PART III

RESULTS
CHAPTER 3: TETRACHLORODIBENZO-P-DIOXIN (TCDD) INHIBITS DIFFERENTIATION AND INCREASES APOPTOTIC CELL DEATH OF PRECURSOR T CELLS IN THE FETAL MOUSE THYMUS

E.G. Besteman*1, K.L. Zimmerman1, S.D. Holladay1

1Virginia Tech
College of Veterinary Medicine
Duckpond Drive
Blacksburg, VA  24061-0442

*Corresponding author: address shown above
Telephone: 540-231-6471
FAX: 540-231-6033

ebestema@vt.edu

Running title: TCDD increases fetal thymocyte apoptosis

Published in J Immunotox 2:107-114, 2005
3.1 **Abstract** 2,3,7,8-Tetrachlorodibenzo-\(p\)-dioxin (TCDD) causes thymic atrophy as well as alterations in thymocyte maturity in mice. Multiple mechanisms for thymic hypocellularity have been suggested, and include an increase in thymocyte apoptosis, a maturation arrest of thymocyte development, inhibited thymocyte proliferation, and a diminution of seeding of the thymus by the hematopoietic progenitors in the fetal liver or adult bone marrow. Fetal mice are highly sensitive to hypocellularity induction by TCDD when the chemical is administered during the window of thymic development, between days 10 and 18 of gestation. Treatment of pregnant C57Bl/6 mice in the present experiments with doses of 5 or 10 \(\mu\)g/kg TCDD by oral gavage on gestation days 14 and 16 severely depressed day 18 thymic cellularity. Histopathologic evaluation of day 18 fetal thymi showed disruption of the normal organ architecture with loss of clear distinction between cortical and medullary regions after TCDD. A decrease in thymocyte density was noted in all regions, and was most dramatic in the cortical zones where pyknotic cells were increased by TCDD treatment. Using day 18 thymocyte suspensions and flow cytometry, the marker 7-AAD showed a decrease in viable thymocytes from TCDD-treated fetal mice, and a concomitant and dose-related increase of thymocytes in early apoptosis. Specifically, relative to control, thymocytes from the 5 and 10 \(\mu\)g/kg TCDD exposure groups displayed 1.9% and 5.3% respective increases in early apoptotic cells. When thymocytes were co-identified by CD4 and CD8 cell surface antigen expression, the enhanced apoptosis occurred in the CD4\(^+\)CD8\(^+\) phenotype with no significant apoptosis seen in the CD4\(^-\)CD8\(^-\), CD4\(^+\)CD8\(^-\), or CD4\(^-\)CD8\(^+\) thymocytes. Given the rapid clearance of apoptotic cells from the thymus, these histopathologic and cytometric data suggest increased thymocyte apoptosis contributes to fetal thymic atrophy after TCDD exposure.
3.2 Introduction

2,3,7,8-Tetrachlorodibenzo-\textit{p}-dioxin (TCDD), an environmental contaminant, is one of the most immunotoxic of the halogenated aromatic hydrocarbons. TCDD results as an inadvertent by-product of chemical synthesis, combustion, and manufacturing. Environmental or occupational human TCDD exposure, or accidental exposures at higher levels such as those in Seveso, Italy, lead to concern for the effects of this agent which is also a known carcinogen, teratogen, and endocrine disrupter (reviewed by Birnbaum and Tuomisto, 2000; reviewed by Mann, 1997).

TCDD has multiple immunotoxic effects, influencing both humoral and cell-mediated immunity. These effects are demonstrated in many animal species (reviewed by Couture et. al., 1990). Developing T cell mediated immune function may be especially sensitive to TCDD (Holladay and Luster, 1994; Holladay and Smialowicz, 2000). TCDD crosses the placenta and is present in milk, leading to routes of pre- and postnatal exposure for the fetus and neonate (Nau and Bass, 1981; Nau et al., 1986).

Prothymocytes of fetal liver origin enter the outer cortex of the thymus and slowly migrate to the medulla, undergoing changes in surface receptors and markers which are characteristic of their maturation (Suniara et al., 2000; reviewed by Manley, 2000; reviewed by Owen et al., 2000). Interactions between the thymocytes and the other cells of the thymus, the stromal cells, epithelial cells, and dendritic cells, then lead to selection of thymocytes. Autoreactive cells are either deleted via apoptosis or silenced in their activity. Since only 2% of
these cells that enter the thymus survive selection and emigrate to the periphery, this organ has a well developed phagocytic function for eliminating apoptotic cells (Elgert, 1996).

Multiple mechanisms have been offered as the cause(s) of TCDD-induced thymic atrophy, and include increased thymocyte apoptosis (Kamath et al., 1997, 1999; Dearstyne and Kirkvliet, 2002; Camacho et al., 2004), alterations of normal thymocyte differentiation (Holladay et al., 1991; Blaylock et al., 1992), antiproliferative activity (Comment et al., 1992), and reduction in fetal thymus hematopoietic progenitors in the fetal liver (Fine et al., 1988; 1990). Regarding the effects on hemopoietic precursors, progenitor T cell targeting by TCDD resulted in decreased seeding of the thymus, an event clearly related to thymic atrophy (Fine et al., 1990). The contribution of additional mechanisms to thymic atrophy caused by TCDD, including a possible enhancing effect on thymocyte apoptosis, has remained less clear. Large numbers of thymocytes move through the thymus during perinatal establishment of this organ, thus we evaluated developmental TCDD exposure as a model for detection of an in vivo effect of TCDD on thymocyte apoptosis.

3.3 Materials and Methods

Animal Model. Eight-week-old C57Bl/6 timed pregnant female mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) on the morning of gestational day 14 (gd 14). These mice were arbitrarily distributed into control and treatment groups, with 5 in the control group and 6 in the TCDD treated group for each experiment. The mice were housed from gestational days 14-16 in groups of 3-5 per cage and provided with Harlan 2018 Teklab Global 18% Protein Rodent Diet (Harlan) and distilled water ad libitum. A constant temperature of 21 ± 2 °C was
maintained, and a 14.5/9.5 hour light/dark cycle used in the facility. On day 16, mice were weighed prior to final dosing and then housed individually until time of sacrifice on gestational day 18. These and all other procedures for these mice were reviewed and approved by the Virginia Tech Animal Care and Use Committee prior to initiation of the experiments.

Chemical Exposure. TCDD (AccuStandard, Inc., New Haven, CT) was dissolved in corn oil (Sigma Aldrich, St. Louis, MO) to concentrations of 0.9 µg/mL (for the 5 µg/kg exposure) and 1.8 µg/mL (for the 10 µg/kg exposure) and administered in a volume of 120 – 200 µL by oral gavage. Doses were administered on gd 14 and 16 based on the body weight of the mice. Control mice were administered comparable volumes of plain corn oil by oral gavage on the same dates.

Tissue Collection. On gd 18, mice were sacrificed by cervical dislocation and fetal mice collected. Total fetal weight per litter as well as total fetal number per litter were recorded. Fetal thymi were removed and placed in pre-weighed culture dishes containing 2 mL RPMI (Sigma Aldrich, St. Louis, MO). Dishes were re-weighed (Mettler Toledo PB303, Carlton Scale, Roanoke, VA), and then stored in the refrigerator until all thymi were collected from all fetal mice. Two thymi from each litter were then arbitrarily removed from culture dishes and submersed in 10% neutral buffered formalin for later histopathologic evaluation.

Cell Enumeration and Flow Cytometry. Thymocytes from remaining thymi of each litter were released by mechanical disruption using curved forceps on steel sieve screens (Sigma Aldrich) and the cells collected into 2 mL of RPMI. One mL of RPMI was added and the cells washed at
1200 rpm for 5 min (IEC Centra GP8R, International Equipment Company, Needham Heights, MA). Cells were washed once more in 3 mL of RPMI and then resuspended in Hanks Buffered Salt Solution (Cambrex BioScience Walkersville, Inc., Walkersville, MD) for counting on a CASY 1 cell counter (Cell Tools Inc., San Francisco, CA). After enumeration, cells were resuspended at a concentration of 5 x 10^6 cells per mL. One hundred µL of cells (0.5 x 10^6) from each sample were placed into individual tubes containing anti-mouse CD4 antibodies labeled with phycoerythrin (PE; 0.2 µg/tube diluted in 100 µL HBSS) and anti-mouse CD8 antibody labeled with fluorescein isothiocyanate (FITC; 1 µg/tube diluted in 100 µL HBSS) and incubated for 30 minutes on ice in the dark. Two mL of HBSS were then added after incubation and the cells washed and resuspended in 400 µL of HBSS. Two and one half µL 7-AAD diluted in 100 µL HBSS were then added to each tube, and tubes were incubated for 20 minutes in the dark on ice. The pooled fetal thymocytes from each litter were then evaluated by flow cytometry (Coulter Epics XL, Miami, FL).

Histopathology. Fetal thymi were fixed in 10% neutral buffered formalin for a minimum of 24 hours prior to processing, embedded in paraffin, sectioned at 6 microns, and stained with hematoxalin and eosin. Thymi were then evaluated microscopically. Sections were assessed for alterations in architecture or cellularity at 100x and 600x magnification, as well as for nuclear condensation (pyknosis) indicative of apoptosis. Mitotic indices in the different cell populations were assessed. Evaluation of the surface epithelial layer as well as the underlying cortex and medulla was performed separately. Proportion of the cortex to the medulla was evaluated as well as the maintenance of architecture including the distinct
cortical/medullary regions. A minimum of four (4) fields of 600x magnification was used to evaluate mitotic indices, pyknotic cells, and cellular density.

The quality of tissue preparation and staining may affect interpretation, therefore those sections considered of poor quality due to fractures in the tissue, folds of tissue, autolysis, or inadequate staining were excluded when evaluating architecture and other indices.

Statistics
The two-tailed Student's *t*-test with \( p < 0.05 \) was applied to flow cytometric data, cellularity data, weight data, and data involving litter size, and histopathologic findings that were numerically evaluated. Data means described as different in this report were significantly different. Some observations, such as alterations of cellular density were not enumerated. Such findings were assessed as being present or absent and thus described.

3.4 Results

*Organ Weights and Cellularity*. Thymic weights were diminished by TCDD treatment at both doses, as was thymic cellularity (Table 3.1). No significant differences in fetal weights or fetal numbers were present in TCDD treated mice when compared with control animals.

*Fetal Thymocyte Expression of CD4 and CD8 antigens and 7-AAD fluorescence*: In addition to severe fetal thymocyte depletion, the TCDD treated cells demonstrated altered patterns of intrathymic differentiation as defined by the percentage of thymocytes expressing surface antigens CD4 and CD8 (Table 3.2). Percentages of cells in the CD4^−^8^−^ and CD4^+^8^+^ populations were increased in a dose-dependent manner by TCDD, while percentages of CD4^”^8^”^ cells were
decreased in a dose-dependent manner by TCDD. Total numbers of cells in all thymocyte phenotypes defined by CD4 and CD8, calculated as the percentage in each phenotype times total thymic cellularity, decreased as a result of the overall hypocellularity caused by TCDD (Table 3.3). This outcome was similar to a previous report where TCDD was given daily throughout a longer period of fetal thymus development (Holladay et al., 1991). TCDD exposure also caused marginally decreased thymocyte viability, as indicated by 7-AAD fluorescence in fetal thymocytes, and a corresponding increase in the percentage of thymocytes in early apoptosis (Figure 3.1A). No statistically significant differences in late apoptotic/necrotic cells were apparent by 7-AAD fluorescence. Widely used as a marker for apoptosis (Konemann et al., 2005; Hayashi et al., 2004; George et al., 2004; Eckstein et al 2005), 7-AAD has been utilized successfully in thymocytes for the detection of apoptosis (Dearstyne and Kirkvliet, 2002; Olgun et al, 2004). Characterization of the apoptotic cells by CD4 and CD8 surface antigens in concert with 7-AAD staining demonstrated a dose-dependent, statistically significant increase in apoptosis in the CD4^+CD8^+ cell population of TCDD treated fetal thymocytes relative to controls (Figure 3.1B). In both control and TCDD treated fetal mice, the CD4^+CD8^+ cell population was the only one to demonstrate significant detectable early apoptosis via 7-AAD staining.

**Histopathology:** TCDD treated thymi were substantially smaller than control thymi as assessed by the proportion of the field covered by tissue at 40x magnification (Figure 3.2B). Control fetal thymi were densely cellular organs within a thin connective tissue capsule (Figures 3.2A, 3.3A and 3.4A). A thin subcapsular population of large polygonal epithelial cells was present forming the lobules of this tissue. This epithelial cell layer surrounded a predominant cortical region that was densely cellular and consisted of deeply staining thymocytes. Nucleoli were present in
approximately half of these cells. The mitotic indices for control thymi were 2.66 ± 0.39 per 600x magnification field. Pyknotic nuclei or tingible bodies were also present at approximately 1-2 per 600x magnification field. A few pale histiocytes cells were found in the cortical region as were scattered vascular channels. The medullary region was much smaller and less cellular and consisted of pale staining histiocytes cells with fewer numbers of thymocytes. Widely scattered large epithelial cells with increased cytoplasmic eosinophilia were noted in the medulla (Hassall's corpuscles).

In TCDD treated thymi, the normal architecture was disrupted with a loss of clear distinction between the cortical and medullary regions (Figure 3.3B and 3.4B). The cortical:medullary ratios were also altered as the cortical area was relatively diminished. A decreased density of cells was noted in all areas, but was particularly marked in the cortical region. Increased numbers of pyknotic nuclei and tingible bodies were seen with an average of 6 per 600x magnification field in the cortical region. Decreased mitoses were noted with only 0.88 ± 0.22 mitotic figures per 600x magnification field (significantly different at p<0.05). In the TCDD treated thymus, epithelial cells had a diffuse vacuolation and an increased cytoplasmic eosinophilia with increased amounts of cytoplasm and larger nuclei. No inflammatory response was noted within the organ.

3.5 Discussion

Fetal thymic atrophy with pronounced thymocyte depletion and a shift of thymocyte phenotype toward immaturity were caused by the present developmental TCDD exposure. These results agreed with previous studies, and provided a model system of induced thymic
hypocellularity during a time of highly active thymocyte seeding and maturation, that might facilitate \textit{in vivo} detection of an effect of TCDD on thymocyte apoptosis.

Enhanced apoptosis plays a part in deletion of peripheral (i.e., mature) T-cells after TCDD exposure (Dearstyne and Kerkvliet, 2002; Camacho et al., 2001, 2004). However the literature is divided regarding the contribution of increased pre-T cell (thymocyte) apoptosis to TCDD-induced thymic atrophy. Early studies of rat thymocytes exposed \textit{in vitro} to TCDD suggested that apoptosis may be a contributing factor to thymocyte hypocellularity (McConkey et al., 1988). The rat thymocyte suspensions, when incubated with TCDD, had increased cytosolic \textit{Ca}^{2+} concentration and DNA fragmentation that were consistent with increased apoptotic cell death. Treatment with \textit{Ca}^{2+} free media or protein synthesis inhibitors (blocking endonuclease activation) negated these effects. Later authors suggested they were not able to fully replicate these results, however (Comment et al., 1992).

Kamath et al. (1997) later reported increased thymocyte apoptosis at 8-12 hours after TCDD administration in C57Bl/6 mice, but not at 24 hours or beyond. In that maximal thymic atrophy occurs in these mice about 96 hours after the TCDD exposure, the authors suggested that rapid clearance of apoptotic cells by phagocytic cells \textit{in vivo} might explain the lack of detected increased apoptosis after 12 hours. Subsequently, Kamath et al. (1999) dosed C57Bl/6 wild-type (+/+), Fas-deficient (lpr/lpr) and Fas-ligand defective (gld/gld) mice with TCDD, and found TCDD induced thymic atrophy in the wild-type mice but not the mice with compromised apoptotic pathways. These authors noted in the same report that caspase inhibitors blocked TCDD-induced thymocyte apoptosis, both \textit{in vivo} and \textit{in vitro}.

In contrast to the above reports, Staples, et al. (1998) used \textit{lck}^{pr\cdot}bcl-2 transgenic mice to evaluate the protective capability of this anti-apoptotic gene in TCDD treated mice. Over-
expression of bcl-2 did not abate the thymic atrophy induced by TCDD, leading the authors to suggest thymocyte apoptosis may not make a meaningful contribution to total thymic atrophy in these mice. In a related study, Lai et al (2000) compared CH3/bcl-2 transgenic mouse fetal thymus organ culture with wild type fetal thymus culture after treatment with TCDD. Thymocyte development inhibition was noted in both cultures, however reduction of cell number was similar regardless of bcl-2 status. TCDD did not induce increased thymocyte apoptosis in these cultures as measured by the TUNEL assay and corroborated by pulse field gel electrophoresis at post-exposure time points of 48, 72 and 120 hours. TCDD did block entry of the cell into S-phase of the CD4 CD8\(^{-}\) population. Regarding such transgenic models that over-express bcl-2, however, it should be considered that multiple pro- and anti-apoptotic genes are affected by TCDD and a single over-expressed anti-apoptotic gene may not adequately represent complex molecular events regulating entry of cells into apoptosis.

Using developmental exposure similar to the present report, Camacho et al. (2004) investigated apoptosis in the fetal mouse thymus at daily time points following single-dose TCDD administration on gd14. Triple staining for TUNEL, CD4 and CD8 showed increased apoptosis after TCDD in all subpopulations defined by CD4 and CD8, with CD4\(^{+}\)CD8\(^{-}\) cells demonstrating the greatest (three-fold) increase in apoptosis. Control thymocytes in this report also demonstrated marked apoptosis in all subpopulations, with the CD4 CD8\(^{-}\) population showing greatest apoptosis (68.5\%) and CD4\(^{+}\)CD8\(^{+}\) cells showing the least (8.2\%). These results in control thymocytes differ somewhat from previous reports of limited apoptosis in all but the CD4\(^{+}\)CD8\(^{+}\) thymocyte population (Jiang et al., 1999; Small and Kraal, 2003), as well as from the present control thymocytes that showed limited apoptosis in non- CD4\(^{+}\)CD8\(^{+}\) thymocyte
populations. The observation of TCDD-increased fetal thymocyte apoptosis in non-CD4⁺CD8⁻ populations also was different from the present report.

Camacho et al. (2004) also demonstrated several pro-apoptotic genes including death receptor genes and pro-apoptotic members of the bcl-2 family, as well as death receptor ligands, that were upregulated in fetal C57Bl/6 mouse thymocytes by TCDD. In contrast, decreased levels of the pro-apoptotic p53 gene, in DBA/2 mice, have been associated with a relative resistance to thymic atrophy caused by TCDD (Yang et al., 1999). Using cDNA microarrays, Fisher et al. (2004) found upregulation of thymocyte genes involved in negative selection after TCDD exposure, including Fas, CD30, and LIGHT. These results may support induction of an apoptotic pathway as a mechanism by which TCDD reduces thymocyte number, however, again, pro-survival genes were also induced by TCDD. Increased expression of anti-apoptotic genes (e.g., bcl-2), may over-ride the apoptotic affect of increased p53 expression (Sharova et al., 2000).

Histopathologic evaluation of the present TCDD treated fetal thymi indicated that TCDD treatment affects the thymic epithelial layer, the cortical region, and the medulla of the thymus, with the most marked changes occurring in the cortical region. The relative depletion of the cortical area leading to a decrease in the cortical:medullary ratio and a loss of architectural distinction of the fetal mouse organ was similar to studies involving adult Wistar rats (DeHeer et al, 1995). In the latter study, SCID mice received human fetal thymus grafts that, after TCDD administration, exhibited similar morphometric changes. The increase in Hassall's corpuscles in TCDD treated fetal mouse thymi is thus consistent with effects in rats and in human fetal thymus grafts into SCID mice. Increased numbers of pyknotic nuclei in the present mice provided visual support for the cytometric observation of increased apoptosis in CD4⁺CD8⁻ cells after TCDD
administration, whereas decreases in mitotic rate indicate that reduced cellular proliferation by
TCDD may also contribute to thymic atrophy.

In summary, the literature has been inconclusive concerning the possibility that enhanced
thymocyte apoptosis may contribute to thymic atrophy after TCDD exposure. The present study
used a sensitive in vivo developmental mouse model to investigate a potential role of apoptosis in
thymic atrophy. Both cytometric and histopathologic evaluation detected a limited shift toward
increased apoptosis. The cytometric increase in apoptotic cells in TCDD treated fetal mice was
not large (5.3%) when compared with the marked decrease in thymic cellularity and organ
weight. However, a continuous diminution in cell viability may have a cumulative effect over
time and thus, apoptosis of fetal thymocytes may have a greater impact on cellularity and organ
atrophy than initial interpretation might suggest. These collective results support the idea that
thymic atrophy production by TCDD is a multifactorial process, and that enhanced thymocyte
apoptosis is a contributing factor.
References:


Kamath, A. B., I. Camacho, P. S. Nagarkatti, M. Nagarkatti (1999) Role of Fas-Fas ligand interactions in 2,3,7,8-tetrachlorodibenzo- p-dioxin (TCDD)-induced immunotoxicity: increased resistance of thymocytes from Fas-deficient (lpr) and Fas ligand-defective (gld) mice to TCDD-induced toxicity. *Toxicol Appl Pharmacol* 160:141-55.


**Table 3.1.** The effect of gestational exposure to TCDD on thymic weight and cellularity.

Thymic weights are in micrograms. Thymic cellularity is x 10⁶ cells per thymus. Weights and cellularity are presented on a per organ basis (mean ± SEM) as the total is divided by the number of individuals in the litter. (A) Comparison of control and 5 µg/kg TCDD treatment. (B) Comparison of control and 10 µg/kg TCDD treatment.

Fetal litter weight, number of pups/litter, individual fetal thymic weight and cellularity on gestation day 18

<table>
<thead>
<tr>
<th></th>
<th>Litter weights (mg)</th>
<th>Number of pups/litter</th>
<th>Thymic weights (µg)</th>
<th>Cellularity¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.5 ± 2</td>
<td>7 ± 2</td>
<td>2.86 ± 0.39</td>
<td>3.80 ± 1.24</td>
</tr>
<tr>
<td>5 µg/kg TCDD</td>
<td>8.1 ± 2.4</td>
<td>6.8 ± 2.1</td>
<td>1.65 ± 0.44*</td>
<td>1.35 ± 0.69*</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.9 ± 0.8</td>
<td>6.8 ± 1.5</td>
<td>3.01 ± 0.75</td>
<td>4.26 ± 1.50</td>
</tr>
<tr>
<td>10 µg/kg TCDD</td>
<td>9.2 ± 2.2</td>
<td>7.8 ± 1.7</td>
<td>1.17 ± 0.10*</td>
<td>1.18 ± 0.57*</td>
</tr>
</tbody>
</table>

¹ Cellularity is x 10⁶ cells

*Significantly different from control, p <.05.
### Table 3.2

The effect of gestational exposure to TCDD on thymocyte populations defined by CD4 and CD8 cell surface antigens. Numbers are percentages (mean ± SEM) of cells within each phenotype. (A) Controls compared to 5 µg/kg TCDD. (B) Controls compared to 10 µg/kg TCDD.

#### Fetal thymocyte surface antigen expression

<table>
<thead>
<tr>
<th>A</th>
<th>CD4^8^-</th>
<th>CD4^8^+</th>
<th>CD4^*8^-</th>
<th>CD4^*8^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.13 ± 3.87</td>
<td>1.83 ± 0.22</td>
<td>2.18 ± 1.30</td>
<td>84.86 ± 3.68</td>
</tr>
<tr>
<td>5 µg/kg TCDD</td>
<td>18.46 + 2.17*</td>
<td>3.69 + 0.76*</td>
<td>1.79 + 0.87</td>
<td>76.04 + 2.26*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>CD4^8^-</th>
<th>CD4^8^+</th>
<th>CD4^*8^-</th>
<th>CD4^*8^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.67 ± 4.25</td>
<td>1.90 ± 0.39</td>
<td>1.38 ± 0.48</td>
<td>86.08 ± 3.75</td>
</tr>
<tr>
<td>10 µg/kg TCDD</td>
<td>25.80 + 11.0*</td>
<td>5.04 + 1.21*</td>
<td>1.72 + 0.75</td>
<td>67.42 + 9.80*</td>
</tr>
</tbody>
</table>

*Significantly different from control, \( p < .05 \).
Table 3.3. Cellularity in thymocyte phenotypes defined by CD4 and CD8 expression, calculated as total cellularity multiplied by the percentage of cells within each phenotype. Numbers are $\times 10^6$ cells per thymus.

Fetal thymocyte numbers in populations defined by CD4 and CD8 expression

<table>
<thead>
<tr>
<th></th>
<th>CD4$^-$</th>
<th>CD4$^+$</th>
<th>CD4$^+$</th>
<th>CD4$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.42 ± 0.15</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.05</td>
<td>3.22 ± 0.14</td>
</tr>
<tr>
<td>5µg/kg TCDD</td>
<td>0.26 ± 0.03*</td>
<td>0.05 ± 0.01*</td>
<td>0.02 ± 0.01*</td>
<td>1.02 ± 0.03*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CD4$^-$</th>
<th>CD4$^+$</th>
<th>CD4$^+$</th>
<th>CD4$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.45 ± 0.18</td>
<td>0.08 ± 0.02</td>
<td>0.06 ± 0.02</td>
<td>3.67 ± 0.16</td>
</tr>
<tr>
<td>10µg/kg TCDD</td>
<td>0.28 ± 0.12</td>
<td>0.06 ± 0.01*</td>
<td>0.02 ± 0.01*</td>
<td>0.82 ± 0.11*</td>
</tr>
</tbody>
</table>

*Significantly different from control, $p<0.05$. 
Figure 3.1A. Representative histograms from control and dioxin exposed gestation day 18 mice, showing 7-AAD fetal thymocyte fluorescence in all CD4CD8 cell compartments. Figure 3.1B. Representative histograms from control and dioxin exposed gestation day 18 mice, showing 7-AAD fluorescence in CD4⁺CD8⁺ fetal thymocytes. Abbreviations: V- viable cells; EA- early apoptotic cells; LA/N- late apoptotic/necrotic cells. Numbers immediately above labeled compartments represent percentages of cells in each compartment. * significantly different at p<0.05
Figure 3.2A and 3.2B. Representative gestation Day 18 Fetal Thymi, 40x magnification. Figure 3.2A is a control thymus, while Figure 3.2B is a 10 µg/kg TCDD-exposed thymus. Note the increased cellularity and size of the control thymus.
Figure 3.3. Gestation day 18 fetal thymus at 100x magnification. (A) is the control thymus, (B) is the 10 µg/kg TCDD-exposed thymus. The cortex is labeled C and the medulla is labeled M. Note the clear demarcation of the cortical/medullary junction for the control thymus.
Figure 3.4A and 3.4B, Gestation Day 18 Fetal Thymus, 500x magnification. Figure 3.4A is the control thymus, Figure 3.4B is the 10 µg/kg TCDD-exposed thymus. The long thin arrows indicate mitotic figures seen in the control thymus, while the short thick arrows indicate pyknotic nuclei in the TCDD-exposed thymus.