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PRENATAL TCDD IN MICE INCREASES ADULT AUTOIMMUNITY

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Abstract

Two immunologically-different mouse strains, C57BL/6 and SNF₁, were exposed to a mid-gestation dose of TCDD. The C57BL/6 mouse has a high-affinity aryl hydrocarbon receptor (*AhR*) and is sensitive to TCDD. The SNF₁ mouse has a low-affinity *AhR* but spontaneously develops autoimmune nephritis. Autoreactive V β ⁺CD4⁺17a and V β ⁺CD3⁺ T cells were increased at 24-weeks-of-age in offspring of C57BL/6 mice, more so in females than males. The cytokine IFN- γ was elevated in the females, while IL-10 was elevated in males. Phenotypic changes in B-lineage cells were present in bone marrow and spleen, and circulating autoantibodies were increased after prenatal TCDD. Kidneys of males showed significant anti-IgG and anti-C3 deposition, suggesting early-stage autoimmune disease. The SNF₁ offspring similarly showed increased peripheral V β ⁺ cells in the females, increased autoantibody production in both sexes, and increased IFN- γ production in females. Male SNF₁ mice had increased anti-IgG and anti-C3 deposition in kidneys. Both mouse models therefore showed clear signatures of enhanced autoimmunity after prenatal TCDD.

Keywords

TCDD; autoimmunity; developmental; C57BL/6; SNF₁; lupus nephritis

1.1 Introduction

Reports evaluating the developmental immunotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin; TCDD) began appearing in the literature about 1977, when perinatal exposure of mice was found to produce a more severe thymic atrophy than a similar exposure in the adult [1]. Subsequent studies in mice described transient postnatal T-lineage phenotypic changes and suppressed cytotoxic T lymphocyte responses, which were reported to resolve by 8–10 weeks after birth [2,3]. Gehrs and Smialowicz [4] later reported permanent suppression of the delayed-type hypersensitivity (DTH) response in rats after prenatal exposure to TCDD. In the latter studies, F344 rats were dosed on gestation day 14 with 0.1 μ g/kg TCDD, and the male offspring displayed significant inhibition of DTH responses as late as 14 months of age, the last endpoint examined. The female offspring also showed depressed DTH responses at 14 months, but were not as sensitive as males, requiring a 3-fold higher (0.3 μ g/kg) maternal TCDD dose.

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Beyond immune suppression, a progression of reports in the literature suggests TCDD may increase risk of autoimmune responses by altering T cell selection. Greenlee et al. [5] and Schuurman et al. [6] described thymic epithelial targeting by TCDD, which led both to propose that TCDD may have potential to alter epithelium-dependent deletion of autoreactive thymocytes. DeWaal et al. [7] observed altered thymic epithelial distribution of major histocompatibility complex (MHC) class II molecules in TCDD-treated mice, leading these authors to also suggest possible altered thymocyte deletion. Fisher et al. [8] later reported enhanced thymic negative selection of T cells and increased autoreactive T cell release from the thymus of mice treated with TCDD. Given these observations, it may be noteworthy that similar patterns of inhibited thymic T cell differentiation occur spontaneously in some autoimmune mouse models, in mice treated *in vivo* with monoclonal antibodies to the thymic MHC class I and class II molecules that govern selection, and in TCDD-treated mice [9]. We therefore hypothesized that prenatal exposure of mice to TCDD may, in addition to selective immunosuppression, produce immune modulation suggestive of enhanced autoimmunity.

1.2 C57BL/6 and SNF₁ mouse studies

The present studies used two immunologically different mouse models in an attempt to mimic humans who may display both a high susceptibility to TCDD, and a genetic predisposition for developing a lupus-like autoimmune disease. The C57BL/6 mouse has a high affinity aryl hydrocarbon receptor (*AhR*), and as such is highly sensitive to TCDD and related ligands of the *AhR*. The SNF₁ mouse has a low affinity *AhR* (*Ah^{dd}*), making it less sensitive to TCDD, but spontaneously develops lupus-like autoimmune nephritis. Thus, if C57 mice showed an enhanced autoimmune profile at low doses of TCDD, and SNF₁ mice did the same at higher doses of TCDD, this outcome would suggest an individual who has both a high-affinity *AhR* and genetic programming toward autoimmune disease, may be at higher risk for immune modulation than an individual showing only one of these predispositions.

The thymus was collected from C57BL/6 mice that were prenatally-exposed to 0, 2.5 or 5.0 mg/kg TCDD, and screened MHC class II antigen protein expression. This analysis was performed by Western blot using an affinity-purified anti-mouse MHC class II antibody tagged with a secondary HRP goat anti-rat IgG. The MHC antigen expression numerically and visibly increased in the mice that received developmental TCDD exposure (Fig. 1). Western blot analyses of protein from TCDD-treated thymic stromal tissue at 10, 24 and 36 weeks yielded the same trend toward increasing expression of MHC Class II (data not shown). These data contribute to the above reports suggesting the possibility of altered thymic selection in mice developmentally exposed to TCDD.

The relative percent expression of specific autoreactive T cell clones was next examined in the spleen and thymus of the study mice, to detect possible altered T cell selection. The autoreactive phenotypes chosen for evaluation were CD4⁺ 17a variable beta (V β) and CD3⁺ V β T cells. Silverstone et al. [10] previously found increased numbers of these T cell phenotypes in the livers of young adult mice dosed with TCDD, which was the basis for the present selection. Table 1 shows that both V β phenotypes were increased in the periphery of adult C57BL/6 mice that were prenatally exposed to TCDD. These data exhibit a dose-response pattern almost in every case, with the females being more affected than males. There was as much as a 4-fold increase of autoreactive cells in the spleen of treated female mice. This could be an important observation, in that a small number of these cells may be required to manifest an autoimmune response, if activated. The female tendency for increased autoreactive T cells is in contrast to the reduced male response in DTH activity,

reported by Gehrs and Smialowicz [4], but is consistent with the general increased tendency of females to express autoimmune diseases.

An important question that remains to be answered is the origin of increased peripheral V β + T cells in the 24-week-old study mice that were prenatally exposed to TCDD. Given the above-described effects of TCDD on thymic epithelium and MHC antigen expression, a persistent effect on thymocyte negative selection may exist and result in elevated peripheral V β + cells. Alternately, Silverstone et al. [10] hypothesized that TCDD may drive T cell development, including negative selection, into extrathymic compartments where deletion of autoreactive cells is inefficient. With this in mind, routine histopathology in the present C57BL/6 mice detected atypical centers of lymphocytic infiltration in the livers at adulthood (Fig. 2). It is not yet known if these centers may include extrathymic T lymphopoiesis.

The presence of phenotypic differences in T cells at 24 weeks in the prenatal TCDD-exposed mice suggested possible co-existing functional differences in these cells. Also, a skewed Th1/Th2 cytokine balance has been associated with pathology in several autoimmune disorders. Therefore select cytokines were examined in Con A-activated splenic T cells from prenatally exposed mice. Interferon-gamma (IFN- γ) was increased in the adult female offspring but not in the males (Fig. 3), suggesting a sex-dependent skewing toward Th1 activity. Increased IFN- γ has been linked to disease development in several models of autoimmunity, including lupus nephritis, autoimmune insulinitis, Sjogren's syndrome, and autoimmune arthritis [11–13].

In the male but not female mice, interleukin-10 (IL-10) was also elevated at 24-weeks-of-age (Fig. 3). This cytokine has been considered the hallmark systemic lupus erythematosus (SLE) marker; serum levels of IL-10 are elevated in SLE patients and increased IL-10 correlates well with disease activity [14]. These IFN- γ and IL-10 data infer that a permanent dysregulation of cytokine production was imprinted into T cells by the prenatal TCDD, in a direction that indicates increased risk of disease development.

Very limited information is available in the literature regarding transient or persistent B cell effects from prenatal exposure to dioxin. However, B cells may contribute to autoimmunity by several mechanisms, including autoantibody production, altered antigen presentation, or modulation of other immune cells through inappropriate cytokine production. Four surface markers, B220/CD45R, IgM, CD24, and AA4.1, were selected to provide a preliminary view of B lymphopoiesis in the adult bone marrow. When combined these 4 surface markers allow for the characterization of the multi-lineage and common lymphoid progenitor cells (B220⁻, CD24⁻, IgM⁻, AA4.1⁺), the B lineage progenitor and Pro B Cells (B220^{lo}, CD24^{lo}, IgM⁻, AA4.1⁺), large Pre-B cells (B220^{lo}, CD24^{hi}, IgM⁺, AA4.1⁺) small Pre-B cells (B220^{hi}, CD24^{hi}, IgM⁺, AA4.1⁺) and immature B cells (B220^{hi}, CD24^{hi}, IgM⁺, AA4.1⁻) [23–24]. In females, prenatal TCDD significantly increased B lineage committed progenitor cells, Pro-B cells, and large Pre-B cells, while decreasing the more mature small Pre-B and immature B cell populations. Males were less affected, showing an increase only in B lineage committed progenitors (Fig. 4). These data suggest a permanent change in immature B cell development, including possible inhibited differentiation of these cells toward more mature phenotypes. This profile of effect has some similarities to that seen with T cells, where bone marrow T progenitors (prothymocytes) are decreased [15] and thymic T precursors (thymocytes) are both diminished and display inhibited differentiation [16]. Similar to T cells, critical steps in B cell selection and receptor editing occur during these stages of differentiation, including deletion of lambda or kappa light chains from the B cell receptor [17].

Adult B cell differentiation in the spleen was also altered by prenatal TCDD. Females displayed increased transitional 1 (T1) B cells, while in males, T2 B cells were increased. T1 cells represent recent immigrants from the bone marrow, are believed to be direct precursors of T2 B cells, and are commonly increased in SLE patients [18]. Both sexes also displayed enhanced shifting of B cells to the marginal zone (MZ), identified as B220^{hi}, IgD^{lo}, CD23⁻, CD21^{hi}, and CD1d^{hi}. This splenic phenotype is characterized by a lower activation threshold, and is enhanced in murine models of SLE [19].

Thus, several changes in B cells, after prenatal TCDD, suggested possible enhanced self-directed activity in these cells. We therefore examined the profile of autoantibody production in the 24-week-old mice. In all cases these mice either showed trends toward higher serum autoantibodies against double stranded DNA, single stranded DNA, and cardiolipin, or produced significantly higher levels (Fig. 5). Since immune complex deposition in the kidney is a common signalment in mouse lupus models, immunofluorescence staining to elucidate IgG and C3 deposition was next performed. Female and male mice that received prenatal TCDD showed increasing, dose-related trends for both autoantibody IgG and C3 immune complex deposition at 24-weeks-of-age (Fig. 6). By 48-weeks-of-age, IgG and C3 immune complex deposition were significantly increased in the male offspring, at both the 2.5 and 5.0 $\mu\text{g}/\text{kg}$ prenatal TCDD doses (unpublished data). Such deposition is not a normal observation in C57BL/6 mice, and is visual evidence of initiation of autoimmunity. In particular, autoantibodies produced by B cells tend to display strong polyreactivity to DNA and glomerular substrate, and are frequently localized as immune deposits in kidneys [20].

Therefore, C57BL/6 mice that received a single prenatal dose of TCDD displayed several signs of autoimmunity in adulthood. These included increased autoreactive V β T cells in the periphery, altered cytokine profile suggestive of enhanced immune reactivity, increased autoantibody production by B cells, and signs of an immune-mediated autoimmune disease or glomerulonephritis in a murine strain not normally known to express this condition. Given these observations, prenatal TCDD exposure was next evaluated in SNF₁ hybrid mice.

1.3 Prenatal TCDD exposure in autoimmune SNF1 mice

The incidence of nephritis is low in autoimmune NZB mice, but when this strain is crossed with normal SWR mice, the female SNF₁ hybrids develop glomerulonephritis that is fatal by about 8 months of age. Onset of clinical disease is much slower in the male offspring, beginning at about 1 year of age. The progression of disease in both sexes is then critically dependent on accelerated antibody production subsequent to inappropriate activity of CD4⁺ T helper cells, and histopathologically characterized by IgG deposition in kidney glomeruli [21]. This genetic predisposition to a disease that showed signs of being induced in non-autoimmune C57BL/6 mice after prenatal TCDD, made the SNF₁ model of value for parallel developmental studies.

Using the same study design as for the C57BL/6 mice, pregnant female SWR \times NZB:F₁ (SNF₁) mice were administered a single oral dose of 40 or 80 mg/kg TCDD on gestation day 12. The TCDD-exposed SNF₁ offspring showed a number of noteworthy immune changes at 24 weeks of age, including increased peripheral V β ⁺ T cells in the females, increased autoantibody production in both sexes, and increased IFN- γ production by T cells in females [22]. Histopathologically, the control female mice were in early stages of autoimmune nephritis, while control males showed almost no autoantibody IgG or C3 immune complex deposition, consistent with normal progression of disease in these mice (Fig. 7). The female mice showed a visual suggestion of increased deposition, and also numeric, but non-

significant, increased deposition of both immune complexes. For males, both autoantibody IgG or C3 immune complex deposition showed dose-dependent and significant increases. These results suggest prenatal TCDD may exacerbate autoimmune nephritis in SNF₁ lupus-like female mice, and induce early expression of disease in the males. This pattern of result was almost identical to that displayed by the C57BL/6 mice, where females showed trends toward increased nephritis and males showed significantly enhanced disease.

1.4 Conclusions

As initially stated, the C57BL/6 and the SNF₁ mouse models were selected to represent TCDD-sensitive and genetically autoimmune segments of the human population, respectively. Both models showed clear signatures of enhanced autoimmunity after prenatal TCDD. This outcome suggests that prenatal exposure to TCDD or other environmental *AhR* ligand, during development of the immune system, may elevate risk of autoimmune responses in individuals who express the combination of a high-affinity *AhR* and a genetic predisposition for autoimmune disease.

Abbreviations

AhR	aryl hydrocarbon receptor
DTH	delayed-type hypersensitivity
IFN-γ	interferon-gamma
IL	interleukin
MHC	major histocompatibility complex
SLE	systemic lupus erythematosus
T1	transitional 1
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
Th	T helper
Vβ	variable beta

Acknowledgments

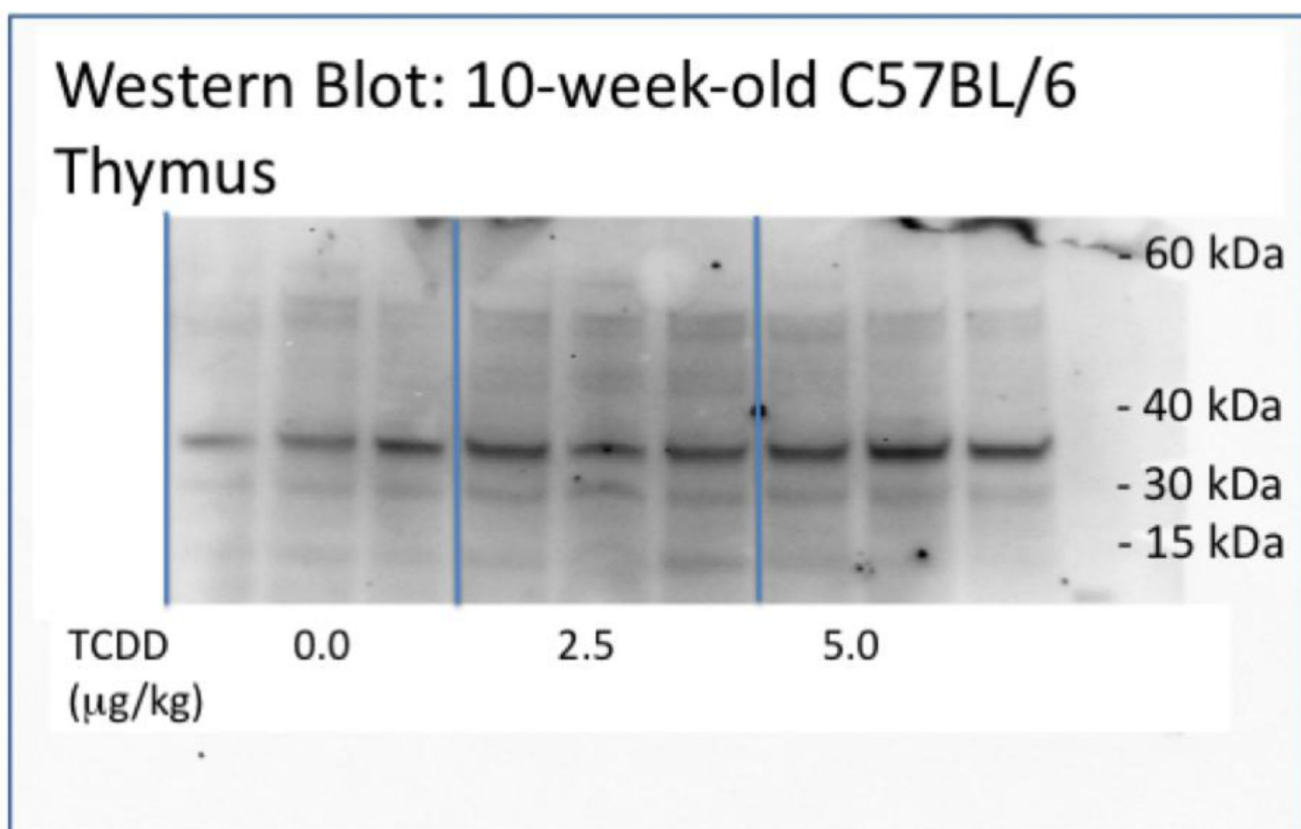
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(TCDD) alters adult B cell lymphopoiesis and exacerbates autoimmune lupus in 24-week-old SNF₁ mice. *Toxicological Sci.* 2009b; 112:133–143.



Treatment	0.0 µg/kg	2.5 µg/kg	5.0 µg/kg
Density (# of pixels)	24504 ± 5353	37251 ± 1061	35782 ± 3250

Figure 1. Representative western blot of the thymus. Thymic stromal tissue was collected from 10-week-old C57BL/6 mice prenatally exposed to 0.0, 2.5 or 5.0 µg/ml TCDD, and stored at -80°C in RNAlater. Protein was isolated from each tissue, quantitated and normalized across all samples. The protein samples were separated on a NuPage Novex Bis-Tris mini gel, blot transferred, probed with an affinity-purified anti-mouse MHC class II antibody and tagged by a secondary HRP goat anti-rat IgG. (n=3 mice/treatment)

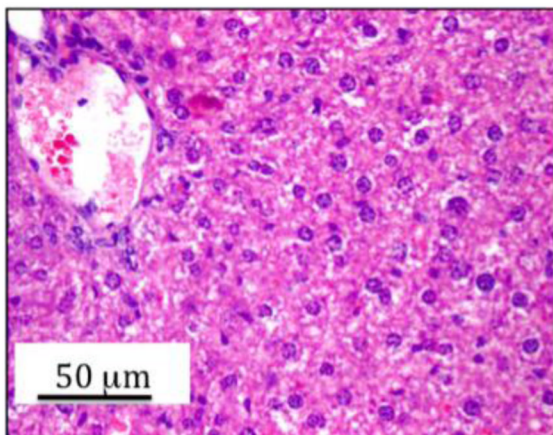
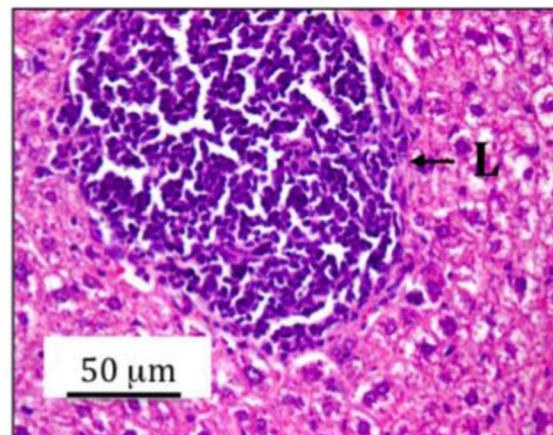
0.0 $\mu\text{g}/\text{kg}$ TCDD**5.0 $\mu\text{g}/\text{kg}$ TCDD**

Figure 2. The livers from 24-week-old C57BL/6 mice that were prenatally exposed to 0.0 or 5.0 $\mu\text{g}/\text{kg}$ TCDD were collected, fixed, sectioned and H&E stained. The right image shows a representative lymphocytic center of infiltration (L) in the TCDD group.

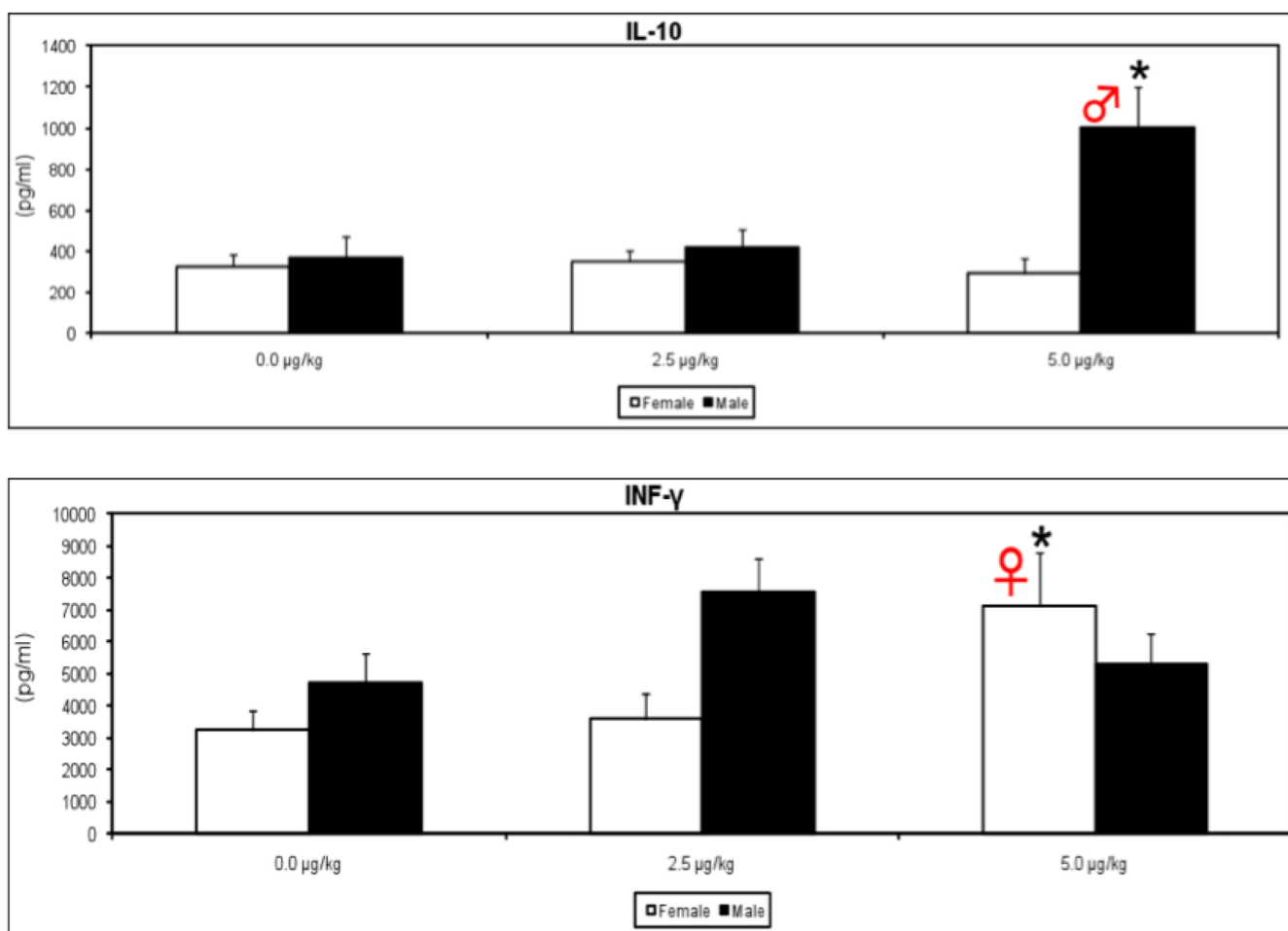


Figure 3.

Supernatants were collected from splenocytes of 48-week-old mice that were prenatally exposed to 0.0, 2.5 or 5.0 $\mu\text{g}/\text{kg}$ TCDD, after culturing for 48h with Con A (10 $\mu\text{g}/\text{mL}$). The levels of IL-10 and INF- γ were determined using a commercially-available murine cytokine ELISA kit. (n=5 mice/treatment/gender, * $p \leq 0.05$, Dunnett's t -test).

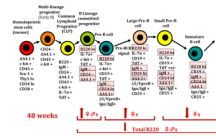


Figure 4. B-cell lymphopoiesis in the bone marrow. The cell-surface markers highlighted under the specific cell types were the ones evaluated in this study. Arrows pointing up indicate significantly increased, and arrows pointing down indicate significantly decreased (n=5 mice/treatment/gender, * $p \leq 0.05$, Dunnett's t -test). Modified from Mustafa et al., 2008 [23].

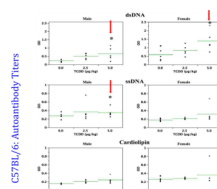
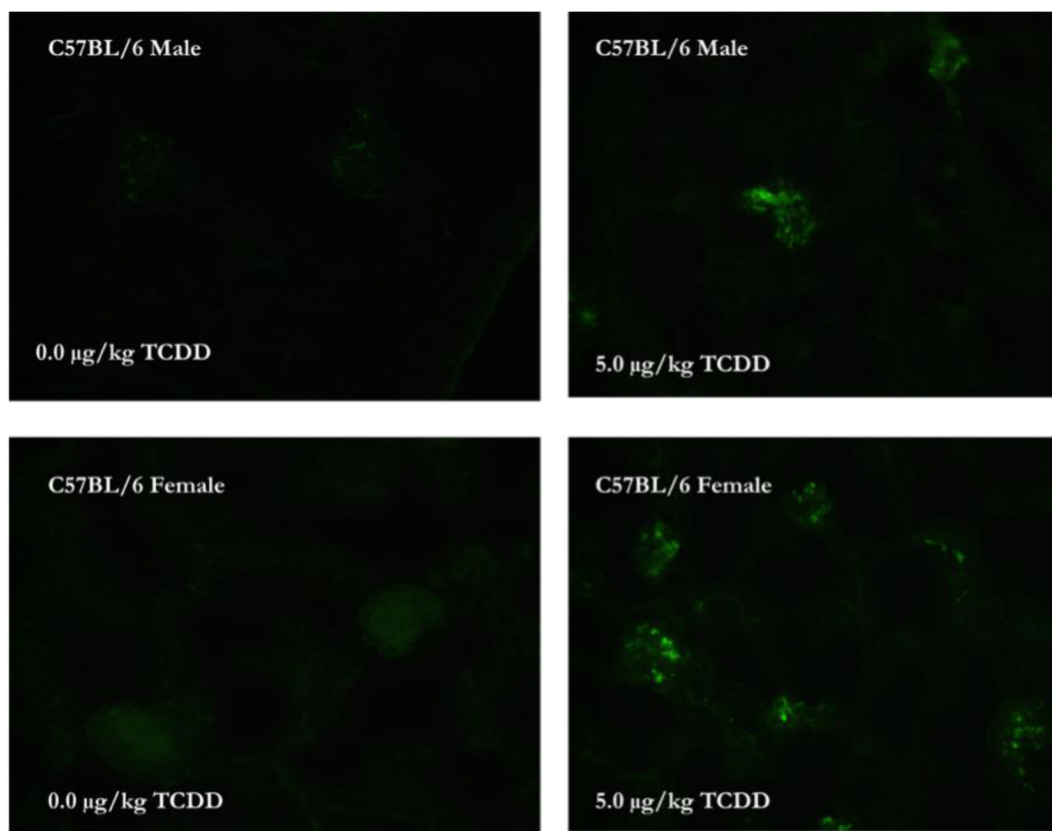


Figure 5.

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 (A), ssDNA (B) and cardiolipin (C), (n=5/gender; * $p \leq 0.05$, Dunnett's t -test). Each horizontal line represents the arithmetic mean for each treatment group. Modified from Mustafa et al., 2008 [23].



IgG deposition

TCDD dose	Mean±SE ♀s	Mean±SE ♂s
0 µg/kg	0.4±0.24	0.4±0.24
2.5 µg/kg	1.0±0.31	0.6±0.24
5.0 µg/kg	1.2±0.37	1.2±0.31

C3 deposition

TCDD dose	Mean±SE ♀s	Mean±SE ♂s
0 µg/kg	0.4±0.24	0.2±0.20
2.5 µg/kg	0.6±0.24	0.6±0.24
5.0 µg/kg	1.2±0.37	1.0±0.31

Figure 6.

The kidneys from 24-week-old C57BL/6 mice that were prenatally exposed to 0.0 or 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 (bottom left) and male (top left) or 5.0 µg/kg TCDD-exposed female (bottom right) and male (top right) mice. The table data show the mean±SEM of the IgG and C3 disposition scores of 5 mice/treatment/gender. Modified from Mustafa et al., 2008 [23].

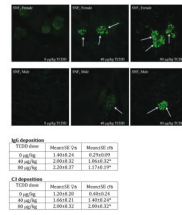


Figure 7. Kidneys from 24-week-old SNF1 mice that were prenatally exposed to 0, 40, or 80 μg/kg TCDD were collected, fixed, sectioned, and stained with FITC-labeled anti-IgG or anti-C3. The above are representative images of kidneys stained with FITC-anti-IgG based on treatment and sex. Arrows indicate regions of heavy glomerular IgG deposition. The data are based on five mice per treatment per sex * $p \leq 0.05$, Dunnett's t -test). Modified from Mustafa et al., 2009b [24].

Table 1

Variable-beta positive T cells in the spleen and lymph nodes of C57BL/6 mice following prenatal exposure to TCDD. Modified from Mustafa et al., 2008 [23].

Splenic T cells			
	0 µg/kg	2.5 µg/kg	5.0 µg/kg
Vβ17a ⁺ (%)	♀ 2.76±0.54 ♂ 3.67±0.58	5.44±1.25 4.82±0.48	7.83±1.94* 4.76±0.29
Vβ3 ⁺ (%)	♀ 2.02±0.50 ♂ 2.92±0.71	4.56±0.73 5.02±0.96	10.08±2.49* 5.32±0.54
Lymph node T cells			
Vβ17a ⁺ (%)	♀ 6.05±0.28 ♂ 3.55±0.28	7.25±0.90 4.93±1.02	8.73±0.84* 5.35±0.54
Vβ3 ⁺ (%)	♀ 5.07±0.40 ♂ 5.23±0.41	6.35±1.04 6.35±1.04	8.24±1.23* 8.08±1.03*

Data are mean±SE for N=5 mice/treatment/sex;

* $p \leq 0.05$, Dunnett's *t*-test