) F₁ mice to a mid-gestation dose of 10 µg/kg TCDD. These authors reported significantly reduced time to postnatal onset of glomerulonephritis in the male SNF₁ offspring. Mechanisms underlying this induced autoimmunity in the normally resistant males are not known. Both SWR and NZB mice are of the Ah^{dd} phenotype (low affinity *AhR*/less sensitive to TCDD) whereas C57BL/6 mice are of the Ah^{bb} (sensitive) phenotype (Holsapple et al., 1991). The present experiments therefore used developmental exposure in TCDD- and immunologically-sensitive C57BL/6 mice to detect postnatal changes in T and B cells that may relate to increased risk of autoimmune disease.

The ability of TCDD to alter T cell maturation and differentiation may negatively impact central tolerance. Damage to thymic epithelium by TCDD has been detected, leading to suggestions that thymic epithelium-dependent selective events may be impaired, through which thymocytes expressing autoreactive T cell receptors (TCR) are deleted (De Waal et al. 1992; Schuurman et al. 1992). Thymic epithelial distribution of MHC class II molecules was also altered in TCDD-treated mice, an additional effect hypothesized as having potential to cause defective thymocyte selection (De Heer et al. 1994). These reports may support the present observation of dramatically increased autoreactive T cells expressing $V\beta 17a^+$ and $V\beta 3^+$ TCR in the spleens of male TCDD-treated mice. The marked difference between $V\beta$ TCR expression by gender in C57BL/6 mice, with females showing no increased expression, suggests possible influence of endogenous hormones. Whether these $V\beta$ T cells are increased in SNF₁ males and may contribute to TCDD-induced glomerulonephritis is not yet known.

Previous studies have shown that thymic atrophy, thymocyte hypocellularity and delayed thymocyte maturation rebound shortly after birth in offspring of TCDD-treated pregnant mice (Fine et al. 1990; Holladay et al. 1991). These thymic effects were no longer apparent by postnatal day 14 in mice exposed to 1.5 or 3.0 mg/kg/day TCDD from gd 6-14 (Holladay et al. 1991). Rats exposed to 3 mg/kg TCDD on gd 14 had decreased percentages of CD4-8⁻ thymocytes lasting until three weeks of age and decreased thymic/body weight ratio lasting until 14 weeks of age, in both genders (Gehrs et al., 1997). These rats showed persistent and gender-dependent T cell function as indicated by significant depression of the delayed-type

hypersensitivity (DTH) response lasting to 4 months of age in females and 19 months of age in males (Gehrs and Smialowicz, 1999). The present mice also responded differently by gender to early TCDD exposure, and differently from the above rats, with decreased thymic weight, decreased percentages of CD4⁺CD8⁺ thymocytes, and increased CD4⁺CD8⁻ thymocytes occurring in only the female offspring.

In addition to increased V β TCR expression in splenocytes, male but not female offspring of TCDD-treated dams showed decreased CD4⁻CD8⁺ T cells in the spleen. Hess and colleagues (1990) demonstrated that the therapeutic immunosuppressive drug cyclosporine A (CsA) interfered with negative selection of autoreactive thymic T cells in Lewis rats following irradiation and syngeneic bone marrow reconstitution, an effect they were able to relate to a TCDD-like down-regulation of thymic MHC antigen presenting molecules. These authors did not examine V β TCR expression, however found that autoreactivity, in the form of a syngeneic graft-vs-host response, could be adoptively transferred into naïve hosts by injection of CD4⁻8⁺ splenocytes from the CsA-treated rats. The present male mouse spleens contained approximately 6% CD4⁻8⁺ splenocytes and approximately 31% CD4⁺8⁻ splenocytes, suggesting the observed increase in V β TCR expression occurred primarily in the Th lymphocyte population. These data suggest the possibility of defective thymic Th lymphocyte selection (MHC class II restricted) in males caused by prenatal TCDD, which may be supported by the observation of De Heer et al. (1994), that distribution of thymic MHC class II molecules is altered by TCDD.

Disruption in normal B cell selection or function can lead to autoimmunity, in which B cells do not merely serve as passive producers of autoantibodies but also have important roles in antigen presentation and cytokine production (Fujimoto & Sato 2007). The majority of studies assessing developmental immunotoxicity of TCDD have focused on T cells. However, autoimmune glomerulonephritis in SNF₁ mice has a major B cell component and was induced by prenatal TCDD (Silverstone et al., 1998). Prenatal TCDD exposure also targets terminal deoxynucleotidyl transferase (TdT)-positive bone marrow cells that include progenitor T and B cells in mice (Fine et al., 1989). Further, the lack of studies investigating altered B cell function after early TCDD exposure is a recognized important data gap in the literature (Luster et al., 2003; Holsapple et al., 2005). For these collective reasons, B cell endpoints were also evaluated in the present mice at 24 weeks-of-age.

Male offspring but not females showed increased percentages of bone marrow CD24⁺B220⁺ B cell progenitors at 24 weeks-of-age, while both genders had reduced numbers of B cells in the spleen. These effects suggest long-lasting dysregulation of B lymphopoiesis by developmental TCDD. Autoantibody production against dsDNA was increased in both the male and female TCDD mice, and against ssDNA in males at 5 µg/kg TCDD. These are the first data showing persistent alterations in B-lineage cells, and in humoral immune function, as a consequence of TCDD exposure during immune system development.

In summary, male and female C57BL/6 mice showed persistent changes in both T and B cells as a consequence of gd 12 exposure to TCDD. Among these were numerous gender-specific effects, suggesting possible interactions with endogenous

hormones, which may not manifest until puberty when hormone levels significantly shift. In this regard, Karpuzoglu-Sahin et al. (2001) reported an immune system imprinting effect of prenatal diethylstilbestrol (DES), such that T cells in late-adult mice over-produced the cytokine IFN- γ , but only following a secondary exposure to this estrogenic compound. The present mice displayed a clear autoimmune profile, including increased V β TCR expression, increased autoantibody production, and increased IgG and C3 disposition in kidney glomeruli. The TCDD-related histopathologic changes in kidneys were suggestive of progressing autoimmunity and may be viewed as surprising, in that C57BL/6 mice typically do not express autoimmune disease or glomerulonephritis. Collectively, these data suggest that developmental exposure to TCDD, during ontogenesis of the immune system, may increase risk of autoimmune disease.

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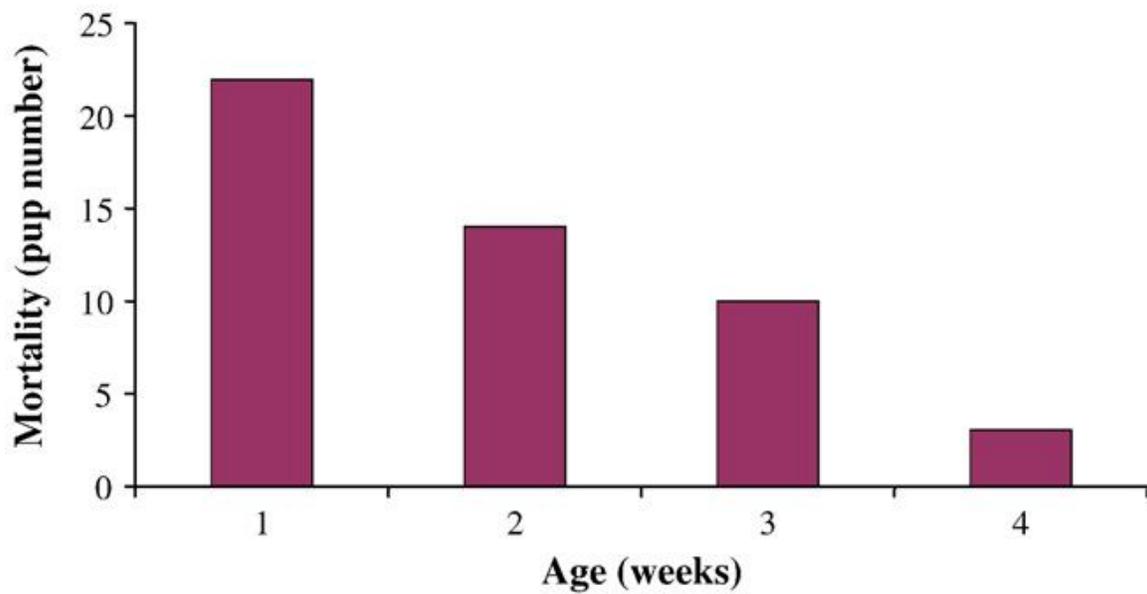


Figure 1. The incidence of mortality in C57BL/6 pups born from dams prenatally-exposed to 10 $\mu\text{g}/\text{kg}$ TCDD. The data represent the number of pup deaths observed up to 30 days post-parturition. No significant mortality was observed in the other treatment groups.

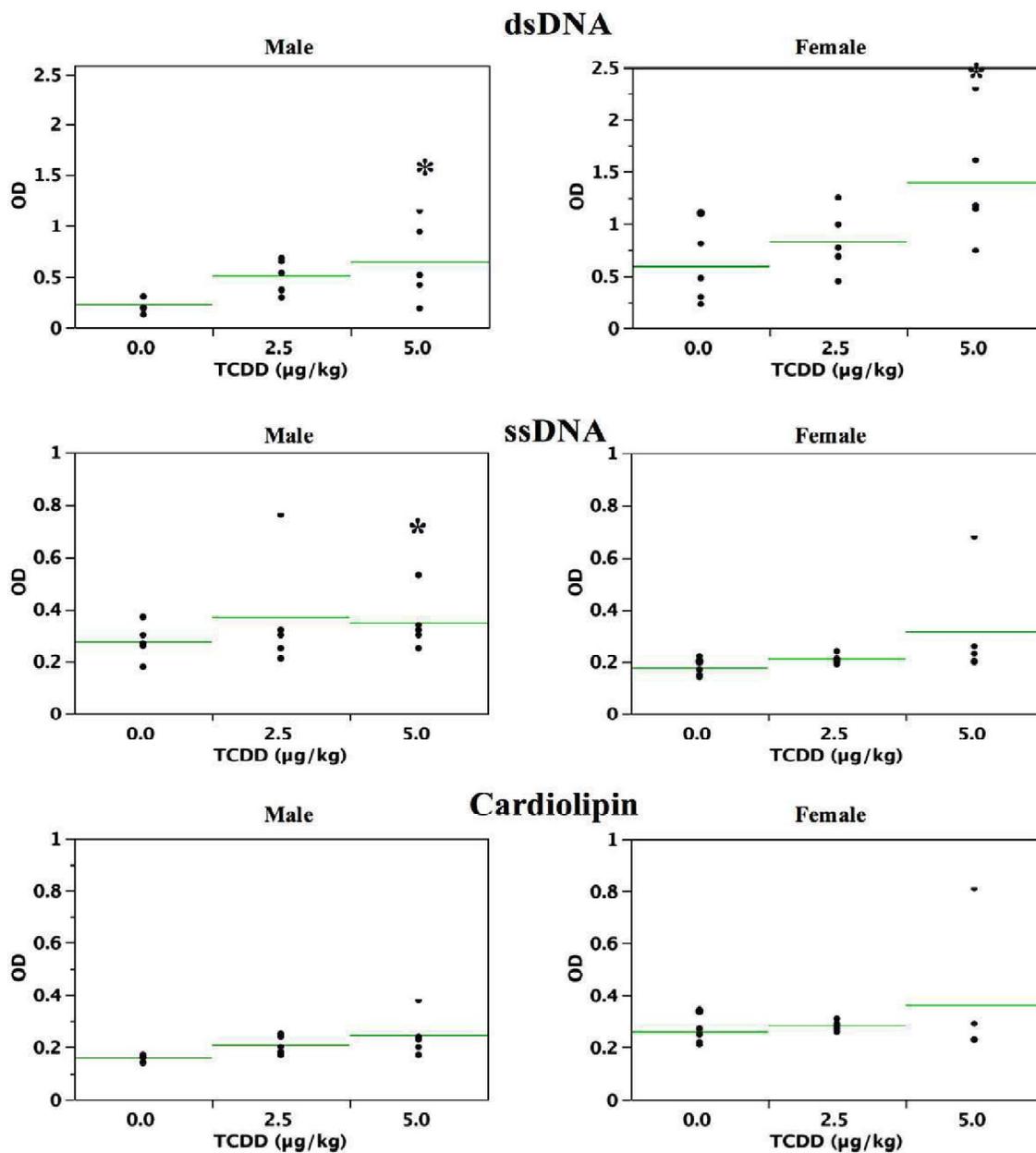
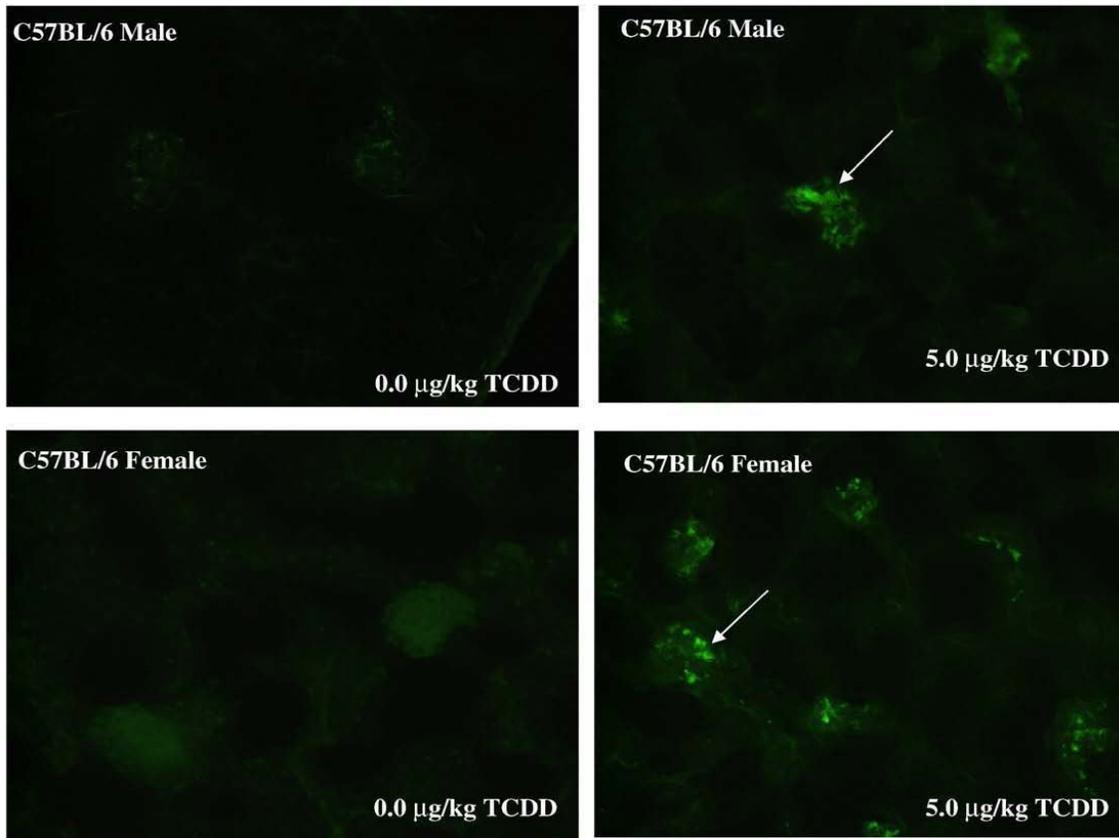


Figure 2. Sera from 24 week-old C57BL/6 mice that were prenatally exposed to 0.0, 2.5 and 5.0 µg/kg TCDD were analyzed for the presence of autoantibodies to dsDNA, ssDNA and cardiolipin (n = 5/sex; * $p \leq 0.05$, Dunnett's test). Each horizontal line represents the arithmetic mean for each treatment group.



IgG deposition

TCDD- Male	N	Mean	SEM
0.0 µg/kg	5	0.40	0.24
2.5 µg/kg	5	0.60	0.24
5.0 µg/kg	5	1.20	0.20

TCDD- Female	N	Mean	SEM
0.0 µg/kg	5	0.40	0.24
2.5 µg/kg	5	1.00	0.31
5.0 µg/kg	5	1.20	0.37

C3 deposition

TCDD- Male	N	Mean	SEM
0.0 µg/kg	5	0.20	0.20
2.5 µg/kg	5	0.60	0.24
5.0 µg/kg	5	1.00	0.31

TCDD- Female	N	Mean	SEM
0.0 µg/kg	5	0.40	0.24
2.5 µg/kg	5	0.60	0.24
5.0 µg/kg	5	1.20	0.37

Figure 3. The kidneys from 24 week-old C57BL/6 mice that were prenatally exposed to 0.0, 2.5 and 5.0 µg/kg TCDD were collected, fixed, section and stained with FITC-labeled anti-IgG. The above figures are representative of kidneys stained with FITC-anti-IgG from control female (A) and male (C) or 5.0 µg/kg TCDD-exposed female (B) and male (D) mice. The table data show the mean± SEM of the IgG and C3 disposition scores of 5 mice/treatment/sex.

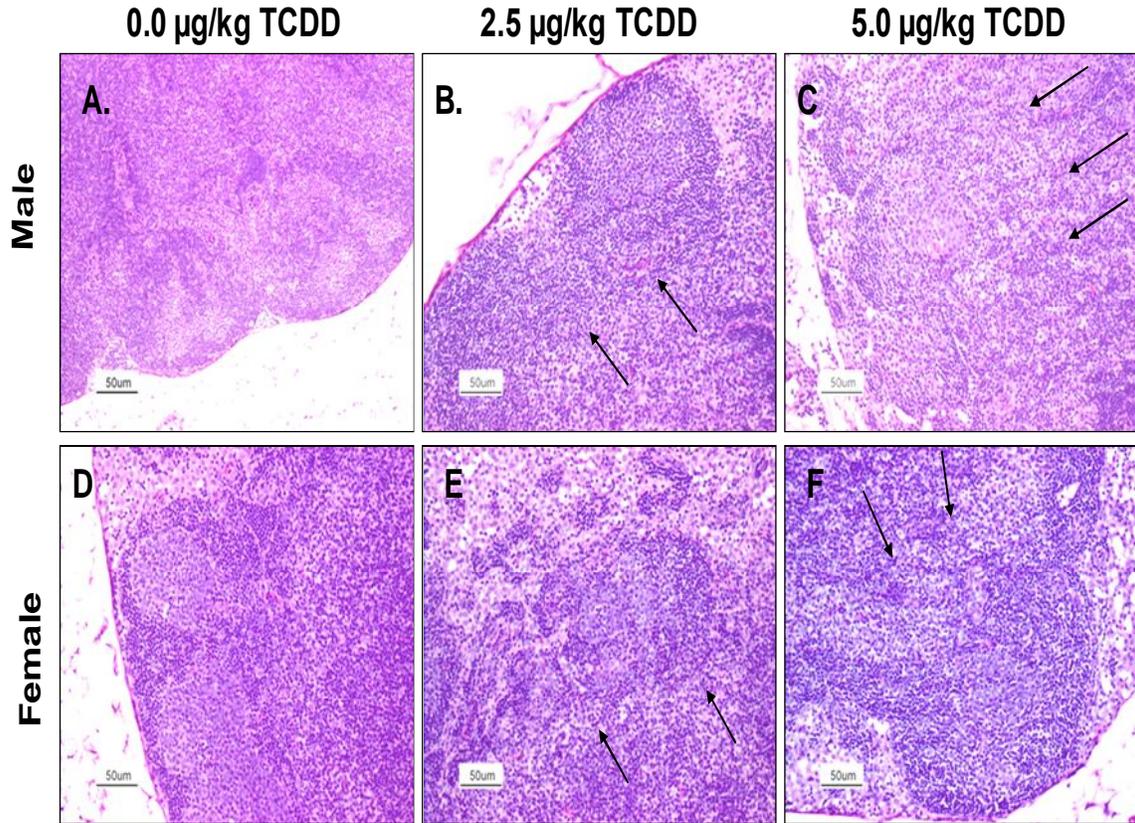


Figure 4. The lymph nodes from 24 week-old C57BL/6 mice that were prenatally exposed to 0.0, 2.5 or 5.0 $\mu\text{g}/\text{kg}$ TCDD were collected, fixed, section and stained with H&E stain. The above images are representative of control male (A) and female (D), 2.5 $\mu\text{g}/\text{kg}$ TCDD male (B) and female (E) and 5.0 $\mu\text{g}/\text{kg}$ TCDD male (C) and female (F) lymph nodes. The lymph nodes from male mice prenatally-exposed to either dose of TCDD showed disruption of architecture compared to controls. Lymph nodes from the prenatally-exposed TCDD female mice had regions of diminished follicular activity when compared to controls.

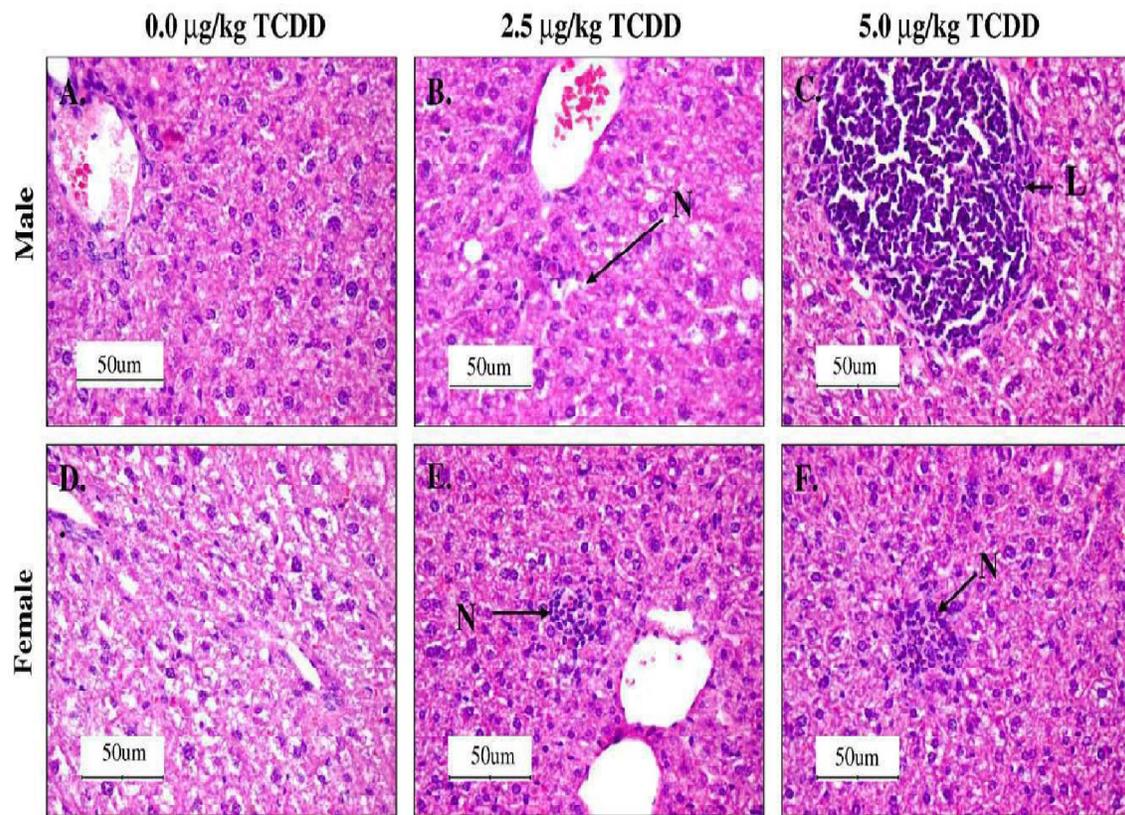


Figure 5. The livers from 24 week-old C57BL/6 mice that were prenatally exposed to 0.0, 2.5 and 5.0 $\mu\text{g}/\text{kg}$ TCDD were collected, fixed, section and stained with H&E stain. The above images are representative of control male (A) and female (D), 2.5 $\mu\text{g}/\text{kg}$ TCDD male (B) and female (E) and 5.0 $\mu\text{g}/\text{kg}$ TCDD male (C) and female (F) liver sections. Livers from male mice prenatally-exposed to 5.0 $\mu\text{g}/\text{kg}$ TCDD showed infiltration of lymphocytes (L) round central veins compared to control group. Livers from the 2.5 $\mu\text{g}/\text{kg}$ prenatally-exposed TCDD males and 5.0 $\mu\text{g}/\text{kg}$ TCDD females contained foci of necrosis (N) with inflammatory cells not evident in the controls.

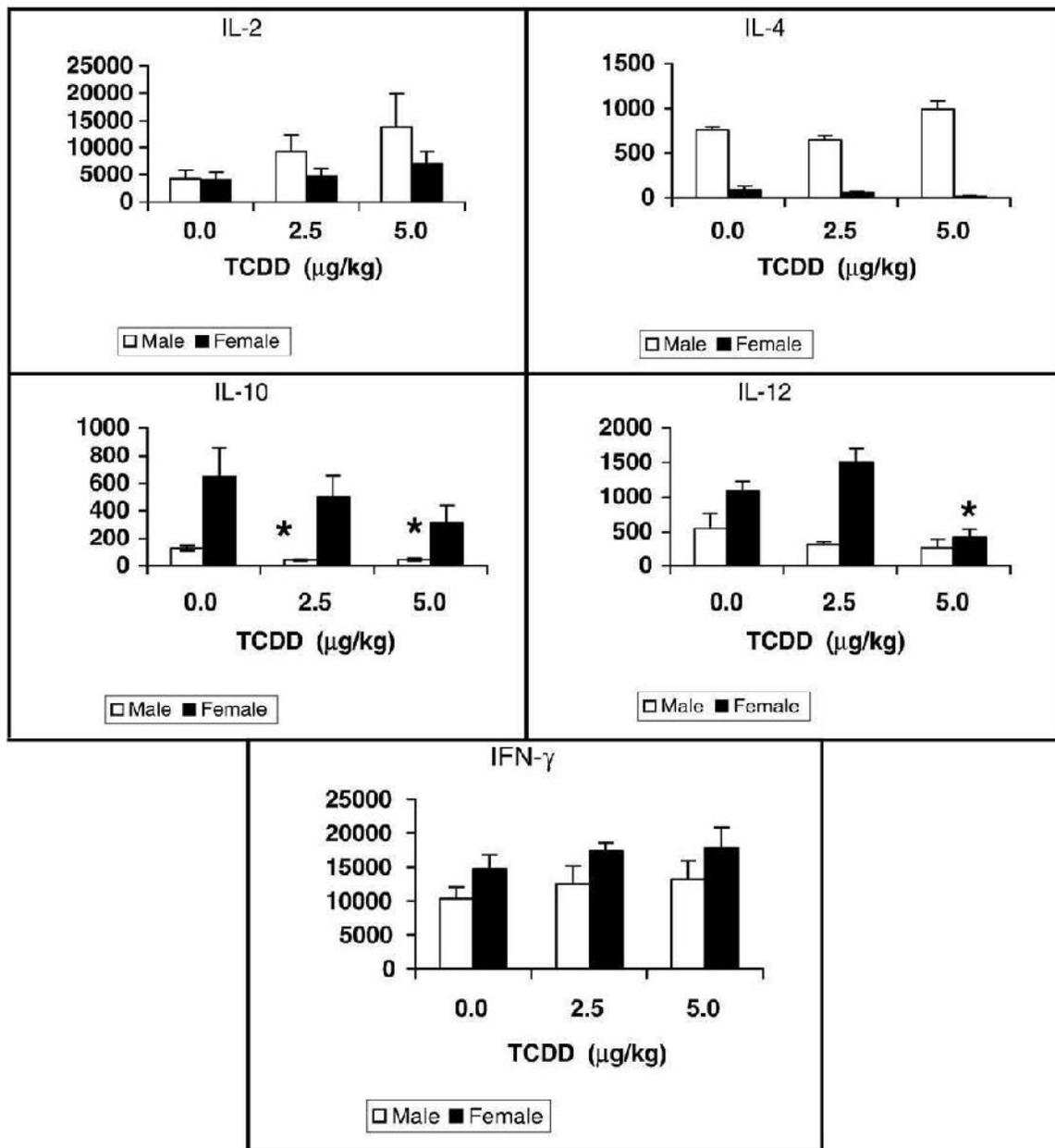


Figure 6. Supernatants were collected from splenocytes, of 24 week-old mice that were prenatally exposed to 0.0, 2.5 and 5.0 $\mu\text{g}/\text{kg}$ TCDD, cultured for 48 hr with Con A (10 $\mu\text{g}/\text{mL}$). The levels of IL-2, IL-4, IL-10, IL-12 and INF- γ were determined using commercially available murine cytokine ELISA kits. The data are presented in a histogram analysis with mean bars (n = 5 mice/treatment/gender, * = $p \leq 0.05$, Dunnett's test).

Table 1. Body Weight, Organ Weight and Total Cellularity

		0.0 ug/kg Mean ± SEM	2.5 ug/kg Mean ± SEM	5.0 ug/kg Mean ± SEM
Body Weight Grams	Male	37.71 ± 0.96	33.26 ± 1.51*	35.05 ± 0.90
	Female	25.43 ± 0.49	22.83 ± 0.60*	25.27 ± 1.06
Thymic Weight Grams	Male	0.061 ± 0.004	0.049 ± 0.006	0.049 ± 0.003
	Female	0.072 ± 0.003	0.055 ± 0.003*	0.061 ± 0.003*
Thymus:Body Weight Ratio	Male	0.0016 ± 0.0001	0.0015 ± 0.0002	0.0014 ± 0.0001
	Female	0.0028 ± 0.0001	0.0024 ± 0.0001*	0.0024 ± 0.0001*
Thymic Cellularity Cells (Millions)	Male	87.60 ± 9.82	79.40 ± 9.30	75.40 ± 7.19
	Female	73.26 ± 7.37	49.02 ± 2.91*	55.20 ± 6.08*
Splenic Weight Grams	Male	0.09 ± 0.01	0.08 ± 0.00	0.09 ± 0.00
	Female	0.15 ± 0.01	0.17 ± 0.01	0.16 ± 0.01
Spleen:Body Weight Ratio	Male	0.0024 ± 0.0002	0.0025 ± 0.0001	0.0025 ± 0.0002
	Female	0.0058 ± 0.0004	0.0072 ± 0.0004	0.0061 ± 0.0005
Splenic Cellularity Cells (Millions)	Male	65.68 ± 13.09	61.41 ± 7.61	59.81 ± 7.48
	Female	67.36 ± 8.42	62.87 ± 13.41	58.19 ± 7.03

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 2. Thymus T cell Differentiation

Thymus		0.0 µg/kg Mean ± SEM	2.5 µg/kg Mean ± SEM	5.0 µg/kg Mean ± SEM
CD4+CD8+ (%)	Male	76.08 ± 1.41	70.18 ± 3.53	77.04 ± 1.39
	Female	79.90 ± 0.87	75.84 ± 1.49	71.35 ± 3.20 *
CD4 - CD8 - (%)	Male	2.49 ± 0.11	11.58 ± 5.40	3.00 ± 0.84
	Female	3.74 ± 0.52	4.33 ± 0.87	7.12 ± 3.53
CD4+CD8 - (%)	Male	20.02 ± 1.22	16.96 ± 1.95	18.56 ± 1.04
	Female	14.70 ± 1.29	17.04 ± 0.44	19.38 ± 1.05 *
CD4 - CD8+ (%)	Male	1.41 ± 0.15	1.27 ± 0.11	1.39 ± 0.16
	Female	1.76 ± 0.29	2.70 ± 0.35	2.10 ± 0.21

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 3. Splenic T cell Subset Analysis

Spleen		0.0 ug/kg Mean ± SEM	2.5 ug/kg Mean ± SEM	5.0 ug/kg Mean ± SEM
CD4+CD8- (%)	Male	27.31 ± 0.80	27.15 ± 0.54	30.58 ± 1.11
	Female	26.91 ± 1.04	25.14 ± 0.86	29.26 ± 1.80
CD4CD8+ (%)	Male	10.69 ± 0.56	10.34 ± 0.26	5.67 ± 0.49 *
	Female	12.19 ± 0.59	11.87 ± 0.27	13.42 ± 0.89
Vβ17aTCR+ (%)	Male	2.76 ± 0.54	5.44 ± 1.25	7.83 ± 1.94 *
	Female	3.67 ± 0.58	4.82 ± 0.48	4.76 ± 0.29
Vβ3 TCR+ (%)	Male	2.02 ± 0.50	4.56 ± 0.73	10.08 ± 2.49 *
	Female	2.92 ± 0.71	5.02 ± 0.96	5.32 ± 0.54
CD4+CD25+ (%)	Male	2.11 ± 0.17	2.23 ± 0.07	2.00 ± 0.12
	Female	2.19 ± 0.24	1.90 ± 0.06	2.62 ± 0.29

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 4. Lymph Node T cell Subset Analysis

Lymph Nodes		0.0 µg/kg Mean ± SEM	2.5 µg/kg Mean ± SEM	5.0 µg/kg Mean ± SEM
CD4+ CD8- (%)	Male	38.20 ± 1.41	35.46 ± 1.80	38.98 ± 0.66
	Female	42.77 ± 0.78	40.96 ± 1.90	34.33 ± 2.52 *
CD4- CD8+ (%)	Male	20.76 ± 1.85	19.88 ± 1.43	18.86 ± 0.74
	Female	22.37 ± 2.15	20.32 ± 1.72	15.45 ± 1.88
CD4- CD8- (%)	Male	39.94 ± 2.93	43.46 ± 2.96	41.12 ± 0.63
	Female	33.70 ± 1.86	37.62 ± 3.61	49.40 ± 4.20 *
CD4+ CD25- (%)	Male	31.48 ± 0.59	31.98 ± 0.86	31.20 ± 0.69
	Female	36.52 ± 0.88	34.30 ± 0.59	30.64 ± 1.80 *
CD4+ CD25+ (%)	Male	1.46 ± 0.13	1.30 ± 0.15	1.37 ± 0.24
	Female	2.26 ± 0.15	2.20 ± 0.19	1.98 ± 0.09

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 5. Splenic B cell Subset Analysis

Spleen		0.0 µg/kg Mean ± SEM	2.5 µg/kg Mean ± SEM	5.0 µg/kg Mean ± SEM
CD24+ B220+ (%)	Male	44.6 ± 0.9	47.5 ± 1.6	20.9 ± 8.5 *
	Female	40.5 ± 1.3	38.8 ± 4.0	30.7 ± 2.0 *
CD24- B220- (%)	Male	49.3 ± 0.7	45.4 ± 2.2	64.5 ± 5.1 *
	Female	51.1 ± 1.5	49.4 ± 3.7	53.5 ± 2.4
CD24- B220+ (%)	Male	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.1 *
	Female	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
CD23- CD1+ (%)	Male	2.8 ± 0.3	4.0 ± 0.3	9.4 ± 0.5 *
	Female	2.7 ± 0.4	4.0 ± 0.5	8.8 ± 0.4 *
CD24- CD21+ (%)	Male	6.6 ± 1.0	8.8 ± 0.9	12.7 ± 0.7 *
	Female	5.7 ± 0.6	6.7 ± 0.6	11.0 ± 0.5 *
CD138+ (%)	Male	6.0 ± 0.7	8.6 ± 0.7	14.1 ± 2.5 *
	Female	5.9 ± 0.7	8.0 ± 1.0	8.3 ± 0.5

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 6. Bone Marrow Analysis

Bone Marrow		0.0 µg/kg Mean ± SEM	2.5 µg/kg Mean ± SEM	5.0 µg/kg Mean ± SEM
CD24+ B220+ (%)	Male	32.30 ± 1.15	31.20 ± 0.47	23.70 ± 1.03 *
	Female	28.16 ± 2.47	26.98 ± 1.71	22.00 ± 1.45 *
CD24+ B220- (%)	Male	55.12 ± 1.48	56.22 ± 0.55	62.56 ± 1.48 *
	Female	54.08 ± 3.21	56.24 ± 2.07	63.98 ± 2.49 *
CD24- B220+ (%)	Male	0.50 ± 0.12	0.50 ± 0.12	1.06 ± 0.14 *
	Female	0.68 ± 0.20	0.68 ± 0.20	0.76 ± 0.18
CD24- B220- (%)	Male	12.08 ± 0.61	11.68 ± 0.39	12.68 ± 1.10
	Female	17.06 ± 1.63	16.06 ± 1.59	13.30 ± 1.37

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

**CHAPTER 3. DEVELOPMENTAL EXPOSURE TO 2,3,7,8-TETRACHLORODIBENZO-
P-DIOXIN EXACERBATES POSTNATAL AUTOIMMUNE LUPUS IN 24-WEEK-OLD
SNF₁ MICE**

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Running title: Effects of prenatal TCDD in 24-week-old SNF₁ mice

Keywords: TCDD, prenatal exposure, autoimmunity, SNF₁ mouse

Abstract

We recently observed that a single prenatal dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6 mice resulted in serologic, cellular, and histopathologic changes in the 24-week-old offspring, characteristic of a lupus-like immune-mediated disease. In the present study, SWR female mice were mated with NZB males to yield SNF₁ offspring. More than 95% of such female SNF₁ hybrids spontaneously develop a fatal lupus-like glomerulonephritis by 8 months-of-age, whilst disease onset in males is much slower. The time-pregnant dams (10/treatment) were gavaged on gestational day (GD) 12 with 0, 40 or 80 µg/kg TCDD, and the offspring again evaluated at 24-weeks-of-age. Females showed a significant increase in the percentage of autoreactive CD4⁺Vβ17a⁺ mesenteric lymph node T cells. Con-A stimulated splenocytes from prenatal TCDD-treated mice produced decreased IL-17 in the females while males showed increased IL-2 and IFN-γ, and diminished IL-4. Splenic marginal zone (MZ) B cells (CD21^{hi}CD24^{low-int}) were decreased and follicular (FO) B cells (CD21^{int}CD24^{low}) were increased across sex and treatment, while transitional-2 B cells (CD21^{int}CD24^{hi}) (CD23^{low-int} CD1^{low-int}) were significantly decreased in males only. Mitogen-stimulated pan-lymphoproliferative responses as well as antibodies to dsDNA were significantly increased across sex by TCDD. Anti-IgG and anti-C3 immune complex renal deposition was visibly worsened in females, and present in the TCDD-treated males. Collectively, these data suggest that developmental TCDD exposure can permanently alter establishment of the immune system, beyond the classic immune suppression profile, to also include exacerbation and induction of a type III

hypersensitivity lupus-like autoimmune disease in genetically pre-disposed individuals.

Introduction

Prenatal exposure of rodents to a number of immunotoxicants results in persistent immune function deficits in the offspring (Holladay & Smialowicz 2000; Dietert & Piepenbrink 2006). For instance, exposure of pregnant mice to TCDD caused, in offspring, depressed T lymphocyte proliferation (Vos et al. 1974), inhibited graft-versus-host reactivity and skin graft rejection times (Vos et al. 1974), and reduced cytotoxic T-lymphocyte (Holladay et al. 1991) and delayed-type hypersensitivity (DTH) responses (Faith & Moore 1977; Blaylock et al. 1992; Gehrs et al. 1997; Gehrs & Smialowicz 1997). Dosing of mid-gestation pregnant rats with as little as 0.1 µg/kg TCDD similarly depressed T lymphocyte activity, measured by the DTH response, an effect still present in late-adulthood (Gehrs & Smialowicz 1999).

The effects of TCDD on the immune system are primarily mediated through the AhR (Hundeiker et al. 1999; Hogaboam et al. 2008). Constant or cyclic AhR activation during development can result in permanent immune changes that persist into adulthood. These effects are greatly influenced by the affinity of the AhR (high vs. low) relative to the dose of TCDD encountered. For example, C57BL/6 mice express a high affinity AhR and are highly sensitive to TCDD. SNF₁ mice express a low affinity AhR and require an approximately 8-fold increase in TCDD dose to equal the C57BL/6 response (Mustafa et al., 2008).

Besides causing immune suppression, several reports suggest TCDD may increase the risk of autoimmunity. Mice treated *in vivo* with monoclonal antibodies to MHC class I and class II molecules displayed inhibited thymocyte differentiation, similar to that occurring spontaneously in murine models of autoimmune disease

(Kruisbeek et al. 1985; Kakkanaiah et al. 1990) or after TCDD (Blaylock et al. 1992). These thymic MHC class I and class II antigens are required for normal thymocyte differentiation as well as for deletion of autoreactive cells (Blaylock et al. 1992). (De Waal et al. 1992) observed reduced thymic epithelial MHC class II antigen expression in TCDD-treated mice, while (Dong et al. 1997) found that TCDD down-regulated an MHC class I gene (*Q1^b*) in a mouse hepatoma cell line. The MHC *Q1^b* cDNA encodes for the $\alpha 3$ domain and transmembrane domain of the *Q1^b* class I protein, implying that the MHC gene product could interact with $\beta 2$ -microglobulin, and as such function in antigen presentation. These effects on MHC class I and II molecules by TCDD raised questions regarding the ability of TCDD to impair autoreactive thymocyte deletion (Holladay 1999). In support of the possibility TCDD may impair T cell selection, Silverstone et al. (1994) observed T cells expressing elevated levels of CD4⁺ V β 17a and V β 3⁺ TCR in the livers of TCDD-treated mice. Such TCR variable β (V β) chains are usually deleted in the thymus by reaction with self-MHC and minor lymphocyte stimulatory antigens (Okuyama et al. 1992; Hanawa et al. 1993) and have been associated with autoimmunity in experimental mouse models (Rocha et al. 1992).

The incidence of nephritis is low in autoimmune NZB mice, but when this strain is crossed with normal SWR mice, almost 100% of the female (SWR x NZB) F₁ hybrids (SNF₁) develop a fatal glomerulonephritis by about 8 months-of-age. The progression of autoimmune disease in lupus-like SNF₁ mice, as in the human version of the disease, is critically dependent on accelerated antibody production subsequent to inappropriate activity of CD4⁺ T helper cells. This autoimmune

nephritis is then characterized by IgG deposition in kidney glomeruli (Mohan et al. 1993). Silverstone et al. (1998) exposed autoimmune-predisposed SNF₁ (low affinity AhR) mice to a mid-gestation dose of 10 µg/kg TCDD, and reported an accelerated postnatal onset of glomerulonephritis in the male SNF₁ offspring compared to controls. Male rats have similarly been found to be more sensitive than females to the immunosuppressive effects of developmental TCDD (Gehrs & Smialowicz 1999).

Mechanisms underlying the early induction of autoimmunity in male SNF₁ mice by TCDD are not known, and are important for understanding environmental influences on disease initiation and expression. In the current study, we hypothesized that a prenatal exposure to TCDD in an autoimmune-prone murine strain (SNF₁) would generate permanent defects in T and B cell development, maturation and function leading to acceleration or exacerbation of autoimmune lupus. Previous work in our laboratory (Mustafa et al. 2008) and by others (Nishijo et al. 2007; Aragon et al. 2008) has indicated that prenatal TCDD exposure can affect many different immune cell types and compartments throughout the body. We therefore performed a broad survey of the immune system of an autoimmune-prone murine strain to identify specific TCDD-responsive targets and to direct follow-up mechanistic studies. Detecting these changes is a necessary first step toward determining how TCDD and other environmental agents may act as potential risk factors in the development of autoimmune diseases.

Methods and Materials

Mice and TCDD exposure. SWR x NZB (SNF₁) mice were obtained by breeding male NZB mice with female SWR/J mice, both from Jackson Laboratories (Bar Harbor, ME). Mice (4-5 weeks-of-age) were acclimated to the animal care facility for at least 2 weeks prior to breeding. Briefly, 60 SWR females were bred to 30 NZB males overnight in cages containing one NZB male per two SWR females. Plug positive mice, evaluated the next morning, were designated gestation day (GD) 0. Pregnant SWR mice were orally gavaged on GD 12 with 0, 40 or 80 µg/kg TCDD dissolved in corn oil (N= 10 pregnant mice/treatment). The SNF₁ offspring were weaned at 20-21 days and separated by treatment, allowed to mature to 24 weeks-of-age, and evaluated for changes in immune status. All animals were fed a commercial pelleted diet, provided water *ad libitum*, and housed under controlled conditions of temperature (22 °C), humidity (40–60%), and lighting (12:12 light:dark cycle). Animal maintenance, care and use were approved prior to initiation of experiments and at all times were in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines at Virginia Tech.

Body weights and tissue collection. Mice at 24 weeks-of-age were euthanized by cervical dislocation and weighed. At 24 weeks, the untreated females were in the early stages of lupus nephritis and males were free of clinical signs (Eastcott et al. 1983). The thymus, spleen, bone marrow, axillary and inguinal lymph nodes were immediately collected post-euthanasia under aseptic conditions, using dissection scissors and curved forceps. The spleen and thymus were weighed and then all

tissues placed individually into pre-labeled sterile Petri dishes (Corning, Corning, NY), containing 8 mL of RPMI-1640 culture medium (Mediatech, Herndon, VA). Dishes were placed on ice until tissue dissociation.

Cell dissociation and enrichment. Each organ was gently dissociated over a stainless steel sieve screen (Sigma, St. Louis, MO) using curved forceps. Cells were then pipetted through the sieve screen following dissociation to remove debris. Cells were washed in RPMI-1640 for 10 min, 240 x g, and 23 °C. The supernatant was discarded and, with the exception of spleen, the cell pellet was resuspended in 8 mL of RPMI-1640. Spleen cells were resuspended in 1 mL incomplete RPMI-1640. To each tube, 2 mL of 0.83 % ammonium chloride lysis buffer (ACK, pH 7.29) were added, to lyse red blood cells, and tubes were incubated for 5 min at 23 °C. After lysis incubation, the cells were resuspended in 5 mL of incomplete RPMI-1640 and washed twice (7 min, 290 x g and 7 °C). The splenic leukocyte-rich cells were then resuspended in 5 mL complete RPMI-1640 media containing 10% heat-inactivated FBS (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/mL penicillin (ICN), and 50 mg/mL of streptomycin (ICN), and maintained at 7-10 °C. For bone marrow isolation, femurs were removed and the bone marrow cavities flushed with 2% FBS-PBS, washed once (7 min, 290 x g and 7 °C), resuspended in 1 mL incomplete RPMI-1640 media and stored at 4 °C.

Cell enumeration. Cells were enumerated and size-analyzed using a Beckman Multisizer 3[®] Cell Coulter (Beckman Coulter, Fullerton, CA) according to the

manufacturer's protocol. Briefly, a 10 μ L aliquot of enriched cell suspension was transferred to a plastic counting-chamber containing 10 mL of PBS (Mediatech). The plastic chamber was capped, mixed by repeated gentle inversion, and counted. The cells were enumerated and adjusted to 5.0×10^6 cells/mL in complete media.

Flow cytometric evaluation of cell-surface markers. Cell suspensions ($5 \times 10^5/100$ μ L) from the thymus, spleen, lymph node and bone marrow were dispensed into individual wells of a 96-well round-bottom tissue culture plate (Corning). Monoclonal antibodies (mAbs) with phycoerythrin (PE) fluorescent labels were used according to manufacturer's (BD Pharmingen; San Diego, CA) recommendation at a concentration of 0.2 μ g/ μ L; mAbs with fluorescein isothiocyanate (FITC) fluorescent labels were similarly used at the recommended concentration of 0.5 μ g/ μ L. Cells were stained as previously described (Gogal et al. 2001; Klein et al. 2006). Briefly, lymphocyte aliquots (5×10^5 cells/ 100 μ L) from thymus, spleen, lymph node and bone marrow were incubated with the following primary mAbs: PE-anti CD4, FITC-anti CD8, FITC-anti CD19, PE-anti CD5, FITC-anti CD25, PE-anti CD45/B220, PE-Cy5 anti CD93 (AA4.1), PE-Cy5 anti CD45/B220 (Ebioscience, San Diego, CA); FITC-anti V β 3 TCR (KJ25), FITC-anti V β 17^a TCR (KJ23), FITC-anti CD45/B220, PE-anti CD24 (HSA), FITC-anti CD1, PE-anti CD23 (BD Pharmingen). For double or triple staining protocols, mAbs with different fluorescent labels were simultaneously added to the sample. For bone marrow analysis, aliquots of 5×10^5 cells were pre-blocked with anti-Fc γ III/IIR (clone 2.4G2, Rat IgG2_b). Following staining, cells were washed and evaluated on a Coulter Epics XL flow cytometer

(Beckman Coulter). From each sample, 10000 events were collected and analyzed using the FlowJo software (Tree Star, San Carlos, CA). Dead cells, clumps, and debris were excluded electronically by gating on forward scatter (FSC) versus side scatter (SSC). The distribution of B cell subsets analysis were gated as previously described (Grimaldi et al. 2001).

ELISA for autoantibodies to double-stranded DNA, single-stranded DNA and cardiolipin. To detect the presence of dsDNA antibody, 96-well medium-binding microtiter plates (Corning) were coated with heat-denatured calf thymus DNA (100 µg/mL; Sigma). For ssDNA antibody titers, DNA from calf thymus was used (10 µg/mL; Sigma), and for autoantibodies to cardiolipin a solution from bovine heart was used (10 µg/mL; Sigma). All pre-coated plates were incubated at 4 °C overnight. The plates were washed thrice with 300 µL PBS/0.05 % Tween 20 (Mediatech), blocked with 1% BSA (Sigma) for 2 h at 23 °C, washed and then incubated with diluted serum samples to be tested (1/100). For all three ELISA assays, serial dilutions of each mouse serum were performed to optimize optical density readings. Serum diluted 1/100 was determined to be the optimal dilution to attain a wide spectrum in the optical density among the samples. After 3 h at 23 °C, the serum-coated plates were washed thrice with 300 µL of PBS-0.05% Tween 20. To each well, 0.2 mL of diluted alkaline phosphatase-conjugated anti-IgG antibody (1/3000) (Sigma) were added and the plates were incubated for 60 min at 23 °C. The plates were then washed thrice with 300 µL of PBS-0.05 % Tween 20. To each well, 0.2 mL prepared substrate for alkaline phosphatase conjugated secondary

antibody (SIGMA FAST™ p-Nitrophenyl phosphate tablets) were added and allowed to develop for 45 min at 23 °C before adding 50 µL of 3 M NaOH stop solution (Sigma). The absorbance (A_{405}) of the initial dilution was measured. Optical density (OD) readings represent the average from sera from each mouse performed in duplicate.

Histology of the kidneys, thymus, and spleen. Kidneys were collected at the time of euthanasia, sectioned for histopathology and immunohistochemistry. The histopathology section was immediately fixed in 10 % formalin (Fisher Scientific, Pittsburgh, PA) for 48 h and the immunohistochemistry section was embedded in OCT media (Miles, Elkhart, IN) and frozen at -80 °C for cryosections. For histopathology, the thymus and spleen were also collected from littermates of the above mice (n=4 mice/treatment) and immediately fixed in 10 % formalin. After 48 h in formalin, the tissues were removed, routinely processed and embedded in a paraffin block. Following embedding, a 5 µm section was cut from each tissue block, and stained with hematoxylin and eosin (H&E, Richard-Allen Scientific, Kalamazoo, MI) using standard histologic methods. The prepared slides were then evaluated, with a light microscope, in a blinded-manner by a veterinary pathologist (co-author PS). For each kidney, 100 consecutive renal cortex glomeruli were evaluated. Each glomerulus was scored for the presence of fibrinoid necrosis or crescents and extent of lymphocytic infiltration. For the thymus, all fields of each section were evaluated. The thymic evaluation included changes in the cortex and medulla as well as the proportion of tissue in the cortex to the medulla. In the spleens, all fields were

examined. The spleens were evaluated for changes within splenic follicles including the presence or absence of germinal centers, periarteriolar cuffs including cellular density, evidence of cell death and overall architecture.

Immunohistochemistry of the kidneys: C3 and IgG deposition. Frozen kidneys were cut into 5 μm sections and stained with FITC conjugated antibodies. Briefly, tissue sections were thawed at room temperature and dried for 30 min (Xie et al. 2003; Bagavant & Fu 2005; Mustafa et al. 2008). Slides were fixed in acetone for 10 min and then washed with PBS thrice for 3 min/wash. Goat anti-mouse IgG diluted 1:100 (MP Biomedicals, Santa Ana, CA) or goat anti-mouse C3 diluted 1:100 (MP Biomedicals) were incubated with tissues sections in a humid chamber for 60 min at 23 °C. The sections were then rinsed thrice for 5 min/wash with PBS. The slides were mounted using Vectashield™ mounting media (Vector Labs, Burlingame, CA) and then examined using an Olympus BX-60 fluorescence microscope (Center Valley, PA). The severity of glomerulonephritis and immune complex deposition was scored using a range from 0 to 3+, where 0 corresponded to a non-autoimmune healthy mouse and 3+ to the maximal alteration observed in the study. All slides were scored in a blinded manner independently by two experienced investigators (co-authors CR and RG). Scores were averaged for the final tissue score.

Lymphocyte proliferation assay. Splenocytes were plated into each well (5×10^5 cells/ 100 μL per well) of a 96-well round-bottom tissue-culture plate (Corning Cell Wells™, Corning). Cells were exposed to mitogens as follows: 100 μL of:

Concanavalin A (Con A, 10 µg/mL, Sigma), lipopolysaccharide (LPS, 50 µg/mL, Sigma), or phorbol myristate acetate (PMA, 10 ng/mL, Sigma) plus ionomycin (0.5 µg/ml, Sigma) in complete media (Klein et al. 2006; Mustafa et al. 2008). Non-stimulated control cultures contained 100 µL of complete media alone. Triplicate wells were used for each stimulant. Following 48 h incubation, 20 µL of Alamar Blue™ dye (Serotec, Raleigh, NC) (10 % of incubation volume) were added to each well of the culture plates. At 24 and 48 h post addition, degree of absorbance was determined under dual wavelength (570 and 600 nm) using a Molecular Devices plate reader (Menlo Park, CA).

Cytokine ELISA Assays. Splenocytes were plated into each well (1 mL in complete media; 5 x 10⁶ cells per well) of a 24-well tissue-culture plate (Corning). Cells were co-cultured with 1 mL Con A (10 µg/mL) and incubated at 37 °C, 5 % CO₂ for 48 h. The plates were centrifuged (7 °C, 250 x g, 7 min), and the supernatants were transferred to sterile 12 x 75 mm cultured tubes (Fisher). Supernatants were stored at -80 °C until use. The levels of interleukin 2 (IL-2), IL-4, IL-10, IL-12, and interferon-gamma (INF-γ) were determined using ELISA kits (Ready-to-use; Ebioscience) according to the manufacturer's instructions. IL-17 levels were measured using the Searchlight Mouse Cytokine Array (Pierce Biotechnology Inc. Rockford IL) according to the manufacturer's instructions and imaged using a cooled CCD camera and analyzed using the Arrayvision 8.0 software.

Statistical analysis. Data were expressed as arithmetical mean ± SEM. Analysis of variance (ANOVA) was used with Dunnett's *t*-test to establish significant differences

in the same sex groups between treatment groups and control. The pregnant dam was maintained as the statistical unit in all cases such that each offspring analyzed represented a separate dam (one pup/gender/treatment). Group size was five SNF₁ offspring per sex for all experiments (n=5) except spleen histology (n=4). Results described as different in this report indicate significantly different at $p < 0.05$.

Results

Body and organ weights, and organ cellularity. Body weight of the 24-week-old adult SNF₁ offspring was decreased in the males by prenatal exposure to 40 and 80 µg/kg TCDD and 80 µg/kg TCDD in the females. Thymic weight was decreased by 80 µg/kg TCDD in the male offspring only, while thymic cellularity was decreased in both sexes by 80 µg/kg prenatal TCDD. There were no significant differences in splenic weight or the spleen/body weight ratio across treatment groups. In contrast, splenic cellularity was increased, in males but not females, by 80 µg/kg TCDD (Table 1).

B lymphoid progenitors in bone marrow. At 24 weeks-of-age, the 80 µg/kg TCDD females showed a significant decrease in total B220 cells (B220⁺). The percentage of these B220⁺ cells that were B220^{hi}, representing both small pre-B cells that phenotypically immediately precede immature B lymphocytes as well as the immature B cells, was significantly decreased while the percentage that were B220^{low}, representing B lineage committed progenitors, pro-B cells and large pre-B cells, was significantly increased in these females. The percentage of B220^{lo}CD24⁺ cells that expressed AA4.1 (B220^{low}CD24⁺AA4.1⁺), representing B lineage committed progenitors, was significantly increased in the females. Prenatal TCDD-treated males did not show changed bone marrow total B220⁺ expression or B220^{low} expression. However, the percentage of B220^{hi} cells that were CD24^{low} was significantly decreased, and the percentage of B220^{lo} CD24^{low} cells, representing B lineage committed progenitors and pro-B cells, was significantly decreased in 80

$\mu\text{g}/\text{kg}$ TCDD males. Interestingly, the percentage of $\text{B220}^{\text{low}}\text{CD24}^-$ cells was significantly increased in both $40 \mu\text{g}/\text{kg}$ and $80 \mu\text{g}/\text{kg}$ TCDD males. The percentage of $\text{B220}^{\text{lo}}\text{CD24}^- \text{AA4.1}^+$ cells was not changed in males by TCDD (**Table 2**).

Thymic T cell differentiation. Female, but not male, offspring of dams dosed with TCDD exhibited significant thymic phenotypic changes at 24 weeks-of-age. The relative expression of thymic $\text{CD4}^+\text{CD8}^+$ cells was decreased in females by the $80 \mu\text{g}/\text{kg}$ prenatal TCDD dose. In addition, $\text{CD4}^-\text{CD8}^-$ thymocytes were significantly increased in females at this same TCDD exposure level (**Table 3**).

Phenotype of spleen B and T cells, and migration of mature B cells in the spleen.

The relative percentage of splenic leukocytes that expressed B220 did not differ across treatment or sex compared to controls. However, the relative percentage and absolute number of $\text{CD19}^+\text{CD5}^+$ B cells (representing B-1a cells, a B cell subset of fetal origin only, rather than bone marrow) were significantly increased in $40 \mu\text{g}/\text{kg}$ TCDD females, whilst the absolute number of cells expressing the $\text{CD19}^+\text{CD5}^+$ phenotype was increased in the $40 \mu\text{g}/\text{kg}$ TCDD females and $80 \mu\text{g}/\text{kg}$ TCDD males (**Table 4**). Although the relative percentage of splenic B cells was unchanged, there was a shift in the immature and mature B cell compartments with reduced numbers of $\text{B220}^+\text{CD24}^{\text{hi}}$ transitional B cells and increased numbers of $\text{B220}^+\text{CD24}^{\text{low-int}}$ mature B cells. Additionally, there was a significant difference within B cell subsets expressing $\text{CD21}/\text{CD24}$. The $80 \mu\text{g}/\text{kg}$ females displayed decreased percentages of B marginal zone cells ($\text{CD21}^{\text{int}}\text{CD24}^{\text{low-int}}$), and increased relative percentages of FO B

cells (CD21^{int}CD24^{low}). In TCDD males, the CD21^{int}CD24^{low-int} MZ cells and B transitional-2 cells (CD21^{int}CD24^{hi}) were significantly decreased, whereas the percentage of CD21^{int}CD24^{low-int} FO cells significantly increased. Furthermore, B transitional-2 cells (CD23^{low-int} CD1^{low-int}) were significantly decreased in males whilst other B cell subsets expressing CD1/CD23 were not different compared to the control groups (**Table 5**). The relative percentage of T cells expressing CD4/CD8 markers did not change in any treatment group, in either sex. However, the absolute number of T cells expressing CD4 was significantly increased in the 40 µg/kg TCDD females and 80 µg/kg TCDD males when compared to corresponding controls (**Table 6**).

T cell subset expression in lymph nodes. The combined axillary and inguinal lymph node CD4/CD8 T cell phenotypes were not different across treatment group or sex compared to controls. However, the 80 µg/kg TCDD females showed significantly increased relative percentages of CD4⁺Vβ17^{a+} TCR T cells (**Table 7**). The T cells expressing CD4⁺CD25⁺, which includes T regulatory and activated T cells, were increased in the 80 µg/kg female and male treatment groups.

Antibody titers to ssDNA, dsDNA and cardiolipin. To assess the extent of autoantibody production to dsDNA, ssDNA and cardiolipin autoantigens following a prenatal TCDD exposure, ELISA assays were performed. Anti-dsDNA antibodies were increased in both male and female offspring by 80 µg/kg TCDD. Anti-ssDNA

and anti-cardiolipin titers showed numeric but non-significant increasing trends in both the male and female offspring (**Figure 1**).

Kidney pathology. Since immune complex deposition in the kidney is a common signalment in lupus patients, histopathologic examination of the kidney was performed. Glomeruli that manifested with fibrinoid necrosis or crescents (**C**) showed an increasing non-significant trend by treatment and sex. The mean number of mononuclear inflammatory cell (**I**) foci also numerically increased, non-significantly, by treatment and sex (**Figure 2**).

Deposition of anti-IgG and anti-C3 immune complexes in the kidney. Based on the histopathologic changes seen with light microscopy and H&E staining of the TCDD kidneys, immunofluorescent staining to elucidate IgG and C3 involvement in immune complex deposition was performed. Kidney sections from 24-week-old SNF₁ offspring showed a TCDD dose-dependent increasing trend in deposition of immune complexes, for both anti-IgG and anti-C3 probes, which was significant in the males (**Figure 3**).

Thymic and splenic pathology. Based on the changes in cytology and phenotypic analysis of the T and B cells in mice prenatally exposed to TCDD, histopathologic analysis of the thymus and spleen was performed. Microscopic analysis of the thymic sections showed no significant changes in cortical necrosis or cortical-medullary ratio based on treatment or sex (data not shown). Analysis of the splenic

sections showed large active follicles in the females for all treatment groups. In the male spleens, 75% of 40 µg/kg TCDD mice displayed small germinal centers while in the 80 µg/kg TCDD mice the follicular architecture detail was diminished, and the follicles were large and coalescing (**Figure 4**).

Mitogen-stimulated splenic lymphocyte proliferation. Mitogen stimulation of enriched, cultured splenic lymphocytes was employed to assess the influence of prenatal TCDD on lymphocyte functionality in the adult mouse. Prenatal TCDD had a selective effect on splenic lymphoproliferative responses of 24 week-old SNF₁ offspring. No significant effects on stimulation responses were seen using ConA or LPS in splenocytes, at 48 or 72 h. However, the mitogenic response to PMA/Ionomycin (P/I) was significantly enhanced in females at both TCDD exposures, at 48 and 72 h. Enhanced proliferation was also seen in 80 µg/kg TCDD P/I males, at 48 h (**Figure 5**).

Th1/Th2 cytokine balance. In males, prenatal exposure to TCDD caused a shift toward a Th1 cytokine profile in Con-A-stimulation splenic lymphocytes collected from 24-week-old mice. The 80 µg/kg TCDD males showed enhanced IL-2 and IFN-γ production, and diminished IL-4 production relative to controls. In the female mice, IL-17 production followed a decreasing trend reaching significance at 80 µg/kg TCDD (**Figure 6**).

Discussion

T cells, which may be preferentially targeted by developmental exposure to TCDD (Vos et al. 1974; Faith & Moore 1977; Luster et al. 1980), were initially proposed to not play a direct role in tissue damage in SNF₁ lupus-like nephritis. However, the proliferation of glomerulus-specific autoantibodies may require interaction between autoreactive T-helper (Th) cells and autoreactive B cells (Mohan et al. 1993). An increase in the CD4 to CD8 ratio of IdLNF1-reactive T cells, suggestive of increased Th cells, was also detected at 22-24 weeks of age in sera of SNF₁ mice and coincided with increased IdLNF1 Ig (IgG + IgM) deposition in the kidneys (O'Garra et al. 1992). Further, the presence of pathogenic autoantibody-inducing Th cells specific for chromatin subparticles or histones has been noted in human patients with systemic lupus erythematosus as well as in SNF₁ mice (Fournel et al. 2003). Thus, the more current data would suggest that the early onset of autoimmune nephritis in SNF₁ mice after TCDD may be influenced by a T cell lesion. This would appear to be supported by a study showing that postnatal day 3 thymectomy protects SNF₁ mice from glomerulonephritis which would suggest T cell involvement in the pathogenesis of this disease (Bagavant et al. 2002).

In contrast to uncertain contributions by T cells, autoantibodies produced by B cells display strong polyreactivity to DNA and glomerular substrate, and dominate immune deposits in lupus kidneys (Xie et al. 2003). There has been a lack, however, of studies investigating B cell function following developmental TCDD exposure (Kantor et al. 1997; Sato et al. 2004). We therefore examined B cell lymphopoiesis,

autoantibody production, and immune complex deposition in kidneys of the present SNF₁ mice that received prenatal TCDD. The age of 24-weeks was selected because SNF₁ females are then in early stages of autoimmune disease, while the males do not begin to show signs until about 6 months thereafter (Eastcott et al. 1983).

In this study, SNF₁ mice exposed developmentally to TCDD varied considerably, by sex and compartment, in both type and severity of immune lesions. Thymic cellularity was approximately equally diminished in the male and female offspring. Mechanisms underlying this effect are not known, but may include bone marrow progenitor T cell damage by TCDD (Fine et al. 1989) or enhancement of precursor T cell death at the thymic level (Shivakumar et al. 1989; Rajagopalan et al. 1990). Female offspring, but not males, showed a dramatic decrease in CD4⁺8⁺ cells and increase in CD4⁺8⁻ cells. These data suggest prenatal TCDD caused permanent postnatal alterations in thymic T cell maturation in female offspring, in addition to thymocyte hypocellularity. T cell parameters in the secondary lymphoid organs were not changed by TCDD, with two exceptions. Total spleen CD4⁺ Th cells tended toward increased numbers in 80 mg/kg TCDD females and were significantly increased, by 79%, in males at the same dose level. Lymph node V β TCR expression was unchanged in females, however in males CD3⁺V β 3⁺ TCR expression tended toward increase while CD4⁺V β 17a⁺TCR expression increased significantly, by 115%. The increased peripheral V β ⁺ Th cells may indicate that TCDD compromised thymic deletion of autoreactive TCR (negative selection), a possibility previously suggested based on diminished thymic MHC class II antigen expression by TCDD (Holladay 1999).

Disruption in normal B cell function can either initiate or promote autoimmunity, in which B cells may act via nonconventional mechanisms, e.g., in addition to autoantibody production, including through altered antigen presentation or cytokine production (Fujimoto & Sato 2007). In female SNF₁ mice dosed with 80 µg/kg TCDD, bone marrow cells expressing the pan B cell marker (B220⁺) were decreased, while B220^{low} B cell progenitors and B220^{low}CD24-AA4.1⁺ early B cell progenitors were increased. Male B cell lymphopoiesis was also affected but not as dramatically, showing decreased large pre-B cells (B220^{lo}CD24^{hi}) and small pre-B and/or IgM⁺ B cells (B220^{hi}). These results suggest that prenatal TCDD may permanently dysregulate B cell lymphopoiesis, similar to thymic T cell maturation (Faith & Moore 1977).

There was a dose-related trend toward increased spleen B cell numbers in both sexes. Total numbers of CD5⁺ B cells increased significantly in both sexes after TCDD, which may be noteworthy. B-1a cells are distinguished from conventional B cells (B-2) by their fetal developmental origin, their surface marker expression of CD5, their FO location versus MZ and their function (Duan & Morel 2006). These are long-lived, non-circulating B lymphocytes that display reduced B cell receptor diversity and affinity compared to B-2 cells (Kantor et al. 1997), and produce circulating low affinity and polyreactive IgM “natural antibodies” that function as a first line of defense against bacterial pathogens (O'Garra et al. 1992). The polyreactivity of these antibodies also mediates the recognition of autoantigens, which serves in the clearance of apoptosis products (Carroll & Prodeus 1998). The unique autoreactivity of B-1a cells may play a role in autoimmune pathogenesis, as

may production of high levels of IL-10 and enhanced antigen presentation capacities by these cells (O'Garra et al. 1992). Further, high levels of B-1a cells have been detected in patients with SLE, Sjogren's syndrome and rheumatoid arthritis, and numerous associations between expansion of this cell compartment and systemic autoimmunity have been found in murine models (Karpuzoglu-Sahin et al. 2001; Pillai et al. 2004). B-1a cells also express high levels of costimulatory molecules B7-1, B7-2 and display an enhanced antigen presentation capacity (Mohan et al. 1998). Accumulation of B1a cells in target organs has been observed in aged (NZB x NZW) F1 mice (Ishikawa et al. 2001). Thus, it is possible that B-1a cells may activate autoreactive T cells and produce autoantibodies against specific target organs, contributing to immune-complex mediated pathology (Sato et al. 2004).

Production of anti-nuclear autoantibodies in human and mouse SLE requires interactions between select populations of autoimmune Th cells and B cells. These pathogenic antibodies are usually highly somatically mutated (O'Keefe et al. 1996) and the products of T-dependent FO B cell activity. In particular, autoantibody-inducing Th cell clones isolated from SNF₁ mice with lupus nephritis rapidly induce immune complex glomerulonephritis when transferred into young pre-autoimmune mice (Shivakumar et al. 1989; Rajagopalan et al. 1990; Naiki et al. 1992; Mohan et al. 1993). In the absence of these Th cells, the autoantibody-producing B cells were not sustained and underwent apoptosis (Kalled et al. 1998). Further, in murine SLE models, B cells are needed to prime pathogenic T cells, for instance B-cell deficient MRL/lpr mice do not show expansion of activated T cells (Leitges et al. 1996). However, MZ B cells are more efficient than FO B cells at priming naïve T cells and

are over-represented in lupus-prone strains, suggesting that MZ B cells help trigger the T-cell dependent disease (Cariappa et al. 2001). Thus, whereas MZ B cells may be important in initiating autoimmune disease, Th cells and FO B cells appear to be important in sustaining and promoting such disorders. Prenatal TCDD also caused decreased expression of transitional 2 (T2) B cells in spleens of the present mice. The significance of this finding is not yet known, however at least two developmentally and functionally distinct types of T2 cells have been identified (Cariappa & Pillai 2002; Pillai et al. 2004). These collective B cell results suggest these cells may play a role in the early induction of SLE-like symptoms in male mice and exacerbation of SLE symptoms in the females after prenatal TCDD.

SNF₁ female mice spontaneously produce moderate levels of autoreactive IgG antibodies by 24 weeks of age, whereas males produce low levels of autoreactive IgG antibodies at the same age. Both sexes of prenatal TCDD-exposed SNF₁ mice produced significantly increased anti-dsDNA antibody, with the basal and TCDD-induced levels being higher in the females. These results suggest that perinatal TCDD exposure caused exacerbated autoantibody formation in the 24 week-old SNF₁ females, and induced early autoantibody production in the SNF₁ males. Kidney histopathology supported this finding, in which immune complex IgG and C3 deposition was significantly worsened in the TCDD-exposed males and displayed a dose-dependent numeric increase in females. Females and males both also showed dose-related trends toward increases in autoimmune-related kidney pathology, in the form of fibrinoid necrosis, crescents and inflammatory cells.

Altered T cell maturation including increased $V\beta^+$ Th cells in the present mice suggested prenatal TCDD exposure may increase SLE-like symptoms in SNF₁ mice by mechanisms involving inappropriate T cell function. The secreted levels of five major cytokines from Con A-stimulated splenocytes were therefore determined. Developmental TCDD increased the level of IFN- γ and IL-2, and decreased IL-4, in male but not female SNF₁ mice at 24 weeks-of-age. This suggests permanent dysregulation of cytokine production by T cells is caused by prenatal TCDD, including a skew towards Th1 activity in the males. At this age, SNF₁ males usually do not manifest SLE-like symptoms, however, higher levels of INF- γ may be of concern for driving autoimmune disease expression (Karpuzoglu-Sahin et al. 2001; Karpuzoglu-Sahin et al. 2001). For example, increased IFN- γ has been associated with development of autoimmune insulinitis (Campbell et al. 1991), lupus nephritis (Haas et al. 1997), Sjogren's syndrome (Hayashi et al. 1996) and autoimmune arthritis (Billiau 1996). Beyond altered cytokine production, T cells from both sexes of the present TCDD-exposed SNF₁ mice displayed enhanced lymphoproliferative responses to mitogen stimulation at 24 weeks-of-age, suggesting over-activity in these cells.

In summary, male and female SNF₁ mice showed persistent changes in both T and B cells as a consequence of GD 12 exposure to TCDD. Among these were numerous sex-specific effects, suggesting possible interactions with endogenous hormones. The TCDD-exposed mice displayed a clear enhanced autoimmune profile, including increased $V\beta^+$ Th cells, increased T cell proliferative capacity, dysregulated cytokine production toward Th1, increased splenic CD5⁺ and FO B cells, increased

autoantibody production, and increased autoimmune kidney lesions. These collective data suggest that developmental exposure to TCDD permanently alters the postnatal immune system, in a manner beyond the well-established profile of immune suppression, to include increased risk of autoimmune responses.

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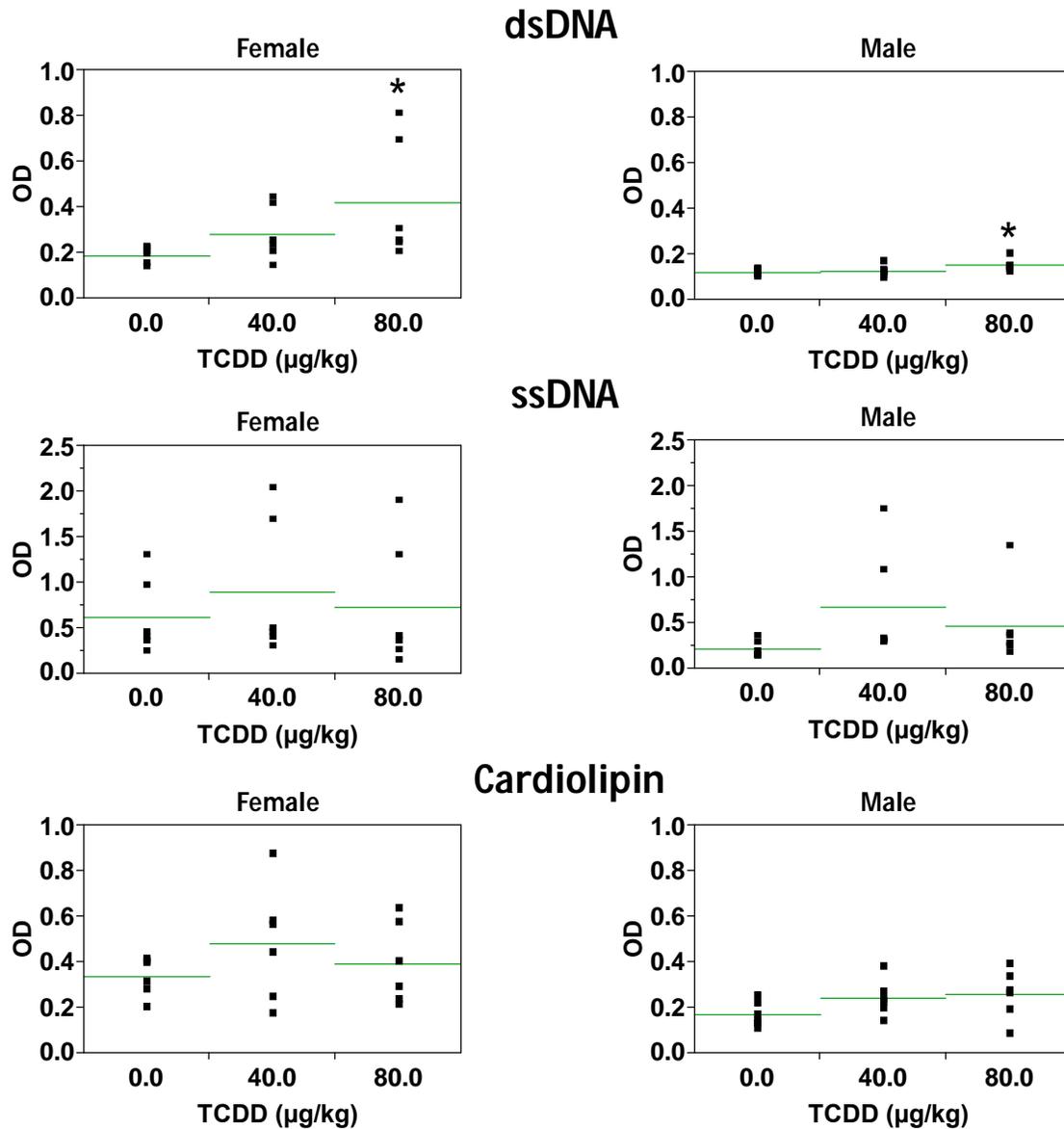


Figure 1. Sera from 24-week-old SNF₁ mice that were prenatally exposed to 0, 40 or 80 µg/kg TCDD were analyzed for the presence of autoantibodies to dsDNA, ssDNA and cardiolipin. The data are arranged by sex and are based on 5 mice/treatment/sex (* = $p < 0.05$, Dunnett's test). Each horizontal line represents the arithmetic mean for each treatment group.

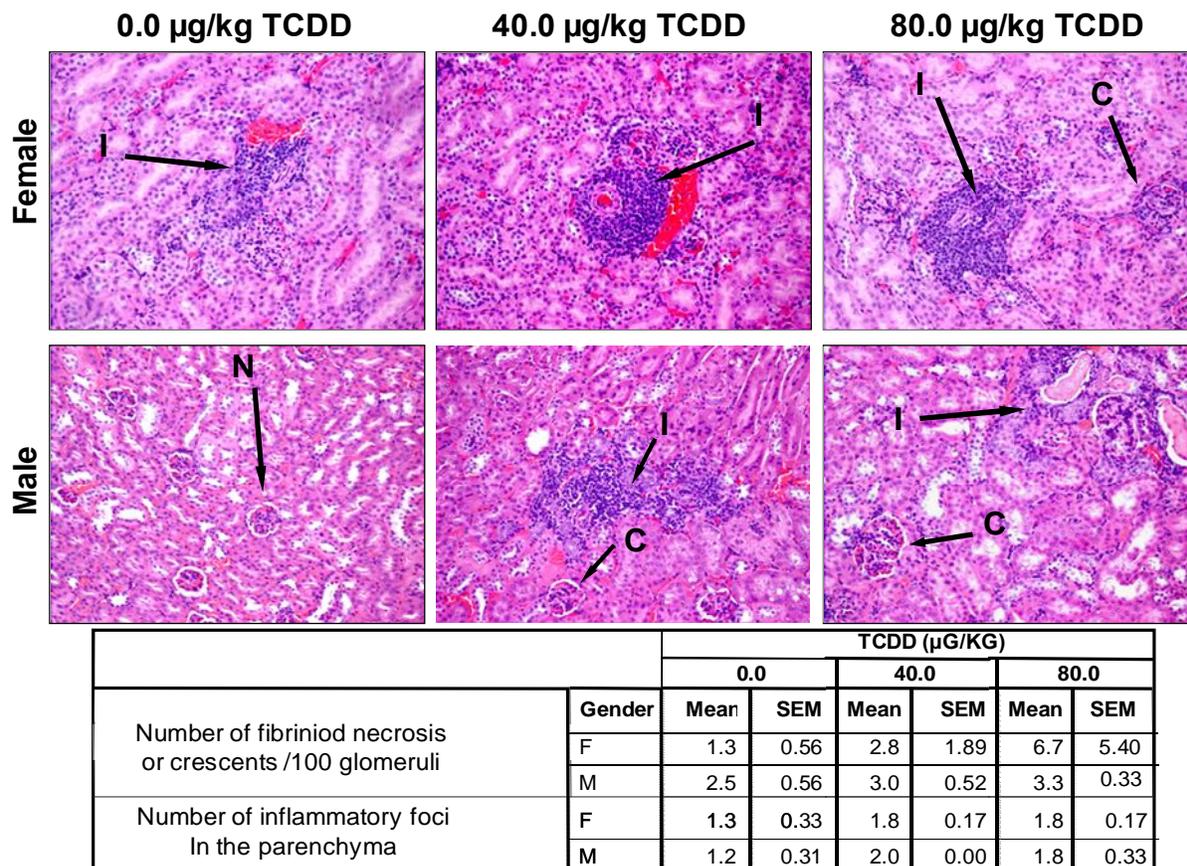


Figure 2. The kidneys from 24-week-old SNF₁ mice that were prenatally exposed to 0, 40 or 80 µg/kg TCDD were collected, fixed, sectioned and stained with H&E. Images are representative of renal sections by treatment and sex. All sections were scored for number of fibrinoid necrosis cells or crescents/100 glomeruli and number of inflammatory foci in the parenchyma. Normal glomerulus (N), lymphocytes infiltration (I), glomerular crescent (C). The data are based on 5 mice/treatment/sex (* = $p < 0.05$, Dunnett's test).

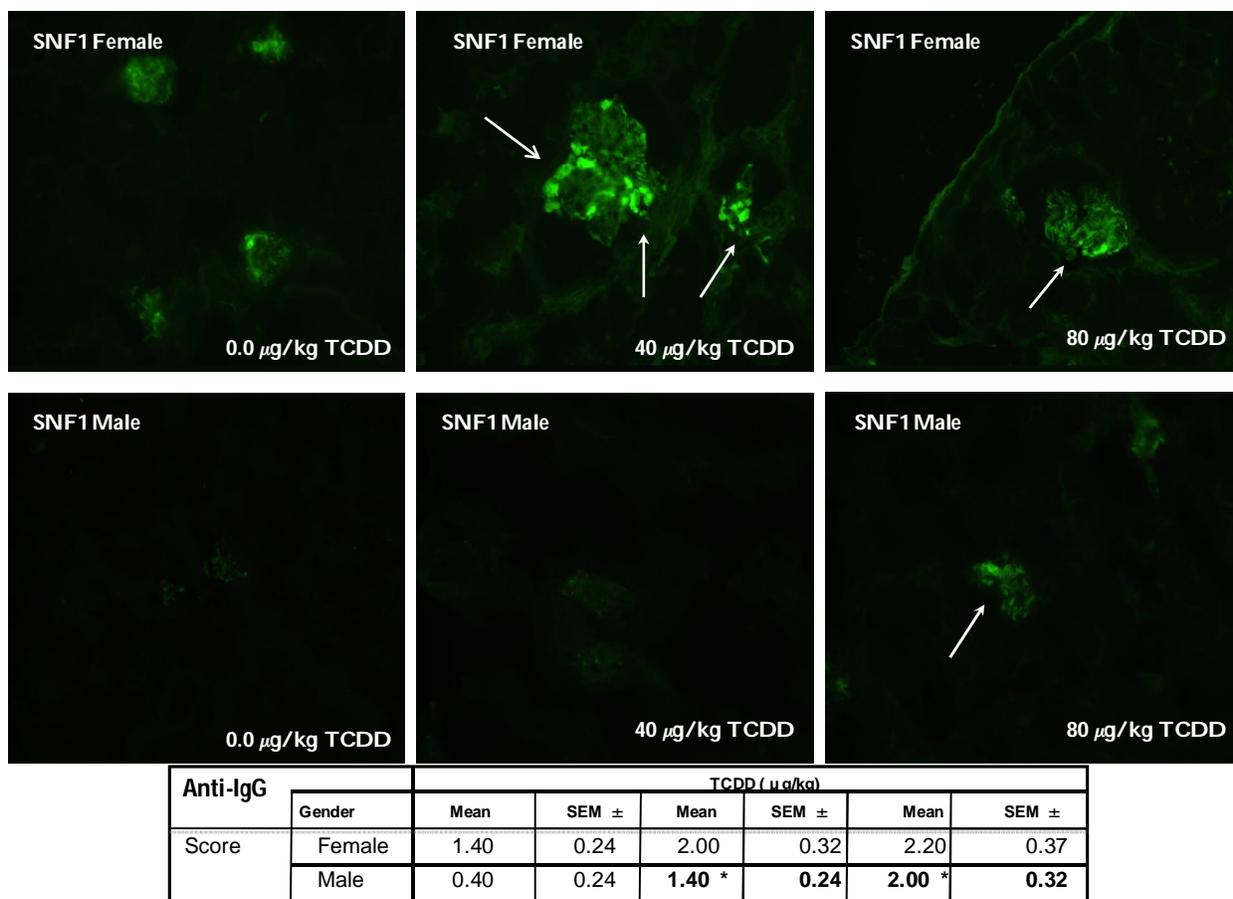


Figure 3. The kidneys from 24-week-old SNF₁ mice that were prenatally exposed to 0, 40 or 80 µg/kg TCDD were collected, fixed, section and stained with FITC-labeled anti-IgG and anti-C3. The above are representative images of kidneys stained with FITC-anti-IgG based on treatment and sex. The data are based on 5 mice /treatment/sex (* = $p < 0.05$, Dunnett's test).

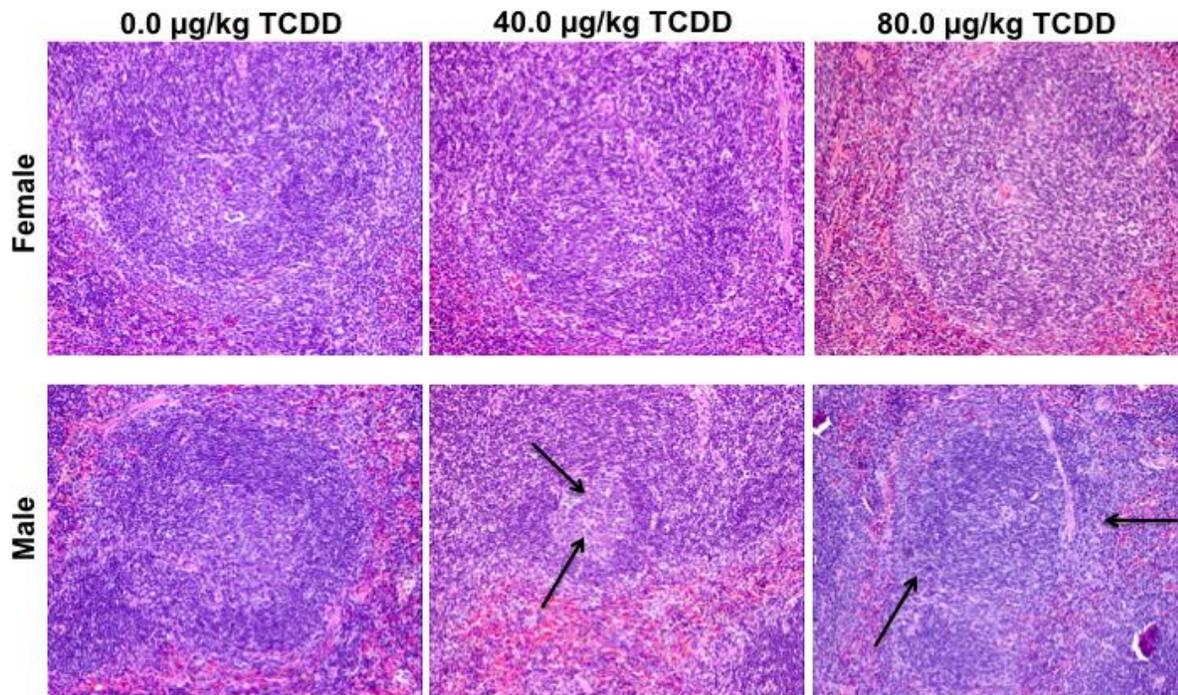


Figure 4. The spleens from 24-week-old SNF₁ mice that were prenatally exposed to 0, 40 or 80 µg/kg TCDD were collected, fixed, sectioned and stained with H&E. Images are representative of splenic sections presented by treatment and sex. Spleens from male mice prenatally exposed to 40 µg/kg TCDD frequently displayed follicles with small germinal centers (arrows). In the 80 µg/kg TCDD male mice, the germinal centers were few and discrete with coalescing of follicles and a loss of follicular architecture (arrows).

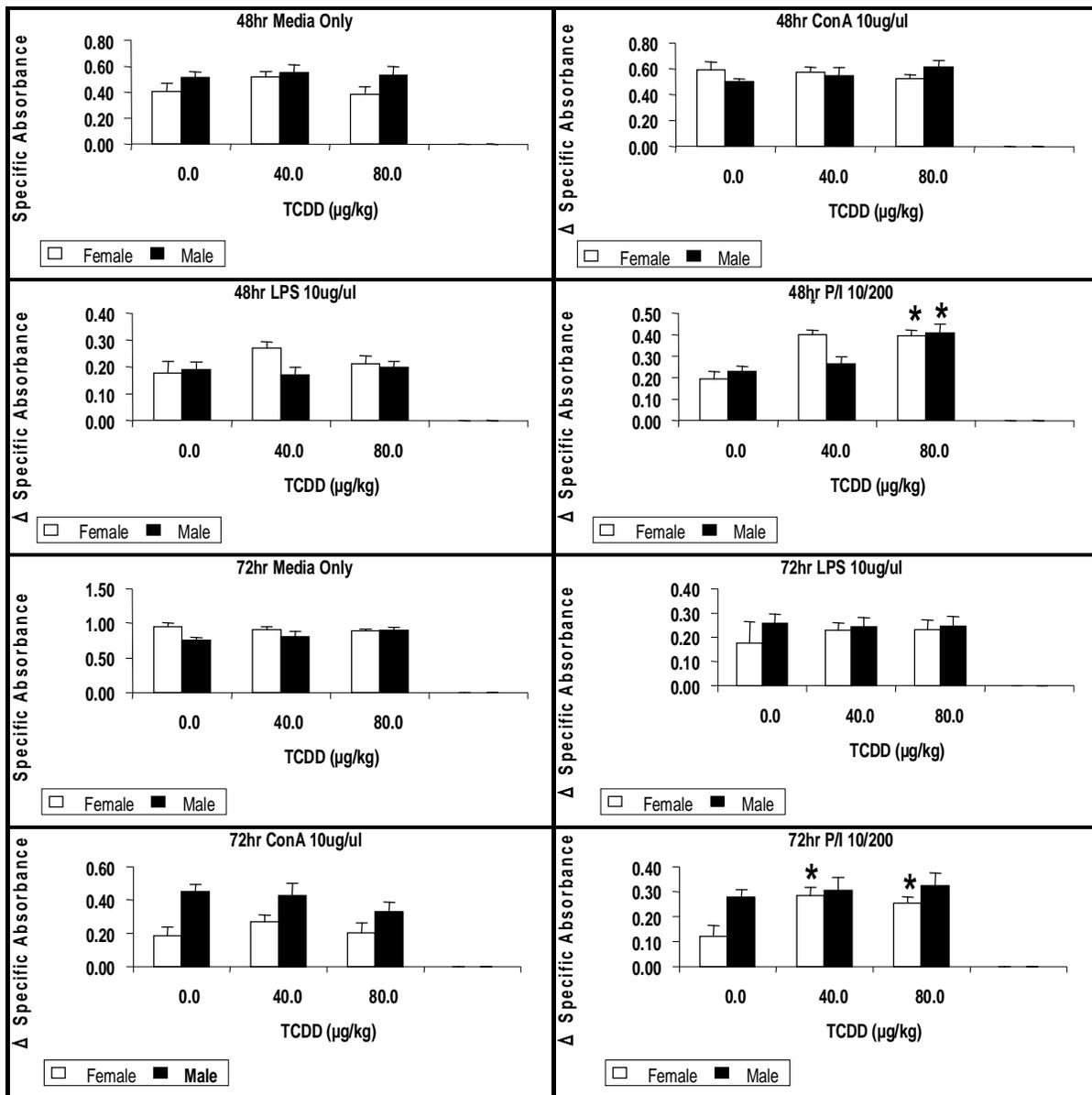


Figure 5. The lymphoproliferative responses of cultured splenocytes in 24-week-old SNF₁ mice exposed prenatally to 0, 40 or 80 μg/kg TCDD. Splenocytes were exposed to Con A, LPS, or P/I for 48 h or 72 h. Values are reported as mean Δ specific absorbance ± SEM. The results are based on 5 mice/treatment/sex (* = $p < 0.05$, Dunnett's test).

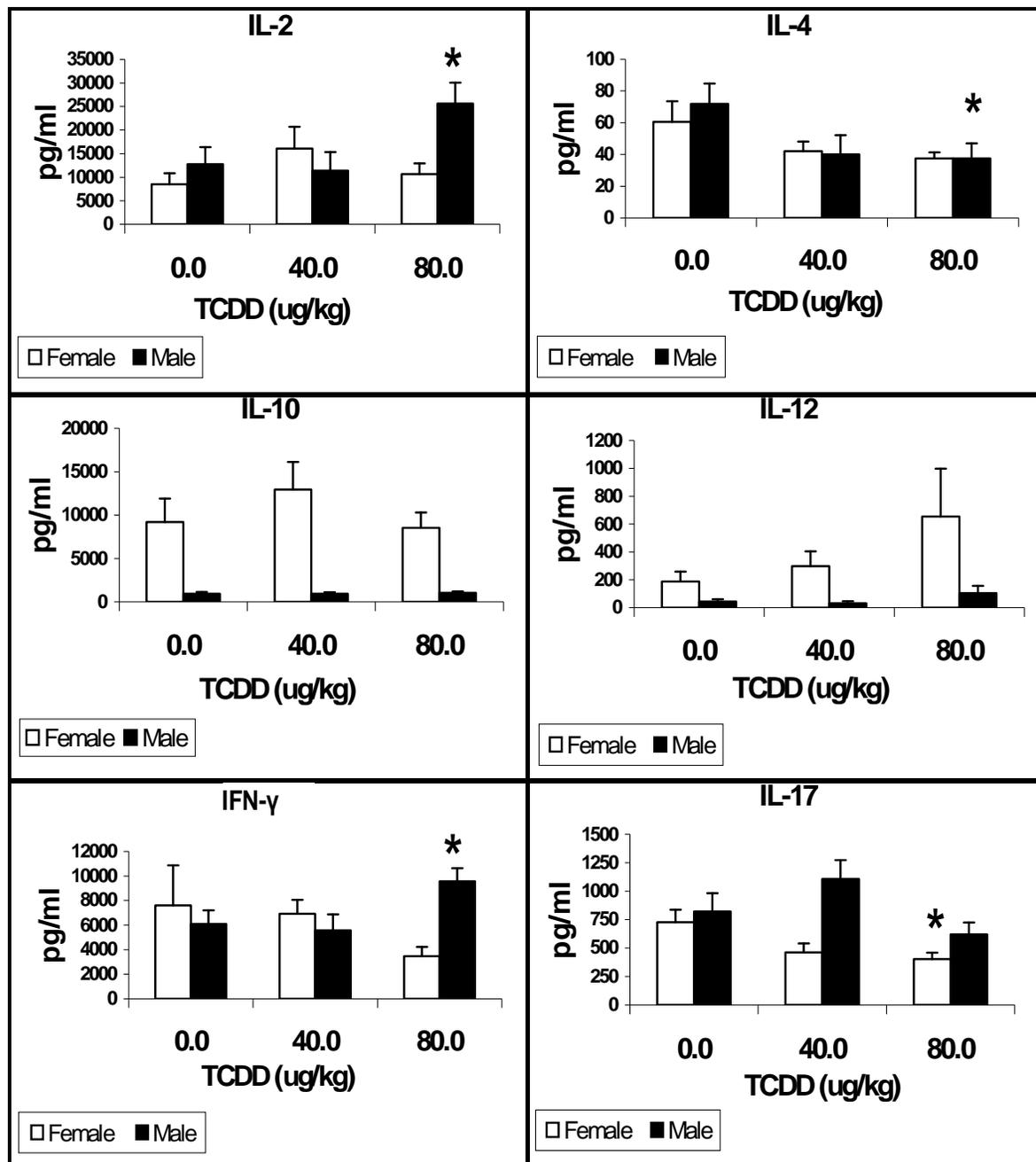


Figure 6. Supernatants were collected from splenocytes, of 24-week-old mice that were prenatally exposed to 0, 40 or 80 µg/kg TCDD, and cultured for 48 h with Con A (10 µg/mL). The levels of IL-2, IL-4, IL-10, IL-12, IL-17 and IFN-γ were determined using commercially available murine cytokine ELISA kits. The data are presented in a histogram analysis with mean bars. The results are based on 5 mice/treatment/sex (* = $p < 0.05$, Dunnett's test).

Table 1. Body Weight, Organ Weight and Total Cellularity

		0.0 µg/kg	40.0 µg/kg	80.0 µg/kg
		Mean ± SEM	Mean ± SEM	Mean ± SEM
Body Weight Grams	<i>female</i>	26.9 ± 0.9	25.9 ± 0.3	24.1 ± 0.6 *
	<i>male</i>	42.0 ± 0.8	36.1 ± 1.3 *	33.7 ± 1.1 *
Thymic Weight Grams	<i>female</i>	0.042 ± 0.004	0.052 ± 0.006	0.038 ± 0.004 *
	<i>male</i>	0.042 ± 0.002	0.038 ± 0.003	0.032 ± 0.001 *
Thymic Cellularity Cells (Millions)	<i>female</i>	35.3 ± 4.6	26.0 ± 3.5	20.9 ± 2.6 *
	<i>male</i>	22.2 ± 1.4	21.2 ± 1.8	14.4 ± 1.8 *
Thymus: Body Weight Ratio	<i>female</i>	0.0015 ± 0.0001	0.0020 ± 0.0002	0.0016 ± 0.0001
	<i>male</i>	0.0010 ± 0.0001	0.0011 ± 0.0001	0.0009 ± 0.0001
Splenic Weight Grams	<i>female</i>	0.190 ± 0.047	0.217 ± 0.040	0.172 ± 0.026
	<i>male</i>	0.114 ± 0.016	0.111 ± 0.008	0.108 ± 0.009
Splenic Cellularity Cells (Millions)	<i>female</i>	106.3 ± 13.4	151.8 ± 22.3	144.5 ± 22.7
	<i>male</i>	64.4 ± 5.8	65.2 ± 4.8	88.8 ± 10.3 *
Spleen: Body Weight Ratio	<i>female</i>	0.007 ± 0.001	0.008 ± 0.002	0.007 ± 0.001
	<i>male</i>	0.003 ± 0.00	0.003 ± 0.00	0.003 ± 0.00

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 2. Bone Marrow Analysis

		0.0 µg/kg		40.0 µg/kg		80.0 µg/kg	
		Mean	+ SEM	Mean	+ SEM	Mean	+ SEM
Total B220	%	<i>female</i>	21.5	+ 0.9	18.5	+1.6	16.6 ±2.2 *
		<i>male</i>	18.6	+ 0.5	19.6	+1.1	19.8 ±1.0
B220 ^{low}	%	<i>female</i>	67.0	+ 1.7	67.7	+3.7	81.3 ±2.1 *
		<i>male</i>	73.0	+ 3.2	67.1	+1.5	68.4 ±2.2
B220 ^{low} CD24 ^{pos}	%	<i>female</i>	75.3	+ 1.3	79.1	+0.7	72.1 ±3.1
		<i>male</i>	79.5	+ 0.4	75.2 ±1.1 *	73.9 ±1.1 *	
B220 ^{low} CD24 ^{hi} % of total B220 ^{low} CD24 ^{pos}		<i>female</i>	8.5	+ 1.0	10.8	+1.1	6.7 ±3.1
		<i>male</i>	12.9	+ 1.1	12.7	+1.2	15.3 ±2.1
B220 ^{low} CD24 ^{low} % of total B220 ^{low} CD24 ^{pos}		<i>female</i>	66.7	+ 1.4	68.3	+0.7	65.4 ±3.8
		<i>male</i>	66.6	+ 1.1	62.5	+1.1	58.6 ±1.3 *
B220 ^{low} CD24 ^{neg}	%	<i>female</i>	26.5	+ 1.1	21.0 ±0.7 *	27.9	+2.0 *
		<i>male</i>	20.5	+ 0.4	24.8 ±1.1 *	26.1 ±1.3 *	
B220 ^{low} CD24 ^{neg} AA4.1 ^{pos} % of total B220 ^{low} CD24 ^{neg}		<i>female</i>	64.6	+ 2.6	63.1	+3.9	74.5 ±3.0 *
		<i>male</i>	61.9	+ 1.9	68.6	+3.0	68.3 ±1.8
B220 ^{hi}	%	<i>female</i>	32.5	+ 2.0	32.4	+3.7	18.7 ±3.0 *
		<i>male</i>	27.0	+ 3.2	32.9	+1.5	31.7 ±2.2
B220 ^{hi} CD24 ^{pos}	%	<i>female</i>	52.6	+ 6.8	61.6	+4.2	39.7 ±5.6
		<i>male</i>	46.1	+ 5.9	35.8	+2.3	29.0 ±1.1 *
B220 ^{hi} CD24 ^{hi} % of total B220 ^{hi} CD24 ^{pos}		<i>female</i>	15.2	+ 4.6	14.4	+3.7	7.3 ±1.4
		<i>male</i>	8.4	+ 1.2	5.8	+0.5	5.9 ±0.8
B220 ^{hi} CD24 ^{low} % of total B220 ^{hi} CD24 ^{pos}		<i>female</i>	37.4	+ 2.4	47.3	+3.7	32.4 ±4.5
		<i>male</i>	37.7	+ 5.6	30.0	+2.3	23.1 ±1.1 *
B220 ^{hi} CD24 ^{neg}	%	<i>female</i>	47.4	+ 6.8	38.4	+4.2	60.3 ±5.6
		<i>male</i>	53.9	+ 5.9	64.2	+2.3	71.0 ±1.5 *

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 3. Thymus T cell Differentiation

Thymus		0.0 µg/kg Mean ± SEM	40.0 µg/kg Mean ± SEM	80.0 µg/kg Mean ± SEM
CD4+ CD8- (%)	<i>female</i>	16.1 ± 3.7	19.0 ± 3.9	9.9 ± 0.9
	<i>male</i>	20.0 ± 4.5	21.8 ± 3.3	14.1 ± 2.2
CD4+ CD8+ (%)	<i>female</i>	31.3 ± 6.2	26.0 ± 3.7	16.2 ± 2.9 *
	<i>male</i>	34.9 ± 8.8	40.8 ± 3.0	42.2 ± 6.5
CD4- CD8- (%)	<i>female</i>	49.2 ± 9.9	50.3 ± 7.6	71.9 ± 3.3 *
	<i>male</i>	43.5 ± 12.1	35.0 ± 1.8	41.8 ± 8.4
CD4- CD8+ (%)	<i>female</i>	3.4 ± 0.7	4.8 ± 1.0	1.9 ± 0.2
	<i>male</i>	1.5 ± 0.3	2.5 ± 0.4	1.8 ± 0.3

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 4. Splenic B cell Subset Analysis

Spleen		0.0 µg/kg Mean ± SEM	40.0 µg/kg Mean ± SEM	80.0 µg/kg Mean ± SEM
B220+ (%)	<i>female</i>	24.6 ± 2.4	23.4 ± 3.0	28.1 ± 3.2
	<i>male</i>	28.7 ± 2.9	30.4 ± 3.3	31.5 ± 1.6
CD19+ CD5+ (%)	<i>female</i>	5.4 ± 0.8	11.0 ± 1.4 *	9.3 ± 2.0
	<i>male</i>	3.4 ± 0.5	3.1 ± 0.6	4.5 ± 0.6
Absolute number B220+	<i>female</i>	2609.6 ± 383.1	3835.7 ± 1066.0	4144.4 ± 972.9
	<i>male</i>	1901.3 ± 320.0	2038.8 ± 372.1	2783.7 ± 333.1
Absolute number CD19+ CD5+	<i>female</i>	599.9 ± 158.8	1719.9 ± 368.4 *	1229.1 ± 220.9
	<i>male</i>	215.1 ± 32.9	198.0 ± 34.7	414.7 ± 86.8 *

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 5. Splenic B cell Subset Analysis

Spleen- % of total CD45RB220		0.0 µg/kg Mean ± SEM	40.0 µg/kg Mean ±SEM	80.0 µg/kg Mean ±SEM
CD24 ^{hi}	<i>female</i>	10.9 ± 0.8	11.6±0.5	7.9 ±1.0 *
	<i>male</i>	8.8 ± 0.6	8.2 ±0.7	6.1 ±0.5 *
CD24 ^{low-int}	<i>female</i>	88.5 ± 0.8	88.0±0.5	91.8±2.1 *
	<i>male</i>	85.6 ± 0.9	86.3±0.9	88.4±0.6 *
CD21 ^{hi} CD24 ^{low-int}	<i>female</i>	15.4 ± 2.9	12.8±0.8	6.4 ±1.0 *
	<i>male</i>	16.9 ± 1.1	11.6±0.9	10.8±1.3 *
CD21 ^{int} CD24 ^{hi}	<i>female</i>	7.4 ± 0.7	7.9±0.5	7.7 ±0.6
	<i>male</i>	8.4 ± 0.6	6.0±0.6 *	4.3 ±0.5 *
CD21 ^{int} CD24 ^{low}	<i>female</i>	65.2 ± 3.4	67.8±1.3	73.4±1.6 *
	<i>male</i>	64.3 ± 1.7	72.1±1.6 *	75.3±1.7 *
CD21 ^{neg-low} CD24 ^{hi}	<i>female</i>	12.9 ± 0.5	12.7±0.5	13.2±0.7
	<i>male</i>	10.9 ± 0.5	10.7±0.7	9.9 ±0.3
CD23 ^{int-hi} CD1 ^{neg}	<i>female</i>	56.0 ± 2.8	56.9±4.1	60.4±1.7
	<i>male</i>	59.7 ± 4.2	55.7±1.1	62.7±2.6
CD23 ^{low-int} CD1 ^{low-int}	<i>female</i>	9.8 ± 1.0	10.1±0.9	11.3±0.5
	<i>male</i>	6.1 ± 0.4	5.5±0.5	3.0 ±0.4 *
CD23 ^{neg} CD1 ^{low}	<i>female</i>	14.7 ± 1.6	15.9±2.5	14.8±1.6
	<i>male</i>	21.5 ± 4.2	26.7±0.9	22.4±2.0
CD23 ^{neg-low} CD1 ^{hi}	<i>female</i>	12.3 ± 0.5	11.2±0.8	10.9±0.8
	<i>male</i>	12.8 ± 0.6	12.3±0.5	12.1±0.7

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 6. Splenic T cell Subset Analysis

Spleen		0.0 µg/kg Mean ± SEM	40.0 µg/kg Mean ± SEM	80.0 µg/kg Mean ± SEM
CD4+ CD8- (%)	<i>female</i>	32.8 ± 3.2	42.6 ± 4.1	34.3 ± 4.2
	<i>male</i>	34.9 ± 3.2	38.6 ± 3.6	43.8 ± 3.0
CD4- CD8+ (%)	<i>female</i>	4.8 ± 0.4	4.9 ± 0.3	4.6 ± 0.3
	<i>male</i>	4.1 ± 0.4	3.6 ± 0.3	4.0 ± 0.5
Absolute number CD4+ CD8-	<i>female</i>	3326.9 ± 236.0	5602.6 ± 821.2	4989.1 ± 1079.6
	<i>male</i>	2195.7 ± 178.4	2462.2 ± 193.4	3921.3 ± 602.0 *
Absolute number CD4- CD8+	<i>female</i>	517.1 ± 88.4	745.3 ± 130.2	660.2 ± 112.5
	<i>male</i>	255.9 ± 18.2	225.7 ± 11.2	344.3 ± 47.4

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 7. Lymph Node T cell Subset Analysis

Lymph nodes		0.0 µg/kg Mean ± SEM	40.0 µg/kg Mean ± SEM	80.0 µg/kg Mean ± SEM
CD4+ CD8- (%)	<i>female</i>	67.6 ± 1.1	71.6 ± 0.9	63.6 ± 2.2
	<i>male</i>	79.4 ± 1.1	79.7 ± 2.5	75.7 ± 0.7
CD4- CD8+ (%)	<i>female</i>	25.1 ± 1.6	22.0 ± 0.9	22.6 ± 2.8
	<i>male</i>	15.2 ± 1.1	11.2 ± 1.8	18.7 ± 1.3
CD4+ CD25+ (%)	<i>female</i>	7.4 ± 0.9	7.4 ± 1.3	15.2 ± 2.8 *
	<i>male</i>	3.5 ± 0.1	4.4 ± 0.4	5.1 ± 0.4 *
CD4+ Vβ17 ^a +TCR (%)	<i>female</i>	3.9 ± 1.0	3.6 ± 0.5	8.4 ± 1.7 *
	<i>male</i>	3.2 ± 0.0	3.2 ± 0.0	3.1 ± 0.0
CD3+Vβ3+TCR (%)	<i>female</i>	2.4 ± 0.5	2.3 ± 0.6	4.7 ± 1.6
	<i>male</i>	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

CHAPTER 4. PERSISTENT MODULATION OF T CELL AND B CELL MATURATION AND FUNCTION IN 48-WEEK-OLD C57BL/6 MICE DEVELOPMENTALLY EXPOSED TO TCDD

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Running title: Immune modulation in prenatal TCDD-exposed mice

Keywords: TCDD, C57BL/6, mice, T cell, B cell, thymus, bone marrow

Abstract

A single prenatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is capable of altering a number of immune pathways and enhancing immune complex formation, and resulted in lupus-like lesions in 24-week-old C57BL/6 offspring. The extent to which prenatal TCDD exposure can permanently alter immunity and affect disease progression during aging is unknown. In this study, 48 week-old offspring of high affinity *AhR* C57BL/6 dams prenatally exposed to oral TCDD (0, 2.5 or 5.0 µg/kg) were evaluated for changes in T cell and B cell maturation and function as well as the progression of immune-mediated renal disease. TCDD-treated females displayed decreased thymic weight and cellularity compared to controls. Axillary and inguinal lymph nodes from TCDD-treated male offspring contained increased percentages of autoreactive CD3⁺Vβ3 TCR⁺ T cells. Con-A stimulated splenocytes from prenatal TCDD-treated female mice produced increased IL-12 and IFN-γ and diminished IL-4 while males showed increased IL-10 and IL-12. Alterations in bone marrow lymphopoiesis were seen in both sexes. Splenic transitional-2 B cells (CD21^{int}CD24^{hi}) were increased in males while transitional-1 B cells (CD23^{neg}CD1^{neg}) were increased in females. Autoantibodies to cardiolipin were significantly increased in the TCDD-treated males. Anti-IgG and anti-C3 immune complex renal deposition was significantly increased in TCDD-treated males, and enhanced in the TCDD-treated females. The findings from this study suggest that developmental TCDD exposure permanently and differently alters immune system ontogeny based on sex. Further, the outcome from this immune dysregulation includes a shift

toward a sustained type III hypersensitivity, lupus-like autoimmune-mediated phenotype in genetically non-predisposed murine strain.

Introduction

Prenatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent environmental contaminant, has been shown to cause numerous immunologic changes that are detectable into adulthood. The majority of these prenatal TCDD studies have focused on suppressive effects on cell-mediated immunity (Vos et al. 1974; Faith & Moore 1977; Luster et al. 1980). Prenatal TCDD exposure affects thymus biology and T cell development and maturation, manifested by severe thymic atrophy and changes in proportion of thymocyte subpopulations (Faith & Moore 1977; Holladay et al. 1991; Blaylock et al. 1992; Gehrs et al. 1997; Gehrs & Smialowicz 1999).

The thymocyte is a highly sensitive target of TCDD insult as demonstrated by inhibited differentiation of CD4⁺CD8⁺ T cells and a block in the transition phase from CD4⁺CD8⁺CD24⁺ to CD4⁺CD8⁺ (Fine et al. 1989; Kerkvliet & Brauner 1990; Lundberg et al. 1990; Holladay et al. 1991; Blaylock et al. 1992; Gehrs et al. 1997; Gehrs & Smialowicz 1997). Later work by Laiosa et al. (2003) has shown that exposure to TCDD results in proliferation arrest of triple negative (TN) (CD4⁻ CD8⁻ CD3⁻) thymocytes especially in the TN3 (CD44⁻CD25⁺) and TN4 (CD44⁻CD25⁻) stages (Laiosa et al. 2003). Other studies have shown that inducing thymic apoptosis via Fas-Fas interactions is one pathway responsible for TCDD induced thymic atrophy (Rhile et al. 1996; Kamath et al. 1997; Zeytun et al. 2002; Camacho et al. 2004; Camacho et al. 2005). Additional modes of action may include an aberrant emigration pattern of immature thymic T cell subpopulations, specifically double

negative (DN) CD4-CD8- cells, and a skew of the thymic output towards CD8⁺ T cells (Majora et al. 2005).

In addition to direct effects on the thymocyte, other studies have shown that thymus epithelial and stroma cells are targeted by TCDD (Nagarkatti et al. 1984; Greenlee et al. 1985; De Waal et al. 1992; Kremer et al. 1995; Camacho et al. 2005). Based on promoter analysis of TCDD-susceptible genes, a recent study indicated the potential for cell-specific transcription factor crosstalk between aryl hydrocarbon receptor (*AhR*) signaling and other pathways such as estrogen signaling in thymus epithelium cells (Frericks et al. 2008). It has been suggested that TCDD exposure elicits thymic toxicity by altering transcription of genes controlling proliferation and trafficking in the most immature thymocyte phenotypes specially TN2 (CD44⁺, CD25⁺) and TN3 (CD44⁻, CD25⁺), and that *AhR* activation is dependent on the cell-type, cell stage and possibly the involvement of other transcription factors (Frericks et al. 2006; McMillan et al. 2007). Therefore, it is reasonable to suggest that TCDD influences multiple mechanisms or pathways in the thymic compartment contributing to thymic atrophy and altered T cell differentiation. Extensive molecular and pharmacological studies have demonstrated that TCDD toxicity is primarily mediated through activation of the *AhR*.

In the thymus, generating a repertoire of T cells capable of recognizing a diverse and broad array of MHC-restricted antigens is critical for survival. Yet, to avoid autoreactivity, the diversity of MHC-restricted antigen recognition is limited through the process of negative selection (Sebzda et al. 1999; Palmer 2003). TCDD appears to alter the dynamics of thymic selection, in part, through dysregulation of

coreceptors, MHC expression and costimulatory molecules that are believed to play an important role in thymic selection (Fisher et al. 2004). Specifically, MHC class I and class II molecules act as thymic self-antigen-presenting molecules in a process whereby thymocytes expressing T-cell receptors (TCRs) with high affinity to self-antigen are eliminated (negative selection).

Targeting of the thymic parenchyma and thymic stroma cells by TCDD led to suggestions that TCDD may have the potential to alter critical, selective and dynamic developmental steps through which thymocytes expressing autoreactive TCRs are deleted via lympho-stromal communication (Okuyama et al. 1992; Hanawa et al. 1993). This would infer that the consequences of developmental exposure to TCDD might not be exclusively immunosuppressive, as thymic negative selection is aberrantly dysregulated. In support of this idea, De Waal and colleagues reported reduced expression of cortical thymic MHC class II molecules in TCDD-treated mice (1992). It has also been noted that similar patterns of inhibited thymic T-cell differentiation occur spontaneously in autoimmune mice (Kakkanaiah et al. 1990), in TCDD-treated mice (Blaylock et al. 1992), and in mice treated *in vivo* with monoclonal antibodies to MHC class I and class II molecules (Kruisbeek et al. 1985). These observations support the importance of these MHC molecules in thymocyte differentiation and maturation.

TCDD has also been shown to down regulate expression of an MHC class I gene (*Q1^b*) in a mouse hepatoma cell line (Dong et al. 1997). These investigators reported that MHC *Q1^b* cDNA encoded for the $\alpha 3$ domain and transmembrane domain of the *Q1^b* class I protein, implying that the MHC gene product could interact with $\beta 2$ -

microglobulin, a component of MHC class I molecules. These authors therefore suggested that TCDD may dysregulate the antigen presentation process in the liver as well as the thymus by targeting MHC Q1^b molecule expression. These same investigators proposed that TCDD may, through shared mechanisms, also down regulate MHC class II molecules, although this hypothesis remains to be tested. Exposure to TCDD also up-regulates the expression of MHC class II molecules on the splenic dendritic cells (Vorderstrasse et al. 2003), further verifying the effect of this agent on these immunologically important molecules.

In addition to potentially facilitating the escape of autoreactive thymocytes from negative selection, TCDD enhanced extrathymic production of T cells in various compartments where negative selection is absent or is less stringent compared to thymus. Silverstone et al. (1994) showed that TCDD induced T cell differentiation in the liver of young adult mice. These extrathymic-derived T cells expressed elevated levels of CD4⁺ V β 17^a and V β 3⁺ TCR. Such TCR variable β (V β) chains are usually deleted in the thymus by reaction with self-MHC and minor lymphocyte stimulatory antigens (Okuyama et al. 1992; Hanawa et al. 1993) and have been associated with autoimmunity in experimental mouse models (Rocha et al. 1992).

Whereas the majority of studies assessing the immunotoxicity of TCDD have focused on cell-mediated immunity, little work has been conducted to address the possible effects on B development, function and maturation after early TCDD exposure (Luster et al., 2003; Holsapple et al., 2005). Recent adult mouse studies suggest the disruption of normal B cell function can precipitate in autoimmunity by production of autoantibodies or potentially by altered mediation of other important immune

events, including antigen presentation, cytokine production and modulation of other immune cells (Fujimoto and Sato 2007). Thus, the study shows that B cells appear to play a more important role in autoimmunity than previously proposed (Martin and Chan 2006).

A recent study from our laboratory has shown an enhanced autoimmune profile accompanied by altered B lymphopoiesis and B cell subpopulations in the spleen of C57BL/6 mice at 24 weeks following a single prenatal dose of TCDD (Mustafa et al. 2008). These findings show that prenatal TCDD exposure alters differentiation and activation of B cells shifting the naïve B cells toward the marginal zone in the spleen. Further, these mice exhibited persistent humoral immune dysregulation as well as altered cell-mediated responses. Although a sex effect in the immune response was apparent, both sexes shared an increased autoimmune disease profile including increased antibody titers to dsDNA, ssDNA and cardiolipin and increased autoantibody to DNA and glomerular substrate dominating the deposits in kidneys. The current study examined the progression of immune changes and renal pathology following prenatal TCDD exposure in C57BL/6 mice at 48 weeks of age. Our hypothesis was that immune-mediated renal disease would progress beyond that which we previously observed at 24 weeks of age (Mustafa et al., 2008), and that the immune mechanisms driving this disease in these now-geriatric mice would change based on sex. We investigated selected immune parameters of the primary and secondary lymphoid organs of both the cell-mediated and antibody-mediated pathways with a more detailed analysis of bone marrow B lymphopoiesis as well as renal pathology in these high affinity *AhR*, prenatally TCDD-exposed C57BL/6 mice.

Materials and Methods

Mice and TCDD exposure. C57BL/6 mice were obtained from Charles River Laboratories (Portage, MA) at 4–5 weeks-of-age. Mice were acclimated to the animal care facility for at least 2 weeks prior to breeding. Briefly, 80 female C57BL/6 mice were bred overnight using one C57BL/6 male per two females, and plug positive mice the next morning were designated gd 0. Pregnant C57BL/6 mice were orally gavaged on gestation day 12 with 0, 2.5 or 5.0 µg/kg TCDD dissolved in corn oil (n= 12 pregnant mice/treatment). The F₁ offspring remained with dams until weaning, then separated by treatment and a randomly selected group of mice from each treatment group were allowed to mature to 48 weeks-of-age. All animals were fed a commercial pelleted diet and provided water *ad libitum*, and were housed under controlled conditions of temperature (22 °C), humidity (40–60 %), and lighting (12:12 light:dark cycle). Animal maintenance, care and use were at all times in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines at Virginia Tech, and approved prior to initiation of experiments.

Body weights and tissue collection. Mice were euthanized by cervical dislocation and weighed. The thymus, spleen, bone marrow and axillary and inguinal lymph nodes (LN) were immediately collected post-euthanasia under aseptic conditions, using dissection scissors and curved forceps. Axillary and inguinal LN were pooled for flow cytometry. Non-LN organs were weighed and then placed individually into pre-labeled sterile Petri dishes (Corning, Corning, NY), containing 8 mL of RPMI-

1640 culture medium (Mediatech, Herndon,VA). Dishes were placed on ice until tissue dissociation.

Cell dissociation and enrichment. Each organ was gently dissociated over a stainless steel sieve screen (Sigma, St. Louis, MO) using curved forceps. Cells were then pipetted through the sieve screen following dissociation to remove debris. Cells were washed in RPMI-1640 for 10 min, 240 x g, and 23 °C. The supernatant was discarded and, with the exception of spleen, the cell pellet was resuspended in 8 mL of RPMI-1640. Spleen cells were resuspended in 1 mL incomplete RPMI-1640. To each tube, 2 mL of 0.83 % ammonium chloride lysis buffer (ACK, pH 7.29) were added, to lyse red blood cells, and tubes were incubated for 5 min at 23 °C. After the lysis incubation, the cells were resuspended in 5 mL of incomplete RPMI-1640 and washed twice (7 min, 290 x g and 7 °C). The splenic leukocyte-rich cells were then resuspended in 5 mL complete RPMI-1640 media containing 10 % heat-inactivated FBS (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/mL penicillin (ICN), and 50 mg/mL of streptomycin (ICN), and maintained at 7-10 °C. For bone marrow isolation, femurs were removed and the bone marrow cavities flushed with 2% FBS-PBS, washed once (7 min, 290 x g and 7 °C), resuspended in 1 mL incomplete RPMI-1640 media and stored at 4 °C.

Cell enumeration. Cells were enumerated and size-analyzed using a Beckman Multisizer 3[®] Coulter cell counter (Beckman Coulter, Fullerton, CA) according to the manufacturer's protocol. Briefly, a 10 µL aliquot of enriched cell suspension was

transferred to a plastic counting-chamber containing 10 mL of PBS (Mediatech). The plastic chamber was capped, mixed by repeated gentle inversion, and counted. The cells were enumerated and adjusted to 5.0×10^6 cells/mL in complete media.

Flow cytometric evaluation of cell-surface markers. Cell suspensions ($5 \times 10^5/100 \mu\text{L}$) from the thymus, spleen, lymph node and bone marrow were dispensed into individual wells of a 96-well round-bottom tissue culture plate (Corning). Monoclonal antibodies (mAbs) with phycoerythrin (PE) fluorescent labels were used according to manufacturer's (BD Pharmingen; San Diego, CA) recommendation at a concentration of $0.2 \mu\text{g}/\mu\text{L}$; mAbs with fluorescein isothiocyanate (FITC) fluorescent labels were similarly used at the recommended concentration of $0.5 \mu\text{g}/\mu\text{L}$. Cells were stained as previously described (Gogal et al. 2001; Klein et al. 2006). Briefly, lymphocyte aliquots (5×10^5 cells/ $100 \mu\text{L}$) from thymus, spleen, lymph node and bone marrow were incubated with the following primary mAbs: PE-anti CD4, FITC-anti CD8, FITC-anti CD25, FITC-anti CD19, FITC-anti IgM, PE-anti CD5, PE-anti CD3, PE-anti CD45/B220, PE-Cy5 anti CD45/B220, PE-Cy5 anti CD93 (AA4.1) (Ebioscience, San Diego, CA); FITC-anti V β 3 TCR (KJ25), FITC-anti V β 17^a TCR (KJ23), FITC-anti CD45/B220, PE-anti CD24 (HSA), FITC-anti CD1, PE-anti CD23, PE-anti CD138 (BD Pharmingen). For double or triple staining protocols, mAbs with different fluorescent labels were simultaneously added to the sample. For bone marrow analysis, aliquots of 5×10^5 cells were pre-blocked with anti-Fc γ III/IIR (clone 2.4G2, Rat IgG2_b). Following staining, cells were washed and evaluated on a Coulter Epics XL flow cytometer (Beckman Coulter). From each

sample, 10000 events were collected and analyzed using the FlowJo software (Tree Star, San Carlos, CA). Dead cells, clumps, and debris were excluded electronically by gating on forward scatter (FSC) versus side scatter (SSC). The distribution of B cell subsets analysis were gated as previously described (Grimaldi et al. 2001).

ELISA for autoantibodies to double-stranded DNA, single-stranded DNA and cardiolipin. To detect the presence of dsDNA antibody, 96-well medium-binding microtiter plates (Corning) were coated with heat-denatured calf thymus DNA (100 µg/mL; Sigma). For ssDNA antibody titers, DNA from calf thymus was used (10 µg/mL; Sigma), and for autoantibodies to cardiolipin a solution from bovine heart was used (10 µg/mL; Sigma). All pre-coated plates were incubated at 4 °C overnight. The plates were washed thrice with 300 µL PBS/0.05% Tween 20 (Mediatech), blocked with 1% BSA (Sigma) for 2 h at 23 °C, washed and then incubated with diluted serum samples collected via retro-orbital bleeding prior to euthanasia. For all three ELISA assays, serial dilutions of each mouse serum were performed to optimize optical density readings. Serum diluted 1/50 was determined to be the optimal dilution to attain a wide spectrum in the optical density among the samples. After 3 h at 23 °C, the serum-coated plates were washed thrice with 300 µL of PBS-0.05 % Tween 20. To each well, 0.2 mL of diluted alkaline phosphatase-conjugated anti-IgG antibody (1/3000) (Sigma) were added and the plates were incubated for 60 min at 23 °C. The plates were then washed thrice with 300 µL of PBS-0.05 % Tween 20. To each well, 0.2 mL prepared substrate for alkaline phosphatase conjugated secondary antibody (SIGMA FAST™ p-Nitrophenyl

phosphate tablets) were added and allowed to develop for 45 min at 23 °C before adding 50 µL of 3 M NaOH stop solution (Sigma). The absorbance (A_{405}) of the initial dilution was measured. Optical density (OD) readings represent the average from sera from each mouse performed in duplicate.

Histology of the kidneys. Kidneys were collected at the time of euthanasia, sectioned for histopathology and immunohistochemistry. The histopathology section was immediately fixed in 10 % formalin (Fisher Scientific, Pittsburgh, PA) for 48 h and the immunohistochemistry section was embedded in OCT media (Miles, Elkhart, IN) and frozen at -80 °C for cryosections. After 48 h in formalin, the tissues were removed, routinely processed and embedded in a paraffin block. Following embedding, a 5 µm section was cut from each tissue block, and stained with hematoxylin and eosin (H&E, Richard-Allen Scientific, Kalamazoo, MI) using standard histologic methods. The prepared slides were then evaluated, with a light microscope, in a blinded-manner by a resident veterinary pathologist (co-author PS). For each kidney, 100 consecutive renal cortex glomeruli were evaluated. Each glomerulus was scored for the presence of fibrinoid necrosis or crescents and extent of lymphocytic infiltration. The perivascular scores were evaluated at 40x. Number of inflammatory foci and their relative size were evaluated and scored together in the following manner; a score of "0" indicated no inflammation associated with vessels (these include both arteries and veins, though usually the veins appear to be the main structure associated with these), a score of "1" indicated small foci and only one or two in the section, a score of "2" indicated more than two, and of a size

that was obvious and a score of "3" indicated widespread, large foci around several vascular structures.

Immunohistochemistry of the kidneys: C3 and IgG deposition. Frozen kidneys were cut into 5 μm sections and stained with FITC conjugated antibodies (Bagavant et al. 2002; Xie et al. 2003; Mustafa et al. 2008). Briefly, tissue sections were thawed at room temperature and dried for 30 min. Slides were fixed in acetone for 10 min and then washed with PBS thrice for 3 min/wash. Goat anti-mouse IgG diluted 1:100 (MP Biomedicals, Santa Ana, CA) or goat anti-mouse C3 diluted 1:100 (MP Biomedicals) were incubated with tissues sections in a humid chamber for 60 min at 23°C. The sections were then rinsed thrice for 5 min/wash with PBS. The slides were mounted using Vectashield™ mounting media (Vector Labs, Burlingame, CA) and then examined using an Olympus BX-60 fluorescence microscope (Center Valley, PA). The severity of glomerulonephritis and immune complex deposition was scored using a range from 0 to 3+, where 0 corresponded to a non-autoimmune healthy mouse and 3+ to the maximal alteration observed in the study. All slides were scored in a blinded manner independently by two experienced investigators (co-authors CR and RG) and averaged for the final score.

Lymphocyte proliferation assay. Splenocytes were plated into each well (5 x 10⁵ cells/ 100 μL per well) of a 96-well round-bottom tissue-culture plate (Corning Cell Wells™, Corning). Cells were exposed to mitogens as follows: 100 μL of: Concanavalin A (Con A, 10 $\mu\text{g}/\text{mL}$, Sigma), lipopolysaccharide (LPS, 50 $\mu\text{g}/\text{mL}$,

Sigma), or phorbol myristate acetate (PMA, 10 ng/mL, Sigma) plus ionomycin (0.5 µg/ml, Sigma) in complete media. Non-stimulated control cultures contained 100 µL of complete media alone. Triplicate wells were used for each stimulant. Following 48 h incubation, 20 µL of alamarBlue™ dye (Serotec, Raleigh, NC) (10 % of incubation volume) were added to each well of the culture plates. At 24 and 48 h post addition, degree of absorbance was determined under dual wavelength (570 and 600 nm) using a Molecular Devices plate reader (Menlo Park, CA)(Klein et al. 2006; Mustafa et al. 2008).

Cytokine ELISA Assays. Splenocytes were plated into each well (1 mL in complete media; 5×10^6 cells per well) of a 24-well tissue-culture plate (Corning). Cells were co-cultured with 1 mL Con A (10 µg/mL) and incubated at 37 °C, 5 % CO₂ for 48 h. The plates were centrifuged (7 °C, 250 x g, 7 min), and the supernatants were transferred to sterile 12 x 75 mm cultured tubes (Fisher). Supernatants were stored at – 80 °C until use. The levels of interleukin 2 (IL-2), IL-4, IL-10, IL-12, and interferon-gamma (IFN-γ) were determined using ELISA kits (Ready-to-use; Ebioscience) according to the manufacturer's instructions.

Statistical analysis. Data were expressed as arithmetical mean ± SEM. Analysis of variance (ANOVA) was used with Dunnett's test to establish significant differences in the same sex groups between treatment groups and control. The pregnant dam was maintained as the statistical unit in all cases such that each offspring analyzed represented a separate dam (one pup/gender/treatment). Group size was six

C57BL/6 offspring per sex for all experiments (n=6). Results described as different in this report indicate significantly different at $p < 0.05$.

Results

Body and organ weights, and organ cellularity.

Body weight was reduced relative to controls in 48-week-old female, but not male offspring of TCDD-treated dams, at the 5.0 µg/kg dose. Thymic weights showed a significant decrease in the 5.0 µg/kg TCDD females but not males. Similarly, mean thymic cellularity decreased significantly in 5.0 µg/kg TCDD females but not males. Thymic weight/body weight ratios showed a numerical trend toward increase in males and decrease in females, at 5.0 µg/kg TCDD. Splenic weight/body weight ratios were near significantly increased ($p= 0.054$) at 48 weeks, in the 5.0 µg/kg TCDD females but not males. Additionally, splenic weight and cellularity showed a numerical trend toward increase ($p= 0.09$) in the 5.0 µg/kg TCDD females (**Table 1**).

B lymphoid progenitors in bone marrow.

At 48 weeks-of-age, the 5.0 µg/kg TCDD females showed a near significant ($p=0.053$) decrease and the 5.0 µg/kg TCDD males showed a significant decrease in total B220 cells (B220⁺). The percentage of these B220⁺ cells that were B220^{hi}, representing small pre-B cells and immature B lymphocytes, was significantly decreased while the percentage that were B220^{low}, representing B lineage committed progenitors, pro-B cells and large pre-B cells, was significantly increased in 5.0 µg/kg TCDD females only. The percentage of B220^{low}CD24⁻ cells that expressed AA4.1 (B220^{low}CD24⁻AA4.1⁺), representing B lineage committed progenitors, was near significantly ($p=0.054$) increased in the 5.0 µg/kg TCDD

females and significantly increased in the 2.5 and 5.0 µg/kg TCDD males (**Table 2A**). The percentage of B220^{low}IgM⁻, representing pro lymphocytes B cells was significantly increased and the percentage of B220^{low}IgM⁺ representing pre lymphocytes B cells was significantly decreased in 5.0 µg/kg TCDD males, not females (**Table 2B**).

Thymic T cell differentiation.

Both female and male offspring of dams dosed with 5.0 µg/kg TCDD exhibited marginal but significant thymic phenotypic changes at 48 weeks-of-age. The percentage of CD4⁻CD8⁻ double negative thymocytes was increased by gd 12 TCDD in 5.0 µg/kg females. In 5.0 µg/kg males, the percentage of CD4⁺CD8⁺ double positive thymocytes was significantly decreased and the percentage of CD4⁺CD8⁻ was near significantly increased ($p=0.54$) (**Table 3**).

Phenotype of spleen B and T cells, and migration of mature B cells in the spleen.

The relative percentage of spleen leukocytes that expressed B220 did not differ across treatment or sex compared to controls (**Table 4**). However, there was a significant difference within B cell subsets expressing CD21/CD24 and CD1/CD23 phenotypes. The 5.0 µg/kg TCDD males showed a significant increased relative percentage of B transitional-2 cells (CD21^{hi}CD24^{hi}). In females, the B transitional-1 cells (CD23^{neg} CD1^{neg}) were significantly increased whilst other B cell subsets expressing CD1/CD23 did not differ from control groups. The percentages of splenic immature B220⁺CD24^{hi} transitional B cells and B220⁺CD24^{low-int} mature B were

unchanged; however, there was a significant decrease in mature IgD⁺ splenic B cells and a significant increase in IgD⁻ splenic B cells at 5.0 µg/kg TCDD regardless the gender (**Table 5**). Additionally, CD138⁺ plasma cells were significantly increased in 5.0 µg/kg TCDD males. The relative percentage of CD19⁺CD5⁺ B cells (representing B-1a cells, a B cell subset of fetal origin only, rather than bone marrow) was unchanged (**Table 4**). Similarly, the relative percentage of T cells expressing CD4/CD8 markers did not change in any treatment group or by sex (**Table 6**).

T cell subset expression in lymph nodes.

Prenatal TCDD at 5.0 µg/kg caused a significant decrease in the percentage of CD4⁻CD8⁺ cells in the lymph nodes of both male and female 48-week-old mice. Further, the percentage of CD3⁺ expressing Vβ3⁺ TCR and the percentage of CD4⁺ expressing Vβ17^{a+} TCR T cells showed a numeric (non-significant) dose-response increasing trend in the female mice. Interestingly, both of these Vβ phenotypes were significantly increased in the 5.0 µg/kg males. CD4⁺CD8⁻ cells in lymph nodes of male, but not female, 5.0 µg/kg mice were significantly increased. The CD4⁺ T cells expressing CD25, which include T regulatory and activated T cells showed a numeric (non-significant) dose-response trend toward decrease by prenatal TCDD in females (**Table 7**).

Antibody titers to ssDNA, dsDNA and cardiolipin.

The detection of dsDNA, ssDNA and cardiolipin autoantigens was performed with ELISA assays to assess the autoreactive functionality of B cells in mice

prenatally exposed to TCDD. Anti-cardiolipin titers antibodies were significantly increased in male but not in female offspring by 5.0 µg/kg TCDD. Anti-ssDNA and anti-dsDNA anti-cardiolipin titers showed numeric but non-significant increasing trends in both the male and female offspring (**Figure 1**).

Kidney pathology.

Since immune complex deposition in the kidney is a common clinical signalment in lupus patients, histopathologic examination of the kidney was performed. Glomeruli evaluated for evidence of fibrinoid necrosis (**N**) or crescents (**C**) showed an increasing non-significant trend by treatment and sex. The mean number of parenchyma mononuclear inflammatory score (**I**) foci also numerically increased, non-significantly, by treatment and sex turning significant by 5.0 µg/kg TCDD males (**Figure 2**).

Deposition of anti-IgG and anti-C3 immune complexes in the kidney.

Based on the histopathologic changes seen with H&E stained TCDD-kidneys, immunofluorescent staining was performed to elucidate IgG and C3 involvement in immune complex deposition. Kidney sections from 48-week-old C57BL/6 offspring showed a TCDD dose-dependent increasing trend in deposition of immune complexes, for both anti-IgG and anti-C3 probes. However, the 5.0 µg/kg TCDD-treated males were significantly increased for both anti-IgG and anti-C3 (**Figure 3**).

Mitogen-stimulated splenic lymphocyte proliferation.

Mitogen stimulation of enriched, cultured splenic lymphocytes was employed to assess the influence of prenatal TCDD on lymphocyte functionality in the adult mouse. Prenatal TCDD had a selective effect on splenic lymphoproliferative responses of 48 week-old C57B/L offspring. There was a numerical non-significant enhanced effect on spontaneous proliferation in 5.0 µg/kg TCDD females at 72 h and a significant suppressed effect on spontaneous proliferation in 5.0 µg/kg TCDD males at 72 h. Surprisingly, 5.0 µg/kg TCDD males group showed a significant enhanced effect on stimulation responses using PMA/ionomycin in splenocytes, at 72 h. In 5.0 µg/kg TCDD females, the responses showed similar increasing trends as 5.0 µg/kg TCDD males with no significant differences compared to control group, at 72 h. Additionally, the mitogenic response were not significant using ConA or LPS or PMA/ionomycin in both genders, at 48 h (**Table 8**).

Th1/Th2 cytokine balance.

In the females, prenatal exposure to TCDD enhanced the Th1 cytokine profile. The 5.0 µg/kg TCDD females showed increased IFN-γ production, increasing trend in IL-12, and diminished IL-4 production relative to controls. In the males, the 5.0 µg/kg TCDD males the cytokine profile was shifted toward a Th2 enhanced IL-10 and IL-12 while the 2.5 µg/kg TCDD males showed enhanced IL-12 and IFN-γ production, relative to controls (**Figure 4**).

Discussion

We recently reported that a single prenatal dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6 mice resulted in serologic, cellular, and histopathologic changes in the 24-week-old offspring, characteristic of a type III hypersensitivity lupus-like autoimmune disease (Mustafa et al. 2008). In the present study, we tracked these immune endpoints in the littermates of these mice to 48 weeks, with a focus on change toward autoimmunity. Toxicity of TCDD is mediated through the *AhR* (Holsapple et al. 1991; Hogaboam et al. 2008). The present C57BL/6 mice are of *Ah^{bb}* phenotype and represent an immunologically sensitive, high *AhR* affinity mouse strain.

Several studies describe targeting of thymocytes or thymic epithelium by TCDD, leading to suggestions that TCDD may have the potential to alter critical epithelium-dependent selective events in the thymus through which developing thymocytes expressing autoreactive T-cell receptors (TCRs) are deleted (De Waal et al. 1992; Schuurman et al. 1992; Blaylock et al., 1992). Another study observed altered thymic epithelial distribution of major histocompatibility complex (MHC) class II molecules in TCDD-treated mice, an effect that was hypothesized as having potential to cause defective thymocytes (De Heer et al. 1994). These observations might explain the elevated levels of autoreactive T cells expressing $V\beta 17a^+$ and $V\beta 3^+$ TCR in spleens of mice in the present study. A similar increase in these autoreactive $V\beta$ T cells has been suggested as a mechanism by which estrogen may promote autoimmunity (Silverstone et al. 1994). Okuyama et al. similarly suggested that these findings with TCDD (increased extrathymic autoreactive T cells) may relate to

ability of this halogenated aromatic hydrocarbon (HAH) to the promote autoimmunity (Okuyama et al. 1992).

At 24 weeks of age, female litter-mates of the 48 week-old mice showed decreased thymic weights and cellularity, decreased CD4⁺CD8⁺ thymocytes, and increased CD4⁺CD8⁻ thymocytes (Mustafa et al., 2008). At 48 weeks, such disruptions in thymocytes again predominantly affected female offspring, however males were beginning to show affects as well. The percentage of CD4⁺CD8⁺ double positive thymocytes was significantly decreased and the percentage of CD4⁺CD8⁻ was near significantly ($p= 0.054$) increased in the male offspring.

Since the T-cell subset phenotypic data suggested that T cells from prenatal TCDD-treated mice might be functionally altered, select cytokines were examined to assess production homeostasis and the Th1/Th2 balance. Similar to effects on thymocytes, prenatal TCDD exposure differentially shifted the cytokine production by mitogen-activated T cells based on sex. IFN- γ and IL-12 levels from 5.0 $\mu\text{g}/\text{kg}$ TCDD females and 2.5 $\mu\text{g}/\text{kg}$ TCDD males were increased, as was IL-10 in the 5.0 $\mu\text{g}/\text{kg}$ TCDD males. These observations may suggest a skew towards Th1 activity in the prenatal TCDD exposed mice. In particular, optimal activation of naive CD4⁺ T cell requires two signals. The first signal is received through the T-cell receptor (TCR)–CD3 complex followed by a second co-stimulatory signal, which can be provided by antigen presenting cells (Lenschow et al. 1996). These signals result in the production of cytokines such as IL-2, which promote the differentiation of cells that secrete cytokines such as IFN- γ (Lenschow et al. 1996; Boehm et al. 1997). IL-12 thereby promotes Th1 activity via the production of IFN- γ .

The increase in IFN- γ caused by perinatal TCDD exposure may be crucial, since this cytokine has been linked to in a number of autoimmune conditions. In several animal models of autoimmunity, IFN- γ appears to play a vital role in the development of lupus nephritis (Haas et al. 1997), autoimmune insulinitis (Campbell et al. 1991), Sjogren's syndrome (Hayashi et al. 1996) and autoimmune arthritis (Billiau 1996). However, IL-10 has been considered the hallmark SLE disease marker. Serum levels of IL-10 are elevated in SLE patients and increased IL-10 correlates well with SLE disease activity (Hagiwara et al. 1996; Horwitz et al. 1998; Park et al. 1998). Thus, it is possible that 5.0 $\mu\text{g}/\text{kg}$ TCDD males exhibited predominant characteristics of a type III hypersensitivity lupus-like autoimmune disease whereas 5.0 $\mu\text{g}/\text{kg}$ TCDD females and 2.5 $\mu\text{g}/\text{kg}$ TCDD males skewed toward other type of autoimmune disease in addition to lupus-like autoimmune or using a different mechanism leading to autoimmunity.

Recent studies have shown that B cells appear to have a more diverse role in immune function than previously recognized (Martin & Chan 2006). The idea that B cells serve merely as passive producers of autoantibodies is not tenable as they appear to play a more pivotal role in autoimmunity (Brummel & Lenert 2005; Lenert et al. 2005; Blenman et al. 2006; Yan et al. 2006). This role appears to be through nonconventional mechanisms, including antigen presentation, cytokine production and modulation of other immune cells (Fujimoto & Sato 2007). Whereas the majority of studies assessing the immunotoxicity of TCDD have focused on T cells, several reports have shown that B cells may also be a target. The majority of these studies have focused on the impact of TCDD on the functionality of mature B

cells, with little work addressing the possible effects on development, function and maturation following prenatal TCDD exposure. The lack of studies investigating altered B cell function after early TCDD exposure is a recognized important gap in the literature. In one study, a single dose of TCDD produced a decrease in immature bone marrow-derived B cells in male C57BL/6 mice at 10 days post treatment (Thurmond & Gasiewicz 2000). Furthermore, this same laboratory showed that TCDD significantly increased pre-pro-B population in cultures with the S17 stromal cell line (Wyman et al. 2002). This increase in cell numbers expressing this phenotype (B220⁺/CD24⁻/CD43⁺) was seen in both wild-type and *AhR*^{-/-} primary bone marrow cell cultures. These results would suggest that the maturing B220⁺ B cell is not the direct target of TCDD-induced bone marrow B-cell alterations. However, this study did not investigate the effects of TCDD on more mature B cells because the cell line system did not support the differentiation of B cells to the stage of IgM expression.

Data from the present study show that one prenatal dose of 5.0 µg/kg TCDD resulted in a significant increase in earliest B progenitor (B220^{low}CD24⁻AA4.1⁺) and a significant decrease in bone marrow-derived lymphoid cells expressing B220. This increase in the earliest B progenitor (B220^{low}CD24⁻AA4.1⁺) is consistent with an observed increased pre-pro-B population in a S17 stromal cell line exposed to TCDD, *in vitro* (Wyman et al. 2002). Presently we have no clear explanation for this phenomenon. However, TCDD is known to modulate a broad range of genes which may also include genes that modulate B cells lymphopoiesis. The expression and activation of the B-cell receptor (BCR) during development determines both positive

and negative selection processes, as well as B cell trafficking from bone marrow. These processes are mediated and modulated by several signal-transduction pathways (Monroe et al. 2003) the proteins of which (e.g., nuclear factor- κ B, CD22 and CD19) may be affected by inappropriate activation of the cellular and stromal *AhR* (Masten & Shiverick 1995; Tian et al. 1999).

CD19, a B cell-specific protein tyrosine phosphatase, is thought to modulate BCR-induced responses (Fearon & Carter 1995; Smith & Fearon 2000). A major binding site for B cell-specific activator protein (BSAP or PAX5) is located in the promoter region of the gene encoding CD19 (Busslinger & Urbanek 1995). Previous data have indicated that the TCDD-activated *AhR* complex binds to the BSAP site on the CD19 gene producing a decreased CD19 mRNA expression in human B-lymphoblast cell lines (Masten & Shiverick 1995). CD19 enhances the BCR-mediated calcium response and the phosphorylation of Src-family kinases. Furthermore, a recent study reported impaired down-regulation of paired box 5 (Pax5), a repressor of B cell differentiation and concomitant suppression of the IgM response by an acute exposure of TCDD in the murine CH12.LX B cell line (Yoo et al. 2004). These authors demonstrated the involvement of altered Pax5 regulation in the suppression of the primary IgM antibody response by TCDD. Similarly, we also observed decreased IgM expression in the bone marrow of TCDD-treated 48 week-old mice. Thus, it is possible that TCDD may alter CD19 expression and subsequently modulate B cell selection processes. In addition, TCDD may directly elicit an arrest in cell cycle at developmental stage (Laiosa et al. 2002; Laiosa et al. 2003). Several reports indicate that the *AhR* may be involved in cell cycle regulation

(Ma & Whitlock 1996; Weiss et al. 1996; Dohr & Abel 1997; Puga et al. 2000; Huang & Elferink 2005).

Recently, one study showed that activation of the *AhR* by TCDD disrupts the circadian rhythm associated with C57BL/6 murine stem cell and hematopoietic precursors (Garrett & Gasiewicz 2006). Other studies have shown that TCDD induces the inhibition of thymic development through altered cell cycle regulation (Kolluri et al. 1999; Laiosa et al. 2002; Laiosa et al. 2003). A block in the continued maturation at the mature B-cell stage may also explain the subsequent changes to the more immature subpopulations. It is possible, for example, that feedback mechanisms, based on the numbers of cells in the mature subpopulation, regulate the numbers of precursors moving into the immature stage. Additional studies are needed to better elucidate the mechanisms in which TCDD dysregulates B lymphopoiesis.

In order to gain more insight into the effects of TCDD on B cell differentiation and maturation, we analyzed the B cell phenotypes in the spleen. In a previous study, prenatal TCDD exposure altered the maturation of splenic B cell precursors and enhanced shifting toward marginal zone (MZ) B cells (Mustafa et al. 2008). Interestingly, at 48 weeks, prenatal TCDD exposure continued to alter the maturation of splenic B cell precursors. During the transitional stage, autoreactive B cells that have not encountered self-antigen in the bone marrow are likely to be important targets for negative selection in the spleen (Sater et al. 1998; Sandel & Monroe 1999). Transitional T1 B cells are recent immigrants from the bone marrow and believed to be direct precursors of transitional T2 B cells (Loder et al. 1999).

Signaling through the B cell receptor initiates signal transduction events that direct the differentiation of transitional B cells into the mature marginal zone or follicular B subset or B1 cell subset (Lai et al. 1997; Martin & Kearney 2000). Interestingly, B cell-activating factor of the TNF family (BAFF) in transgenic (Tg) mice correlates with a lupus phenotype as well as decreased transitional T1 B cells and expansion of transitional T2 and marginal zone B cell compartments (Batten et al. 2000). This appears to be the result of impaired negative selection of autoreactive B cells leading to an increased number of marginal zone B cells in these mice (Batten et al. 2000). Studies have shown that marginal zone B cells in the spleen of NZB x NZW mice, a model of SLE, are the major source of autoreactive B cells and are known to contribute to the pathogenic autoantibody responses (Li et al. 2002; Li et al. 2002; Atencio et al. 2004; Lopes-Carvalho et al. 2005).

Data from the present study also suggest that prenatal TCDD induces a lower threshold stimulation of splenic B cells, characterized by increased production of autoantibodies to mouse dsDNA, ssDNA and cardiolipin. As such we hypothesized that mice exposed to TCDD *in utero* may be more prone to develop autoimmune disease due to inappropriate B cell activity characterized by autoantibody production. This effect may be secondary to alteration in B cell differentiation or to inappropriate T-cell signaling. Autoantibody anti-dsDNA, anti-ssDNA and anti-cardiolipin titers in the TCDD-treated 48-week-old mice showed increasing trends reaching significance in males 5.0 µg/kg TCDD for anti-cardiolipin. C57BL/6 mice spontaneously produce low but detectable levels of autoreactive antibodies (1gM and IgG but not IgA), to dsDNA and cardiolipin. The presence of elevated cardiolipin

antibody titers has been associated with an increased risk of thrombosis, spontaneous abortion, thrombocytopenia, and several other manifestations that have sometimes been referred to as the antiphospholipid antibody in SLE and related disorders (Harris et al. 1985; Thiagarajan et al. 1997). According to the literature, a cumulative dose of 10.5 $\mu\text{g}/\text{kg}$ perinatal TCDD did not diminish postnatal antibody-mediated immune responses to influenza virus in male mice. This may suggest that perinatal exposure might not suppress B cell antibody production (Vorderstrasse et al. 2006). Results from our study suggest that prenatal TCDD exposure exacerbated autoantibody formation in adult C57BL/6 mice in both genders. However, it has been difficult to make comparisons between the dose of TCDD given in our studies and those used in previous studies due to different exposure paradigms.

To summarize, results from this study clearly demonstrate that TCDD, administered during the sensitive time of prenatal immune system development, can cause persistent immunologic changes leading to increased signs of autoimmunity in the adult. Altered lymphocyte cell-surface marker expression in bone marrow and spleen, and increased peripheral expression of autoreactive T cells appear to be sensitive indicators of this TCDD-induced modulation. Together, these observations suggest that developmental exposure to TCDD may produce a fundamental disruption in establishment of central self-tolerance. Such dysregulation in central tolerance would be predicted to increase susceptibility to autoimmune disease. The magnitude of the autoimmunity risk may be influenced by binding capacity of TCDD to the susceptibility allele (*Ahr*), by genetic predisposition

to autoimmune disease, and by sex. These collective data suggest that individuals expressing the combined risk factors, who are exposed to TCDD during gestational and postnatal development, may be at increased risk of developing autoimmune disease.

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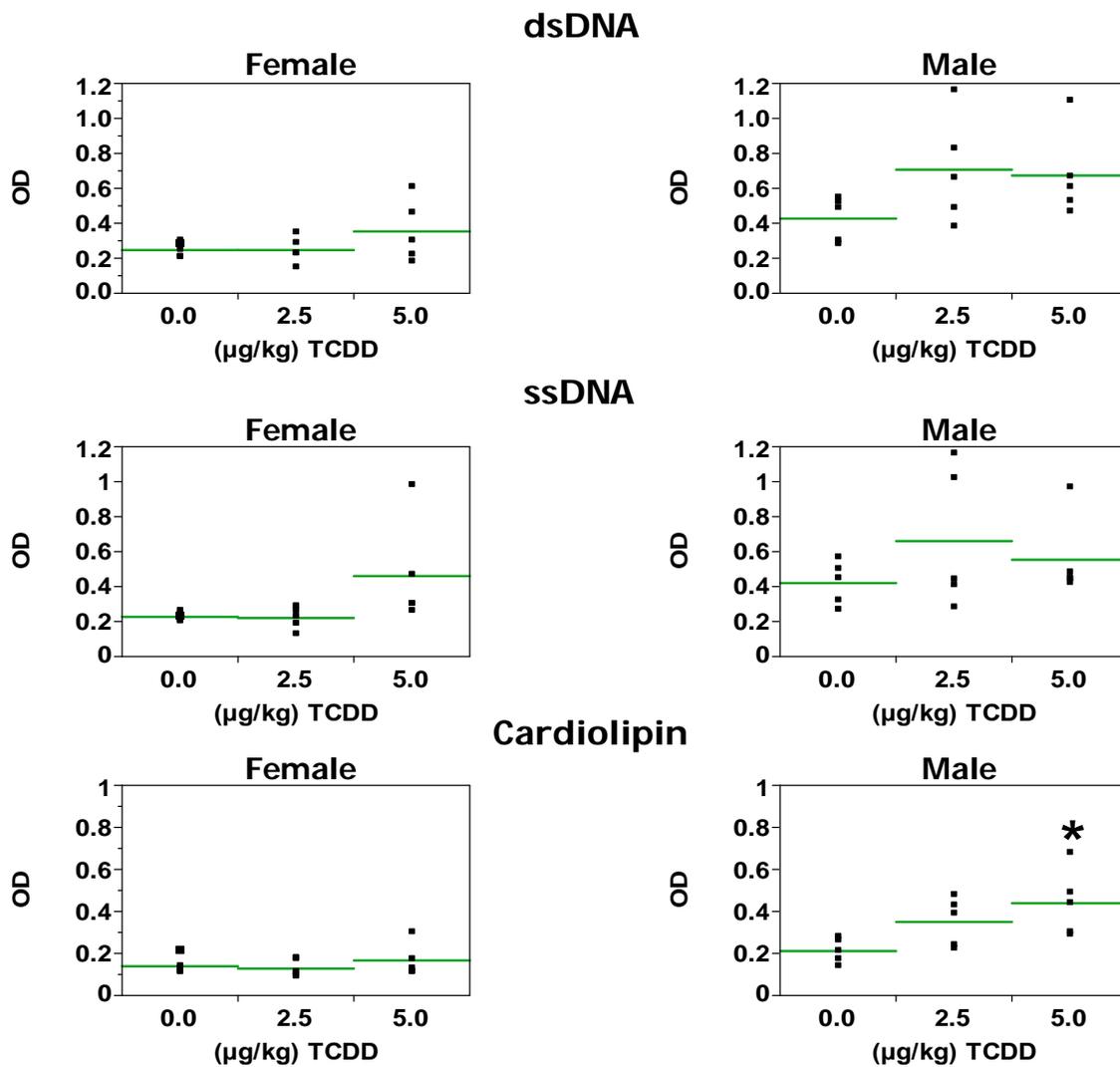
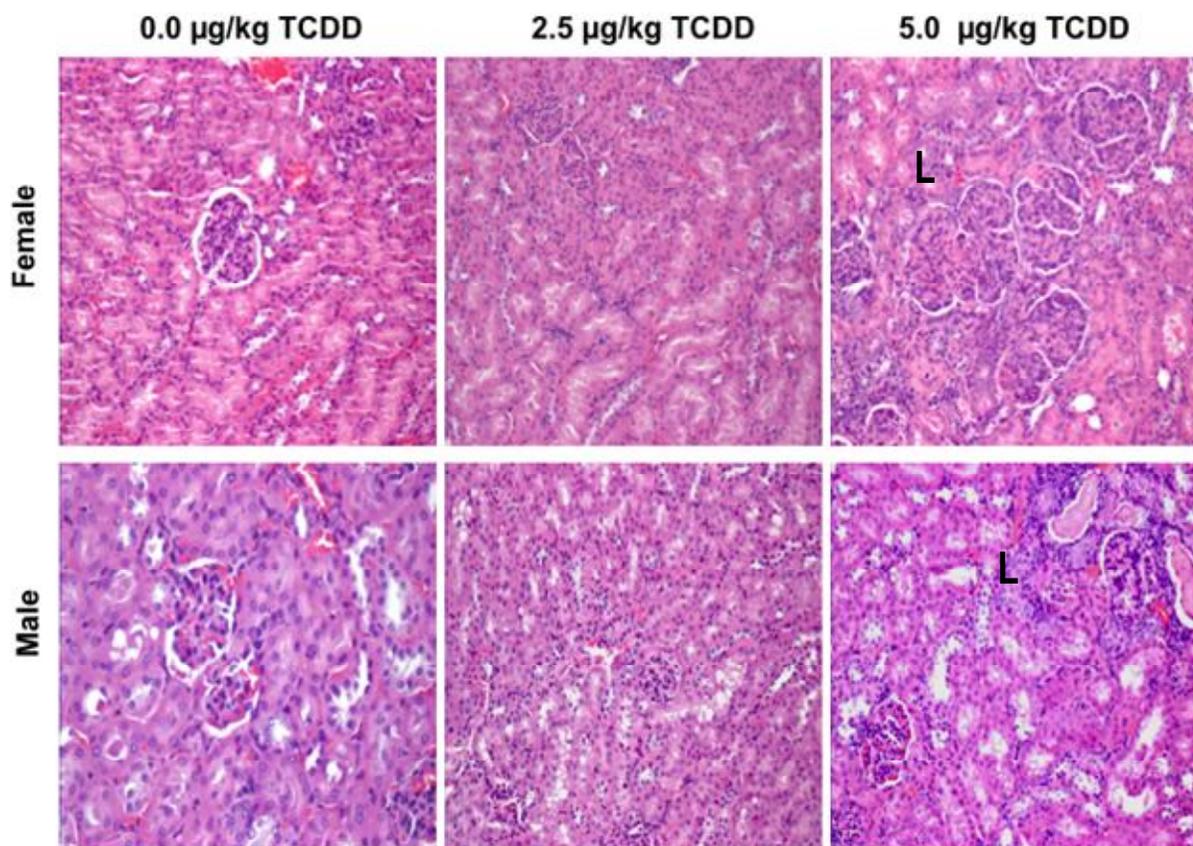


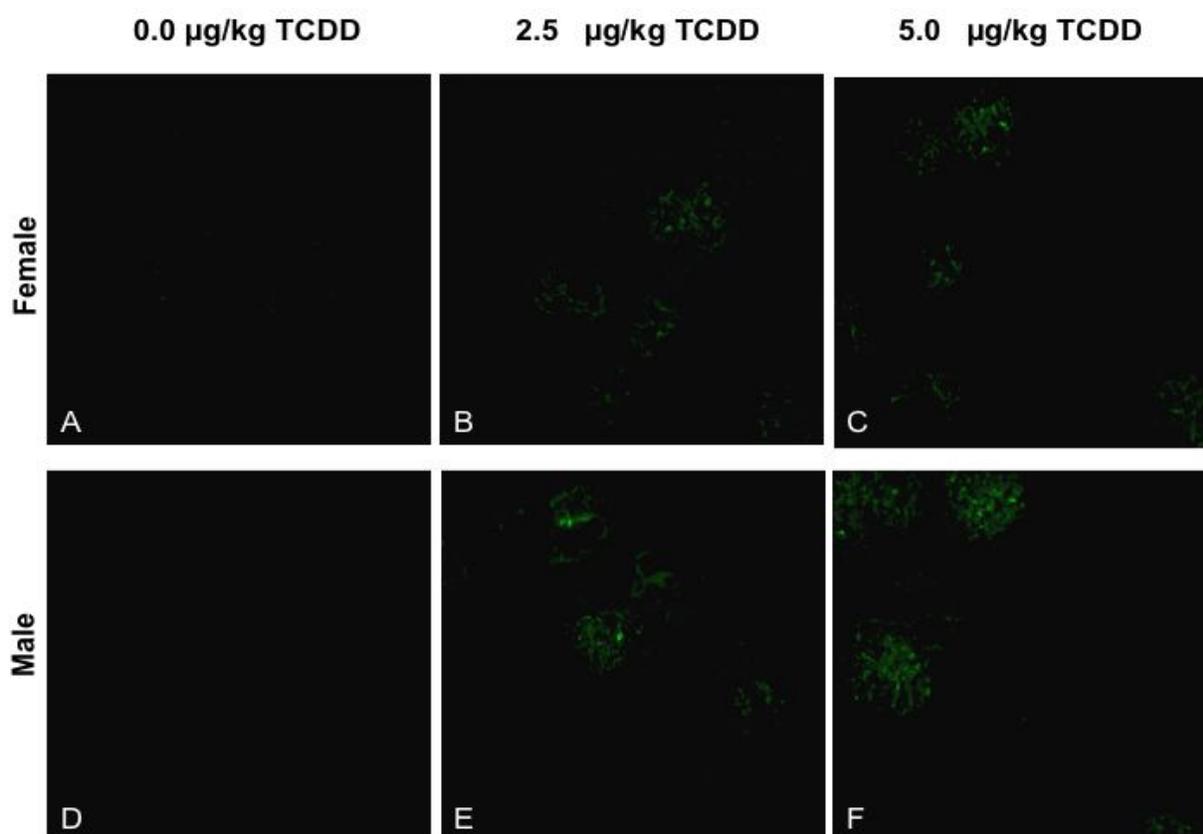
Figure 1. Sera from 48 week-old C57BL/6 mice that were prenatally exposed to 0.0, 2.5 and 5.0 µg/kg TCDD were analyzed for the presence of autoantibodies to dsDNA, ssDNA and cardiolipin, (n = 6/sex; * = $p < 0.05$, Dunnett's test). Each horizontal line represents the arithmetic mean for each treatment group.



RENAL HISTOPATHOLOGY		TCDD (µG/KG)					
		0.0		2.5		5.0	
	Gender	Mean	± SEM	Mean	± SEM	Mean	± SEM
Number of fibrinoid necrosis or crescents /100 glomeruli	F	0.3	0.21	0.5	0.22	1.2	0.30
	M	0.3	0.21	0.8	0.30	1.5	0.6
Number of inflammatory foci In the parenchyma	F	0.0	0.0	0.3	0.21	0.7	0.33
	M	0.17	0.17	0.3	0.21	1.2	0.40*

n = 6 mice/ treatment/ sex (* = $p < 0.05$, Dunnett's test)

Figure 2. The kidneys from 48-week-old SNF₁ mice that were prenatally exposed to 0, 2.5 or 5.0 µg/kg TCDD were collected, fixed, sectioned and stained with H&E. Images are representative of renal sections by treatment and sex. All sections were scored for number of fibrinoid necrosis cells or crescents/100 glomeruli and number of inflammatory foci in the parenchyma. The data are based on 6 mice/treatment/sex (**Bold *** = $p < 0.05$, Dunnett's test). L= lymphocyte infiltration.



RENAL IMMUNE COMPLEX DEPOSITION		0.0 µg/kg (Mean ± SEM)	2.5 µg/kg (Mean ± SEM)	5.0 µg/kg (Mean ± SEM)
Mean Kidney Score - IgG (Score)	<i>Female</i>	0.96 ± 0.23	1.42 ± 0.27	1.50 ± 0.40
	<i>Male</i>	1.21 ± 0.19	2.08 ± 0.20*	1.92 ± 0.08*
Mean Kidney Score - C3 (Score)	<i>Female</i>	0.75 ± 0.09	1.08 ± 0.25	0.83 ± 0.20
	<i>Male</i>	1.25 ± 0.17	1.50 ± 0.22	1.92 ± 0.08*

n = 6 mice/ treatment/ sex (* = p < 0.05, Dunnett's test)

Figure 3. The kidneys from 48 week-old C57BL/6 mice that were prenatally exposed to 0.0, 2.5 and 5.0 µg/kg TCDD were collected, fixed, section and stained with FITC-labeled anti-IgG. The above figures are representative of kidneys stained with FITC-anti-IgG from control female (A) and male (D) or 2.5 µg/kg TCDD-exposed female (B) and male (E) mice or 5.0 µg/kg TCDD-exposed female (C) and male (F) mice. The table data show the mean ± SEM of the IgG and C3 disposition scores of 6 mice/treatment/sex.

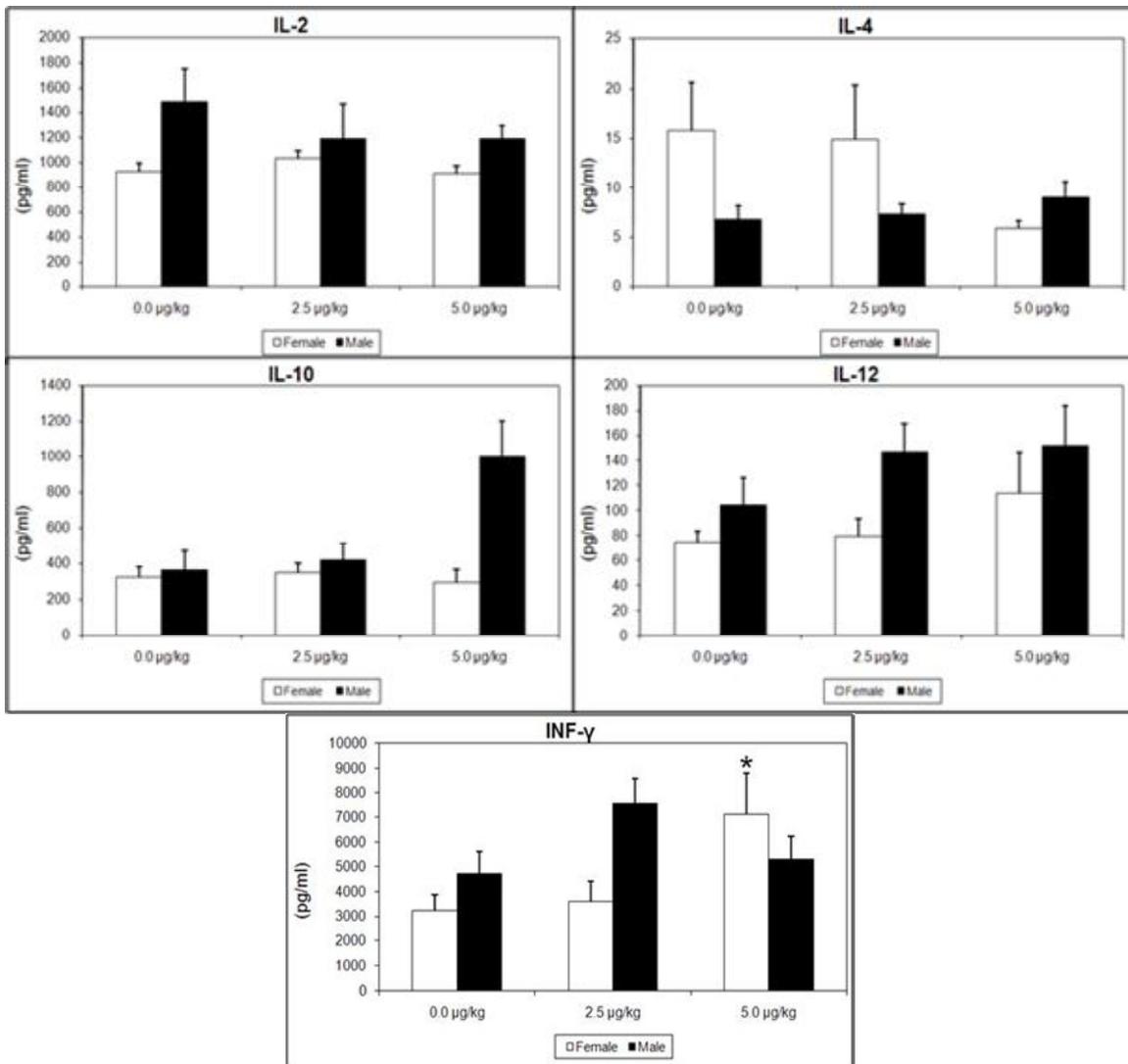


Figure 4. Supernatants were collected from splenocytes, of 48 week-old mice that were prenatally exposed to 0.0, 2.5 and 5.0 μg/kg TCDD, cultured for 48 hr with Con A (10 μg/mL). The levels of IL-2, IL-4, IL-10, IL-12 and INF-γ were determined using commercially available murine cytokine ELISA kits. The data are presented in a histogram analysis with mean bars (n = 6 mice/treatment/gender, * = $p < 0.05$, Dunnett's test).

Table 1. Body Weight, Organ Weight and Total Cellularity

		0.0 µg/kg (Mean±SEM)	2.5 µg/kg (Mean±SEM)	5.0 µg/kg (Mean±SEM)
Body Weight (g)	Female	30.20±0.63	30.70±1.80	27.32±0.72*
	Male	44.47±2.84	41.55±3.28	44.51±0.50
Spleen Weight (g)	Female	0.14±0.02	0.13±0.03	0.18±0.02
	Male	0.11±0.01	0.09±0.01	0.10±0.00
Spleen: Body Weight (% ratio)	Female	0.45±0.07	0.50±0.04	0.64±0.05
	Male	0.25±0.01	0.23±0.03	0.22±0.01
Cellularity: Spleen Cells (millions)	Female	57.71±4.81	54.39±9.22	76.24±3.35
	Male	89.88±5.38	82.11±5.45	91.04±4.72
Thymus Weight (g)	Female	0.06±0.00	0.06±0.00	0.05±0.00*
	Male	0.06±0.01	0.07±0.00	0.08±0.01
Thymus: Body Weight (% ratio)	Female	0.19±0.01	0.19±0.01	0.17±0.01
	Male	0.14±0.01	0.16±0.01	0.19±0.03
Cellularity: Thymus Cells (millions)	Female	76.15±9.66	67.17±6.97	49.20±6.09*
	Male	71.96±6.46	63.48±7.16	71.30±2.64
Cellularity: Bone Marrow Cells (millions)	Female	12.73±1.25	15.24±2.64	14.37±0.99
	Male	9.27±1.73	11.48±0.99	7.84±1.05
Cellularity: Lymph Node Cells (millions)	Female	5.04±0.49	3.26±0.59*	4.86±1.37
	Male	7.79±1.76	7.14±0.60	7.07±1.10

n = 6 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 2A. Bone Marrow Analysis

Bone Marrow		0.0 µg/kg (Mean ±SEM)	2.5 µg/kg (Mean ±SEM)	5.0 µg/kg (Mean ±SEM)
Total B220 (%)	Female	11.80 ±1.46	9.12 ±1.00	8.37 ±0.61
	Male	10.15 ±0.62	8.88 ±0.79	6.67 ±0.43*
B220 ^{low} of total B220 % (%)	Female	47.48 ±1.52	53.65 ±1.89*	58.20 ±2.37*
	Male	46.58 ±0.66	48.47 ±2.25	47.28 ±0.64
B220 ^{low} CD24 ^{pos} of B220 ^{low} % (%)	Female	67.83 ±4.36	66.38 ±3.13	69.73 ±2.09
	Male	71.88 ±2.84	61.72 ±3.27	55.45 ±3.62*
B220 ^{low} CD24 ^{hi} of B220 ^{low} CD24 ^{pos} % (%)	Female	26.67 ±2.50	27.62 ±3.24	33.03 ±2.35
	Male	32.80 ±3.74	24.37 ±1.69*	29.23 ±2.00
B220 ^{low} CD24 ^{low} of B220 ^{low} CD24 ^{pos} % (%)	Female	73.33 ±2.50	72.38 ±3.24	66.97 ±2.35
	Male	67.20 ±3.74	75.63 ±1.69*	70.77 ±2.00
B220 ^{low} CD24 ^{neg} of B220 ^{low} % (%)	Female	32.17 ±4.36	33.62 ±3.13	30.27 ±2.09
	Male	28.12 ±2.84	38.28 ±3.27	44.55 ±3.62*
B220 ^{low} CD24 ^{neg} AA4.1 ^{pos} of B220 ^{low} CD24 ^{neg} % (%)	Female	68.23 ±2.13	71.30 ±4.98	80.42 ±3.04
	Male	73.30 ±6.39	90.75 ±1.59*	91.73 ±1.59*
B220 ^{hi} of total B220 % (%)	Female	52.52 ±1.52	46.35 ±1.89*	41.80 ±2.37*
	Male	53.42 ±0.66	51.53 ±2.25	52.72 ±0.64
B220 ^{hi} CD24 ^{pos} of B220 ^{hi} % (%)	Female	90.92 ±1.38	92.58 ±0.82	93.47 ±0.45
	Male	70.65 ±1.59	61.22 ±4.41*	57.47 ±1.57*
B220 ^{hi} CD24 ^{hi} of B220 ^{hi} % (%)	Female	30.63 ±3.69	35.28 ±2.51	35.85 ±2.36
	Male	28.03 ±3.07	24.55 ±2.35	19.15 ±1.64*

n = 6 mice/treatment/sex, * = p < 0.05, Dunnett's test

Table 2B. Bone Marrow Analysis

Bone Marrow		0.0 µg/kg (Mean ±SEM)	2.5 µg/kg (Mean ±SEM)	5.0 µg/kg (Mean ±SEM)
B220 ^{hi} IgM ⁺ of B220 ^{hi} (%)	Female	86.47 ±1.59	87.38 ±1.38	86.87 ±0.98
	Male	91.58 ±0.52	92.50 ±0.62	91.77 ±0.47
BM-B220 ^{hi} IgM ⁻ of B220 ^{hi} (%)	Female	13.48 ±1.63	12.42 ±1.28	13.13 ±1.23
	Male	8.42 ±0.52	7.50 ±0.62	8.23 ±0.47
B220 ^{low} IgM ⁺ of B220 ^{low} (%)	Female	32.38 ±5.04	35.00 ±4.91	33.65 ±2.87
	Male	40.43 ±0.98	36.65 ±2.52	34.88 ±0.70*
B220 ^{low} IgM ⁻ of B220 ^{low} (%)	Female	67.62 ±5.04	65.00 ±4.91	66.35 ±2.87
	Male	59.57 ±0.98	63.35 ±2.52	65.12 ±0.70*

n = 6 mice/treatment/sex, * = p < 0.05, Dunnett's test

Table 3. Thymus T cell Differentiation

Thymus		0.0 µg/kg (Mean±SEM)	2.5 µg/kg (Mean±SEM)	5.0 µg/kg (Mean±SEM)
CD4+CD8 ⁻ (%)	Female	9.98 ± 1.03	14.20 ± 1.71	12.40 ± 1.31
	Male	14.15 ± 0.37	14.72 ± 0.50	16.80 ± 1.21
CD4+CD8 ⁺ (%)	Female	67.75 ± 1.55	63.33 ± 1.38	62.25 ± 2.01
	Male	65.00 ± 1.09	64.72 ± 1.29	58.82 ± 1.43*
CD4 ⁻ CD8 ⁻ (%)	Female	17.13 ± 0.64	18.57 ± 1.19	21.77 ± 1.22*
	Male	13.17 ± 0.81	12.18 ± 0.79	15.02 ± 1.16
CD4 ⁻ CD8 ⁺ (%)	Female	5.10 ± 1.02	3.92 ± 0.27	3.60 ± 0.18
	Male	7.68 ± 0.30	8.40 ± 0.59	9.38 ± 0.73

n = 6 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 4. Splenic B cell Subset Analysis

Spleen		0.0 µg/kg (Mean ±SEM)	2.5 µg/kg (Mean ±SEM)	5.0 µg/kg (Mean ±SEM)
Total B220 ^{pos} (%)	Female	35.38 ± 1.49	36.53 ± 3.11	33.83 ± 2.12
	Male	27.32 ± 0.78	24.97 ± 1.42	25.92 ± 1.49
B220 ^{pos} CD24 ^{hi} of B220 ^{pos} (%)	Female	21.08 ± 2.31	23.22 ± 2.82	20.13 ± 1.81
	Male	25.13 ± 2.04	29.60 ± 2.37	20.45 ± 1.34
B220 ^{pos} CD24 ^{low-int} of B220 ^{pos} (%)	Female	78.90 ± 2.31	76.77 ± 2.83	79.85 ± 1.81
	Male	74.87 ± 2.04	70.38 ± 2.37	79.53 ± 1.34
CD19 ^{pos} (%)	Female	42.02 ± 4.49	42.65 ± 3.94	36.93 ± 4.09
	Male	40.92 ± 2.12	38.37 ± 1.76	37.63 ± 0.92
CD19 ^{pos} CD5 ^{pos} of CD19 ^{pos} (%)	Female	5.73 ± 2.35	9.00 ± 1.93	4.70 ± 1.49
	Male	6.62 ± 0.59	7.63 ± 0.72	7.42 ± 0.77
CD138 ^{pos}	Female	8.95 ± 1.05	11.27 ± 1.75	12.67 ± 1.06
	Male	3.80 ± 1.77	5.68 ± 1.97	13.03 ± 3.50*

n = 6 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 5. Splenic B cell Subset Analysis

Spleen		0.0 µg/kg (Mean ±SEM)	2.5 µg/kg (Mean ±SEM)	5.0 µg/kg (Mean ±SEM)
CD23 ^{hi} CD1 ^{hi} (%)	Female	8.22 ±0.81	8.68 ±1.86	5.03 ±0.56
	Male	10.58 ±0.64	10.13 ±0.63	9.47 ±1.04
CD23 ^{pos} CD1 ^{low-int} (%)	Female	58.12 ±3.34	59.75 ±2.15	52.00 ±3.31
	Male	60.02 ±2.81	58.30 ±1.68	60.90 ±1.45
CD23 ^{neg-low} CD1 ^{hi} (%)	Female	8.80 ±0.99	7.83 ±1.18	8.58 ±1.27
	Male	5.22 ±0.54	6.07 ±0.61	5.78 ±0.38
CD23 ^{neg} CD1 ^{neg} (%)	Female	23.50 ±2.27	22.05 ±2.56	33.57 ±3.09*
	Male	25.78 ±1.79	27.22 ±1.00	25.63 ±1.22
CD21 ^{int} CD24 ^{low-int} (%)	Female	60.67 ±2.87	64.74 ±0.47	66.78 ±3.57
	Male	60.78 ±1.47	57.97 ±2.95	58.58 ±1.33
CD21 ^{hi} CD24 ^{int} (%)	Female	13.57 ±0.83	11.94 ±0.92	11.28 ±1.28
	Male	11.80 ±0.79	11.58 ±0.61	10.93 ±0.53
CD21 ^{neg} CD24 ^{hi} (%)	Female	12.40 ±1.02	13.12 ±1.08	11.70 ±1.61
	Male	16.23 ±0.97	17.03 ±1.34	14.43 ±0.43
CD21 ^{hi} CD24 ^{hi} (%)	Female	13.02 ±1.47	9.92 ±0.41	9.85 ±1.87
	Male	10.72 ±1.39	13.03 ±1.49	15.30 ±0.95*
B220 ^{pos} IgD ^{hi} (%)	Female	69.25 ±2.44	62.78 ±3.24	60.15 ±1.36*
	Male	58.53 ±0.89	58.63 ±1.48	53.20 ±1.34*
B220 ^{pos} IgD ^{Neg-low} (%)	Female	30.75 ±2.44	37.22 ±3.24	39.85 ±1.36*
	Male	41.47 ±0.89	41.37 ±1.48	46.80 ±1.34*

n = 6 mice/treatment/sex, * = p < 0.05, Dunnett's test

Table 6. Splenic T cell Subset Analysis

Spleen		0.0 µg/kg (Mean ±SEM)	2.5 µg/kg (Mean ±SEM)	5.0 µg/kg (Mean ±SEM)
CD4+CD8- (%)	Female	16.92 ±1.48	17.70 ±1.47	16.85 ±1.98
	Male	29.12 ±0.99	31.53 ±1.49	31.27 ±1.24
CD4-CD8- (%)	Female	75.10 ±1.91	75.12 ±2.20	76.67 ±2.58
	Male	61.23 ±1.49	57.87 ±1.74	59.22 ±1.40
CD4-CD8+ (%)	Female	7.67 ±0.70	6.80 ±1.00	6.12 ±0.84
	Male	12.37 ±0.79	12.92 ±0.51	11.02 ±0.56

n = 6 mice/treatment/sex, * = p < 0.05, Dunnett's test

Table 7. Lymph Node T cell Subset Analysis

Lymph Node		0.0 µg/kg (Mean±SEM)	2.5 µg/kg (Mean±SEM)	5.0 µg/kg (Mean±SEM)
CD4+CD8- (%)	Female	27.68±3.11	27.32±1.22	24.40±1.93
	Male	28.42±1.07	32.02±2.33	37.67±1.06*
CD4-CD8- (%)	Female	51.40±6.35	58.28±2.51	63.94±2.16
	Male	43.18±2.61	39.98±2.91	39.72±1.30
CD8+CD4- (%)	Female	20.10±3.39	13.70±1.45	11.08±0.86*
	Male	24.43±2.00	24.55±1.45	19.30±1.03
CD4+Vβ17 TCR+(% of CD4+ (%)	Female	3.55±0.28	4.93±1.02	5.35±0.54
	Male	6.05±0.28	7.25±0.90	8.73±0.84*
CD3+Vβ3 TCR+ (%) of CD3+ (%)	Female	5.23±0.41	6.35±1.04	8.08±1.03
	Male	5.07±0.40	6.35±1.04	8.42±1.23*
CD4+CD25+ (%) Of CD4+ (%)	Female	8.66±0.97	6.82±0.96	6.14±0.41
	Male	10.18±1.18	12.97±1.96	7.22±1.78

n = 6 mice/treatment/sex, * = p < 0.05, Dunnett's test

Table 8. Lymphoproliferative Responses of Cultured Splenocytes

Proliferation		0.0 µg/kg (Mean ± SEM)	2.5 µg/kg (Mean ± SEM)	5.0 µg/kg (Mean ± SEM)
Spontaneous Proliferation (48hr) Specific Absorbance	Female	0.62±0.04	0.67±0.01	0.68±0.07
	Male	0.65±0.04	0.62±0.03	0.59±0.03
ConA @ 10.0 µg/ml (48hr) Δ Specific Absorbance	Female	0.26±0.05	0.19±0.01	0.23±0.04
	Male	0.38±0.05	0.44±0.06	0.45±0.02
LPS @ 10.0 µg/ml (48hr) Δ Specific Absorbance	Female	0.12±0.02	0.09±0.02	0.14±0.03
	Male	0.14±0.02	0.16±0.02	0.14±0.02
P/I @ 20.0/400 µg/ml (48hr) Δ Specific Absorbance	Female	0.11±0.04	0.13±0.03	0.21±0.04
	Male	0.16±0.04	0.21±0.03	0.23±0.02
Spontaneous Proliferation (72hr) Specific Absorbance	Female	1.03±0.04	1.12±0.01	1.12±0.04
	Male	1.05±0.02	1.03±0.02	0.94±0.02*
ConA @ 10.0 µg/ml (72hr) Δ Specific Absorbance	Female	0.18±0.03	0.09±0.01	0.14±0.04
	Male	0.25±0.03	0.28±0.04	0.27±0.03
LPS @ 10.0 µg/ml (72hr) Δ Specific Absorbance	Female	0.14±0.03	0.06±0.02	0.12±0.04
	Male	0.16±0.02	0.18±0.02	0.19±0.02
P/I @ 20.0/400 µg/ml (72hr) Δ Specific Absorbance	Female	0.11±0.03	0.08±0.02	0.14±0.03
	Male	0.13±0.03	0.15±0.03	0.22±0.01*

n = 6 mice/treatment/sex, * = p < 0.05, Dunnett's test

CHAPTER 5. PRENATAL TCDD EXPOSURE AND POSTNATAL IMMUNOMODULATION IN A 36-WEEK-OLD LUPUS-NEPHRITIS STRAIN

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Running title: Developmental exposure to TCDD in 36 week-old SNF₁ Mice

Keywords: TCDD, prenatal exposure, autoimmunity, mouse

Abstract

Generally, it is recognized that the developmental stage is the most sensitive period for exposure to environmental contaminants, like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Further, the adverse effects associated with this prenatal TCDD exposure can affect multiple sites (i.e. immune system, endocrine system, integument) in the body and persist into adulthood. Previous studies in our laboratory have shown that an acute prenatal exposure altered adaptive immunity differently across sex in a lupus –nephritis strain, SWR X NZB F₁ (SNF₁), exacerbating the disease in the females and accelerating it in the males. The extent to which prenatal TCDD exposure can affect immunity and immune-mediated renal pathology during aging is unknown. In this study, 36-week-old SNF₁ mice prenatally-exposed to TCDD (0, 40 or 80 µg/kg) at gestational day 12 were evaluated for changes in T cell and B cell maturation and function as well as the progression of immune-mediated renal disease. TCDD-treated males displayed decreased thymic weight while females displayed altered CD4/CD8 thymocyte surface expression compared to controls. TCDD-treated offspring contained increased percentages of autoreactive CD4⁺Vβ17⁺ TCR spleen T cells. Alterations in bone marrow B cell lymphopoiesis were seen in both sexes. Splenic B transitional-1 cells (T1) (CD23^{neg} CD1^{neg}) were increased in treated females while B marginal zone (MZ) cells (CD24^{int}CD21^{hi}) (CD23^{neg} CD1^{hi}) were increased in treated males. Anti-IgG and anti-C3 immune complex renal deposition showed trends toward enhancement in TCDD-treated mice in both genders, reaching significant for anti-C3 in females. Together, these findings support and extend our previous work in showing that

prenatal TCDD exposure in an autoimmune-prone strain permanently alters the adult immune system. Additionally, these immune signatures or profiles differ significantly across sex resulting in different manifestation of immune-mediated nephritis.

Introduction

The chemical 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) is an environmental contaminant, classified as the most potent of toxic polyhalogenated aromatic hydrocarbons (PHAHs), with a toxicity equivalency factor (TEF) of 1.0. This potent toxicant has continued to attract a great deal of attention because of its persistence as a global environmental toxicant and its ability to bio-accumulate within the food chain. Prenatal TCDD exposure in human infants is a major concern because dioxins can directly affect the developing fetus, as demonstrated by their detection in placenta and cord blood (Wang et al. 2004). In laboratory animals, TCDD has been shown to cause histological and physiological alterations in the placenta, leading to fetal death (Ishimura et al. 2002; Kawakami et al. 2006). Developmentally, TCDD can induce teratogenic and developmental defects in many tissues and body organs including palate, urinary system, neural system, reproductive system, immune system and endocrine system (Vos et al. 1974; Birnbaum et al. 1985; Fine et al. 1989; Couture-Haws et al. 1991; Theobald & Peterson 1997; Collins et al. 2008).

A majority of the animal studies involving the immune system report a high sensitivity to prenatal TCDD during the developmental stage resulting in persistent immune changes into adulthood (Gehrs et al. 1997; Gehrs & Smialowicz 1999). These immune changes tend to involve both arms of adaptive immunity toward suppression (Faith & Moore 1977; Thomas & Hinsdill 1979; Gehrs & Smialowicz 1997; Gehrs & Smialowicz 1999). This infers that such impairment in the developing immune system could alter an individual's susceptibility to infections, cancer, and autoimmunity during aging (Vos et al. 1974; Faith & Moore 1977;

Luster et al. 1980). Additionally, studies have shown that prenatal TCDD exposure results in thymic atrophy and altered thymocyte differentiation/maturation as indicated by changes in proportion of thymocyte subpopulations in both mice and rats (Faith & Moore 1977; Holladay et al. 1991; Blaylock et al. 1992; Gehrs et al. 1997; Gehrs & Smialowicz 1999; Camacho et al. 2004). This effect has been reported to persist into late adulthood and is accompanied by depressed T cell responses (Gehrs et al. 1997; Gehrs & Smialowicz 1999). Overall, these prenatal TCDD effects appear to drive suppression of cell-mediated immunity (Vos and Moore, 1974; Faith and Moore, 1977; Luster et al., 1980b) manifested by decreased T-cell mitogen responses (Luster et al. 1979), skin graft rejection times, graft-versus-host reactivity (Vos et al. 1974), delayed hypersensitivity (Faith & Moore 1977; Blaylock et al. 1992; Gehrs et al. 1997; Gehrs & Smialowicz 1997), and cytotoxic T-lymphocyte responsiveness (Holladay et al. 1991).

Recently, a few studies suggest that the consequences of developmental exposure to TCDD may not be limited to immunosuppression, but rather also may manifest as immune hyperactivity. For example, offspring lactationally exposed to TCDD showed higher levels of serum interferon gamma (IFN- γ) and higher tumor necrosis factor alpha (TNF- α) following infection with *Listeria monocytogenes* (Sugita-Konishi et al. 2003). Additionally, offspring from TCDD-treated dams exhibited an increased number of neutrophils and higher IFN- γ levels in their lungs after infection with influenza virus (Lawrence & Vorderstrasse 2004; Vorderstrasse et al. 2006). These observations suggest that prenatal TCDD exposure not only adversely influences adaptive immune responses, but also affects innate immune system

function. Thus, TCDD-induced immunomodulation can possibly enhance responsiveness to self-antigen and nonpathogenic antigens, as is the case of autoimmune disease and hypersensitivity reactions, respectively (Mustafa et al. 2008).

The lack of studies investigating altered B cell function after early TCDD exposure has been identified as an important data gap worthy of further investigation (Luster et al., 2003; Holsapple et al., 2005). Traditionally, immunotoxicity studies examining B cell function have primarily focused on antibody production. Recent adult mouse studies suggest B cells may play a more important role in autoimmune responses than previously perceived, including through mechanisms other than autoantibody production (Martin and Chan 2006). For example, B cells can function as potent antigen presenting cells, cytokine producers and modulators for other immune cells. These multiple functional roles allow B-cells to orchestrate or stage inflammatory responses such as regulating T cell and dendritic cell (DC) expansion and activation through the production of cytokines or regulatory antibodies (Fujimoto and Sato 2007).

The toxicity of TCDD is mediated through the aryl hydrocarbon receptor (*AhR*) (Poland & Knutson 1982; Safe 1995; Hogaboam et al. 2008). Interestingly, *AhR* gene expression is heterogenous across mammalian tissues. For example in the immune system, immune cells can differentially express *AhR* across closely related cell subsets within a single organ such as thymus (Ackermann et al. 1989; Lawrence et al. 1996; Williams et al. 1996; Lang et al. 1998; Yamamoto et al. 2004; Majora et al. 2005; Frericks et al. 2006). Dioxin-dependent gene regulation also shows a high

degree of cell specificity in response to *AhR* activation (Majora et al. 2005; Frericks et al. 2006). The polymorphisms that govern the loci for *AhR* and its nuclear transport protein, Arnt, also govern the affinity and the efficacy necessary for ligand binding that reflects the polymorphisms in dioxin's ability to elicit toxicity. As a result, inbred mouse strains can differ both quantitatively and qualitatively in their responsiveness to TCDD. For example, a high *AhR* affinity C57BL/6 mouse is approximately 8-10x more sensitive to TCDD than a low affinity *AhR* mouse such as DBA/2 or SNF₁ (Knutson & Poland 1982; Nohara et al. 2006; Mustafa et al. 2008). Thus, *Ah^{bb}* C57BL/6 mice represent the prototypic responsive strain and are the most sensitive to TCDD toxicity, whereas *Ah^{dd}* SNF₁ mice are a nonresponsive strain and require higher doses of TCDD to produce the same toxic effect.

SNF₁ progeny derived from crossing autoimmune NZB with normal SWR mice uniformly develop lethal immune complex glomerulonephritis by about 6-8 months of age, showing similar pathology to humans with systemic lupus erythematosus (SLE) (Eastcott et al. 1983). The SNF₁ females die of severe nephritis usually by age 12 months. However, SNF₁ males develop similar renal lesions and die 4 to 6 months later than females (Eastcott et al. 1983; Mohan et al. 1995). In many lupus mouse models, genetic differences among the strains can influence the susceptibility and severity of the autoimmune disease. Spontaneous SLE in either mice or humans does not appear to be linked to a single gene mutation or aberrant gene expression but rather to a complex of genetic interactions regulating spontaneous SLE (Bagavant & Fu 2005).

We recently observed an exaggerated autoimmune profile accompanied by altered B lymphopoiesis and B cell splenic subpopulations in 24-week-old SNF₁ mice following a single prenatal dose of TCDD (unpublished data). These findings suggested that prenatal TCDD exposure altered differentiation and activation of B cells, skewing the naïve B cells toward the follicular B cells in the spleen and leading to heightened autoreactivity. Further, these mice exhibited persistent humoral immune dysregulation as well as altered cell-mediated responses. Although a sex effect in the immune response was apparent, both sexes shared an enhanced autoimmune disease profile including increased antibody titers to dsDNA, ssDNA and cardiolipin and increased autoantibody polyreactive responses to DNA and glomerular substrate dominating the deposits in kidneys. In the current study, we examined the progression of immune changes and renal pathology following prenatal TCDD exposure in the littermates of the 24-week-old mice, to determine persistence and advancement of the immune lesions. Our hypothesis was that immune-mediated renal disease would progress, however, the immune mechanisms driving this disease in these mice would vary based on sex. We investigated an expanded panel of immune parameters, including primary and secondary lymphoid organs of both the cell-mediated and antibody-mediated pathways. Bone marrow B lymphopoiesis as well as renal pathology were also examined in these immune-prone low affinity AhR, developmentally (*in utero* and *via* lactation) TCDD-exposed SNF₁ mice.

Methods and Materials

Mice and TCDD exposure. SWR x NZB (SNF₁) mice were obtained by breeding male NZB mice with female SWR/J mice, both from Jackson Laboratories (Bar Harbor, ME). Mice (4-5 weeks-of-age) were acclimated to the animal care facility for at least 2 weeks prior to breeding. Briefly, 60 SWR females were bred to 30 NZB males overnight in cages containing one NZB male per two SWR females. Plug positive mice, evaluated the next morning, were designated gestation day (GD) 0. Pregnant SWR mice were orally gavaged on GD 12 with 0, 40 or 80 µg/kg TCDD dissolved in corn oil (N= 10 pregnant mice/treatment). The SNF₁ offspring were weaned at 20-21 days and separated by treatment, allowed to mature to 36 weeks-of-age, and evaluated for changes in immune status. All animals were fed a commercial pelleted diet, provided water *ad libitum*, and housed under controlled conditions of temperature (22 °C), humidity (40–60 %), and lighting (12:12 light:dark cycle). Animal maintenance, care and use were approved prior to initiation of experiments and at all times were in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines at Virginia Tech.

Body weights and tissue collection. Mice at 36 weeks-of-age were euthanized by cervical dislocation and weighed. By 36 weeks-of-ages, the untreated SNF₁ females will be in middle stages of lupus nephritis and males the early stages of lupus nephritis (Eastcott et al. 1983). The thymus, spleen, bone marrow, axillary and inguinal lymph nodes were immediately collected post-euthanasia under aseptic conditions, using dissection scissors and curved forceps. The spleen and thymus

were weighed and then all tissues placed individually into pre-labeled sterile Petri dishes (Corning, Corning, NY), containing 8 mL of RPMI-1640 culture medium (Mediatech, Herndon,VA). Dishes were placed on ice until tissue dissociation.

Cell dissociation and enrichment. Each organ was gently dissociated over a stainless steel sieve screen (Sigma, St. Louis, MO) using curved forceps. Cells were then pipetted through a sieve screen following dissociation to remove debris. Cells were washed in RPMI-1640 for 10 min, 240 x g, and 23 °C. The supernatant was discarded and, with the exception of spleen, the cell pellet was resuspended in 8 mL of RPMI-1640. Spleen cells were resuspended in 1 mL incomplete RPMI-1640. To each tube, 2 mL of 0.83 % ammonium chloride lysis buffer (ACK, pH 7.29) were added, to lyse red blood cells, and tubes were incubated for 5 min at 23 °C. After lysis incubation, the cells were resuspended in 5 mL of incomplete RPMI-1640 and washed twice (7 min, 290 x g and 7 °C). The splenic leukocyte-rich cells were then resuspended in 5 mL complete RPMI-1640 media containing 10 % heat-inactivated FBS (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/mL penicillin (ICN), and 50 mg/mL of streptomycin (ICN), and maintained at 7-10 °C. For bone marrow isolation, femurs were removed and the bone marrow cavities flushed with 2% FBS-PBS, washed once (7 min, 290 x g and 7 °C), resuspended in 1 mL incomplete RPMI-1640 media and stored at 4 °C.

Cell enumeration. Cells were enumerated and size-analyzed using a Beckman Multisizer 3[®] Coulter cell counter (Beckman Coulter, Fullerton, CA) according to the

manufacturer's protocol. Briefly, a 10 μ L aliquot of enriched cell suspension was transferred to a plastic counting-chamber containing 10 mL of PBS (Mediatech). The plastic chamber was capped, mixed by repeated gentle inversion, and counted. The cells were enumerated and adjusted to 5.0×10^6 cells/mL in complete media.

Flow cytometric evaluation of cell-surface markers. Cell suspensions ($5 \times 10^5/100 \mu$ L) from the thymus, spleen, lymph node and bone marrow were dispensed into individual wells of a 96-well round-bottom tissue culture plate (Corning). Monoclonal antibodies (mAbs) with phycoerythrin (PE) fluorescent labels were used according to manufacturer's (BD Pharmingen; San Diego, CA) recommendation at a concentration of 0.2μ g/ μ L; mAbs with fluorescein isothiocyanate (FITC) fluorescent labels were similarly used at the recommended concentration of 0.5μ g/ μ L. Cells were stained as previously described (Gogal et al. 2001; Klein et al. 2006). Briefly, lymphocyte aliquots (5×10^5 cells/ 100μ L) from thymus, spleen, lymph node and bone marrow were incubated with the following primary mAbs: PE-anti CD4, FITC-anti CD8, FITC anti- $V\beta$ 8.2, 8.3 TCR, FITC-anti CD19, FITC-anti IgM, PE-anti CD5, FITC-anti CD25, PE-anti CD45/B220, PE-Cy5 anti CD45/B220 (Ebioscience, San Diego, CA); FITC-anti $V\beta$ 3 TCR (KJ25), FITC-anti $V\beta$ 17^a TCR (KJ23), FITC-anti CD45/B220, PE-anti CD24 (HSA), FITC-anti CD1, PE-anti CD23 (BD Pharmingen). For double or triple color staining protocols, mAbs with different fluorescent labels were simultaneously added to the sample. For bone marrow analysis, aliquots of 5×10^5 cells were pre-blocked with anti-Fc γ III/IIR (clone 2.4G2, Rat IgG2_b). Following staining, cells were washed and evaluated on a

Coulter Epics XL flow cytometer (Beckman Coulter). From each sample, 10000 events were collected and analyzed using the FlowJo software (Tree Star, San Carlos, CA). Dead cells, clumps, and debris were excluded electronically by gating on forward scatter (FSC) versus side scatter (SSC). Distribution and gating analysis of B cell subsets was as previously described (Grimaldi et al. 2001).

ELISA for autoantibodies to double-stranded DNA, single-stranded DNA and cardiolipin. To detect the presence of dsDNA antibody, 96-well medium-binding microtiter plates (Corning) were coated with heat-denatured calf thymus DNA (100 µg/mL; Sigma). For ssDNA antibody titers, DNA from calf thymus was used (10 µg/mL; Sigma), and for autoantibodies to cardiolipin a solution from bovine heart was used (10 µg/mL; Sigma). All pre-coated plates were incubated at 4 °C overnight. The plates were washed thrice with 300 µL PBS/0.05% Tween 20 (Mediatech), blocked with 1% BSA (Sigma) for 2 h at 23 °C, washed and then incubated with diluted serum samples to be tested (1/100). For all three ELISA assays, serial dilutions of each mouse serum were performed to optimize optical density readings. Serum diluted 1/100 was determined to be the optimal dilution to attain a wide spectrum in the optical density among the samples. After 3 h at 23 °C, the serum-coated plates were washed thrice with 300 µL of PBS-0.05% Tween 20. To each well, 0.2 mL of diluted alkaline phosphatase-conjugated anti-IgG antibody (1/3000) (Sigma) were added and the plates were incubated for 60 min at 23 °C. The plates were then washed thrice with 300 µL of PBS-0.05 % Tween 20. To each well, 0.2 mL prepared substrate for alkaline phosphatase conjugated secondary

antibody (SIGMA FAST™ p-Nitrophenyl phosphate tablets) were added and allowed to develop for 45 min at 23 °C before adding 50 µL of 3 M NaOH stop solution (Sigma). The absorbance (A_{405}) of the initial dilution was measured. Optical density (OD) readings represent the average from sera from each mouse performed in duplicate.

Histology of the kidneys. Kidneys were collected at the time of euthanasia, sectioned for histopathology and immunohistochemistry. The histopathology section was immediately fixed in 10 % formalin (Fisher Scientific, Pittsburgh, PA) for 48 h and the immunohistochemistry section was embedded in OCT media (Miles, Elkhart, IN) and frozen at -80 °C for cryosections. After 48 h in formalin, the tissues were removed, routinely processed and embedded in a paraffin block. Following embedding, a 5 µm section was cut from each tissue block, and stained with hematoxylin and eosin (H&E, Richard-Allen Scientific, Kalamazoo, MI) using standard histologic methods. The prepared slides were then evaluated, with a light microscope, in a blinded-manner by a veterinary pathologist (co-author KZ). For each kidney, 100 consecutive renal cortex glomeruli were evaluated. Each glomerulus was scored for the presence of fibrinoid necrosis or crescents, extent of lymphocytic infiltration, glomerular sclerosis and proliferative changes in mesangial and/or peripheral regions. The perivascular scores were evaluated at 40x. Each of the above-mentioned changes was scored from 0 to 4, with 0 indicating minimal pathologic changes, and 4 representing most dramatic pathologic changes.

Immunohistochemistry of the kidneys: C3 and IgG deposition. Frozen kidneys were cut into 5 μm sections and stained with FITC conjugated antibodies (Bagavant et al. 2002; Xie et al. 2003; Mustafa et al. 2008). Briefly, tissue sections were thawed at room temperature and air-dried ($23\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) for 30 min. Slides were fixed in acetone for 10 min and then washed with PBS thrice for 3 min/wash. Goat anti-mouse IgG diluted 1:100 (MP Biomedicals, Santa Ana, CA) or goat anti-mouse C3 diluted 1:100 (MP Biomedicals) were incubated with tissues sections in a humid chamber for 60 min at 23°C . The sections were then rinsed thrice for 5 min/wash with PBS. The slides were mounted using Vectashield™ mounting media (Vector Labs, Burlingame, CA) and then examined using an Olympus BX-60 fluorescence microscope (Center Valley, PA). The severity of glomerulonephritis and immune complex deposition was scored using a range from 0 to 3+, where 0 corresponded to a non-autoimmune healthy mouse and 3+ to the maximal alteration observed in the study. All slides were scored in a blinded manner independently by two experienced investigators (co-authors CR and RG). Scores were averaged for the final tissue score.

Lymphocyte proliferation assay. Splenocytes were plated into each well (5×10^5 cells/ $100\text{ }\mu\text{L}$ per well) of a 96-well round-bottom tissue-culture plate (Corning Cell Wells™, Corning). Cells were exposed to mitogens as follows: $100\text{ }\mu\text{L}$ of: Concanavalin A (Con A, $10\text{ }\mu\text{g}/\text{mL}$, Sigma), lipopolysaccharide (LPS, $50\text{ }\mu\text{g}/\text{mL}$, Sigma), or phorbol myristate acetate (PMA, $10\text{ ng}/\text{mL}$, Sigma) plus ionomycin ($0.5\text{ }\mu\text{g}/\text{ml}$, Sigma) in complete media. Non-stimulated control cultures contained $100\text{ }\mu\text{L}$

of complete media alone. Triplicate wells were used for each stimulant. Following 48 h incubation, 20 μ L of Alamar Blue™ dye (Serotec, Raleigh, NC) (10 % of incubation volume) were added to each well of the culture plates. At 24 and 48 h post addition, degree of absorbance was determined under dual wavelength (570 and 600 nm) using a Molecular Devices plate reader (Menlo Park, CA) (Klein et al. 2006; Mustafa et al. 2008).

Cytokine ELISA Assays. Splenocytes were plated into each well (1 mL in complete media; 5×10^6 cells per well) of a 24-well tissue-culture plate (Corning). Cells were co-cultured with 1 mL Con A (10 μ g/mL) and incubated at 37 °C, 5 % CO₂ for 48 h. The plates were centrifuged (7 °C, 250 x g, 7 min), and the supernatants were transferred to sterile 12 x 75 mm cultured tubes (Fisher). Supernatants were stored at – 80 °C until use. The levels of interleukin 4 (IL-4), IL-10, IL-12, and interferon-gamma (IFN- γ) were determined using ELISA kits (Ready-to-use; Ebioscience) according to the manufacturer's instructions

Statistical analysis. Data were expressed as arithmetical mean \pm SEM. Analysis of variance (ANOVA) was used with Dunnett's test to establish significant differences in the same sex groups between treatment groups and control. The pregnant dam was maintained as the statistical unit in all cases such that each offspring analyzed represented a separate dam (one pup/gender/treatment). Group size was five SNF₁ offspring per sex for all experiments (n=5). Results described as different in this report indicate significantly different at $p < 0.05$.

Results

Body and organ weights, and organ cellularity. Body weights of the 36-week-old adult SNF₁ offspring were decreased at 80 µg/kg TCDD in males but did not differ in females. Thymic weight was increased at 40 µg/kg TCDD and decreased at 80 µg/kg TCDD in the male offspring only, while thymic cellularity was not different across treatment groups. Bone marrow cellularity was increased at 40 µg/kg TCDD in the male offspring only. There were no significant differences in splenic weight or the spleen/body weight ratio across treatment groups (**Table 1**).

B lymphoid progenitors in bone marrow. At 36 weeks-of-age, the 80 µg/kg TCDD females showed a significant decrease in total B220 cells (B220⁺), representing B cell lineage from early Pro B to mature B cells and natural killer (NK) cell progenitors. However, there were no significant differences in the percentage of these B220⁺ cells that were B220^{hi} or the percentage of these B220⁺ cells that were B220^{low}. Yet, the percentage of B220^{lo} cells that expressed total CD24⁺ and the percentage of B220^{low} cells that expressed CD24^{hi}, representing large pre-B cells, was significantly decreased in 80 µg/kg TCDD males. The percentage of B220^{hi} cells that were CD24^{low} was significantly decreased in 80 µg/kg TCDD female offspring (**Table 2.A**). The percentage of B220^{low}CD24⁻ cells that expressed AA4.1 (B220^{low}CD24-AA4.1⁺), representing B lineage committed progenitors, was significantly increased in the 80 µg/kg TCDD male offspring and near significantly increased ($p=0.051$) in the by 80 µg/kg TCDD female offspring (**Table 2.B**).

The percentage of B220^{hi}IgM⁻ cells, representing non-B cells (i.e NK cells) was near significantly increased ($p= 0.051$) and the percentage of B220^{hi}IgM⁺ cells, representing immature B cells was near significantly decreased ($p= 0.051$) in 40 $\mu\text{g}/\text{kg}$ TCDD male. The percentage of B220^{low}IgM⁻ cells, representing pro B cells was significantly increased and the percentage of B220^{low}IgM⁺ cells representing pre B cells was significantly decreased in 40 $\mu\text{g}/\text{kg}$ TCDD males (**Table 2.B**).

Thymic T cell differentiation .Female, but not male, offspring of dams dosed with TCDD exhibited significant thymic phenotypic changes at 36 weeks-of-age. The relative expression of thymic CD4⁺CD8⁺ cells was decreased in the 80 $\mu\text{g}/\text{kg}$ TCDD females. In addition, CD4⁺CD8⁻ thymocytes were near significantly ($p= 0.053$) increased in females at this same TCDD exposure level (**Table 3**).

Splenic B and T cells phenotypes, and B cell migration in the spleen.

The relative percentage of splenic leukocytes that expressed B220 did not differ across treatment or sex compared to controls. Although the relative percentage of splenic B cells was unchanged, there was a shift in the immature and mature B cell compartments with increased numbers of transitional B cells (B220⁺CD24^{hi}) and reduced numbers of mature B cells (B220⁺CD24^{low-int}) in females (**Table 4.A**). Additionally, B cell subsets expressing CD21/CD24 were significantly different in males. The 80 $\mu\text{g}/\text{kg}$ males displayed a near significant increased percentage of B marginal zone (MZ) cells (CD24^{int}CD21^{hi}) ($p= 0.051$). B transitional-1 (T1) cells (CD23^{neg} CD1^{neg}) were significantly increased in females whilst B marginal zone

(MZ) cells (CD23^{neg} CD1^{hi}) were significantly increased in 80 µg/kg males (**Table 4.B**). The relative percentage of spleen leukocytes that expressed IgD (IgD⁺) was significantly decreased in 80 µg/kg TCDD males. However, the relative percentage of CD19⁺CD5⁺ B cells (representing B-1a cells, a B cell subset of fetal origin only, rather than bone marrow) was significantly increased in 80 µg/kg TCDD males, whilst no significant change was seen in females (**Table 4.B**). The relative percentage of T cells expressing CD4/CD8 markers did not change in any treatment group, in either sex. The relative percentage of spleen leukocytes that expressed Vβ17^a was significantly increased in 80 µg/kg TCDD males. However, the relative percentage of CD4⁺ cells expressing Vβ17^a was significantly increased at 80 µg/kg TCDD in both genders (**Table 5**).

Antibody titers to ssDNA, dsDNA and cardiolipin. To assess the extent of autoantibody production to dsDNA, ssDNA and cardiolipin autoantigens following a prenatal TCDD exposure, ELISA assays were performed. Anti-cardiolipin and anti-dsDNA titers showed numeric but non-significant trends in both TCDD male and female offspring and a significant increase in 80 µg/kg TCDD male offspring (**Figure 1**).

Kidney pathology. Since immune complex deposition in the kidney is a common signalment in lupus patients, histopathologic examination of the kidney was performed. Glomeruli that manifested with glomerular crescents, glomerular sclerosis, mesangial proliferation, hypertrophy of parietal epithelium and tubular

casts showed non-significant increase in TCDD treatment groups. However, the mean score of periarterial inflammation significantly increased by treatment in males and in 40 µg/kg TCDD females and trended toward increase in 80 µg/kg TCDD females. In addition, the mean score of interstitial inflammation trended toward increase by treatment in males and significantly increased in 40 µg/kg TCDD females (**Figures 2-4, Table 6**).

Deposition of anti-IgG and anti-C3 immune complexes in the kidney. Based on the histopathologic changes seen with light microscopy and H&E staining of the TCDD kidneys, immunofluorescent staining to elucidate IgG and C3 involvement in immune complex deposition was performed. Kidney sections from 36-week-old SNF₁ offspring showed a TCDD dose-dependent increasing trend in deposition of immune complexes, for both anti-IgG and anti-C3 probes regardless the sex. However, the 80 µg/kg TCDD-treated males were significantly increased for anti-IgG and the 80 µg/kg TCDD-treated females were significantly increased for anti-C3 (**Figure 5**).

Mitogen-stimulated splenic lymphocyte proliferation. Mitogen stimulation of enriched, cultured splenic lymphocytes was employed to assess the influence of prenatal TCDD on lymphocyte functionality in the adult mouse. Prenatal TCDD appeared to have a selective effect on splenic lymphoproliferative responses of 36 week-old SNF₁ offspring. LPS or PMA/ionomycin stimulated splenocytes showed increased proliferation across treatment and sex at 48 and 72 h reaching

significance in PMA/ionomycin 40 µg/kg male TCDD at 48 h. However, there was a non-significant inhibition in Con A-induced proliferation in females at 48 and 72 h reaching significance in 40 µg/kg TCDD at 48 h, while a non-significant trend toward effect on stimulation responses was observed using Con A proliferation in males at 48 and 72 h (**Figure 6**).

Th1/Th2 cytokine balance. Prenatal exposure to TCDD caused a suppressed effect on the T_h1 cytokine profile in Con-A-stimulation splenic lymphocytes collected from 36-week-old mice. The 80 µg/kg TCDD males showed enhanced IL-2 production, and diminished IL-4, IL-12 and IFN-γ production relative to controls. Females showed enhanced IL-12 production, and diminished IL-10 and IFN-γ production relative to controls (**Figure 7**).

Discussion

Previous studies in our laboratory have shown that a single exposure to TCDD in an *AhR* responsive murine strain (C57BL/6) was capable of permanently altering the immune system into adulthood, resulting in a lupus-like immune-mediated disease that differed in severity based on sex (Mustafa et al, 2008). Based on this observation, parallel studies were initiated in a murine strain that naturally develops autoimmune lupus nephritis (SNF₁) to explore whether an acute developmental exposure to TCDD would worsen and/or accelerate pathologic changes associated with this disease. At 24 weeks of age these prenatal-exposed SNF₁ mice showed considerable dysregulation of both cell-mediated and humoral immune function, including exacerbation of the type III hypersensitivity lupus-like autoimmune disease (unpublished data). The present studies extend these early results to SNF₁ litter-mates at 36 weeks of age, a time when females are normally actively expressing disease.

Developmental exposure to TCDD resulted in persistent alterations in thymocyte surface antigen expression in SNF₁ females at 36 weeks, an observation consistent with the 24-week-old mice. The paradigm in non-autoimmune mice has been that prenatal TCDD-induced thymic atrophy and thymocyte hypocellularity are accompanied by an increase in the percentage of thymocyte CD4⁻CD8⁻ and immature CD4⁻CD8⁺ (CD3⁻CD8⁺ or $\alpha\beta$ TCR⁻CD8⁺) as well as a decrease in the percentage of thymocyte CD4⁺CD8⁺ T cells by late gestation (Fine et al. 1990; Fine et al. 1990; Holladay et al. 1991). Following prenatal TCDD exposure, CD4/CD8 expression was disrupted but then restored to normal levels by postnatal day (PND 1) however,

thymic atrophy and hypoplasia didn't rebound until PND 14 (Fine et al. 1990; Holladay et al. 1991; Camacho et al. 2004). Other studies using different dosing strategies have shown similar thymic effects (Faith & Moore 1977; Luster et al. 1979; Fine et al. 1989; Blaylock et al. 1992; Gehrs et al. 1997; Vos et al. 1997; Gehrs & Smialowicz 1999). These observations would suggest that late-gestation thymic atrophy, although significant, is transient in mice and the murine thymus recovers shortly after birth.

Thymocyte subset expression profiles were altered in TCDD exposed SNF₁ females after 24 weeks. These changes were characterized by decreased percentages of CD4⁺CD8⁺ thymocytes, and increased CD4⁻CD8⁻ thymocytes. In addition, the TCDD-exposed female mice displayed a decrease in thymic cellularity (unpublished data). In the present 36-week-old mice, thymic phenotypic changes were consistent with the previous females. The percentage of CD4⁺CD8⁺ double positive thymocytes was decreased in 80 µg/kg females, however thymic cellularity was no longer reduced by prenatal TCDD. This effect may be attributable to normal atrophy associated with aging.

In addition to changes in the thymic compartment, TCDD-treated mice showed a significant increase in peripheral autoreactive T cells. Targeting of thymic epithelium including distribution of major MHC molecules by TCDD has been proposed as a mechanism that may increase release of autoreactive T cells (Hanawa et al. 1993; De Heer et al. 1994). TCDD also increases extrathymic T cell development in mice, into compartments (e.g., liver) where negative selection for self-tolerance may be compromised (Silverstone et al., 1994). Such increase in

extrathymic T cells has been reported in aged mice, athymic mice and mice with autoimmune disease in several organ systems (Hanawa et al. 1993). Extrathymic-derived T cells have been characterized by TCR-variable ($V\beta$) chains, which are usually deleted in the thymus by a reaction with self-MHC and minor lymphocyte stimulatory antigens (Okuyama et al. 1992; Hanawa et al. 1993). Numerous investigators working with several mouse models have proposed that increased extrathymic autoreactive T cells, similar to that observed in the present mice, maybe a mechanism by which TCDD promotes autoimmunity (Adams et al. 1990; Okuyama et al. 1992; Rocha et al. 1992; Silverstone et al. 1994). In one study, spleens from SNF₁ mice showed an expansion of a cluster of autoreactive T helper cells with TCR V beta genes including CD4⁺V β 17a⁺ TCR (Adams et al. 1990). Collectively, these studies would appear to support our observations of increased incidence of autoreactive T cells expressing V β 17a⁺ TCR in the spleen of TCDD-treated mice.

In 24-week-old female SNF₁ mice prenatally-exposed to TCDD, bone marrow cells expressing the pan B cell marker (B220⁺) were decreased, while B220^{low} B cell progenitors and B220^{low}CD24-AA4.1⁺ early B cell progenitors were increased. At 36 weeks a similar pattern of expression continued to be present in the females. Interestingly, the TCDD-treated 36-week-old males also showed increased expression of early B cell progenitors (B220^{low}CD24-AA4.1⁺) that was not observed at 24 weeks. Thus, it is possible that prenatal TCDD exposure imprints effects on early B cell progenitors in males similar to that observed in female, that manifest upon disease initiation. This increase in the earliest B progenitor is consistent with a

reported increase in the pre-pro-B population in an S17 stromal cell line exposed to TCDD, *in vitro* (Wyman et al. 2002).

Early work by Thurmond & Gasiewicz (2000) also showed that a single dose of 30 µg/kg TCDD in male C57BL/6 mice resulted in a decrease in immature bone marrow B lymphocytes and an increase in earliest B cell-progenitor subpopulation lasting until day 9 post treatment. These same investigators reported that TCDD significantly increased pre-pro-B cell populations in cultures with the S17 stromal cell line (Wyman et al. 2002). This increase in number of cells expressing the pre-pro-B cell phenotype (B220⁺/CD24⁻/CD43⁺) was seen in cultures of both wild-type and *AhR*^{-/-} primary bone marrow cells. These findings would appear to suggest that the maturing B220⁺ B cell is not the direct target for TCDD-induced bone marrow B-cell alterations. This idea supports previous work by Yamaguchi et al. (1997) who showed that *AhR* activation effects immunosuppression in bone marrow by inducing stromal cells to deliver a death signal to lymphocytes. Collectively, these observations indicate, for the B cell compartment, prenatal TCDD exposure events in stromal cells may underlie persistent dysregulation of B cell lymphopoiesis.

B cell-specific activator protein (BSAP or PAX5), located in the promoter region of the gene encoding CD19, has been shown to play a key role in early B lymphopoiesis including the control of cell proliferation, isotype switching and transcription of the immunoglobulin heavy-chain gene at the late stages of B-cell differentiation (Busslinger & Urbanek 1995). Previous results have indicated that the TCDD-activated *AhR* complex interferes with BSAP-stimulated CD19 gene transcription by competing for a common DNA binding site resulting in diminished

CD19 mRNA expression in human B-lymphoblast cell lines (Masten & Shiverick 1995). Furthermore, a more recent study reported impaired down-regulation of paired box 5 (Pax5), a repressor of B cell differentiation, and concomitant suppression of the IgM response by TCDD in the murine CH12.LX B cell line (Yoo et al. 2004). This study demonstrated the involvement of altered Pax5 regulation in the suppression of the primary IgM antibody response by TCDD. Consistent with these observations, we also observed decreased IgM expression in the bone marrow of TCDD-treated 36 week-old mice. Therefore, it is reasonable to propose that TCDD may alter CD19 expression and subsequently modulate BCR threshold in selection processes. In support of this idea, prenatal TCDD exposure altered the B subpopulation balance in spleens of the present SNF₁ mice, in addition to altering splenic lympho-proliferation responses to LPS stimulation. Recently, a study showed that activation of the *AhR* by TCDD disrupts the circadian rhythms associated with C57BL/6 murine stem cell and hematopoietic precursors (Garrett & Gasiewicz 2006). Taken together, this would appear to suggest that prenatal TCDD exposure modulates programming of bone marrow-derived cells directly or/and indirectly (i.e., bone marrow stromal cells). Future studies are being designed to explore in more detail the mechanisms by which TCDD dysregulates B lymphopoiesis.

Our previous studies indicted the effects of TCDD on B cell differentiation and maturation are not limited to just the bone marrow, but also extend into the secondary lymphoid compartments. In 24-week old mice prenatally exposed to TCDD, we observed altered maturation of splenic B cell precursors and increased

shifting toward follicular (FO) B cells. At 36 weeks-of-age, prenatal TCDD exposure altered the maturation of splenic B cell precursors and enhanced shifting toward marginal zone (MZ) B cells in 80 µg/ml TCDD males, and toward transitional type1 (T1) B cells in 80 µg/ml TCDD females. The decision on whether a cell matures as a follicular zone B cell, marginal zone B cell or B1 cell is determined primarily by the strength of the BCR-mediated signal (Niuro & Clark 2002). Our findings suggest that TCDD alters the BCR-signaling pathway, which results in the development and expansion of MZ B cells in males and T1 B cells in females.

In murine SLE models, B cells are needed to prime pathogenic T cells, as B-cell deficient MRL/lpr mice fail to demonstrate an expansion of activated T cells (Leitges et al. 1996). However, MZ B cells are more efficient than FO B cells at priming naïve T cells, and their over-representation in lupus-prone strains supports the hypothesis that MZ B cells help initiate the T-cell dependent disease. It is believed that the high level of somatically hypermutated antibodies of SLE-prone mice are the product of the germinal center reaction, indicating that the autoantibody-secreting plasma cells would be derived from the germinal center reaction, where MZ B cells have not been shown to reside (Cariappa et al. 2001). Thus as suggested by our studies, whereas MZ B cells appear to be important in initiating the autoimmune disease, Th cells and FO B cells may be necessary in sustaining and promoting such disorders.

We also observed an increase of transitional T1 B cells in females. Transitional T1 B cells are believed to be generated from bone marrow immature B cells and to be direct precursors of transitional T2 B cells (Loder et al. 1999). During the

transitional T1 stage, autoreactive B cells that have escaped negative selection in the bone marrow are eliminated by negative selection in the spleen (Sater et al. 1998; Sandel & Monroe 1999). Interestingly, mice transgenic for B cell activation factor display a lupus phenotype and exhibit an altered transitional T1/T2 B cell balance and an expansion of marginal zone B cells (Batten et al. 2000). This appears to be the result of impaired negative selection of autoreactive B cells and leads to a disproportionate number of MZ B cells in these mice (Batten et al. 2000). MZ B cells contribute to the pathogenic autoantibody responses generated in NZB/NZW F1 mice. In the current study, the increase in transitional T1 B cells may reflect an impaired negative selection of autoreactive B cells. The connection between the transitional T1 B cells to autoimmune pathogenesis needs to be better established and will be the focus of future studies.

We also observed, within the secondary lymphoid compartment, an increase in CD5 B cells in the 80 µg/kg TCDD females, suggesting an increase of the B-1a cell type. B-1a cells are distinguished from conventional B cells (B2) by their fetal developmental origin, their surface marker expression of CD5 and their function (Duan & Morel 2006). B-1a cells are long lasting, habitually stationed, with a lower BCR diversity and affinity compared to B2 cells (Kantor et al. 1997). These cells are the primary source for natural IgM antibodies characterized as low affinity, polyreactive proteins. These natural antibodies are believed to be a first line of defense against bacterial pathogens (O'Garra et al. 1992; Duan & Morel 2006). Also, the polyreactive nature of these antibodies facilitate disposal of apoptotic autoantigens (Carroll & Prodeus 1998). Further, the weak autoreactive function of

the B-1a cells is believed to play an important role in autoimmune pathogenesis. Additionally, their high level of IL-10 production and potent antigen presentation capacities have linked B-1a cells to autoimmunity (O'Garra et al. 1992). Elevated concentrations of B-1 cells have been reported in patients with SLE, Sjogren's syndrome and rheumatoid arthritis. Further, the expansion of this cell compartment and the incidence of systemic autoimmunity have been described in murine models. Thus, the increase in B-1a cells observed in this study would appear to correlate well with the immune-mediated pathogenesis.

In the current study, anti-dsDNA, anti-ssDNA and anti-cardiolipin autoantibody titers were non-significantly increased in the TCDD-treated mice. Although detection of serum autoantibodies is considered a common clinical indicator of systemic lupus erythematosus, recent evidence has shown that autoantibodies to classic lupus antigens are neither required nor capable of inducing end-stage renal failure. For example, congenic NZM2328.C57Lc4 mice develop a fatal glomerulonephritis similar to the parent immune prone NZM2328 mice despite the absence of lupus autoantibodies (Waters et al. 2004). The results of this study would appear to dissociate lupus autoantibody production from lupus glomerulonephritis and raise doubts in the current paradigm that autoantibodies to dsDNA and related autoantigens are the primary pathogenic mediators of disease (Waters et al. 2004). Further, a quantitative analysis of immunoglobulins eluted from lupus kidneys in SLE showed that less than 10% of the total IgG reacts with a multiple of lupus antigens such as DNA, histone, C1q complement, Sm proteins, chromatin, and other antigens (Mannik et al. 2003). Thus, the antigens recognized

by most potentially pathogenic autoantibodies in lupus nephritis have yet to be identified.

Autoimmune disease in lupus-like SNF₁ mice and similar disease in humans is critically dependent on T cell function, including accelerated antibody production subsequent to inappropriate activity of CD4⁺ T helper cells. This observation supports the idea that a pathogenic T-cell response to kidney antigens with cooperation from the kidney itself determines the onset and progression of renal failure in SLE. In experimental models of autoimmune disease, it is well recognized that the glomerular patterns of immune complex-mediated injury are related to the site of immunoglobulin deposition, their antigen specificity, and their affinity to bind and activate complement and other serine proteases, and their ability to initiate a cellular inflammatory response (Fries et al. 1988). These patterns of injury can be divided into three groups: mesangial pattern, endothelial pattern and epithelial pattern. In lupus glomerulonephritis, it is common for several different morphologic patterns to coexist, as seen in the current study. In the current study, the histologic evaluation of kidneys showed evidence of pathologic changes characterized by increased quality and quantity of deteriorated glomeruli accompanied by glomerulosclerosis, fibroid necrosis, crescents and inflammatory cells in TCDD-exposed mice. Interestingly in this study at 36 weeks, the 40 µg/kg TCDD females had the highest pathologic scores in regards to nephritis followed by 80 µg/kg TCDD females. It is possible that the higher dose of TCDD may have initiated or shifted different immune pathways to affect the lupus nephritis in this strain differently during the aging process. The increased deposition of C3 in the glomeruli of TCDD-

treated SNF₁ mice coupled with the observation of extensive apoptotic cells provides immunologic evidence for the pathologic changes in the inflamed kidneys. In these animals, we also observed high serum cardiolipin antibody titers that have been associated with an increased risk of thrombosis, spontaneous abortion, thrombocytopenia, and several other manifestations that have sometimes been referred to as the antiphospholipid antibody in SLE and related disorders (Harris et al. 1985; Thiagarajan et al. 1997). Under normal conditions, cardiolipin is absent from the surface of viable cells, but it is translocated to the external surface of the plasma membrane of cells undergoing apoptosis (Rudel & Bokoch 1997; Sorice et al. 2000). In summary, both TCDD treated females and males showed increased immune-mediated renal pathology, manifested by fibrinoid necrosis, crescents, mesangial proliferation, hypertrophy of parietal epithelium and inflammatory cells.

Given that prenatal TCDD altered maturation and differentiation of the T cells, we next assessed the influence of TCDD on T cell functionality by determining the change in Th₁/Th₂ profile. Our data show that prenatal TCDD exposure decreased the levels of IFN- γ , IL-12 and IL-4 in Con-A-stimulated splenic lymphocytes in the females suggesting a down regulation in Th₁ response. By 36 weeks-of-age, SNF₁ females are in mid -advanced stages of lupus-nephritis and the SNF₁ males are usually in the early stage of the disease manifested by SLE-like symptoms. According to the cytokine profile, prenatal TCDD exposure appears to accelerate the initiation and promote the progression of the disease by producing higher levels of IFN- γ as detected in the previous 24-weeks study. It is well established that optimal activation of naive CD4⁺ T cell requires two signals. The first signal through T-cell

receptor (TCR)–CD3 complex followed by a second co-stimulatory signal via CD28/B7 system, which can be provided by antigen presenting cells (Lenschow et al. 1996). These signals result in the production of cytokines such as IL-2, which promote the differentiation of cells that secrete cytokines such as IFN- γ (Lenschow et al. 1996; Boehm et al. 1997). The increase in IFN- γ caused by prenatal TCDD exposure may be crucial, since this cytokine has been implicated in a number of autoimmune conditions. In several animal models of autoimmunity, IFN- γ appears to play a vital role in the development of lupus nephritis (Haas et al. 1997), autoimmune insulinitis (Campbell et al. 1991), Sjogren's syndrome (Hayashi et al. 1996) and autoimmune arthritis (Billiau 1996). However, the complex role of interferons in lupus has been demonstrated by accelerated disease in type I interferon receptor deficient MRL/lpr mice, while deletion of both type I and II interferon receptors in MRL/lpr mice protected from spontaneous lupus disease (Hron & Peng 2004). Thus, it is possible that type II interferon (IFN- γ) plays an important role initiating the disease, however once inflammation has been initiated type I interferons are necessary to sustain the disease.

It is likely that the role of IL-10 in SLE is also complex. The current belief is that a deficiency in the production of IL-10 may play a role early in disease pathogenesis in SLE, while elevated levels of IL-10 might reflect disease activity once inflammation has been initiated. At present, we have no explanation for the reduction of IL-10 in TCDD treated mice despite the high activity of lupus disease in females. However, at 36-weeks age, SNF₁ females are usually in an advanced stage of the disease. Data from this study have shown that prenatal TCDD appears to induce

a lower threshold stimulation of splenic B lymphocytes, characterized by increased production of autoantibodies to mouse dsDNA and cardiolipin, as well as by an increased lymphoproliferative response via LPS and P/I mitogens. In addition, we have also shown that autoreactive V β TCR T cells were increased in the spleen. Based on these findings, it is likely that prenatal TCDD exposure may enhance antibody production derived via Th₂ cytokines such as IL-6 or IL-5 that affect B cells, an aspect that needs to be investigated.

In the current study, Con A stimulation was employed to specifically evaluate the function of Th cells and thus, the observed cytokine profile is reflective of the T cells only. Further, it is possible that this cytokine profile would shift dramatically if these cells had been cultured with other mitogens (i.e. LPS or PMA/ionomycin or anti-CD40). For example, B cells from several lupus strains produced more IL-10 than controls, however, they failed to produce IL-10 compared to controls when stimulated through BCR or CD40 (Brummel & Lenert 2005; Lenert et al. 2005). B cell production of IL-10 has been shown to be important in controlling inflammation in a number of experimental models of autoimmune disease including collagen-induced arthritis (Mauri et al. 2003), experimental allergic encephalomyelitis (Fillatreau et al. 2002), inflammatory bowel disease (Mizoguchi et al. 2002) and lupus (Lenert et al. 2005).

Alternatively, a different set of cytokines might orchestrate the florid immune response at advanced phases of SLE such as type I IFNs. Recent research has focused on type I IFNs (IFN- α and IFN- β) as there is compelling evidence to suggest that type I IFNs may play a critical role in the pathogenesis of lupus. Recent studies contributed of

the effect of type I IFNs in the pathogenesis of SLE to the peripheral tolerance breakdown through the activation of immature myeloid DCs into their potent presenting stage, capable of efficient T cell activation (Banchereau & Pascual 2006). Other studies have shown that type I IFNs also stimulate upregulation of Toll-Like-Receptor 7 (TLR7) in DCs and B cells, potentially enhancing the response to auto-antigen (Banchereau et al. 2000; Blanco et al. 2001; Steinman et al. 2003; Banchereau et al. 2004). Type I IFNs also induce the secretion of B cell activity factor BAFF by DCs, which may stimulate autoreactive B cell proliferation and survival and enhance the ability of CD4 T cell to provide B cell help (Litinskiy et al. 2002; Le Bon et al. 2006).

In conclusion, results from this study demonstrate that a single dose of TCDD given prenatally at the time of immune system ontogeny can cause persistent immunologic changes in an autoimmune-prone murine strain that can result in either an exacerbation or acceleration of autoimmunity development in adulthood. The TCDD-exposed mice displayed clear fundamental immunogenic changes leading to loss of tolerance to self-antigen. Further, the mechanism underlying the autoimmune pathogenesis appears to differ by sex. Additionally, the immune mechanisms involved in initiating autoimmunity may differ from the immune mechanisms involved in promoting organ damage as the disease progresses. Prenatal TCDD exposure could lead to exacerbated autoimmune SLE-like renal lesions and other autoimmune diseases through several steps. These steps include, but are not limited to, increased $V\beta^+$ Th cells, dysregulated cytokine production, increased splenic B1a, MZ and T1 B cells and increased autoantibody production.

Although these observations suggest that developmental exposure to dioxin produces a fundamental disruption in establishment of central self-tolerance, the mechanisms involved in these observations need to be more fully investigated. Finally, the ability of prenatal dioxin to initiate a hyperactive immune system leading to the onset of autoimmune disease appears to be subject to the interaction between a number of factors including *AhR* susceptibility allele, autoimmunity genetic predisposition and sex.

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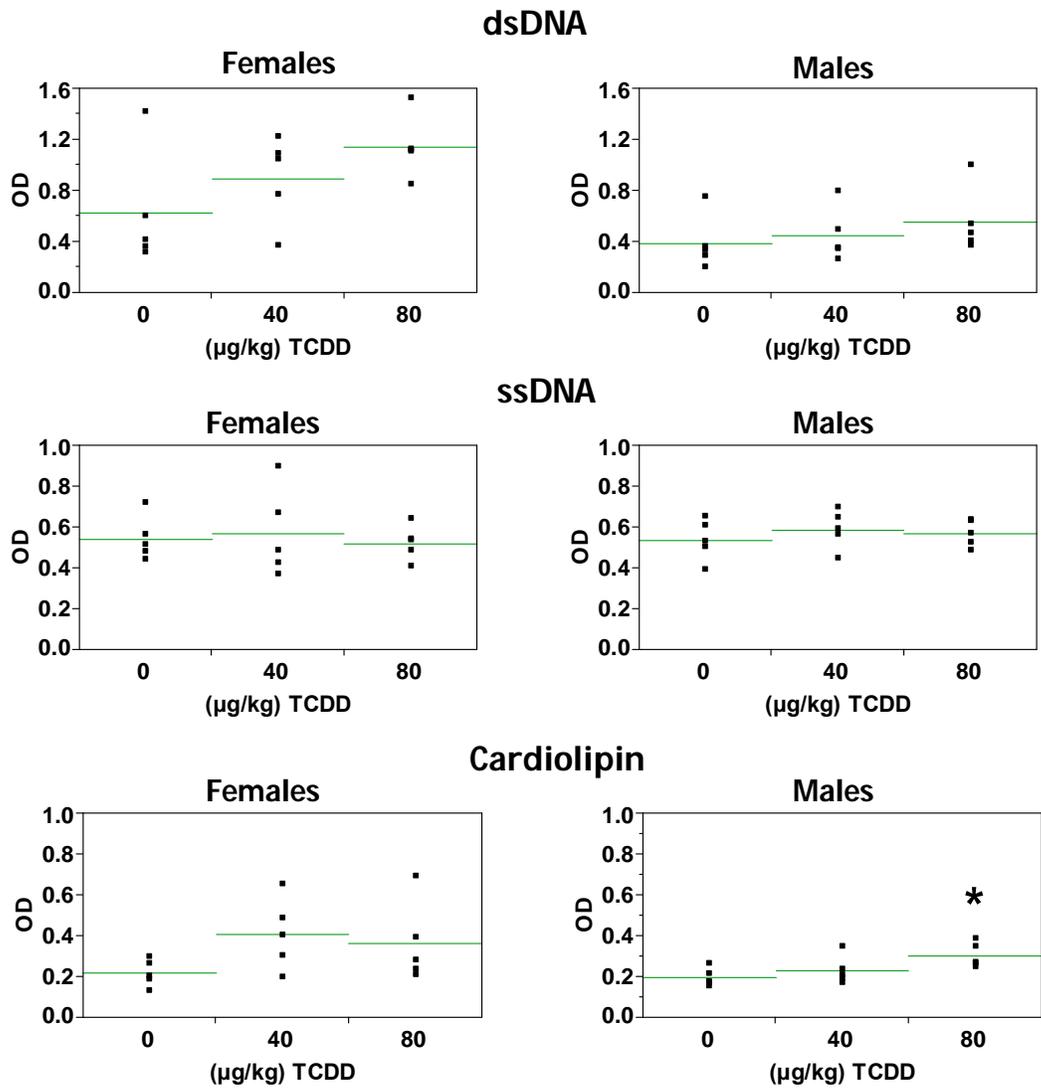


Figure 1. Sera from 36 week-old SNF₁ mice that were perinatally exposed to 0.0, 40.0 and 80 µg/kg TCDD were analyzed for the presence of autoantibodies to dsDNA, ssDNA and cardiolipin. The data are arranged by gender and reflect an N of 5 mice /treatment/sex (* = $p < 0.05$, Dunnett's test). Each horizontal line represents the arithmetic mean for each treatment group.

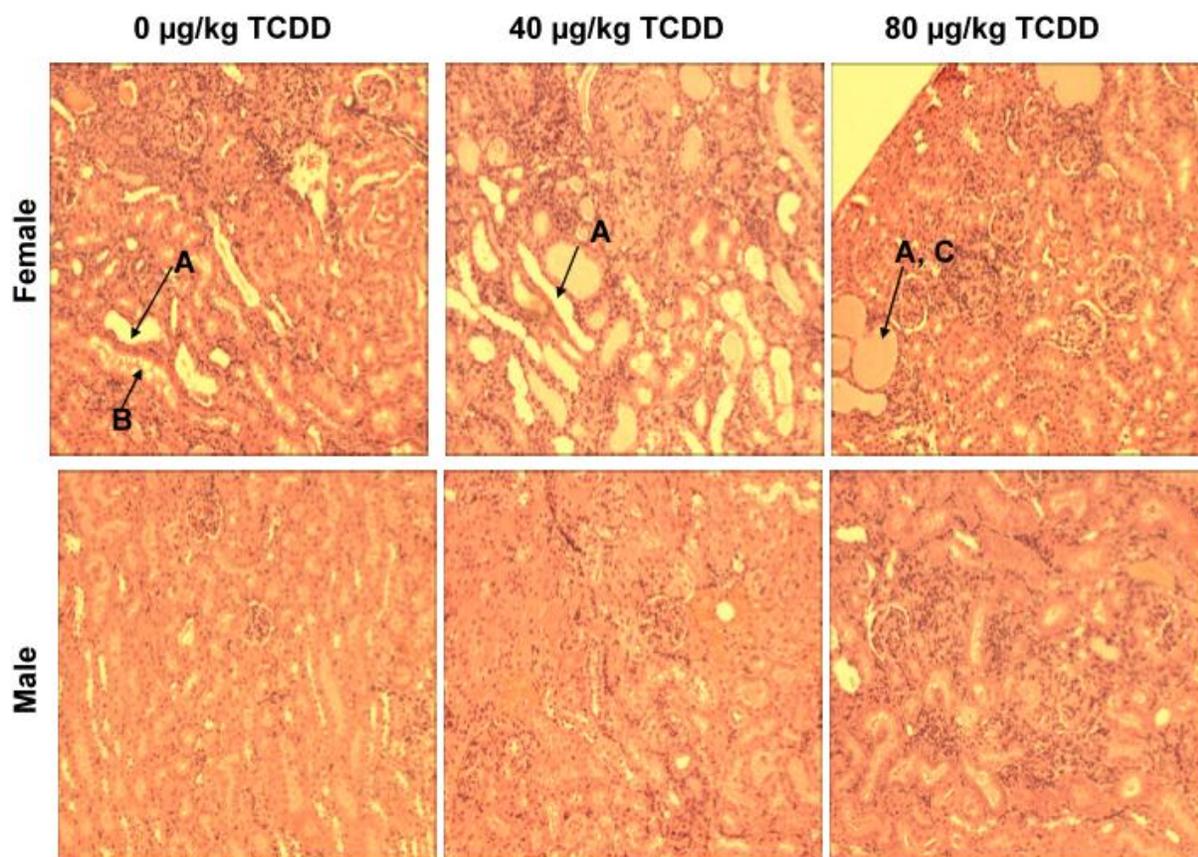


Figure 2. The kidneys from 36-week-old SNF₁ mice that were prenatally exposed to 0, 40 or 80 $\mu\text{g}/\text{kg}$ TCDD were collected, fixed, sectioned and stained with H&E. Images are representative of renal sections by treatment and sex. The data are based on 5 mice/treatment/sex (* = $p < 0.05$, Dunnett's test). Renal cortical tubules with varying degrees of tubular ectasia (A), occasional cellular debris (B), and protein casts (C); tubular changes more prominent in female mice as compared to males with the female 40 $\mu\text{g}/\text{kg}$ TCDD treatment group being most affected; annotations are selected examples of lesion type; hematoxylin and eosin, 100x.

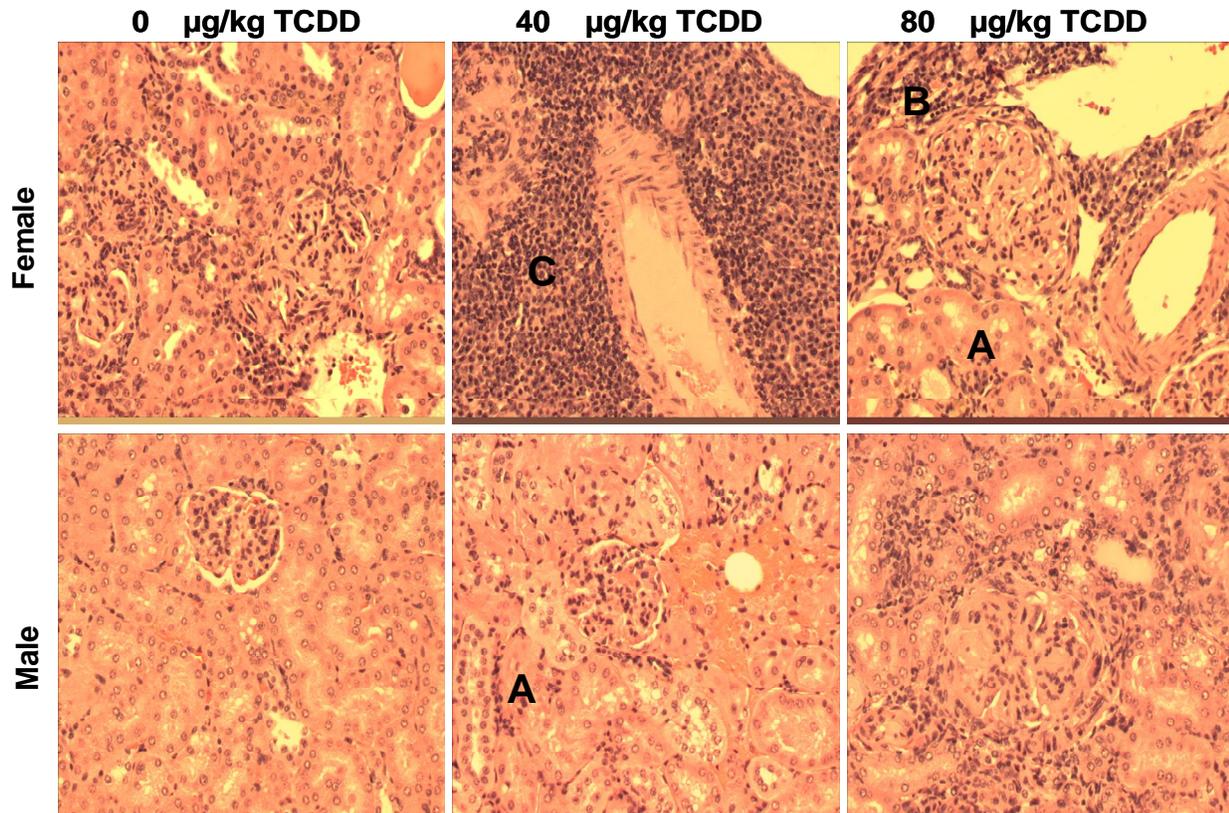


Figure 3. The kidneys from 36-week-old SNF₁ mice that were prenatally exposed to 0, 40 or 80 $\mu\text{g}/\text{kg}$ TCDD were collected, fixed, sectioned and stained with H&E. Images are representative of renal sections by treatment and sex. The data are based on 5 mice/treatment/sex (* = $p < 0.05$, Dunnett's test). Renal cortex showing varying degrees of predominantly lymphocytic inflammation involving the renal interstitium (A), surrounding glomeruli (B), and periarterial (C) regions; inflammation generally more severe in female mice as compared to males with the female 40 $\mu\text{g}/\text{kg}$ TCDD treatment group being most affected, and in the males, the 80 $\mu\text{g}/\text{kg}$ TCDD group; annotations are selected examples of lesion type; hematoxylin and eosin, 200x.

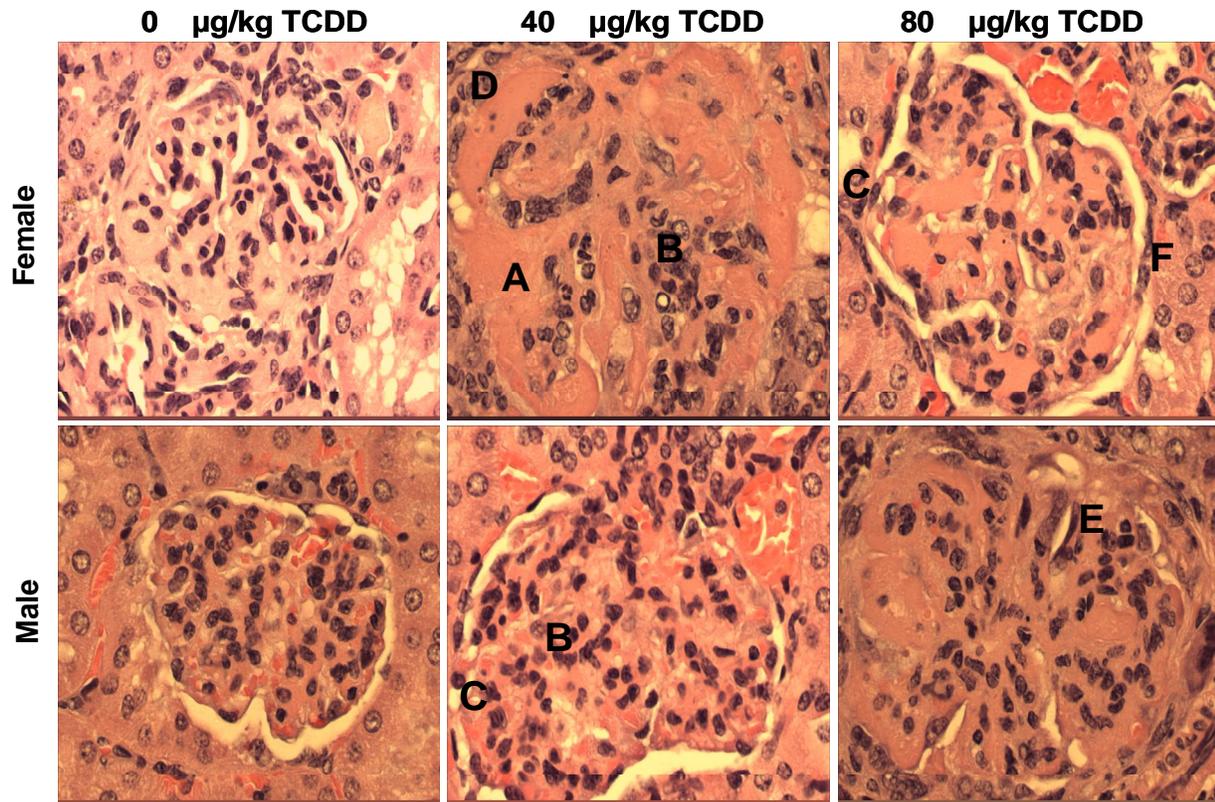
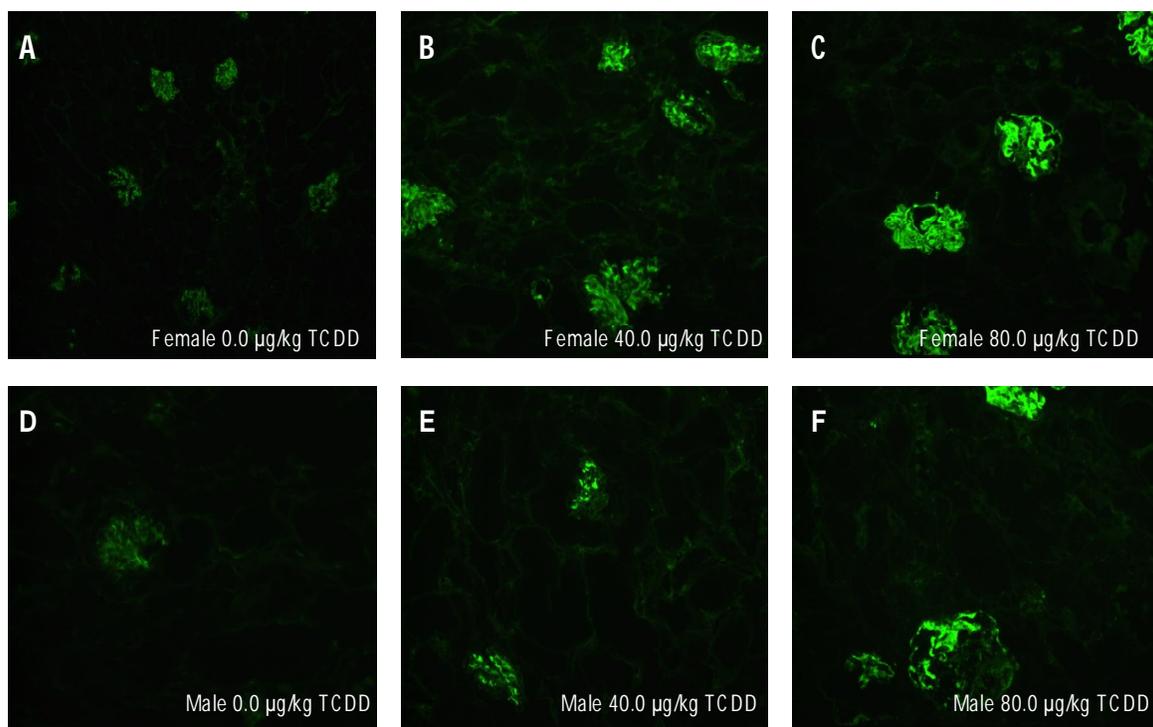


Figure 4. The kidneys from 36-week-old SNF₁ mice that were prenatally exposed to 0, 40 or 80 µg/kg TCDD were collected, fixed, sectioned and stained with H&E. Images are representative of renal sections by treatment and sex. The data are based on 5 mice/treatment/sex (* = $p < 0.05$, Dunnett's test). Renal glomeruli showing one or more of the following changes: sclerosis (A), mesangial proliferation (B), synechia (C), crescents (D), prominent capillary loops (E), hypertrophy of parietal epithelium (F); glomerular changes more severe in female mice as compared to males with the female 40 ug/kg TCDD treatment group being most affected, and in the males, the 80 ug/kg TCDD group; annotations are selected examples of lesion type; hematoxylin and eosin, 500x.



SNF ₁		0 µg/kg Mean ±SEM	40 µg/kg Mean ±SEM	80 µg/kg Mean ±SEM
Complement C3 Score	<i>Female</i>	1.68 ±0.48	2.50 ±0.21	2.70 ±0.18*
	<i>Male</i>	0.70 ±0.29	0.80 ±0.24	1.30 ±0.34
IgG Score	<i>Female</i>	2.25 ±0.33	2.90 ±0.10	2.65 ±0.17
	<i>Male</i>	1.15 ±0.23	1.50 ±0.21	2.25 ±0.16*

Figure 5. The kidneys from 36-week-old SNF₁ mice that were prenatally exposed to 0, 40 or 80 µg/kg TCDD were collected, fixed, section and stained with FITC-labeled anti-IgG and anti-C3. The above are representative images of kidneys stained with FITC-anti-IgG based on treatment and sex. The data are based on 5 mice /treatment/sex (* = $p < 0.05$, Dunnett's test).

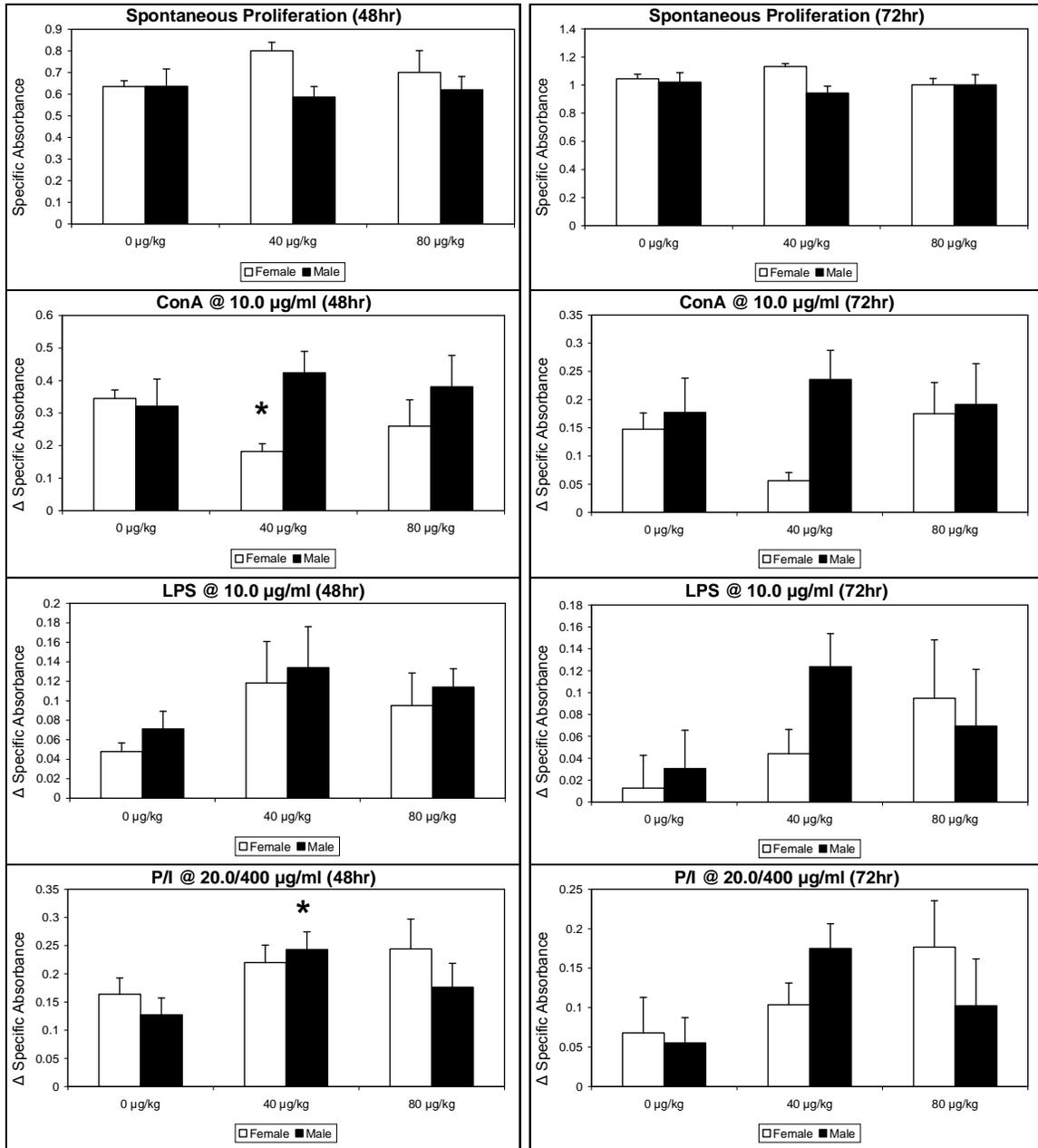


Figure 6. Effects of perinatal exposure to TCDD (maternal doses of 0, 40 and 80 µg/kg TCDD on gestational day 12) on the lymphoproliferative responses of splenocytes from 36-weeks-old SNF₁ mice. Splenocytes were exposed to ConA, or LPS, or P/I for 48hr or 72 hr. Values are reported as mean specific absorbance ± SEM. The results are based on an N of 5 mice/treatment/sex (* = $p < 0.05$, Dunnett's test).

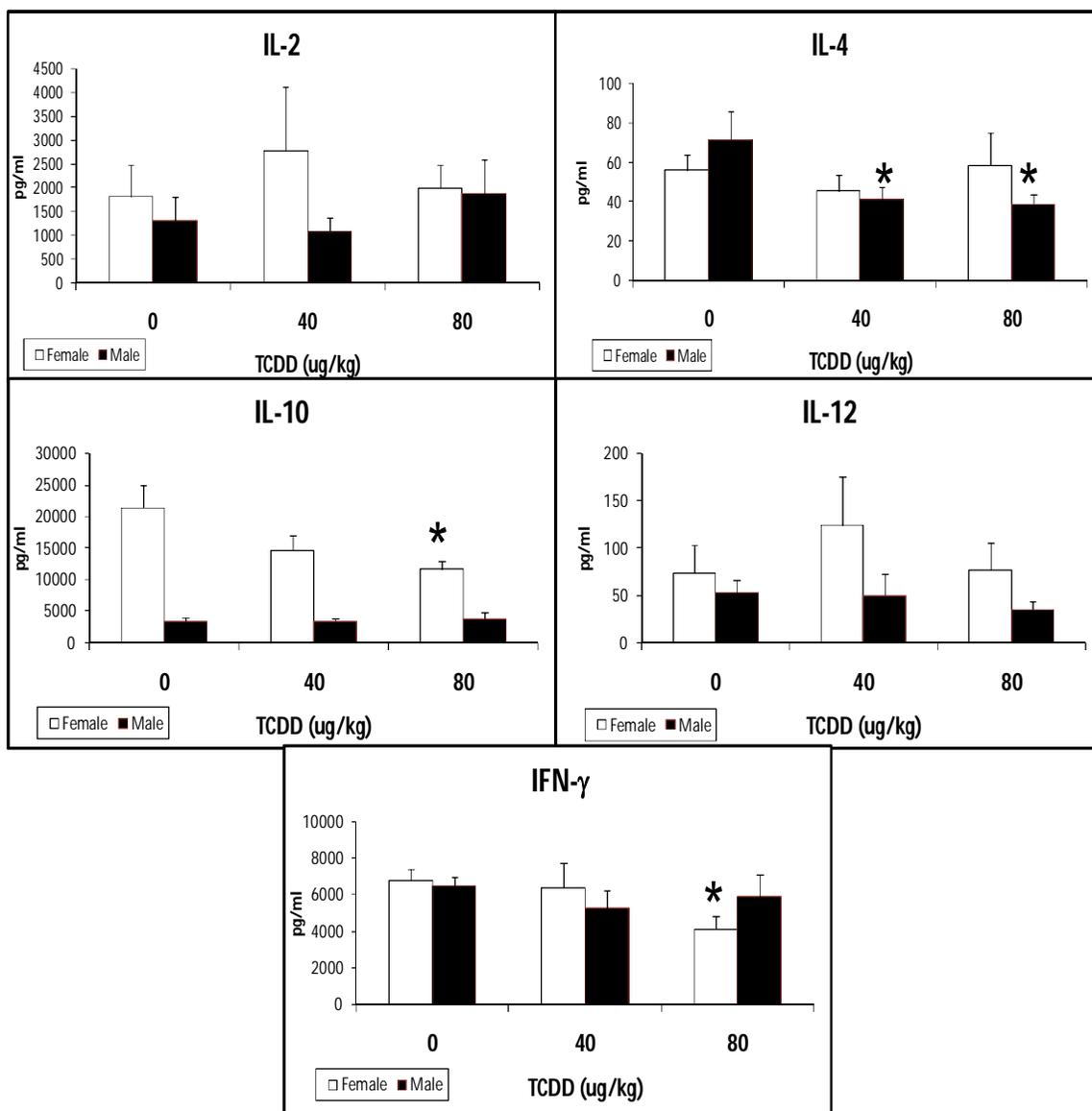


Figure 7. Supernatants were collected from splenocytes, of 36 week-old mice that were perinatally exposed to 0, 40 and 80 μg/kg TCDD, cultured for 48 hr with Con A (10 μg/mL). The levels of IL-4, IL-10, IL-12 and INF-γ were determined using commercially available murine cytokine ELISA kits. The data are presented in a histogram analysis with mean bars. The results are based on an N of 5 mice/treatment/sex (* = $p < 0.05$, Dunnett's test).

Table 1. Body Weight, Organ Weight and Total Cellularity

		0 µg/kg Mean ±SEM	40 µg/kg Mean ±SEM	80 µg/kg Mean ±SEM
Initial Body Weight (g)	<i>Female</i>	27.38 ±0.77	27.93 ±0.75	27.31 ±1.40
	<i>Male</i>	41.28 ±0.97	43.88 ±1.68	36.39 ±1.48*
Spleen Weight (g)	<i>Female</i>	0.12 ±0.01	0.31 ±0.07	0.29 ±0.08
	<i>Male</i>	0.15 ±0.05	0.14 ±0.05	0.11 ±0.01
Thymus Weight (g)	<i>Female</i>	0.04 ±0.00	0.05 ±0.01	0.04 ±0.01
	<i>Male</i>	0.04 ±0.00	0.05 ±0.00*	0.03 ±0.00*
Spleen: Body Weight (% ratio)	<i>Female</i>	0.44 ±0.03	1.10 ±0.23	1.09 ±0.29
	<i>Male</i>	0.36 ±0.12	0.32 ±0.10	0.30 ±0.04
Thymus: Body Weight (% ratio)	<i>Female</i>	0.15 ±0.00	0.17 ±0.03	0.13 ±0.03
	<i>Male</i>	0.10 ±0.01	0.12 ±0.01	0.09 ±0.01
Cellularity: Spleen Cells (millions)	<i>Female</i>	93.80 ±7.28	167.24 ±41.13	158.73 ±35.20
	<i>Male</i>	72.90 ±11.52	84.14 ±21.06	75.93 ±9.60
Cellularity: Thymus Cells (millions)	<i>Female</i>	14.35 ±2.59	18.80 ±6.22	10.83 ±3.23
	<i>Male</i>	16.62 ±3.03	12.63 ±2.20	10.94 ±3.36
Cellularity: Bone Marrow Cells (millions)	<i>Female</i>	9.78 ±1.11	10.61 ±0.88	13.60 ±1.70
	<i>Male</i>	14.32 ±0.68	17.50 ±0.76*	12.71 ±1.01

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 2A. Bone Marrow Analysis

Bone Marrow		0 µg/kg Mean±SEM	40 µg/kg Mean±SEM	80 µg/kg Mean±SEM
Total B220	<i>Female</i>	14.84±1.44	11.08±2.23	7.52±1.35*
	<i>Male</i>	12.44±0.95	12.48±0.65	12.92±0.48
B220 ^{hi} % Total B220	<i>Female</i>	23.54±2.37	32.24±6.77	24.56±2.71
	<i>Male</i>	31.50±3.93	28.12±4.11	28.12±5.41
B220 ^{hi} CD24 ⁺ % B220 ^{hi}	<i>Female</i>	40.64±3.57	34.78±3.76	33.70±3.28
	<i>Male</i>	50.72±8.79	44.14±2.30	42.66±5.72
B220 ^{hi} CD24 ^{hi} % B220 ^{hi}	<i>Female</i>	15.52±3.13	12.80±2.89	11.70±2.63
	<i>Male</i>	18.92±4.96	13.52±1.26	11.74±3.42
B220 ^{hi} CD24 ^{low} % B220 ^{hi}	<i>Female</i>	25.10±1.06	21.96±1.54	20.02±0.77*
	<i>Male</i>	31.84±3.99	30.64±1.89	30.90±2.60
B220 ^{hi} CD24 ⁻ % B220 ^{hi}	<i>Female</i>	59.32±3.57	65.16±3.75	68.16±2.98
	<i>Male</i>	49.18±8.86	55.80±2.31	57.18±5.71
B220 ^{low} % Total B220	<i>Female</i>	76.24±2.35	67.48±6.75	75.12±2.76
	<i>Male</i>	68.42±3.94	71.64±4.15	71.72±5.40
B220 ^{low} CD24 ⁺ % B220 ^{low}	<i>Female</i>	84.44±1.50	81.50±2.59	76.36±5.32
	<i>Male</i>	84.70±1.98	83.66±1.22	77.18±1.21*
B220 ^{low} CD24 ^{hi} % B220 ^{low}	<i>Female</i>	22.78±0.95	27.02±1.78	21.84±2.41
	<i>Male</i>	19.32±1.23	17.56±2.67	12.24±0.37*
B220 ^{low} CD24 ^{low} % B220 ^{low}	<i>Female</i>	62.30±1.27	55.54±2.11	54.38±6.02
	<i>Male</i>	65.64±1.07	66.74±1.99	67.02±1.59

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 2B. Bone Marrow Analysis

Bone Marrow		0 µg/kg Mean±SEM	40 µg/kg Mean±SEM	80 µg/kg Mean±SEM
B220 ^{low} CD24 ⁻	<i>Female</i>	14.56±1.39	17.00±2.37	22.14±5.05
% B220 ^{low}	<i>Male</i>	14.54±1.91	15.66±1.22	21.98±1.28*
B220 ^{low} CD24 ⁺ AA4.1 ⁺	<i>Female</i>	59.92±1.86	72.22±3.69	76.98±7.32
% B220 ^{low} CD24 ⁻	<i>Male</i>	58.54±3.84	58.86±3.25	71.54±2.55*
B220 ^{low} CD24 ⁺ AA4.1 ⁻	<i>Female</i>	39.78±1.75	27.62±3.67	22.90±7.27
% B220 ^{low} CD24 ⁻	<i>Male</i>	41.46±3.84	41.04±3.24	28.28±2.49*
B220 ^{hi} IgM ⁺	<i>Female</i>	66.46±2.57	63.74±2.22	61.46±4.68
% B220 ^{hi}	<i>Male</i>	72.76±1.77	66.34±1.73	72.24±1.97
B220 ^{hi} IgM ⁻	<i>Female</i>	33.54±2.57	36.26±2.22	38.54±4.68
% B220 ^{hi}	<i>Male</i>	27.24±1.77	33.66±1.73	27.76±1.97
B220 ^{low} IgM ⁺	<i>Female</i>	22.26±1.34	25.66±3.12	28.12±4.27
% B220 ^{low}	<i>Male</i>	22.34±0.19	20.52±0.29*	21.92±0.60
B220 ^{low} IgM ⁻	<i>Female</i>	77.74±1.34	74.34±3.12	71.84±4.28
% B220 ^{low}	<i>Male</i>	77.64±0.20	79.48±0.29*	78.08±0.60

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 3. Thymus T cell Differentiation

Thymus		0.0 µg/kg Mean±SEM	40.0 µg/kg Mean±SEM	80.0 µg/kg Mean±SEM
CD8+CD4 ⁻ (%)	<i>Female</i>	4.30±0.96	5.80±1.13	6.86±0.67
	<i>Male</i>	2.60±0.46	2.72±0.34	2.02±0.33
CD4+CD8 ⁺ (%)	<i>Female</i>	53.66±6.67	54.58±5.52	30.32±3.55*
	<i>Male</i>	61.38±7.97	56.92±5.25	49.76±8.64
CD4-CD8 ⁻ (%)	<i>Female</i>	27.94±9.72	20.58±2.17	38.50±4.29
	<i>Male</i>	26.84±9.52	30.94±6.00	39.42±10.14
CD4+CD8 ⁻ (%)	<i>Female</i>	14.12±3.05	19.00±3.17	24.32±2.65
	<i>Male</i>	9.20±1.28	9.42±0.96	8.80±1.28

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 4A. Splenic B cell Subset Analysis

Spleen			0.0 µg/kg Mean ± SEM	40.0 µg/kg Mean ± SEM	80.0 µg/kg Mean ± SEM
Total B220 %	Female		27.02 ± 0.98	31.98 ± 3.36	28.76 ± 4.84
	Male		29.68 ± 3.20	25.84 ± 3.11	25.02 ± 1.78
B220 ^{hi} % Total B220	Female		24.88 ± 1.40	33.86 ± 2.07*	33.40 ± 2.85*
	Male		27.12 ± 2.69	28.98 ± 2.58	29.78 ± 2.60
B220 ^{low-int} % Total B220	Female		75.12 ± 1.40	66.14 ± 2.07*	66.60 ± 2.85*
	Male		72.88 ± 2.69	71.02 ± 2.58	70.22 ± 2.60
CD24 ^{int} CD21 ^{hi} (MZ) % Total B220	Female		13.64 ± 1.35	19.36 ± 3.08	13.22 ± 1.50
	Male		10.36 ± 0.81	13.94 ± 1.40	15.18 ± 1.66
CD24 ^{hi} CD21 ^{hi} (T2) % Total B220	Female		6.36 ± 0.67	5.26 ± 0.85	4.60 ± 0.50
	Male		8.22 ± 1.07	9.32 ± 0.95	8.70 ± 1.60
CD24 ^{hi} CD21 ^{neg} (T1) % Total B220	Female		10.92 ± 0.82	9.24 ± 1.48	8.96 ± 0.50
	Male		14.58 ± 2.07	12.36 ± 1.09	13.72 ± 1.76
CD24 ^{low-int} CD21 ^{int} (FO) % Total B220	Female		63.30 ± 2.18	55.76 ± 3.28	61.62 ± 1.96
	Male		60.92 ± 3.52	56.82 ± 1.98	54.82 ± 4.42

n = 5 mice/treatment/sex, * = p < 0.05, Dunnett's test

Table 4B. Splenic B cell Subset Analysis

Spleen			0.0 µg/kg Mean ± SEM	40.0 µg/kg Mean ± SEM	80.0 µg/kg Mean ± SEM
CD23 ^{hi} CD1 ^{hi} (T2) % Total B220	Female		4.94 ± 0.73	5.74 ± 1.43	4.32 ± 0.55
	Male		4.14 ± 0.43	4.90 ± 0.41	6.18 ± 1.16
CD23 ^{neg} CD1 ^{hi} (MZ) % Total B220	Female		5.94 ± 1.14	7.48 ± 1.52	7.48 ± 2.05
	Male		5.26 ± 0.34	5.90 ± 0.67	8.70 ± 1.24*
CD23 ^{neg} CD1 ^{neg} (T1) % Total B220	Female		20.50 ± 0.29	28.52 ± 2.20*	26.52 ± 1.91*
	Male		29.64 ± 4.42	30.12 ± 1.73	31.10 ± 3.85
CD23 ^{pos} CD1 ^{low-int} (FO) % Total B220	Female		66.14 ± 1.88	55.94 ± 3.31	58.62 ± 4.20
	Male		58.68 ± 4.68	56.90 ± 2.17	51.84 ± 3.78
IgD+ %	Female		35.28 ± 4.40	26.82 ± 1.28	29.34 ± 1.24
	Male		33.42 ± 3.24	31.48 ± 2.59	24.74 ± 0.77*
IgD- %	Female		64.72 ± 4.40	73.18 ± 1.28	70.66 ± 1.24
	Male		66.58 ± 3.24	68.52 ± 2.59	75.26 ± 0.77*
CD19+ CD5+ %	Female		6.82 ± 0.78	5.04 ± 0.40	7.26 ± 1.31
	Male		5.00 ± 0.85	6.52 ± 0.80	12.36 ± 2.40*

n = 5 mice/treatment/sex, * = p < 0.05, Dunnett's test

Table 5. Splenic T cell Subset Analysis

Spleen		0.0 µg/kg Mean ±SEM	40.0 µg/kg Mean ±SEM	80.0 µg/kg Mean ±SEM
CD4+	(%) <i>Female</i>	25.74 ±1.16	20.26 ±1.47	20.78 ±2.44
	<i>Male</i>	19.48 ±2.54	22.62 ±1.90	22.10 ±2.74
CD8+	(%) <i>Female</i>	6.18 ±0.56	5.62 ±0.77	5.24 ±0.77
	<i>Male</i>	5.16 ±1.09	6.72 ±1.04	5.60 ±0.95
CD4-CD8-	(%) <i>Female</i>	27.94 ±9.72	20.58 ±2.17	38.50 ±4.29
	<i>Male</i>	26.84 ±9.52	30.94 ±6.00	39.42 ±10.14
Vβ17 ^a +TCR	(%) <i>Female</i>	3.94 ±0.63	3.52 ±0.49	5.20 ±0.64
	<i>Male</i>	3.22 ±0.12	4.12 ±1.24	8.66 ±0.70*
CD4+Vβ17 ^a +TCR (%)	<i>Female</i>	3.02 ±0.32	2.42 ±0.39	5.40 ±1.02*
	<i>Male</i>	2.88 ±0.36	3.36 ±0.86	8.64 ±0.39*
CD4+Vβ8+	(%) <i>Female</i>	4.30 ±0.42	3.92 ±0.52	3.66 ±0.25
	<i>Male</i>	3.16 ±0.51	2.56 ±0.40	3.44 ±0.45

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

Table 6. Histopathology Scores of the Kidney

Kidney		0 µg/kg Mean ±SEM	40 µg/kg Mean ±SEM	80 µg/kg Mean ±SEM
Perivascular inflammation	<i>Female</i>	1.40 ±0.51	3.00 ±0.32*	1.60 ±0.40
	<i>Male</i>	0.20 ±0.20	1.80 ±0.73*	2.00 ±0.32*
Interstitial inflammation	<i>Female</i>	0.80 ±0.20	2.00 ±0.45*	1.40 ±0.40
	<i>Male</i>	0.60 ±0.24	0.80 ±0.37	1.40 ±0.51
Glomerular sclerosis	<i>Female</i>	1.40 ±0.51	2.60 ±0.60	2.00 ±0.45
	<i>Male</i>	1.40 ±0.24	1.00 ±0.00	2.40 ±0.68
Glomerular crescent	<i>Female</i>	1.40 ±0.51	2.20 ±0.73	1.80 ±0.37
	<i>Male</i>	1.20 ±0.20	1.80 ±0.58	2.40 ±0.68
Glomerular parietal hyperplasia	<i>Female</i>	1.40 ±0.24	2.00 ±0.63	1.80 ±0.37
	<i>Male</i>	2.80 ±0.20	2.40 ±0.60	3.20 ±0.20
Proliferative Glomeruli	<i>Female</i>	3.20 ±0.20	3.40 ±0.24	3.20 ±0.20
	<i>Male</i>	2.20 ±0.20	2.20 ±0.37	2.40 ±0.51
Tubules casts	<i>Female</i>	1.20 ±0.49	2.20 ±0.73	1.60 ±0.24
	<i>Male</i>	0.40 ±0.24	0.40 ±0.24	1.00 ±0.55

n = 5 mice/treatment/sex, * = $p < 0.05$, Dunnett's test

GENERAL CONCLUSIONS AND FUTURE WORK

TCDD continues to generate concern due to its persistence as an environmental toxicant, its mode of bioaccumulation within the food chain (with food from animal origin being the major source), and its broad toxicity. Perinatal exposure of human infants is of particular concern because dioxins have been detected in the placenta and cord blood, demonstrating their ability to target the developing fetus (Wang et al. 2004). Additionally, they are mobilized from adipose tissues and accumulate in stores in the breast milk during lactation, further increasing perinatal exposure. Compared to adults, it is estimated that the daily intake of dioxins and dioxin-like compounds for breast-fed babies is 1–2 orders of magnitude higher (van Leeuwen et al. 2000). Epidemiological studies provide compelling evidence that the risk of developing a chronic non-transmittable disease in adulthood is influenced not only by genetic and adult life-style factors but also by environmental factors to which an individual is exposed during the periconceptual, fetal, and infant phases of life. Collectively, these developmental factors influence the risk for chronic diseases, such as diabetes, cardiovascular disease, and obesity during adulthood (Rhind et al. 2003; Gluckman & Hanson 2004; Jirtle & Skinner 2007).

Environmental factors appear to also influence the development and programming of the immune system (Holladay & Smialowicz 2000; Zhang & Jiang 2005; Dietert & Piepenbrink 2006; Luebke et al. 2006). TCDD has been implicated in a wide range of toxicological biological effects, including alterations in metabolic pathways, immunological changes and immunotoxicity, wasting syndrome, chloracne, hepatotoxicity, gastric lesions, carcinogenesis, reproductive toxicity,

teratogenicity and embryo toxicity (Poland and Knutson, 1982; Maronpot et al., 1986; Safe, 1986; Birnbaum, 1994). TCDD is also a well described developmental toxicant manifested by reduced viability, structural alterations, growth retardation, and functional alterations (Vos et al. 1974; Birnbaum et al. 1985; Fine et al. 1989; Couture-Haws et al. 1991; Peterson et al. 1993; Theobald & Peterson 1997). Concern over the potential toxic effects of dioxins on the immune system is based on the critical role the immune system has on an individual's survival. It is well recognized that a TCDD perinatally-exposed mouse has long-term immunosuppression into adulthood. This would result in an increased incidence and severity of infectious diseases as well as some types of cancer (Vos et al. 1974; Faith & Moore 1977; Thomas & Hinsdill 1979; Holladay et al. 1991; Gehrs & Smialowicz 1999). Conversely, perinatal TCDD exposure can also cause inappropriate enhancement of the immune function or the generation of misdirected immune responses that can precipitate or exacerbate the onset of allergies and autoimmune diseases (Vorderstrasse et al. 2006; Mustafa et al. 2008). Thus, both suppression and hypersensitivity of immune system are possible outcomes and can represent potential immunotoxic effects of TCDD. In the studies reported here, we observed that developmental exposure to TCDD clearly affects permanent immune function later in life (age 24–36-48 weeks) in both C57BL/6 and SNF₁ models in a sex-dependent manner.

At present, it is well documented that the biological and pathological manifestations of dioxins are mediated primarily by the *AhR* (Poland & Knutson 1982; Safe 1990; Hogaboam et al. 2008). In fact, *AhR*-deficient mice (knockout) and

AhR-deficient cell lines are resistant to the biological and pathological manifestations of dioxins even at very high doses, supporting the pathologic role of the *AhR* (Fernandez-Salguero et al. 1996; Schmidt et al. 1996; Thurmond et al. 1999; Tijet et al. 2006). Thus, the central role of the *AhR* in dioxin is to elicit toxicity directly through dioxin response elements (DREs) or indirectly through secondary signaling cascades. (Sun et al. 2004; Majora et al. 2005; Frericks et al. 2006). Additionally, the unique phenotype of *AhR*-deficient (-/-) mice with respect to normal growth, development, differentiation and organ functions supports an important contribution by the *AhR* (Fernandez-Salguero et al. 1995; Schmidt & Bradfield 1996). Additional studies have shown that the absence of the *AhR* alters normal gene expression indicating that constitutive expression of genes can also be dependent on the *AhR* (Karyala et al. 2004; Frericks et al. 2006; Tijet et al. 2006). Therefore, a low affinity *AhR* mouse (i.e. SNF₁) and the high affinity *AhR* mouse (i.e. C57BL/6) differ substantially in their *AhR*-dependent gene expression even without TCDD exposure. Furthermore, the level and activity of the *AhR* appears to be regulated by changes in cell differentiation stages, growth factors, cell activation, diurnal cycle, and prior exposure to receptor agonists (Hayashi et al. 1995; Wanner et al. 1995; Liu et al. 1996; Pollenz 1996; Vaziri et al. 1996; Crawford et al. 1997; Vaziri & Faller 1997; Richardson et al. 1998; Shimba et al. 1998). More importantly, dioxin-dependent gene regulation has shown a high degree of cell specificity in response to the *AhR* even within different cell types of a single organ (i.e., thymus) (Majora et al. 2005; Frericks et al. 2006). Thus, the idea that TCDD has a specific signature across all different cell types is not plausible. Together, these observations

would suggest that dioxin's complex ability to elicit toxicity is not limited to *AhR* affinity and genetic background in a responsive C57BL/6 mice model and a non-responsive SNF₁ mice model, but also to the cell specificity and tissue specificity within the same strain model.

The *AhR* plays a critical role in normal development and physiology including the establishment of a competent immune system. Inappropriate modulation or duration of *AhR* activation during fetal and neonatal development results in alterations in immune function that persist into adulthood. The responsiveness to TCDD exposure is dependent on a variety of factors in addition to the affinity / efficacy of *AhR* for TCDD. Previous studies have shown that TCDD response outcome is influenced by the levels of *AhR* expression in cells, as well as the concentration of the selecting ligand. Thymic atrophy and bone marrow hypocellularity have been observed at higher doses of prenatal TCDD, while at lower doses of prenatal TCDD, functional alterations are manifested in adult offspring in the absence of detectable alterations in the cellular composition of primary and secondary immune organs (Vorderstrasse et al., 2004, 2006). This would suggest that *AhR* signaling is altered during immune system ontogeny. The end result would be a reprogramming of the immune system such that certain responses would be inappropriately elevated and other responses suppressed.

The polymorphism determining the responsiveness to TCDD exposure reflects the heterogeneity of *AhR* concentrations and characteristics of the *AhR* (e.g., affinity and efficacy). Thus, the genetic locus that expresses the physiological form of *AhR* polymorphism also controls the biological responses to TCDD. For instance, a

number of inbred mouse strains differ quantitatively in their responsiveness to TCDD. TCDD elicits its effects at about 8-fold lower concentrations in the more responsive mouse strain C57BL/6 than in the less responsive SNF₁ mouse strain. Similarly, C57BL/6 mice (responsive strain, *Ah^{bb}* genotype) are 10-fold more sensitive to TCDD than DBA/2 mice (nonresponsive strain, *Ah^{dd}* genotype), whereas heterozygous B6D2F1 mice (*Ah^{bd}* genotype) show an intermediate sensitivity (Poland & Glover 1980). Thus, *Ah^{bb}* C57BL/6 mice represent the prototype "responsive" strain and are the most sensitive to TCDD toxicity, whereas *Ah^{dd}* SNF₁ mice represent a "nonresponsive" strain and require considerably higher doses of TCDD to produce the same toxic effect. However, the affinity of the human *AhR* for TCDD appears to be lower compared to rodents. For instance, cultured human embryonic palatal cells were approximately 200 times less sensitive than mouse palatal cells with respect to the inducibility of *CYP1A1* by TCDD (Manchester et al. 1987; Ema et al. 1994; Abbott et al. 1999). Although such observations might imply that human tissues would be less susceptible to the toxic effects of TCDD, the *AhR* in human cells has also been shown to have considerable heterogeneity (Perdew & Hollenback 1995; Wang et al. 1995). Additional studies on the human *AhR* should increase our knowledge of its functional properties and its role in mediating altered cell- and tissue-specific responses elicited by TCDD and related chemicals. This would then allow extrapolation between animal studies and humans.

Prenatal TCDD Exposure Effects on Thymus

Thymic involution is a well known biomarker of TCDD and related HAH exposure in all species examined (Vos et al. 1997). The paradigm has been that perinatal TCDD exposure causes thymic atrophy and thymocyte hypocellularity accompanied by an increase in the percentage of thymocyte CD4⁻CD8⁻ and CD4⁻CD8⁺ (spCD8⁺) as well as a decrease in the percentage of thymocyte CD4⁺CD8⁺ T cells by late gestation (Fine et al. 1990; Fine et al. 1990; Holladay et al. 1991). Further, these studies have shown that by postnatal day (PND) 1, the percentage of cells in CD4/8 subsets in TCDD-treated groups had been restored to normal levels with the exception of a persistent increase in CD4⁻CD8⁺ T cells (Camacho et al. 2004). A change in thymocyte surface antigen expression was not observed at any of the later endpoints (Holladay et al. 1991). The relative thymic weight was significantly reduced in PND 1 pups born from TCDD-treated dams. By PND 14, changes in relative thymic weight were no longer detectable in the mice (Holladay et al. 1991; Camacho et al. 2004). These studies showed that late-gestation thymic atrophy is severe in mice; however this effect is transient and the thymus appears to recover shortly after birth.

In our studies, however, we observed that thymocyte phenotypic expressions were still altered in 24-week-old SNF₁ females after 80.0 µg/kg TCDD prenatal exposure. This was characterized by decreased percentages of CD4⁺CD8⁺ thymocytes, and increased CD4⁻CD8⁻ thymocytes. Similarly, thymocyte phenotypic expressions were altered in 24-week-old C57BL/6 females perinatally exposed to 5.0 µg/kg TCDD, characterized by decreased percentages of CD4⁺CD8⁺ thymocytes,

and increased CD4⁺CD8⁻ thymocytes. In addition, the phenotypic changes in TCDD-exposed female mice were accompanied by a relative decrease in thymic weight and cellularity. At 36 weeks-of-age, SNF₁ mice also responded differently by sex to early TCDD exposure, and to a large degree in a similar manner to the 24 week study. Specifically in females, the percentage of CD4⁺CD8⁺ double positive thymocytes decreased in the 80.0 µg/kg TCDD mice while 48-week-old C57BL/6 mice exhibited increased CD4⁻CD8⁻ thymocytes in females and decreased CD4⁺CD8⁺ thymocytes in males. However, the changes in relative thymic weight and thymic cellularity were no longer detectable in the 36-week-old SNF₁ females that received 80.0 µg/kg prenatal TCDD. We propose a new TCDD prenatal thymic exposure outcome paradigm, characterized by either increased CD4⁻CD8⁻ thymocytes or decreased CD4⁺CD8⁺ thymocytes, or both, and affecting mainly females. Our studies provide compelling evidence that the assumption that the thymus rebounds soon after birth from TCDD prenatal exposure effects is inaccurate and that thymic involution is more dynamic than previously perceived. However, the precise mechanisms leading to this persistent thymic atrophy and phenotypic alteration at this age have yet to be fully investigated.

In this study of SNF₁ and C57BL/6 mice thymic atrophy and thymic hypocellularity did not persist beyond 24 weeks-of-age. While increased apoptosis plays a role in TCDD-induced thymic atrophy in the adult mouse (Kamath et al. 1997), this phenomenon also contributes to thymic atrophy following prenatal exposure to TCDD, and is mediated by the death receptor pathway involving Fas, TRAIL, and DR5 (Camacho et al. 2004). Recently, this same lab demonstrated that

the *AhR* regulates FasL and NF-kappaB in stromal cells, which in turn play a critical role in initiating apoptosis in thymic T cells (Camacho et al. 2005). Since in this case TCDD affected thymic stroma but did not directly affect thymocytes, this supported the findings of (Kremer et al. 1994) who showed that the nonlymphoid compartment (i.e. stroma) rather than the thymocytes were probable targets of these HAHs.

Frazier et al. (1994) showed that TCDD-induced thymic atrophy could be mediated, at least in part, by damage to prethymic T cell progenitor stem cells in both bone marrow and liver. Although our studies showed changes in the bone marrow, our focus was in B cell development and not prethymic T cell progenitor stem cells in bone marrow, a hypothesis yet to be tested in our two models.

Prenatal TCDD exposure induces a severe thymic atrophy and alters thymocyte differentiation and maturation as indicated by decreased thymic weights and changes in expression of thymic differentiation markers and proportion of thymocyte subpopulations, leading to reduced numbers of double positive (DP)(CD4⁺CD8⁻) cells and skewing towards CD8⁺ cells in all laboratory animals examined (Vos et al. 1997). Several hypotheses have been proposed to account for mechanisms underlying the TCDD-elicited thymic phenotype. There is compelling evidence to support the idea that TCDD may directly target intrinsic properties of thymocytes by preventing the differentiation of CD4⁺CD8⁺ T cells and the transition phase from CD4⁺CD8⁺CD24⁺ to CD4⁺CD8⁺ (Fine et al. 1989; Kerkvliet & Brauner 1990; Lundberg et al. 1990; Holladay et al. 1991; Blaylock et al. 1992; Gehrs et al. 1997; Gehrs & Smialowicz 1997). This idea is further supported by the observation

that TCDD treatment can lead to thymic atrophy by altering cell cycle via an increase in the percentage of thymocytes in the G₁ phase of the cell cycle and a significant decrease in the percentage of S + G₂/M thymocytes, especially in the CD4-CD8-CD3- triple-negative intrathymic precursor cell population (Laiosa et al. 2003).

A recent study demonstrated that TCDD not only interferes with the development of fetal double negative (DN) CD4-CD8⁻ thymocytes, but also changes the differentiation and emigration pattern of immature thymic T cell subpopulations, specifically DN CD25^{int/lo}CD44^{hi} cells (Majora et al. 2005). Gene expression analysis of immature thymic T cells has identified an array of gene expression profiles that differ based on specific subpopulations, suggesting that *AhR* activation is dependent on the cell-type, cell stage and possibly the concomitant use of other transcription factors (Hundeiker et al. 1999; Frericks et al. 2006; McMillan et al. 2007). Interestingly, a recent study using a thymic epithelial cell line proposed the existence of crosstalk, at the promotor regions of TCDD-responsive genes involving cell-specific transcription factors, between *AhR* signaling, the estrogen signaling pathway and the hypoxia signaling pathway (Frericks et al. 2008). Clearly, the thymic atrophy induced by TCDD exposure *in vivo* is mediated by multiple mechanisms operating on the thymocytes as well as the thymic epithelial cells. It may be that no single mechanism would explain the long-term thymic atrophy associated with prenatal TCDD exposure. Therefore, it is reasonable to propose that multiple mechanisms may perform in harmony to produce thymic atrophy and delayed thymocyte maturation.

The specific mechanism leading to thymic phenotypic alterations following perinatal TCDD exposure is still unknown. Whether or not a gene becomes a direct target of the activated *AhR* is dependent on the presence of so-called DREs in its promoter, and on cell type and cell differentiation stage, i.e., overall accessibility of a particular locus (Okino & Whitlock 1995). In addition, the activation of the *AhR* can directly target genes via secondary effects. Genes capable of being induced, directly or indirectly, by the *AhR* in thymocytes are, among others, Notch-1, IL-2, bcl-2, CD44 and adseverin, all of which are known for their role in thymocyte differentiation and survival (Kremer et al. 1995; Jeon & Esser 2000; Kronenberg et al. 2000; Svensson et al. 2002). In our study, the thymic parameter changes were more predominant in the females which may raise the question of the role of other factors like estrogen, a hormone that has been demonstrated to alter the thymus (Barr et al. 1982; Rijhsinghani et al. 1996; Zoller & Kersh 2006). For example, estrogen (e.g., 200 µg of β-estradiol-17-valerate) has been demonstrated to induce thymic atrophy, due to effects on thymocytes as well as thymic epithelial cells (Barr et al. 1982; Rijhsinghani et al. 1996; Zoller & Kersh 2006). Thus, it is possible that prenatal TCDD exposure followed by rising endogenous estrogen levels during adulthood could contribute to the immune dysregulation in the thymus. The significant thymic changes might be contributed to TCDD toxicity effects on critical hormones. Several studies have shown that epidermal growth factor, prolactin, thyroid hormones, and growth hormones influence the development of specific organs, and their signaling pathways may be modulated by perinatal exposure to TCDD (Gray et al., 1999).

Prenatal TCDD Exposure: Effects on Autoreactive T cells

The present studies demonstrated that lymph nodes and spleens from two animal models showed an expansion of autoreactive T helper cells with TCR V beta genes including V β 17a⁺ TCR and V β 3⁺ TCR following prenatal TCDD. Investigators have identified several T cells carrying T cell receptor (TCR)-variable regions associated with self-reactivity in TCDD-treated animals. The TCR-variable, (V β) chains are usually deleted in the thymus by reaction with self-MHC and minor lymphocyte stimulatory antigens (Okuyama et al. 1992; Hanawa et al. 1993) and have been associated with autoimmunity in some experimental mouse models (Rocha et al. 1992). Another study observed altered thymic epithelial distribution of major histocompatibility complex (MHC) class II molecules in TCDD-treated mice, an effect that was hypothesized as having potential to cause defective thymocyte selection (De Heer et al. 1994). Furthermore, Dong et al. (1997) found that TCDD down-regulated an MHC class I gene (*Q1^b*) in a mouse hepatoma cell line, while Vorderstrasse et al. (2003) found that exposure to TCDD increased the expression of MHC class II molecules on the splenic dendritic cells (Vorderstrasse et al. 2003). These effects on MHC class I and II molecules by TCDD raised questions regarding the ability of TCDD to impair autoreactive thymocyte deletion (Holladay 1999). These observations might explain the elevated levels of autoreactive T cells expressing V β 17a⁺ and V β 3⁺ TCR in the lymph nodes and spleen in the present mice. Such an increase in T cells associated with autoreactivity has been suggested as a mechanism by which estrogen may promote autoimmunity (Silverstone et al. 1994). Alternatively, the increase in autoreactive T cells has been suggested as a possible

mechanism by which TCDD may promote autoimmunity by inducing extrathymic T-cell differentiation in the liver and other lymphoid organs (Okuyama et al. 1992; Silverstone et al. 1994). Our studies would appear to support the hypothesis of increased extrathymic-derived autoreactive T cells, because we did not observe such increased TCR-variable (V β) chains in thymus, but found these cells infiltrated into the livers of treated C57BL/6 mice.

Prenatal TCDD Exposure: Effects on B Cell Lymphopoiesis

An impetus for our studies was to generate novel data designed to narrow the gap in literature investigating the possible effects on development, function and maturation of B cells following early TCDD exposure. A significant finding in this regard was the persistent alteration of B lymphopoiesis in bone marrow and spleen. The most predominant observations were increased early B cell progenitors (B220^{low}CD24-AA4.1⁺) in the bone marrow, increased splenic marginal zone B cells in C57BL/6 mice at 24 weeks-of-age, increased follicular B cells in SNF₁ mice at 24 weeks of age and alteration of transitional 1 B cells or/and transitional 2 B cells beyond 24 weeks in treated mice. Such observations suggest prenatal TCDD exposure permanently alters BCR signaling. In support of this idea, increased serum autoantibody levels were observed in TCDD-treated mice compared to controls. These findings are significant based on recent reports that have redefined the function of B cells in autoimmunity. The role of the B cell pathogenesis in SLE and similar immune-mediated glomerulonephritis (GN) has conventionally been limited

to antibody production, yet recent studies have suggested that B cells contribute to the pathogenesis of autoimmune diseases through various pathways. They can function as potent antigen-presenting cells (APCs), and their capacity for rapid clonal expansion makes them highly efficient activators of antigen-specific T cells in this role. More recently, new evidence suggests that B cells also play a critical role in the production of cytokines and mediatory regulators required to support an inflammatory immune response (Fujimoto & Sato 2007). Thus, B cells may orchestrate the local expansion of T cells and dendritic cells during a certain stage of inflammation.

Prenatal TCDD Exposure: Effects on B Cell Tolerance

The increased presence of autoantibodies in SLE-like SNF₁ mice and emergence of autoantibody-producing autoreactive B cells in non-immune prone C57BL/6 mice following prenatal TCDD suggest that a major abnormality underlying disease development may be breakdown in one or more of the mechanisms which maintain B cell tolerance. Thus, the failure to purge autoreactive cells during B cell lymphopoiesis, the abnormal exposure to self-antigen and the dysregulation of B cell activation thresholds have all been linked to the pathogenesis of SLE and other autoimmune models. Most, if not all, of the detailed mechanisms implicated in the loss of B cell tolerance, previously mentioned in chapter 1, are potential candidates to explain the mechanism for induction of postnatal autoimmunity by prenatal TCDD. Prenatal TCDD exposure might dysregulate B cell tolerance by modulating programmed cell death (apoptosis) components and signaling (bcl-2 family, Fas-

FasL interaction, FcγRIIb and BAFF family), damaging self apoptotic disposal system like complements, altering BCR signaling apparatus (CD19, CD22, CD21, FcγRIIb), disturbing the endocrine system (estrogen, prolactin, thyroid, testosterone) and modulating antigen presenting molecules (MHC I, MHC II).

Prenatal TCDD Exposure: Effects on Th1/Th2 Balance

The present study also focused on whether prenatal TCDD exposure could alter T cell function by modulating Th1/Th2 balance. To begin to evaluate this, we measured the secreted levels of five major cytokines from Con A-stimulated splenocyte supernatants. Our data clearly show that prenatal TCDD exposure increased the level of IFN-γ in mice at 24 weeks-of-age in Con-A-stimulated splenic lymphocytes. This suggests dysregulation of cytokine production by T cells is caused by prenatal TCDD, including a skew towards Th1 activity especially in the males. At 48 weeks, prenatal TCDD exposure increased the level of IFN-γ and IL-12 in 5.0 μg/kg TCDD female and 2.5 μg/kg TCDD male Con-A-stimulated splenic lymphocytes. On the other hand, levels of IL-10 and IL-12 were increased in 5.0 μg/kg TCDD male Con-A-stimulated lymphocytes compared to control cytokine levels. These observations suggest a skew towards Th1 cells in 5.0 μg/kg TCDD females and 2.5 μg/kg TCDD males.

The increase in IFN-γ caused by perinatal TCDD exposure may be crucial, since this cytokine has been implicated in a number of autoimmune conditions. In several animal models of autoimmunity, IFN-γ appears to play a vital role in the

development of lupus nephritis (Haas et al. 1997), autoimmune insulinitis (Campbell et al. 1991), Sjogren's syndrome (Hayashi et al. 1996) and autoimmune arthritis (Billiau 1996). However, IL-10 is considered the classic SLE disease biomarker. Serum levels of IL-10 are commonly elevated in SLE patients and increased IL-10 correlates well with SLE disease activity (Park et al. 1998). Several studies have reported high levels of IL-10 in SLE patients and in murine models of lupus (Hagiwara et al. 1996; Horwitz et al. 1998). However, recent studies suggest IL-10 may play a more important role in SLE pathogenesis response than previously perceived. B cell production of IL-10 has been shown to be important in controlling inflammation in a number of experimental models of autoimmune disease including collagen-induced arthritis (Mauri et al. 2003), experimental allergic encephalomyelitis (Fillatreau et al. 2002), inflammatory bowel disease (Mizoguchi et al. 2002) and lupus (Lenert et al. 2005). Furthermore, other studies identified IL-10 as a regulatory cytokine that can reduce macrophage-mediated inflammation (Fiorentino et al. 1991) and regulate the Th1/Th2 balance (Pestka et al. 2004). This idea is supported by the observations that continuous overexpression of IL-10 in young mice significantly delayed antinuclear autoantibody production and decreased clinical nephritis in a murine congenic model of SLE (Blenman et al. 2006; Amu et al. 2007). Thus, an increased production of IFN- γ and a decreased production of IL-10 may play a role early in disease pathogenesis in SLE, whilst elevated levels of IL-10 might reflect disease activity and a regulatory mechanism once inflammation has been initiated. Based on these observations, it is possible that 5.0 $\mu\text{g}/\text{kg}$ TCDD males exhibited predominant characteristics of a type III

hypersensitivity lupus-like autoimmune disease whereas 5.0 µg/kg TCDD females and 2.5 µg/kg TCDD males skewed toward other types of autoimmune disease in addition to lupus-like autoimmune, or a different mechanism leading to immune-mediated glomerulonephritis autoimmunity.

Recent studies have focused on the role of type I IFNs (IFN- α and IFN- β) in lupus. Increasing evidence suggests that type I IFNs may play a critical role in the pathogenesis of lupus. Investigators have proposed that type I IFNs are important in the activation of myeloid DCs, heightening their antigen presenting capability and leading to the activation of autoreactive T cells, which in turn leads to SLE pathogenesis (Banchereau & Pascual 2006). Other studies have shown that type I IFNs also stimulate the upregulation of TLR7 in DCs (Siegal et al. 1999; Banchereau et al. 2000; Blanco et al. 2001; Steinman et al. 2003; Banchereau et al. 2004) and may do the same in B cells, potentially enhancing the response to self-antigen. Type I IFNs also induce the production of B cell activity factor BAFF, which may stimulate autoreactive B cell proliferation (Litinskiy et al. 2002) and enhance the ability of CD4 T cell to provide B cell help (Le Bon et al. 2006). Future work in our laboratory will investigate a wide array of cytokines including type I IFNs, IL-5, IL-6, IL-9, IL-13, IL-17 and IL-16 at both molecular and protein levels using multiple specific mitogens targeting T cells, B cells and other specific subpopulations of immune cells including the B1a cell, MZ B cell, FO B cell, CD4⁺ T cell and DC.

In summary, results from current study demonstrate that prenatal TCDD exposure during a sensitive prenatal time of immune system ontogeny, can cause

persistent immunologic changes that are manifested in adulthood. The culmination of our data suggests that developmental exposure to TCDD may produce a fundamental disruption in establishment of central self-tolerance and peripheral self-tolerance. Such dysregulation would be predicted to exacerbate and increase susceptibility to autoimmune disease. The magnitude of the autoimmunity risk and autoimmunity aggravation appears to be influenced by binding capacity of TCDD to the susceptibility allele (*AhR*), by genetic predisposition to autoimmune disease, and to some degree by sex. Collectively, these results suggest that individuals expressing combined risk factors including autoimmune predisposed-genetic factors, who are exposed to TCDD during gestational and postnatal development, may be at increased risk of developing autoimmune disease. Although the regulatory processes and sites of immune ontogeny are similar despite the variation in gestational lengths in mice and humans, careful extrapolation of the current data into a human prenatal TCDD exposure risk assessment will be required.

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