Chapter 3: Characterization of Phenotypic Alterations Induced by TCDD on Thymocytes *in vivo* and its Effects on Apoptosis.

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Abstract

2,3,7,9-Tetrachlorodibenzo-p-dioxin (TCDD) is a highly toxic environmental pollutant and is well known for inducing thymic atrophy in mice although the exact mechanism of its action remains unclear. Recent studies from our laboratory demonstrated that TCDD induces apoptosis in thymocytes and that Fas^- mice (lpr/lpr) were more resistant to TCDD-induced immunotoxicity when compared to the Fas^+ wild-type mice. Inasmuch as, induction of apoptosis is associated with alterations in adhesion molecule expression, in the current study we analyzed the expression of a variety of surface molecules on thymocytes treated with TCDD *in vivo*. Interestingly, in thymocytes from mice treated with a single dose of 50 μg/kg body weight of TCDD, there was a significant increase in the density of expression of CD3, αβTCR, CD44 and IL-2R and a decrease in the expression of J11d, CD4 and CD8 molecules, when compared to the control thymocytes. These alterations were first visible 3 days after TCDD treatment and increased on Days 5 and 10 post-treatment. Furthermore, most of the alterations in the density of expression of various markers were dose dependent with minimal but significant changes at 0.1 μg/kg body weight and maximum alterations at 50 μg/kg body weight of TCDD. At most lower concentrations (0.1-5 μg/kg), TCDD caused alterations in the density of cell surface markers but not in the percentage of cells expressing a specific molecule. It is striking that the phenotypic alterations were similar to those seen in normal thymocytes undergoing spontaneous apoptosis *in vitro* as previously reported. Together, the current study suggests that TCDD treatment induces phenotypic changes in thymocytes that are similar to those seen in normal thymocytes undergoing apoptosis. Also, because detection of apoptosis *in vivo* is difficult, phenotypic alterations in the density of thymocyte surface molecules may serve as a useful biomarker for toxicity involving apoptosis.

Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is well known for its immunotoxic effects, particularly on the thymus. Many hypotheses have been proposed to explain the mechanism by which TCDD induces thymic atrophy. TCDD may destroy committed prethymocytes found in the bone marrow and fetal liver (Fine *et al*., 1989, 1990). TCDD may also inhibit the intrathymic T cell development particularly at the double-positive (DP, $CD4^+CD8^+$) stage of T cell differentiation (Blaylock *et al*., 1992; Holladay *et al*., 1991; Kerkvliet and Brauner, 1990; Lundberg *et al*., 1990). Furthermore, thymic epithelial cells or stromal cells may also be the primary targets of TCDD-induced immunotoxicity (Kremer *et al*., 1995; Greenlee *et al*., 1985; Nagarkatti *et al*., 1984). Recent studies from our laboratory demonstrated that TCDD triggers apoptosis in thymocytes *in vivo*, which can be detected at very early stages but not at later time-points (Kamath *et al*., 1997) possibly due to rapid clearance of apoptotic cells *in vivo* (Savill *et al*., 1993). Also, Fas-deficient mice were more resistant to TCDD-mediated thymic atrophy and immunosuppression.

Most of the immature $CD4^+CD8^+$ T cells in the thymus are known to die due to "neglect" involving failure to receive signals required for positive selection (Robey and Fowlkes, 1994; Surh and Sprent, 1994). Moreover, thymocytes when cultured *in vitro* undergo apoptosis which can be enhanced by corticosteroids and radiation (Cohen and Duke, 1984; Kamath *et al*., 1997; Sellin and Cohen, 1987). Recently, it was demonstrated that apoptosis of thymocytes in culture was associated with marked changes in surface phenotype (Kishimoto *et al*., 1995). In the current study we therefore investigated whether TCDD, which enhances the induction of apoptosis *in vivo*, would induce similar changes. Our studies demonstrate that TCDD brings about changes in thymocyte phenotype similar to those seen in thymocytes undergoing apoptosis *in vitro*. These studies corroborate the ability of TCDD to induce apoptosis *in*

vivo and suggest that phenotypic alterations in thymocytes may serve as a sensitive biomarker for TCDD-induced immunotoxicity.

Experimental Procedure

Mice:

Adult, female C57BL/6 mice were purchased from National Institutes of Health, Bethesda, MD. The mice were housed in polyethylene cages containing wood shavings in laminar flow units (Animal Storage Isolators, Nu Aire Inc., Plymouth, MN) and given rodent chow and tap water *ad libitum.* Mice were housed in rooms maintained at a temperature of 74 ± 2^0F and on a 12-h light/dark cycle.

TCDD exposure:

2,3,7,8-Tetrachlorodibenzo-p-dioxin was generously provided by Dr. K. Chae of NIEHS, Research Triangle Park, NC and stored and -20^0C. TCDD was dissolved in acetone and diluted in corn oil. The solution was gently heated with stirring to evaporate the acetone (Rhile *et al*., 1996). Mice were administered various doses of TCDD ranging from 0.1-50 μg/kg body weight or the vehicle by a single intraperitoneal injection.

Cell preparation:

Mice were euthanized after TCDD or vehicle treatment and the thymus was surgically removed. The thymus was placed in RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 10 mM HEPES, 1mM glutamine, 40 μg/ml of gentamicin sulfate and 50 μM 2-mercaptoethanol, referred to as complete tissue culture medium. Single cell suspensions were made with a laboratory homogenizer (Stomacher, Tekmar Co., Cincinnati, OH) and kept on ice. Cells were pelleted by centrifugation and resuspended in 0.83% ammonium chloride to lyse the erythrocytes. Cells were further washed twice with PBS.

Detection of phenotypic markers on thymocytes:

Two million thymocytes from TCDD or vehicle-treated mice were stained with fluorescein-conjugated monoclonal antibodies to CD3 (hamster IgG), CD4 (rat IgG2a), CD8 (rat IgG2a), CD44 (rat IgG2b), αβTCR (hamster IgG), IL-2R (rat IgG2b), and J11d (rat IgM) (Pharmingen, Torregana, CA). The cells were incubated with the antibodies for 30 minutes on ice and then washed twice with PBS. Negative controls consisted of cells that were not stained with Abs and represented autofluorescence. In addition, cells stained with appropriate fluorescein-conjugated normal antibody isotypes (Pharmingen) were used as negative controls. Cells were then fixed with 1% p-formaldehyde. Fluorescence of the cells was measured by flow cytometry as described (McKallip *et al*., 1995). The analysis was performed with a Coulter Epics V flow cytometer. Five thousand cells were analyzed per sample. The data were analyzed and expressed as percentage positive cells expressing the surface marker. In addition, the mean channel number, which represents the density of expression of the surface marker, was determined for the control and TCDD-treated cells, and the percent change in the mean intensity of fluorescence (M.F.I.) was calculated as follows:

$$\frac{\text{M.F.I. for TCDD histogram - M.F.I. for control histogram}}{\text{M.F.I. for control histogram}} \times 100$$

The data from multiple experiments were pooled and depicted as mean percent change in M.F.I. $\pm$ S.E.M.

Statistical Analysis:

Each vehicle or TCDD-group consisted of 4-5 mice. Each mouse was analyzed individually and the data was expressed as means $\pm$ S.E.M.. The changes in the percent of cells expressing phenotypic markers in the TCDD-treated was compared with the controls, using ANOVA and student's T test and $p<0.05$ was considered to be statistically significant.

Results

Effect of TCDD on the surface phenotype of thymocytes:

To investigate whether TCDD would alter the expression of various surface markers on thymocytes, C57BL/6 mice were injected with 50 μg/kg body weight of TCDD or the vehicle control and 1, 3, 5, or 10 days later the cells were harvested and analyzed. The cells were stained for the expression of CD3, αβTCR, IL-2R, CD44, CD4, CD8 and J11d using mAbs and analyzed using a flow cytometer. In these experiments, groups of 4-5 mice were used, and each mouse thymus was analyzed separately. The data from a representative experiment are depicted in Figs. 3.1 and 3.2, and the pooled data from multiple mice are shown in Table 2. It should be noted that the data in Table 2, depict percentage of cells positive for a marker.

As seen from Figures 3.1 and 3.2 and Table 2, on Day 1 following TCDD administration, no significant changes in the percentages of surface markers were seen, except for an increase in IL-2R, when compared to the controls. However, on Day 3, in addition, significant increases were noted in percentage of cells expressing CD3 and αβTCR. Interestingly, on Day 5 after TCDD administration, significant increase in the percentage of cells expressing IL-2R and CD44 was noticed, while, there was a significant decrease in the percentage of cells expressing CD4, CD8 and J11d. Also, the percentage of cells expressing CD3 and αβTCR returned to normal levels. On Day 10, the increase in the percentage of cells expressing IL-2R and CD44 persisted, while all other markers studied returned to normal levels. In Table 2, data were compared using autofluorescence or isotype-specific antibodies as controls. The data obtained using these controls were comparable, thereby demonstrating the consistency of the phenotypic alterations.

In addition to the percentage, comparisons were also made in the M. F. I. that indicated the density of expression of the surface marker. The M. F. I. obtained with each marker

in the TCDD-treated mice was compared to the M. F. I. of the controls, and the data were expressed as mean percent increase in M. F. I. as discussed in Materials and Methods. Based on this, 100% increase in M. F. I. represents twofold increase in the density of cell surface markers. As seen from the M. F. I. values depicted in Figures 3.1 and 3.2 for individual histograms, and summarized in Figure 3.3, at Day 1 after TCDD injection, there were minor alterations in the density of expression of various markers studied. On Days 3, 5, and 10 following TCDD administration, there was a marked increase in the density of CD3 and αβTCR, IL-2R, and CD44. In contrast, the expression of CD4, CD8 and J11d on these days decreased significantly. These phenotypic alterations increased gradually and peaked on day 10 after TCDD treatment. Overall, the results suggested that TCDD-treatment up-regulates the density of expression of CD3, αβTCR, IL-2R, and CD44 while down-regulating the expression of CD4, CD8, and J11d.

Table 2

TCDD-Induced Alterations in the Percentage of Thymocytes Expressing Various Surface Markers

		Day 1[a]		Day 3		Day 5		Day 10	
Cell Markers	**Controls**	**Vehicle**	**TCDD**	**Vehicle**	**TCDD**	**Vehicle**	**TCDD**	**Vehicle**	**TCDD**
CD3	Auto.[b]	69.3±7.9	71.8±0.9	75.9±2.2	*85.8±2.8	77.3±2.3	77.7±3.8	79.4±3.2	73.2±7.5
	Isotype[c]	66.6±8.3	69.2±1.8	71.2±0.8	*80.5±2.9	74.9±1.2	73.6±2.0	76.8±0.7	78.2±0.4
αβTCR	Auto.	68.6±3.5	73.2±1.8	75.2±2.8	*83.9±1.8	71.5±2.5	73.5±3.8	75.9±0.4	79.4±4.7
	Isotype	67.1±4.7	69.1±0.7	66.6±0.9	*78.0±1.3	69.5±1.2	70.3±1.3	67.5±1.8	69.8±3.2
IL-2R	Auto.	NT[d]	NT	NT	NT	NT	NT	NT	NT
	Isotype	8.8±1.8	*16.2±0.8	9.3±0.7	*17.2±0.9	10.3±0.9	*23.2±2.2	9.8±0.7	*20.0±0.4
CD44	Auto.	65.7±0.8	60.0±4.1	60.0±4.0	68.7±1.4	69.4±1.1	*87.0±1.7	65.1±0.4	*83.7±0.8
	Isotype	66.6±2.0	65.0±0.4	66.8±2.5	68.7±2.2	68.1±1.5	*87.2±0.9	65.4±1.4	*80.4±3.6
CD4	Auto.	96.3±0.1	96.1±0.6	94.9±2.9	96.1±0.9	97.7±0.5	*91.9±0.7	97.8±0.4	96.6±0.2
	Isotype	96.2±0.8	96.6±0.6	95.0±1.3	95.2±1.4	97.4±0.4	*90.6±1.0	97.8±0.4	93.2±2.3
CD8	Auto.	89.6±0.8	89.2±0.1	74.9±1.9	73.5±11.1	87.7±1.8	*81.1±1.4	85.6±1.9	84.0±6.2
	Isotype	87.4±1.4	85.3±2.1	75.4±1.5	64.9±3.9	84.9±1.8	*80.3±0.5	84.9±1.6	84.3±6.5
J11d	Auto.	93.5±1.6	93.2±1.2	84.5±3.7	82.5±7.9	89.7±1.7	*77.4±3.4	89.6±0.6	84.1±6.0
	Isotype	91.4+2.7	91.3+2.3	85.1+4.1	74.3+2.6	88.9+1.5	*76.1+2.7	88.5+1.4	77.9+6.4

* Statistically significant difference when compared to the controls ($p < 0.05$), using ANOVA.

[a] Groups of four to five C57BL/6 mice were injected with TCDD (50 μg/kg body weight) or the vehicle (control). Thymocytes were harvested on various days and stained with mAbs against surface molecules. Data represents the mean percentage of thymocytes expressing a particular marker ± SEM.

[b] Percentages calculated in comparison to autofluorescence.

[c] Percentages calculated in comparison to the isotype control.

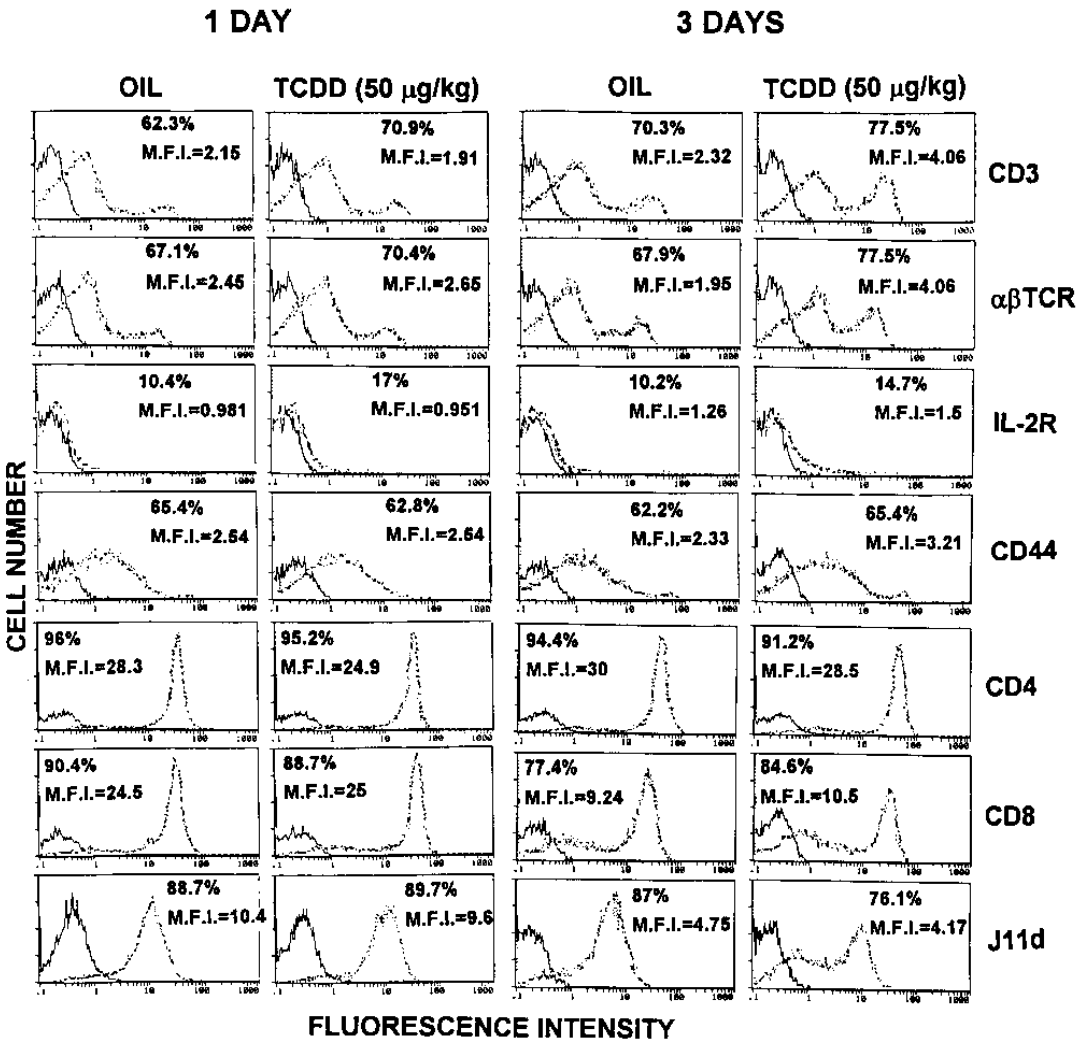

Figure 3.1: **<u>Effect of TCDD-administration on the surface phenotype of thymocytes:</u>** C57BL/6 mice were injected with 50 μg/kg body weight of TCDD or vehicle (corn oil) and 1 or 3 days later the thymocytes were harvested and stained with FITC-conjugated mAbs against CD3, αβTCR, IL-2R, CD44, CD4, CD8, and J11d. Cells were analyzed using a flow cytometer. The bold histogram represents antibody isotype controls and the broken histogram represents thymocytes stained for various surface markers. The percent positive cells and M.F.I. for stained cells has been depicted for each histogram. The figure represents data from a single representative experiment.

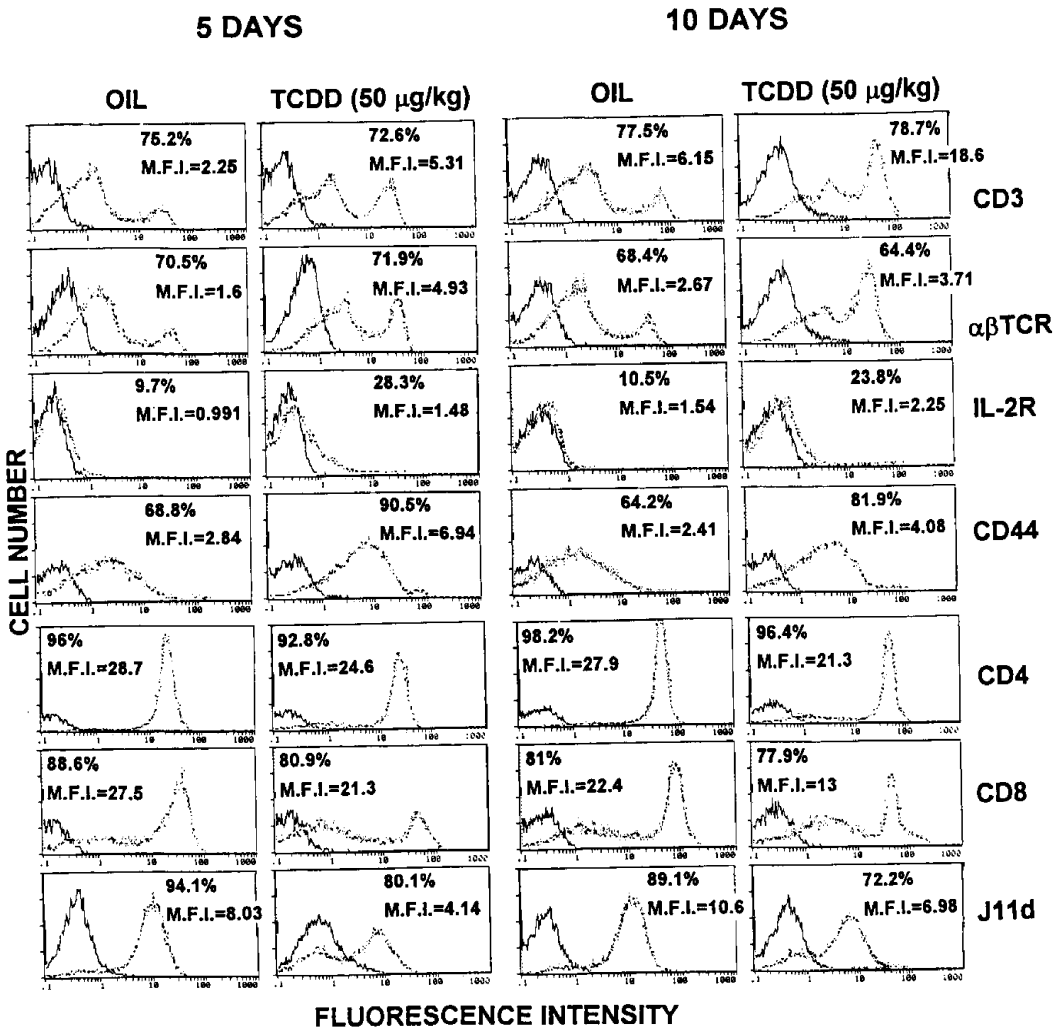

Figure 3.2: **<u>Effect of TCDD-administration on the surface phenotype of thymocytes on Days 5 and 10:</u>** TCDD or the vehicle (corn oil) was injected into C57BL/6 mice as described in Fig. 3.1. After 5 or 10 days, the thymocytes were harvested, stained with the various adhesion molecules. Data were analyzed as described in Fig. 3.1. The bold histogram represents antibody isotype controls, and the broken histogram represents thymocytes stained for various surface markers. The percent positive cells and M.F.I. for stained cells has been depicted for each histogram. The figure represents data from a single representative experiment.

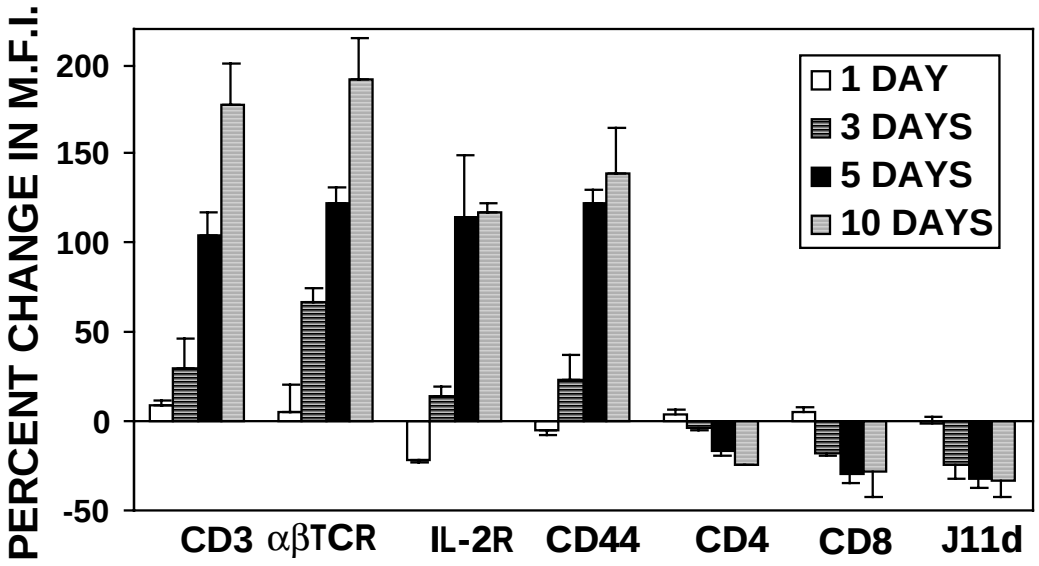

Figure 3.3: **Changes in the density of surface marker expression upon TCDD treatment:** The thymocytes were stained for various markers as described in Fig. 3.1. The M.F.I. obtained with each marker in TCDD-treated mice were compared to the M.F.I. of the controls, and the data were expressed as mean percent change in M.F.I. as described in Materials and Methods. The negative controls consisted of appropriate fluorescent antibody isotypes. The percent change in M.F.I. from four to five individual experiments were pooled to obtain the mean ± S.E.M. and plotted.

Dose-dependent alterations in surface markers on TCDD-treated thymocytes:

We next investigated whether TCDD would induce dose-dependent alterations in the thymocyte surface markers. In these experiments, the thymocytes were analyzed on Day 5 using autofluorescence as a control. When M. F. I. was compared (Fig. 3.4), TCDD was found to cause significant alterations in the density of various markers. Overall, the density of expression of CD3 and CD44 in TCDD-treated mice exhibited a dose-dependent increase, whereas the expression of CD4, CD8, and J11d, although not dose-dependent, exhibited significant decrease at >1 μg/kg of TCDD. The expression of αβTCR was slightly decreased at 0.1-5 μg/kg body weight of TCDD, whereas at 50 μg/kg, there was marked up-regulation. The expression of IL-2R showed an inverse relationship to the dose, with lower concentrations of TCDD decreasing the density of IL-2R, while 50 μg/kg concentration of TCDD caused an increase in the density of IL-2R. When percentages of cells expressing various markers were studied, there were no significant changes seen following administration of 0.1 and 1 μg/kg body weight of TCDD. However, at 5 μg/kg body weight, TCDD caused a significant increase in the percentage of IL-2R and CD44 (data not shown). It should be noted that although minimal changes were noted in the percentage cells expressing the surface markers, significant alterations in the density of most molecules were seen even at lower concentrations of 0.1-5 μg/kg body weight of TCDD. These data suggested that the density of expression of surface markers rather than the percentage of cells expressing a particular molecule, may serve as a biomarker for immunotoxicity. Similar studies were carried out on peripheral T cells and it was observed that TCDD did not cause any significant changes in the percentage or the density of expression of various markers (data not shown).

Effect of TCDD on T cell subsets in the thymus:

To rule out the possibility that the TCDD-induced alterations in the expression of surface markers resulted from selective loss of a specific subpopulation of T cells in the thymus, double-staining procedure was used to enumerate the four subpopulations of T

cells. To this end, groups of five mice were injected with TCDD (50 μg/kg) or the vehicle as described above, and on Day 1, 3, 5, and 10 the thymocytes were stained with FITC-anti-CD4 and PE-anti-CD8 mAbs. The dual parameter histograms were gated based on negative controls consisting of fluorescenated normal isotype antibodies. Figure 3.5 shows dual parameter histograms with percentages of $CD4^+$, $CD8^+$, $CD4^+CD8^+$, and $CD4^-CD8^-$ T cell subsets in the thymus. These data indicated that TCDD did not cause any significant alterations in the T cell subsets at all days tested. These experiments were repeated twice with consistent results.

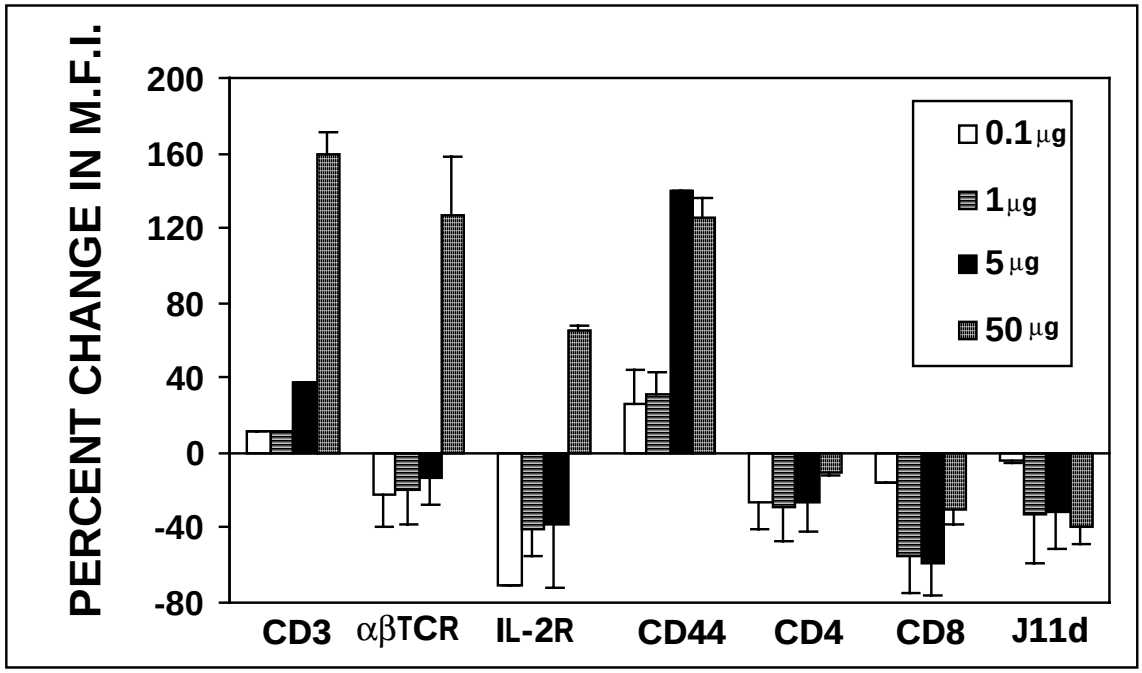

Figure 3.4: **Dose-dependent alterations in the density of surface marker expression upon TCDD treatment:** Mice were injected with various concentrations of TCDD. Thymocytes were processed as described in Fig. 3.1. Comparisons were made in the mean fluorescent intensity (M.F.I.) as described in Fig. 3.3. In this experiment,the negative controls consisted of autofluorescence.

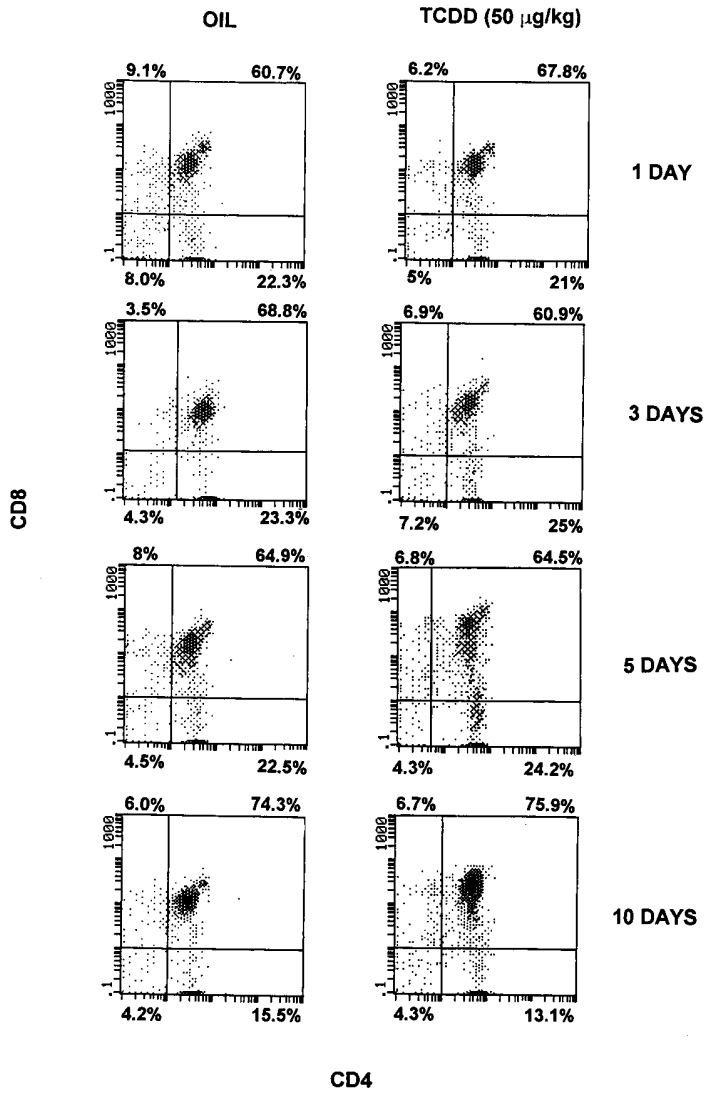

Figure 3.5: **<u>Flow cytometric analysis of T cell subsets in the thymus:</u>** Mice were injected with 50 μg/kg body weight of TCDD or the vehicle as described in Figure 3.1. At different days post-treatment, the thymocytes were harvested as stained with FITC-anti-CD4 and PE-anti-CD8 mAbs. The cells were analyzed using a flow cytometer. The dual parameter histograms were gated based on negative controls consisting of cells stined with FITC- or PE- conjugated normal antibody isotypes. The upper left quadrant represents $CD8^+$ T cells; upper right, $CD4^+CD8^+$, lower left, $CD4^-CD8^-$, and lower right $CD4^+$ T cells.

Discussion

In the current study, we observed that C57BL/6 mice treated with a single dose of TCDD demonstrated significant alterations in the expression of various surface receptors on thymocytes. In general, we found that TCDD-treatment led to marked enhancement in the density of expression of CD3, αβTCR, IL-2R, and CD44 while CD4, CD8, and J11d were down-regulated. The alterations in surface markers were seen on Day 3 after TCDD treatment and peaked around Day 5. At lower concentrations of TCDD (0.1-5 μg/kg body weight), although the percentage of cells expressing various markers remained relatively unaltered, the density of expression of most molecules on the thymocytes was significantly altered.

The nature of phenotypic changes that occur in thymocytes undergoing apoptosis is not well understood. Recently, Kishimoto *et al*. (1995) demonstrated that thymocytes cultured *in vitro* undergo apoptosis and that this is associated with marked changes in cell surface phenotype. The apoptotic cells up-regulated CD3/TCR, CD69, and IL-2R and down-regulated CD4, CD8, CD45, and J11d. In the current study, it is striking to note that TCDD caused almost identical changes. These studies further corroborate our findings that TCDD triggers apoptosis of thymocytes *in vivo* (Kamath *et al*., 1997).

It is not clear whether the phenotypic changes occur as a result of apoptosis or whether they precede programmed cell death of thymocytes. It has been speculated that down-regulation of surface markers on apoptotic cells might reflect progressive degradation of cell components as a byproduct of apoptosis and that up-regulation of surface markers may be due to a breakdown in intracellular trafficking leading to an accumulation of markers on the cell surface (Kishimoto *et al*., 1995). The up-regulation in certain cell surface markers can also be explained by the fact that TCDD is known to be a potent transcriptional regulator of several genes in a variety of tissues (Poland and Knutson, 1982; Sutter *et al*., 1991). On the basis of our earlier studies that mice deficient in Fas

are more resistant to TCDD-induced thymic atrophy (Rhile *et al*., 1996), we have suggested that TCDD may up-regulate Fas and/or FasL expression. Recent studies revealed that Fas expression as detected by flow cytometry was not significantly altered in TCDD-exposed thymocytes, while we detected increased levels of FasL in the serum of TCDD-treated mice (unpublished data).

Thymus is an active site where T cells undergo apoptosis if they fail to receive a positive stimulus (death by neglect) or following negative selection (activation-induced apoptosis) (Robey and Fowlkes, 1994; Surh and Sprent, 1994). Inasmuch as surface molecules including the TCR, MHC, CD44, CD45, CD4, and CD8 are actively involved in positive and negative selection of thymocytes (Poggi *et al*., 1996; Ayroldi *et al*., 1995; Conroy and Alexander, 1996), alterations in such molecules may decrease positive selection or increase negative selection leading to induction of apoptosis. For example, up-regulation of TCR may facilitate negative selection and lead to increased apoptosis.

Several studies have addressed the effect of TCDD on the various subsets of T cells in the thymus based on the expression of CD4 and CD8. TCDD was shown to cause a significant decrease in the percentage of double-positive ($CD4^+CD8^+$) thymocytes (Kremer *et al*., 1995) and an increase in double-negative ($CD4^-CD8^-$) thymocytes (Blaylock *et al*., 1992). Such alterations were particularly conspicuous after prenatal exposure to TCDD (Blaylock *et al*., 1992; Luster *et al*., 1992) and were less marked in adult mice (Kerkvleit and Brauner, 1990). In an earlier study, we observed that TCDD, administered orally at 0.1-5 μg/kg body weight of TCDD daily for 11 days, caused marked thymic atrophy without causing significant changes in T cell subsets (Rhile *et al*., 1996). In the current study, we repeated these experiments with single dose of 50 μg/kg body weight of TCDD, and we found that TCDD did not cause any selective loss of a subset of T cells. This ruled out the possibility that the changes in the density of adhesion molecules seen on TCDD-treated thymocytes resulted from the selective loss

of immature and enrichment of mature T cells. Moreover, the thymic atrophy induced by TCDD is seen on Day 5, but not 3, after TCDD treatment (Kamath *et al*., 1997). These observations rule out the possible loss of a T cell subset as the cause of phenotypic alterations.

It should be noted that while some investigators have reported a decrease in the percentage of $CD4^{-}CD8^{+}$ T cells (Kerkvleit and Brauner, 1990), others reported a significant increase (Holladay *et al*., 1991). In earlier studies, however, the density of cell surface markers as indicated by the M. F. I. was not studied. In the current study, we noted that although the percentages of various T-cell subsets were not altered markedly at lower doses of TCDD, there were major alterations in the density of expression of various markers following TCDD injection. Thus, the density rather than the proportion of cells expressing a specific marker may serve as an indicator for chemically induced immunomodulation, and flow cytometry may constitute an important tool to delineate such changes (Burchiel *et al*., 1997). In the current study, we noted that TCDD induced alterations in the surface markers of only thymocytes but not peripheral T cells, similar to previous reports (Kerkvleit and Brauner, 1990). This may be because TCDD affects differentiating cells *in vivo* such as the T cells in the thymus but not the naive T cells in the periphery (Rhile *et al*., 1996).

In an earlier study, we found that TCDD-induced apoptosis was detected within the first 12 hours but not subsequently, whereas, thymic atrophy peaked on Day 5 (Kamath *et al*., 1997). This may be because the apoptotic cells are rapidly cleared by phagocytic cells (Savill *et al*., 1993). This is also evident from our earlier study in which we noted that dexamethasone, a potent inducer of thymic apoptosis, caused detectable apoptosis only in the first 12 hours of administration and not at subsequent time points (Kamath *et al*., 1997). In the current study we noted that the expression of CD3/αβTCR was up-regulated on Day 3 and thereafter after TCDD treatment. This may facilitate positive selection as suggested by other investigators (Kremer *et al*., 1995). On the other hand,

because TCR is also involved in negative selection of thymocytes, up-regulation in the TCR may also facilitate negative selection and trigger increased apoptosis leading to thymic atrophy by day 5.

In summary, the current study demonstrates that TCDD induces phenotypic changes in thymocytes *in vivo* particularly in the density of expression of surface receptors. Such alterations are identical to those seen in thymocytes that undergo spontaneous apoptosis following *in vitro* culture (Kishimoto *et al*., 1995). These data corroborate the ability of TCDD to trigger apoptosis *in vivo* and suggest that such phenotypic changes may serve as an important biomarker for *in vivo* induced apoptosis that is otherwise difficult to detect. Also, while some phenotypic changes may result from induction of apoptosis, others may precede cell death and may contribute to thymic atrophy by influencing negative selection of thymocytes. Inasmuch as positive and negative selection play an important role in the development of T cell repertoire and induction of tolerance, the current study suggests that TCDD may interfere with these important regulatory mechanisms.