Chapter 4: 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 Apoptosis.

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Abstract

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a highly toxic environmental pollutant well known for its toxicity to the thymus. Recent studies from our laboratory demonstrated that TCDD induces apoptosis in thymocytes. In the current study, we investigated the mechanism of TCDD-induced apoptosis. Administration of a single dose of TCDD at 0.1, 1, 5 or 50 μg/kg body weight intraperitoneally, into C57BL/6 +/+ (wild-type) mice caused a dose-dependent decrease in thymic cellularity. In contrast, a similar treatment with TCDD in Fas-deficient C57BL/6 *lpr/lpr* (*lpr*) or Fas-ligand defective C57BL/6 *gld/gld* (*gld*), mice failed to induce thymic atrophy at 0.1-5 μg/kg body weight of TCDD. In *lpr* and *gld* mice, significant thymic atrophy was seen only at 50 μg/kg body weight of TCDD. Injection of TCDD caused apoptosis only in wild-type but not in *lpr* or *gld* mice. The sera from TCDD-treated wild-type mice exhibited increased levels of soluble Fas ligand, inasmuch as, incubation of Fas^+ but not Fas^- cells with the sera, triggered apoptosis. Also, TCDD-induced apoptosis in thymocytes, was inhibited both *in vitro* and *in vivo* by caspase inhibitors. TCDD-treatment caused significant up-regulation in the expression of FasL but not Fas mRNA in the thymocytes of wild-type mice. Also, such thymocytes exhibited marked alterations in the surface markers, characteristic of cells undergoing apoptosis. In contrast, TCDD-treatment caused minimal phenotypic changes in thymocytes from *lpr* and *gld* mice. Together, the current study demonstrates that Fas-Fas ligand interactions play an important role in TCDD-mediated induction of apoptosis and immunotoxicity.

Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is well-known for its immunotoxic effects particularly on the thymus. Despite extensive research, the exact mode of action of TCDD on thymocytes is not clear. Many hypotheses have been proposed to explain the mechanism of TCDD-induced thymic atrophy. First, it has been demonstrated that TCDD may destroy committed prethymocytes found in the bone marrow and fetal liver (Fine *et al*., 1989, 1990). Secondly, TCDD may also directly affect the thymocytes by either inhibiting the differentiation of double-positive T cells (Blaylock *et al*., 1992, Holladay *et al*., 1991, Kerkvliet and Brauner, 1990, Lundberg *et al*., 1990) or by altering the functions of the thymic epithelial or stromal cells (Greenlee et al., 1985, Nagarkatti *et al*., 1984, Kremer *et al*., 1995). Moreover, recent studies from our lab demonstrated that TCDD may directly affect the thymus by inducing apoptosis in thymocytes (Kamath *et al*., 1997). Furthermore, we noted that the thymocytes from TCDD-treated mice had characteristic phenotypic alterations typical of cells undergoing apoptosis (Kamath *et al*., 1998).

The exact mechanism by which TCDD triggers apoptosis in thymocytes is not clear. Based on the fact that Fas-deficient mice (C57BL/6 *lpr/lpr*) were more resistant to TCDD-induced thymic atrophy and immunosuppression (Rhile *et al*., 1996), we hypothesized that TCDD may induce apoptosis by up-regulating the expression of Fas and/or Fas ligand (FasL) and that the ligation of Fas would induce apoptosis in such cells. To further test this hypothesis, in the current study, we used C57BL/6 *gld/gld* mice which are known to have a functional defect in the FasL. Our results demonstrated that TCDD was less toxic to the thymus of *gld* mice. Furthermore, both *lpr* and *gld* mice exhibited resistance to TCDD-induced apoptosis and phenotypic changes when compared to the wild-type (C57BL/6 +/+) mice. Also, the C57BL/6+/+ mice injected with TCDD exhibited increased levels of FasL. Together our studies demonstrate that

TCDD may up-regulate the expression of FasL which may be responsible for inducing apoptosis in Fas^{+} thymocytes.

Experimental Procedures

Mice:

Adult, female C57BL/6 +/+ mice were purchased from the National Institutes of Health (Bethesda, MD). C57BL/6 *lpr/lpr* (*lpr*) and C57BL/6 *gld/gld* (*gld*) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and bred in our animal facilities. The *lpr* and *gld* mice used were 4-6 weeks of age, prior to the onset of lymphoproliferative disorder. 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 water *ad libitum* (Hammond *et al*., 1993). Mice were housed in rooms maintained at a temperature of 74 $\pm$ 2^0F and on a 12-hr. 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 at -20^0C. TCDD was dissolved in acetone and diluted in corn oil. The solution was gently heated with stirring to evaporate the acetone (Kamath *et al*., 1997,1998, Rhile *et al*., 1996). Mice were administered various doses of TCDD 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 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 10 mM Hepes, 1 mM glutamine, 40 μg/ml 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 in medium.

Total cellularity:

Thymocytes were prepared as described above and resuspended in medium. Twenty-five microliters of the single-cell suspension was added to 100 or 500 μl of trypan blue dye and viable cells were enumerated by exclusion of trypan blue under a phase contrast microscope.

Detection of apoptosis using the TUNEL method:

The FITC-dUTP nick end labeling kit was used to detect and quantitate apoptosis at the single cell level, which is based on labeling of DNA strand breaks using terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP, commonly called the TUNEL technique (Boehringer Mannheim, Indianapolis, IN) (Kamath *et al*., 1997). Briefly, the cells (2×10^6) were washed twice with phosphate-buffered saline (PBS) and fixed with 4% p-formaldehyde for 30 minutes at room temperature. The cells were next washed with PBS, permeabilized on ice for 2 minutes and incubated with FITC-dUTP for an hour in the incubator. Fluorescence of the cells was measured by flow cytometry as described (Kamath *et al*., 1997). The analysis was performed by a Coulter Epics V flow cytometer. Five thousand cells were analyzed per sample.

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 stained with appropriate fluorescein-conjugated normal antibody isotypes (Pharmingen, San Diego, CA). Cells were then fixed with 1% p-formaldehyde.

Fluorescence was measured by flow cytometry as described. The data were analyzed and expressed as percent 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 (MFI) was calculated as follows:

$$\frac{\text{MFI for TCDD histogram - MFI for control histogram}}{\text{MFI for control histogram}} \times 100$$

The data from multiple experiments were pooled and depicted as mean percent change in MFI $\pm$ SEM.

Detection of soluble FasL:

Groups of 5 mice were injected with vehicle control or TCDD (50 μg/kg). After 1 or 5 days, the mice were bled and the sera were collected and pooled from each group. To detect FasL, the sera at 1:10 dilution were added to Fas^+ or Fas^- target cells and cultured *in vitro* for 12 or 24 hours. The cells were then stained with FITC-dUTP to detect apoptosis as described above.

RT-PCR Analysis of Fas and FasL Gene Expression:

Total RNA was isolated from the thymus TCDD or vehicle-treated wild-type mice using Trizol Reagent (Life Technologies, Frederick, MD). Ten micrograms of total RNA from each sample was used for reverse transcription and the cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for Fas, FasL or β-actin as an internal control as described (14). The condition of the PCR used is as follows: denaturation at 94^0C for 30 sec; annealing at 56^0C for 1 min; extension at 72^0C for 2 min. Totally, 30 cycles were performed. The primer sequence for β-actin was 5' - TATCCTGACCCTGAAGTACCCCATT and 3' - AGCACAGCTTCTCTTTGATGTCACG. The primer sequence for Fas was 5' - GCACAGAAGGGAAGGAGTAC and 3' -GTCTTCAGCAATTCTCGGGA. The

sequence of the specific primer for Fas ligand was, 5'-
GACCGCAAGGTCCAACAGGTCAGC and 3'-
CTAGCTTATCAGTCAAATGAGCCTCC. The PCR products were electrophoresed through a 1.5% agarose gel containing ethidium bromide. The quantitation of PCR product was performed by using a digital camera and AlphaImager™ (Alpha Innotech Corporation, San Leandro, CA). The density of the bands for oil versus TCDD treatment was calculated and expressed as a ratio.

Use of inhibitors of apoptosis:

Caspase inhibitor, YVAD-CMK (100 μM); caspase-3 inhibitor, DEVD-CHO (100 μM); calpain inhibitors I and II (10 μg/ml), and aurintricarboxylic acid (ATA) (1 μM) were used to block TCDD-mediated apoptosis *in vitro*. The negative control consisted of cells incubated with DMSO, because the inhibitors were dissolved in DMSO. For *in vivo* inhibition of TCDD-induced apoptosis, mice were injected with 40 mg/kg body weight of YVAD-CMK and TCDD(50 μg/kg) and after 12 or 24 hours, the thymocytes were studied for apoptosis. These thymocytes were also cultured *in vitro* for 24 hours and then stained for apoptosis. As a control, DMSO, the vehicle used for dissolving YVAD-CMK, was similarly injected in TCDD-treated mice.

Statistical analysis:

Each experiment was repeated at least thrice and each vehicle or TCDD group consisted of four to six mice. The changes in the percentage of cells expressing phenotypic markers in the TCDD-treated group was compared with the controls, using ANOVA and $p<0.05$ was considered statistically significant.

Results

Thymic cellularity in C57BL/6 +/+, C57BL/6 lpr/lpr and C57BL/6 gld/gld mice treated with TCDD:

In the first set of experiments, we compared the ability of TCDD to induce thymic atrophy in the wild-type, *lpr* and *gld* mice. To this end, groups of 5-6 mice were injected with either the vehicle or with 0.1, 1, 5 or 50 μg/kg body weight of TCDD and on day 5, the mice were sacrificed and the thymic cellularity was calculated as described (Kamath *et al*., 1997). The total number of cells/thymus has been depicted in Table 3. In C57BL/6 +/+ mice, TCDD at all concentrations tested, caused a significant decrease in thymic cellularity when compared to the thymocytes from vehicle-treated mice. Interestingly, in both *lpr* and *gld* mice the lower concentrations of TCDD (0.1, 1 and 5 μg/kg body weight) failed to cause a significant decrease in thymic cellularity. However, when TCDD was injected at a concentration of 50 μg/kg body weight, both *lpr* and *gld* mice exhibited a significant decrease in cellularity. These data together demonstrated that *lpr* and *gld* mice were more resistant to TCDD-induced thymic atrophy particularly at lower concentrations, when compared to the C57BL/6 +/+ mice.

Detection of apoptosis in thymocytes of C57BL/6 +/+, lpr and gld mice:

To further investigate whether the resistance of thymocytes from TCDD-treated *lpr* and *gld* mice was due to the inability of these thymocytes to undergo apoptosis, the thymocytes from TCDD-treated *lpr* and *gld* mice were stained for apoptosis and were compared to the wild-type mice. To this end, C57BL/6 +/+, *lpr* and *gld* mice were injected with 50 μg/kg body weight of TCDD and 12 hrs later, the thymocytes were harvested and stained immediately for the presence of apoptotic cells or were cultured for 24 hours *in vitro* and then analyzed for the presence of apoptotic cells. The data shown in Fig. 4.1 indicated that the wild-type (+/+) mice showed significant levels of apoptosis (Fig. 4.1A) when the thymocytes were directly stained 12 hrs after *in vivo*

TCDD exposure, whereas, thymocytes from TCDD-treated *lpr* (Fig. 4.1B) and *gld* (Fig 4.1C) mice failed to exhibit apoptosis. It should be noted that in our previous study we found that TCDD-induced apoptosis can be detected only at early stages, such as 12 hr, but not subsequently because of rapid clearance of apoptotic cells by the phagocytes (Kamath *et al*., 1997, Savill *et al*.,1993). To overcome this problem, in the previous study, we incubated the *in vivo* exposed thymocytes in culture for 24 hrs and found that the TCDD-treated thymocytes underwent increased apoptosis when compared to thymocytes from vehicle-treated mice (Kamath *et al*., 1997). In the current study, we used a similar approach to compare the TCDD-induced apoptosis in +/+, *lpr* and *gld* mice. The data shown in Fig. 4.1D, indicated that when the thymocytes from TCDD-treated C57BL6 +/+ mice were cultured for 24 hrs *in vitro,* they exhibited increased levels of apoptotic cells consistent with our previous studies (Kamath *et al*., 1997). Also, such cells exhibited increased intensity of fluorescence indicating the presence of late apoptotic cells (Kamath *et al*., 1997) when compared to the thymocytes from vehicle treated controls. In contrast, thymocytes from TCDD-treated *lpr* (Fig. 4.1E) and *gld* (Fig. 4.1F) mice failed to exhibit increased percentage of apoptotic cells when compared to thymocytes from vehicle-treated mice, when cultured for 24 hrs. In addition, we also determined whether lower concentrations of TCDD such as 5 μg/kg body weight would induce apoptosis in *lpr* and *gld* mice. In these experiments, the thymocytes from mice treated with 5 μg/kg body weight, were incubated for an additional 24 hrs in culture. The data shown in Fig. 4.1 indicated that the thymocytes from TCDD-treated *lpr* (Fig. 4.1H) and *gld* (Fig. 4.1I) mice failed to exhibit significant increase in apoptosis when compared to the thymocytes from vehicle-treated mice. In contrast, thymocytes from TCDD-treated +/+ mice underwent increased apoptosis when compared to the controls after culture (Fig. 4.1G). Together, these data demonstrated that the thymocytes from TCDD-treated *lpr* and *gld* mice failed to undergo apoptosis when tested immediately or following culture *in vitro,* whereas similar cells from TCDD-treated wild type mice exhibited significant levels of apoptosis.

Table 3

Effect of TCDD on cellularity of the thymus in C57BL/6+/+,C57BL/6 *lpr/lpr* and C57BL6 *gld/gld* mice[a].

	Vehicle	**TCDD**			
		0.1μg	**1.0μg**	**5.0μg**	**50μg**
C57BL/6+/+	94.16±1.81	66.45±8.31*	68.95±3.75*	46.0±4.05*	11.81±2.83*
C57BL/6*lpr/lpr*	143.54±7.01	155.0±15.81	141.0±8.86	148.54±11.0	51.68±7.57*
C57BL/6*gld/gld*	159.0±20.8	183.6±17.0	103.8±11.74	107.5±9.3	26.5±11.2*

[a] Groups of 5-6 C57BL/6-+/+, C57Bl/6-*lpr/lpr* and C57BL/6-*gld/gld* mice were administered either vehicle or 0.1, 1.0, 5.0 and 50 μg/kg body weight of TCDD intraperitoneally and cellularity of the thymus determined 5 days later. Mean cellularity ($x10^6$) ± S.EM. is expressed.

* Denotes statistically significant differences ($p<0.05$).

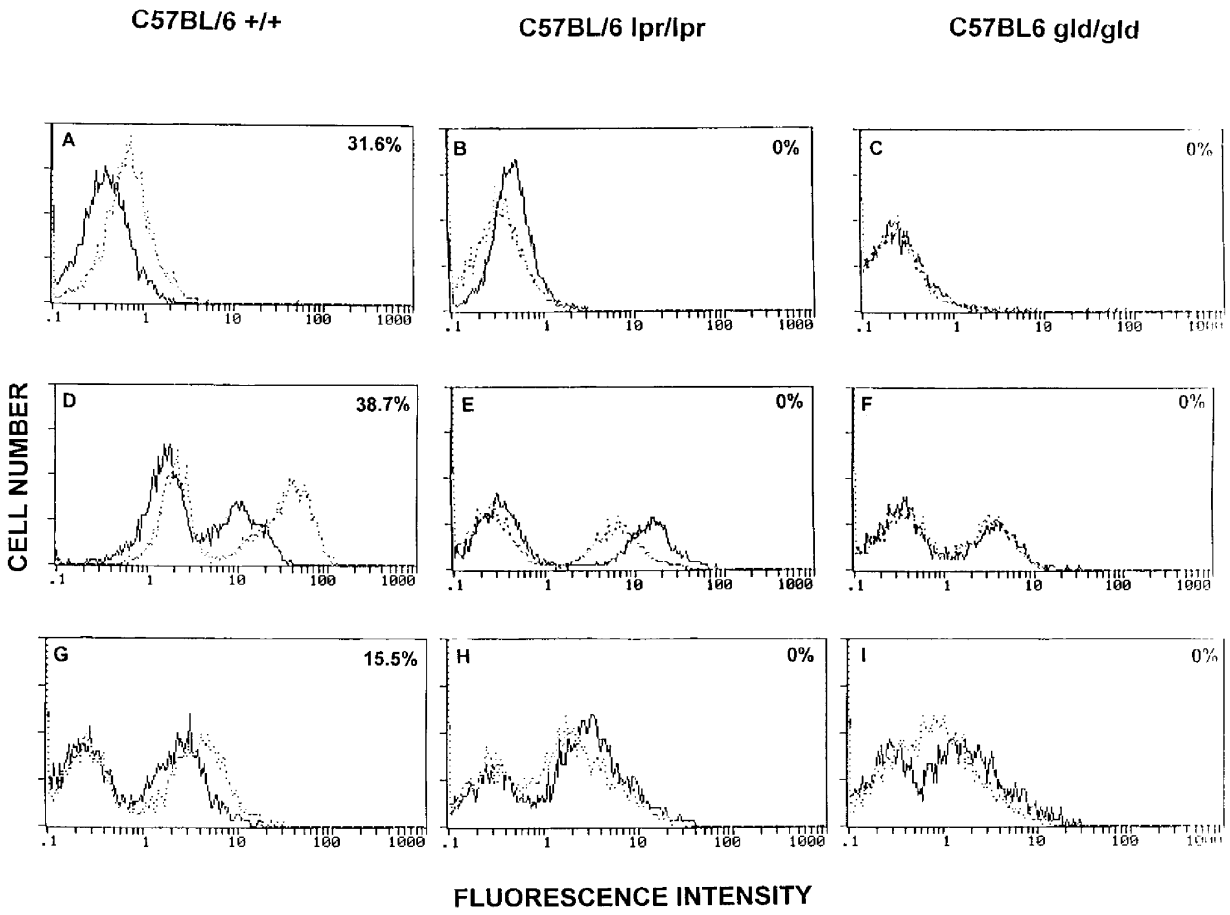

Figure 4.1: <u>TCDD triggers apoptosis only in thymocytes from C57BL/6 +/+ but not *lpr/lpr* or *gld/gld* mice:</u> Groups of 4-5 C57BL/6 +/+ (panels A, D and G), *lpr/lpr* (panels B, E and H) or *gld/gld* (panels C, F and I) mice were injected with TCDD. Twelve hours following treatment with 50 μg/kg of TCDD or the vehicle, the thymocytes were harvested and stained immediately with FITC-dUTP for detecting apoptosis (panels A, B and C) or following *in vitro* culture for 24 hours at 37^0C (panels D, E and F). In panels G, H and I thymocytes from mice treated with 5 μg/kg body weight of TCDD or the vehicle, were cultured for 24 hrs and then analyzed for apoptosis. The bold histogram depicts thymocytes from vehicle-treated mice and the broken histograms represents the thymocytes from TCDD-treated mice. The bold histogram was subtracted from the broken histogram and the difference in percentage of cells expressed in each panel.

Detection of soluble FasL in the serum of TCDD-treated mice:

Recent studies have shown that FasL is produced in soluble form (Sato *et al*., 1996, Nagata, 1997). To investigate whether the TCDD-treated mice would exhibit increased levels of soluble FasL in the serum, we incubated the thymocytes from wild-type mice that express high levels of Fas, with the serum (1:5 dilution) collected from TCDD-treated or vehicle-treated mice, 1 or 5 days after treatment. The cells were incubated at 37^0C for 12 or 24 hrs and stained for apoptosis. The data shown in Fig. 4.2, indicated that the thymocytes treated with the serum from TCDD-exposed mice underwent increased apoptosis when compared to the thymocytes incubated with serum from vehicle-treated mice. This was seen using sera collected on days 1 and 5 following treatment as well as after 12 and 24 hours of *in vitro* culture. These data suggested that the sera from TCDD-exposed mice contained a factor which was capable of inducing apoptosis in thymocytes.

To further delineate whether this factor was soluble FasL, we incubated Fas^+ and Fas^- target cells with sera obtained from TCDD-treated or vehicle-treated mice and tested for apoptosis. We used thymocytes from wild-type (+/+) mice and L1210 tumor cells transfected with the Fas gene, as a source of Fas^+ cells. Furthermore, as a source of Fas^- cells, thymocytes from *lpr* mice and L1210 tumor cells transfected with Fas antisense were used (Zeytun *et al*., 1997). As shown in Fig. 4.3, L1210 Fas^+ but not Fas^- (Fig. 4.3B and Fig. 4.3A respectively) tumor targets when cultured in the presence of serum from TCDD-treated mice exhibited high levels of apoptosis when compared to similar cells incubated with serum from vehicle-treated mice. Similarly, thymocytes from +/+ (Fig. 4.3D) but not *lpr* mice (Fig. 4.3C) exhibited significant apoptosis when incubated with sera from TCDD-treated mice. These data together suggested that the factor found in the serum of TCDD-treated mice inducing apoptosis was indeed FasL.

To further confirm the role of FasL-induced apoptosis, the effect of addition of caspase inhibitors was studied. To this end, normal thymocytes from wild-type mice were incubated with sera obtained from TCDD-treated mice and such cultures were either incubated in the presence of caspase inhibitors such as YVAD-CMK and DEVD-CHO

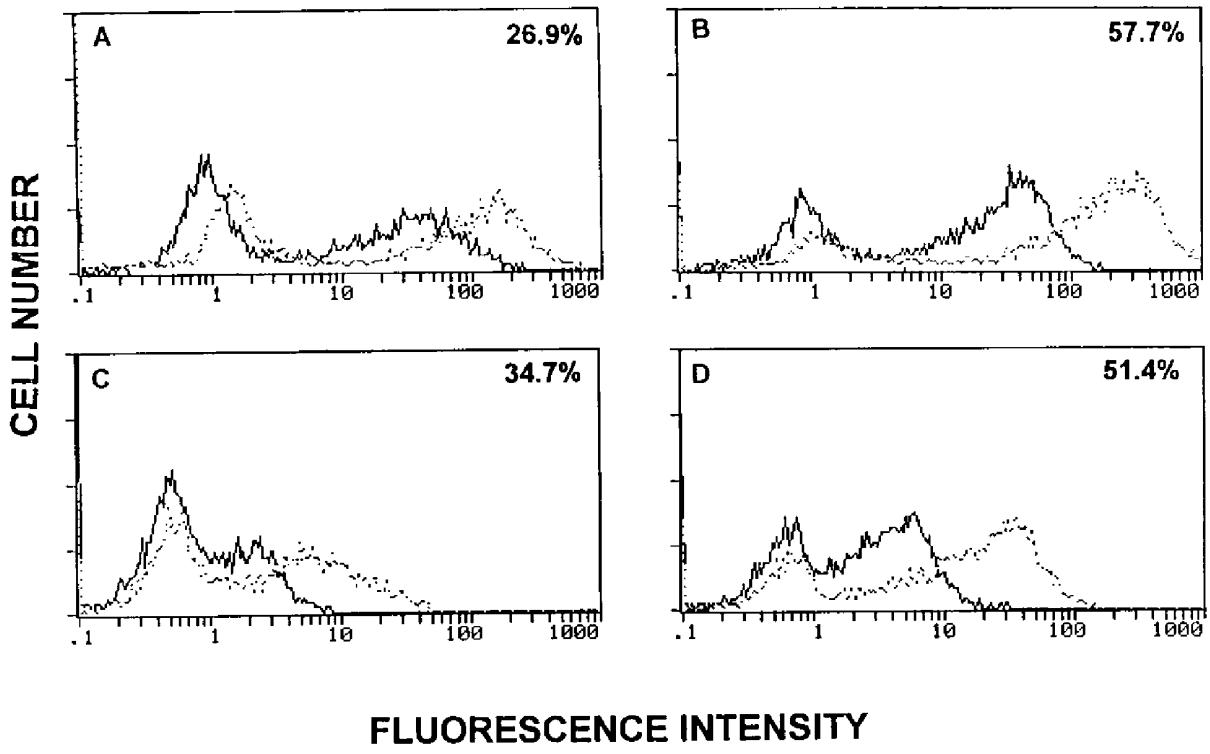

Figure 4.2: **<u>Detection of soluble FasL in the serum of TCDD-treated mice:</u>** Groups of 4-5 C57BL/6 +/+ mice were injected with 50 μg/kg body weight of TCDD or the vehicle and 1 (panels A and B) or 5 (panels C and D) days later, the sera were collected and pooled for each group. Next, Fas^{+} thymocytes from C57BL/6 +/+ mice were incubated with 1:5 dilution of serum from oil or TCDD-treated mice. The cells were cultured for 12 (panels A and C) or 24 (panels B and D) hours *in vitro* at 37^{0}C and stained with FITC-dUTP and analyzed flow cytometrically to detect apoptosis. The bold histogram represents +/+ thymocytes cultured with sera from vehicle-treated mice and the broken histogram represents the thymocytes cultured with sera from TCDD-treated mice. The data were analyzed as described in Fig. 4.1.

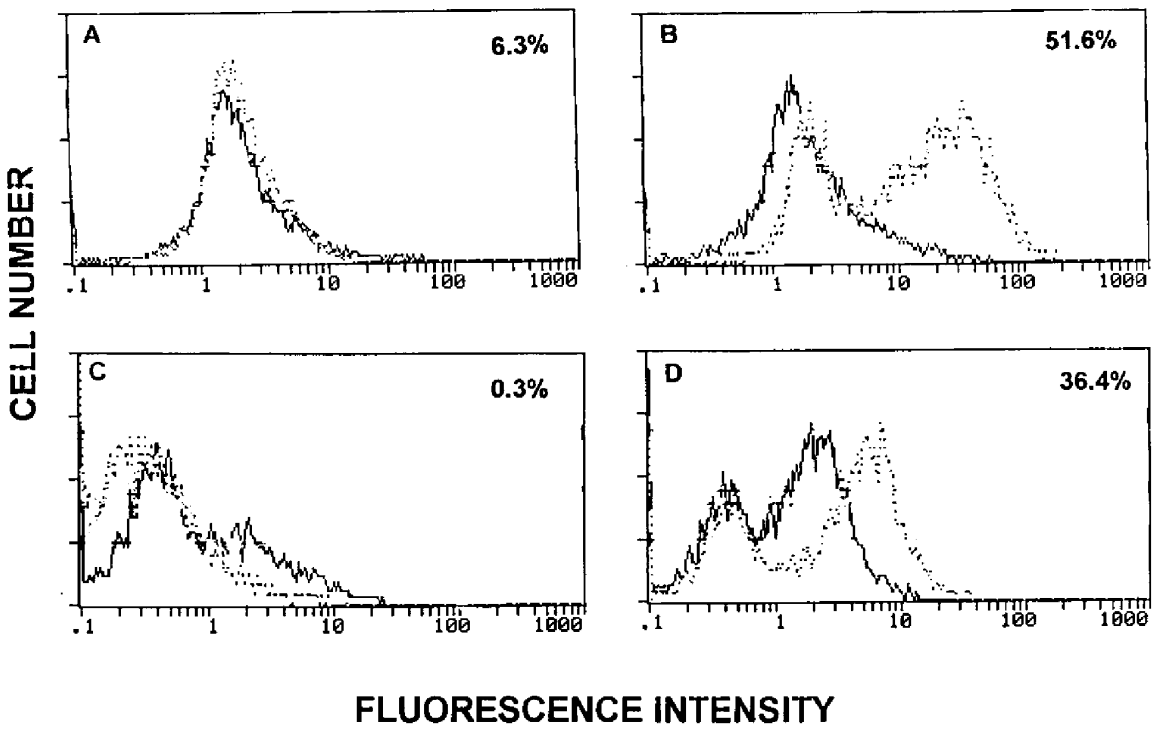

Figure 4.3. <u>Sera from TCDD-treated mice induce apoptosis only in Fas$^+$ but not Fas$^-$ targets:</u> Sera were collected from mice 1 day after vehicle or TCDD treatment of C57BL/6 +/+ mice, as described in Figure 4.2. L1210Fas$^-$ (panel A), L1210Fas$^+$ (panel B), Fas$^-$ thymocytes from *lpr* mice (panel C) and Fas$^+$ thymocytes from +/+ mice (panel D), were next incubated with the sera for 24 hours and the cells were studied for apoptosis as described in Figure 4.2. Bold histograms cells incubated with sera from vehicle-treated mice and broken histograms represent the cells incubated with TCDD-treated sera.

or in the presence of vehicle that was used in dissolving these compounds. The cultures were incubated for 24 hrs *in vitro* and tested for apoptosis. As seen from Fig. 4.4, addition of YVAD-CMK and DEVD-CHO caused significant inhibition of apoptosis-induced by serum from TCDD-treated mice. Calpain inhibitor I and II caused partial inhibition of apoptosis.

Expression of Fas and FasL upon TCDD-treatment:

To investigate whether TCDD up-regulates the expression of Fas or FasL genes, RNA from thymocytes was extracted 1 or 5 days after C57BL/6 +/+ mice were injected with oil or 50 μg/kg body weight of TCDD and RT-PCR analysis was carried out.. The data shown in Fig. 4.5 indicated that 1 or 5 days after TCDD administration, there was increased expression of FasL when compared to the vehicle control. This was evident from the comparison of the density of bands for TCDD versus oil-treated groups, expressed as a ratio. While this ratio was close to 1 for β-actin, thereby suggesting similar expression in oil and TCDD treated groups, the expression of FasL was clearly increased in TCDD-treated groups. Also, FasL expression was up-regulated to a greater extent on day 1 when compared to day 5, following TCDD-treatment. In contrast to FasL, the expression of Fas in TCDD-treated groups was similar to that seen in vehicle controls. These data were consistent with our earlier studies using flow cytometry that Fas expression on thymocytes remained unaltered following TCDD-treatment. (Kamath *et al*., 1998). It should be noted that the normal thymocytes do not express FasL which may account for the failure to observe a significant signal in oil-treated mice on days 1 and 5.

Effect of caspase inhibitors on TCDD-induced apoptosis in thymocytes:

To further investigate the effect of various inhibitors of apoptosis on the toxicity induced by TCDD in the thymocytes, C57BL/6+/+ wild-type mice were injected with 50 μg/kg body weight of TCDD and 1 or 5 days later, the cells were harvested and cultured in the presence or absence of various inhibitors of apoptosis. As a control, such

thymocytes were incubated with the vehicle used for dissolving various inhibitors of apoptosis. The data shown in Fig. 4.6 indicated that all the inhibitors tested, such as, YVAD-CMK, DEVD-CHO, calpain inhibitor I, calpain inhibitor II and aurintricarboxylate were able to induce significant suppression of apoptosis induced by TCDD on days 1 and 5, which was evident by the decreased levels of fluorescence.

Furthermore, we tested whether YVAD-CMK would block apoptosis induced by TCDD *in vivo*. To this effect, two mice were injected with 50 μg/kg body weight of TCDD and simultaneously with YVAD-CMK and 12 or 24 hrs later, the cells were harvested and tested either directly for apoptosis or following *in vitro* culture for 24 hrs. The data shown in Fig. 4.7 indicated that YVAD-CMK was able to significantly inhibit the apoptosis-induced by TCDD in freshly isolated thymocytes (Fig. 4.7A and C) and in cells that were cultured *in vitro* for 24 hrs (Fig. 4.7B and D). These data together demonstrated that the apoptosis induced by TCDD could be inhibited significantly by various inhibitors of apoptosis, particularly the caspase inhibitors.

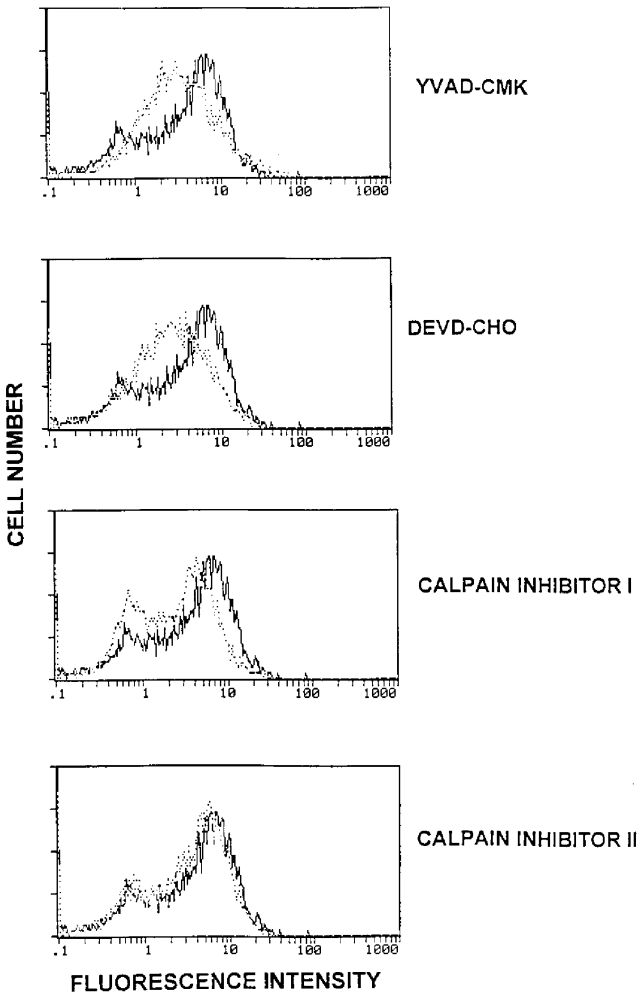

Figure 4.4. **<u>Effect of the addition of caspase inhibitors to cultures of thymocytes incubated with TCDD-treated sera:</u>** Groups of 3-4 C57BL/6 +/+ mice were injected with TCDD (50 μg/kg body weight) or the vehicle. After 1 day, the sera were collected and pooled for each group as described in Fig. 4.2. The sera were added to normal thymocytes from wild-type mice and such cultures were either incubated in the presence of inhibitors of apoptosis or in the presence of the vehicle that was used to dissolve these compounds. The bold histograms represent normal thymocytes cultured with TCDD-treated sera in the presence of the vehicle used to dissolve the inhibitors and the broken histogram depicts the normal thymocytes cultured with TCDD-treated sera in the presence of the various inhibitors.

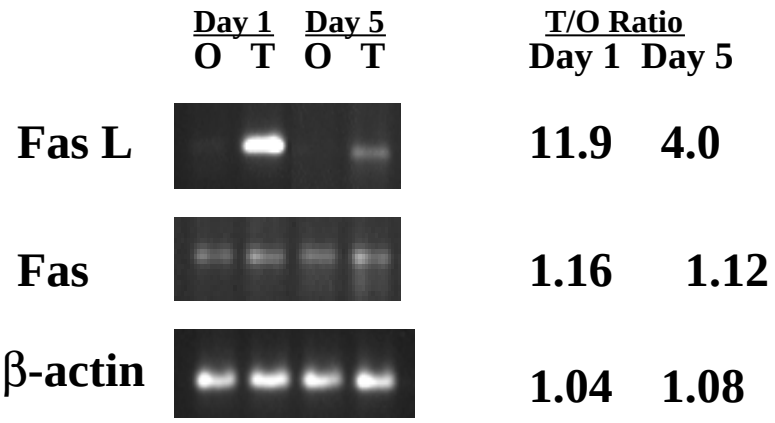

Figure 4.5: **Expression of Fas and FasL genes upon TCDD-treatment:** Total RNA was extracted from the thymus of TCDD (50 μg/kg) (T) or oil (O)-treated C57BL/6 +/+ mice 1 or 5 days after treatment and reverse transcribed. The cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for Fas, FasL or β-actin as an internal control. The PCR products were electrophoresed through a 1.5% agarose gel containing ethidium bromide and quantitated using a digital camera. The data were expressed as a ratio of the density of PCR product in TCDD-treated versus control-treated groups.

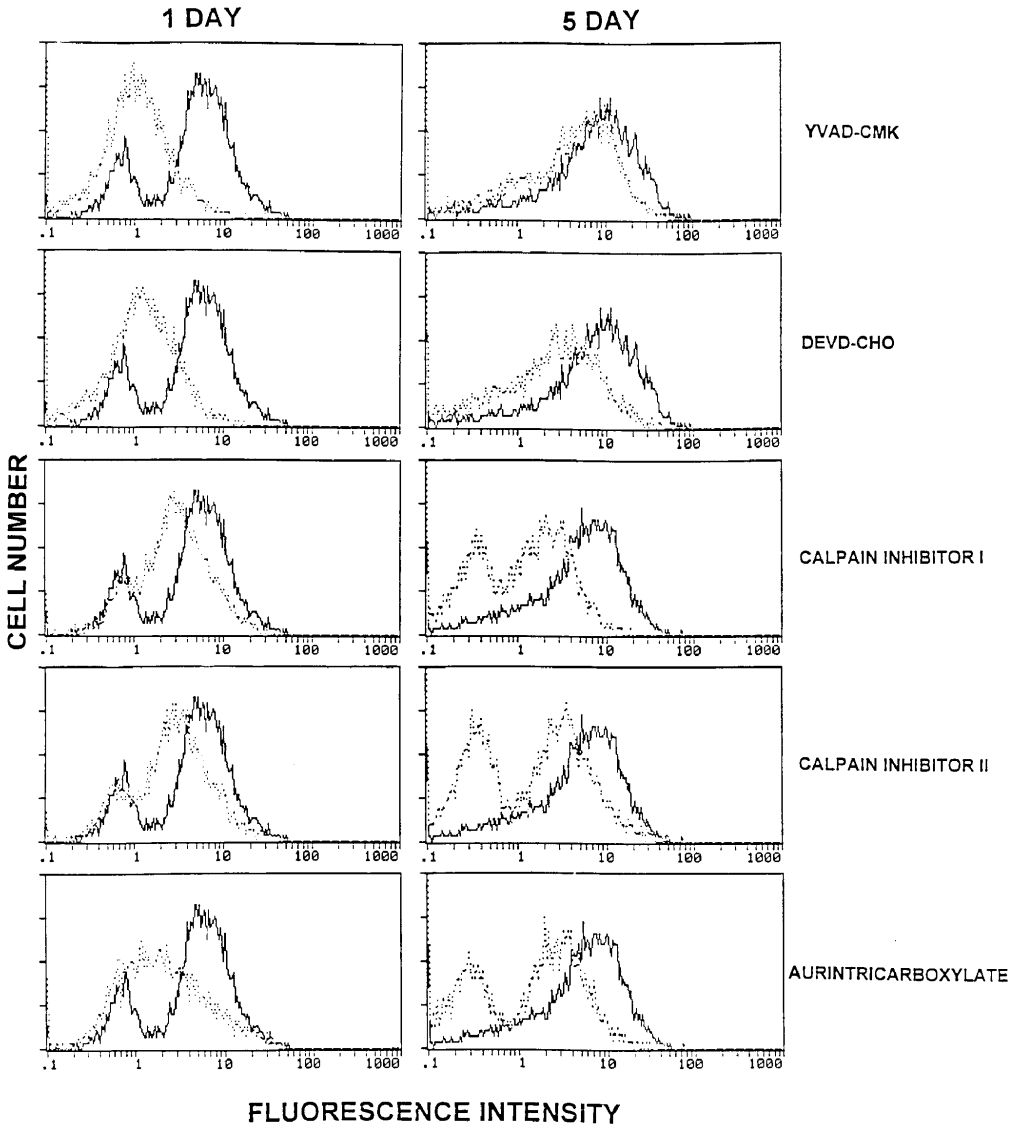

Figure 4.6: **<u>TCDD-mediated apoptosis can be blocked by inhibitors of apoptosis *in vitro*</u>:** One or 5 days after TCDD (50 μg/kg body weight) administration, thymocytes from C57BL/6 +/+ mice were harvested and cultured for 24 hours in the presence or absence of the inhibitors. The thymocytes were then stained with FITC-dUTP to detect apoptosis. The bold histograms represent thymocytes from mice treated with TCDD, cultured with DMSO (control). The broken histograms depict thymocytes from mice treated with TCDD, cultured with various inhibitors.

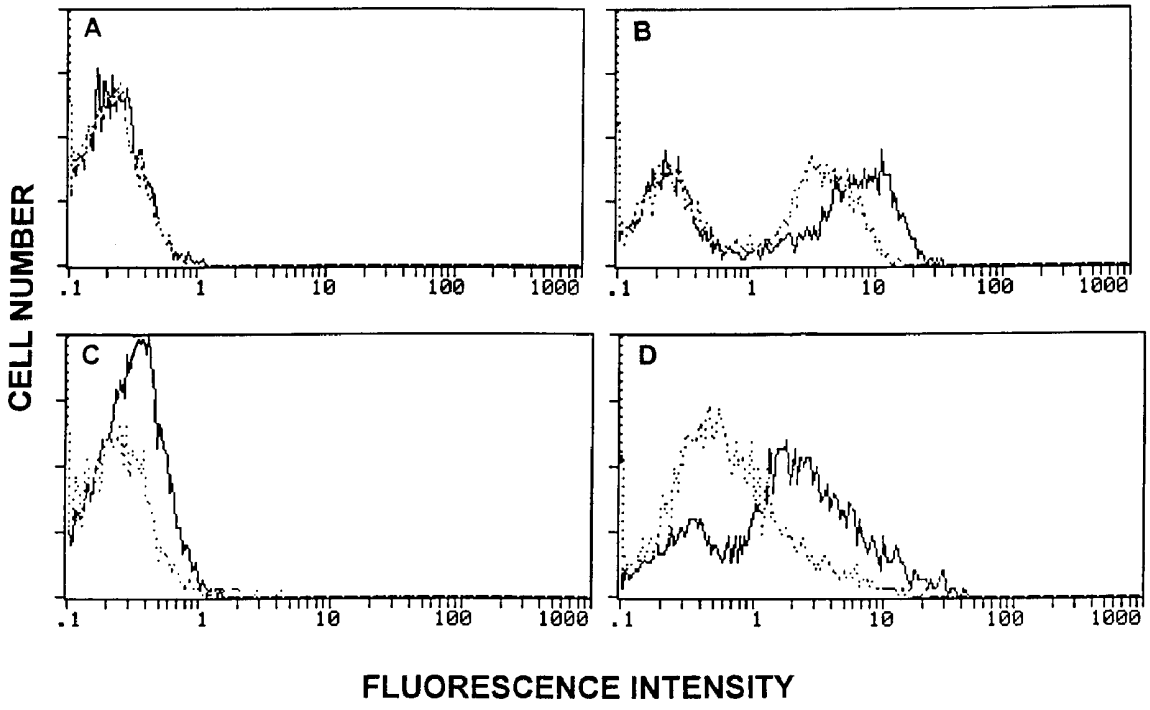

Figure 4.7: <u>TCDD-mediated apoptosis can be blocked by inhibitors of apoptosis *in vivo:*</u> Groups of 2-3 C57BL/6 +/+ mice were injected with 50 μg/kg body weight of TCDD. Caspase inhibitor, YVAD-CMK, was injected at 40 mg/kg body weight and after 12 (panels A and B) or 24 (panels C and D) hours, the thymocytes were stained for apoptosis immediately following isolation (panels 7A and C) or following *in vitro* culture for 24 hrs (panels 7B and D). The bold histograms represent the thymocytes from mice treated with TCDD and DMSO (control) while the broken histograms represent thymocytes from mice treated with TCDD and the inhibitor, YVAD-CMK.

Phenotypic alterations in thymocytes from +/+, lpr and gld mice following TCDD-treatment:

In previous studies, we noted that TCDD-treatment caused characteristic alterations in the density of expression of surface markers on thymocytes (Kamath *et al*., 1998). Furthermore, such alterations were identical to thymocytes undergoing spontaneous apoptosis (Kishimoto *et al*., 1995). In the current study, we therefore tested whether thymocytes from *lpr* and *gld* mice which were more resistant to TCDD-induced apoptosis, would fail to exhibit phenotypic alterations. To this end, C57BL/6 +/+, *lpr* and *gld* mice were injected with 50 μg/kg body weight of TCDD and on day 5, the thymocytes were analyzed for the expression of various surface molecules as described (Kamath *et al*., 1998). The data from a representative experiment have been shown in Fig. 4.8, 4.9 and 4.10 and pooled data from multiple experiments have been depicted in Fig. 4.11. The data shown in Fig. 4.8 indicated that in +/+ mice, there was a significant increase in the density of expression of CD3, αβTCR, IL-2R, CD44 and a decrease in the expression of CD4, CD8 and J11d. Furthermore, the percentage of thymocytes expressing IL-2R and CD44 increased significantly, while the percentage of CD4, CD8 and J11d decreased following TCDD-treatment. These phenotypic alterations were identical to those reported in an earlier study (Kamath *et al*., 1998). In contrast, in the *lpr* (Fig. 4.9) and *gld* (Fig. 4.10) mice, there were less marked phenotypic alterations. For example, the percentage of thymocytes expressing various surface markers in *lpr* (Fig. 4.9) and *gld* (Fig. 4.10) mice following TCDD-treatment, was found to be similar to the vehicle-treated controls. A notable exception was the increase in the percentage of $CD44^+$ thymocytes in both *lpr* and *gld* mice which was comparable to that seen in wild-type mice (Fig. 4.8). In Fig. 4.11, the data were expressed as a percent change in M. F. I. in +/+, *lpr* and *gld* mice treated with TCDD. From these data it was striking that *lpr* and *gld* thymocytes showed minor up-regulation of CD3, αβTCR and IL-2R when compared to the +/+ mice which exhibited marked up-regulation of these molecules. The alterations in CD44 in *lpr* and *gld* mice were somewhat similar to that

seen in wild-type mice. Also, in wild-type mice, TCDD-treatment caused a decrease in the density of expression of CD4, CD8 and J11d. In contrast, in *lpr* and *gld* mice such alterations were noted to a lesser extent. In *gld* mice, TCDD-treatment caused a dramatic up-regulation of J11d, the exact reason for which was not clear. Together these data demonstrated that thymocytes from *lpr* and *gld* mice were more resistant to phenotypic alterations induced by TCDD when compared to the wild-type mice.

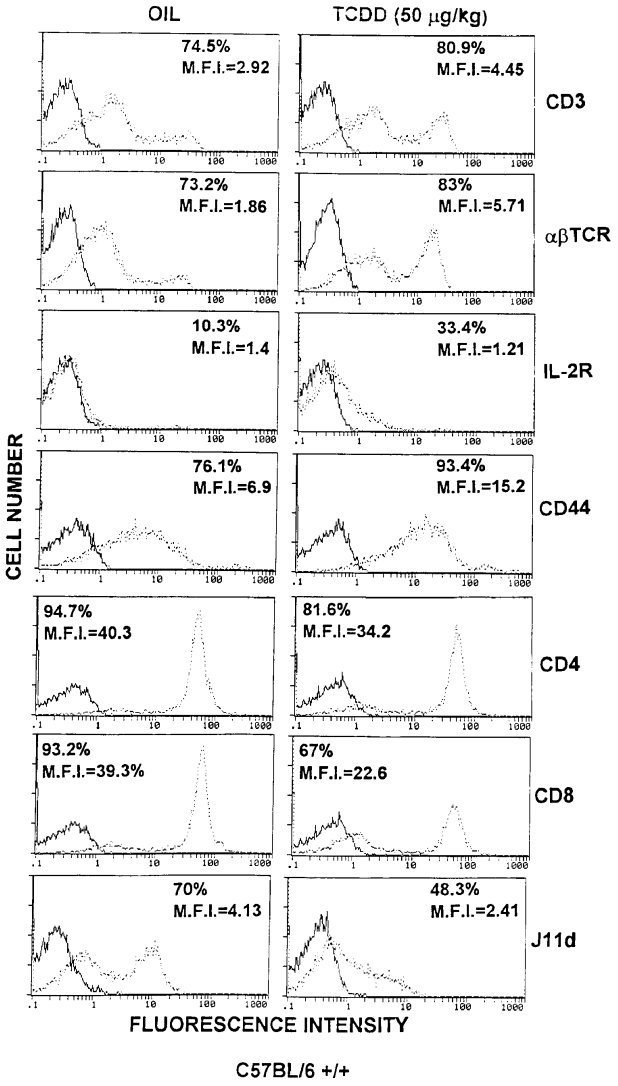

Figure 4.8: <u>Effect of TCDD-administration on the surface phenotype of thymocytes from C57BL/6 +/+ mice:</u> TCDD (50 μg/kg) or the vehicle (corn oil) was injected intraperitoneally into C57BL/6 +/+ mice and 5 days later, the thymocytes were harvested and stained with FITC or PE-conjugated mAbs against CD3, αβTCR, IL-2R, CD44, CD4, CD8 or J11d. Cells were analyzed using a flow cytometer. The bold histogram represents antibody isotype controls and broken histogram represents thymocytes stained for various surface markers. The percent positive cells and mean intensity of fluorescence (M.F.I.) for stained cells has been depicted for each histogram. The figure represents data from a single representative experiment.

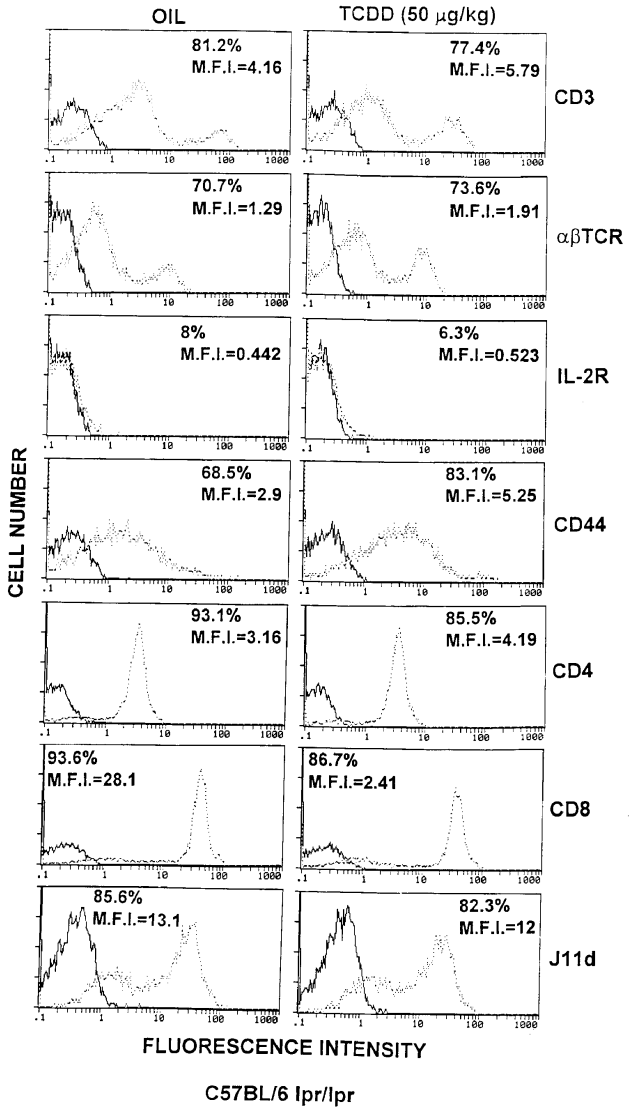

Figure 4.9: <u>Effect of TCDD-administration on the surface phenotype of thymocytes from *lpr* mice:</u> *lpr* mice were injected with 50 μg/kg body weight of TCDD or the vehicle (corn oil) intraperitoneally and 5 days later the thymocytes were harvested and stained with various surface molecules as described in Fig. 4.8. The bold histogram represents antibody isotype controls, and the broken histogram represents thymocytes stained for various surface markers. The percentage positive cells and M.F.I. for stained cells has been depicted for each histogram. The figure represents data from a single representative experiment.

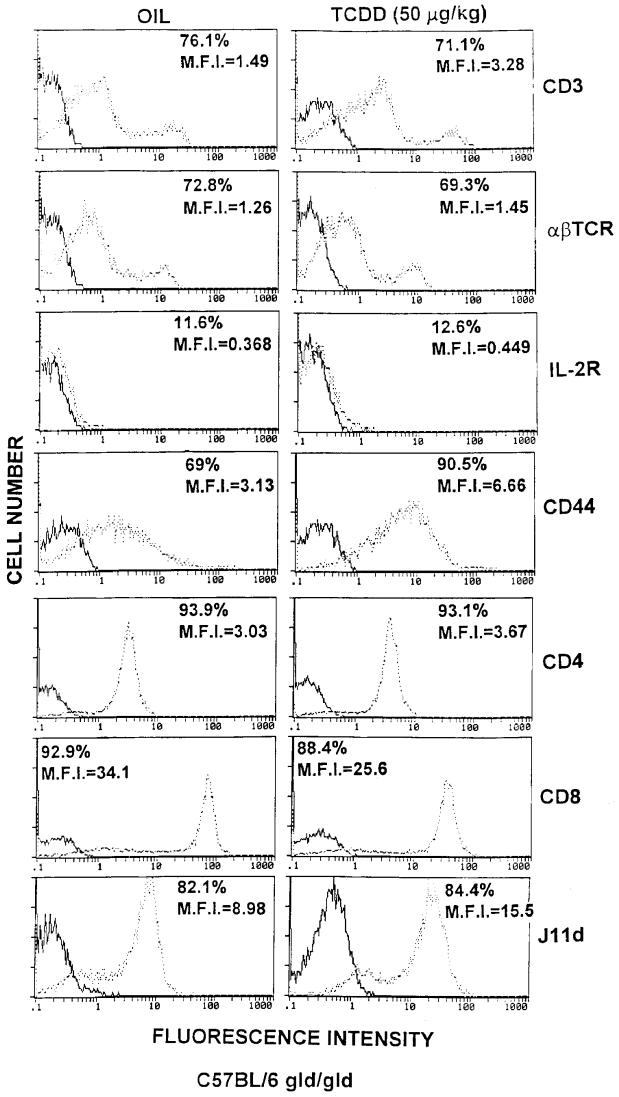

Figure 4.10: **<u>Effect of TCDD-administration on the surface phenotype of thymocytes from *gld* mice:</u>** *gld* mice were injected intraperitoneally with 50 μg/kg body weight of TCDD or the vehicle (corn oil) intraperitoneally and 5 days later the thymocytes were harvested and stained with various surface molecules as described in Fig. 4.8. The bold histogram represents antibody isotype controls, and the broken histogram represents thymocytes stained for various surface markers. The percentage positive cells and M.F.I. for stained cells has been depicted for each histogram. The figure represents data from a single representative experiment.

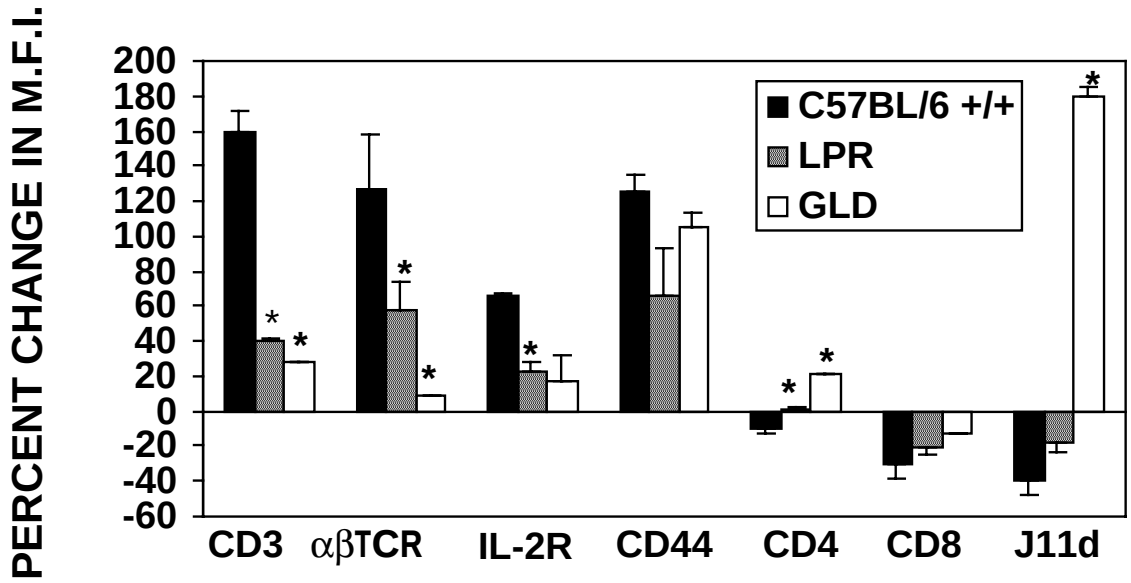

Figure 4.11: **Changes in density of surface marker expression upon TCDD-treatment in +/+, *lpr* or *gld* mice:** The thymocytes were stained for various markers as described in Fig. 4.8. The M.F.I. obtained with each marker in TCDD-treated mice were compared to the M.F.I. of the vehicle controls and the data were expressed as mean percent change in M.F.I. as described in the methods. The negative controls consisted of the appropriate fluorescent antibody isotypes. The percent change in M.F.I. from 4-5 individual experiments were pooled to obtain the mean ± S.E.M. and plotted. Data showing statistically significant difference ($p<0.05$) when compared to controls, have been depicted with an asterisk.

Discussion

In the current study, we noted that thymocytes from *lpr* and *gld* mice were more resistant to TCDD-induced toxicity and apoptosis particularly at concentrations of 0.1-5 μg/kg body weight. The thymocytes from TCDD-treated *lpr* and *gld* mice failed to undergo increased apoptosis when tested immediately following isolation as well as following *in vitro* culture when compared to the thymocytes from vehicle-treated wild-type mice. This was corroborated by the observation that significant phenotypic alterations, characteristic of apoptotic cells, were seen in wild-type but not in *lpr* or *gld* mice. The serum from TCDD-treated wild-type mice contained higher levels of soluble FasL when compared to serum samples from vehicle-treated mice. Furthermore, sera from TCDD-treated but not vehicle-treated mice were able to induce apoptosis in Fas^+ but not the Fas^- target cells, thereby indicating that TCDD induces the production of soluble FasL. Moreover, TCDD-treatment caused significant increase in the expression of FasL but not Fas gene in the thymus. The TCDD-induced apoptosis in the thymocytes could be blocked both *in vitro* and *in vivo* by a variety of inhibitors of apoptosis. These data together demonstrated that TCDD-treatment induces marked apoptosis and thymic atrophy through Fas-FasL interactions, in wild-type mice.

In the current study, it was observed that TCDD at concentrations of 0.1, 1 and 5 μg/kg body weight caused significant thymic atrophy only in wild-type but not in *lpr* or *gld* mice. However, at 50 μg/kg body weight, TCDD caused significant thymic atrophy in all 3 strains of mice. These data demonstrated that at lower concentrations, TCDD may exclusively use the FasL-based pathway to induce apoptosis in thymocytes. However, at higher concentrations such as 50 μg/kg body weight, in addition to FasL-based mechanism, it may use alternative mechanisms such as inducing toxicity in the bone marrow precursor cells thereby preventing the seeding of the T cells in the thymus as suggested in other studies (Fine *et al*., 1989, 1990). This may contribute towards

thymic involution. This notion was supported by the observation that thymocytes from wild-type mice treated with 50 μg/kg body weight of TCDD demonstrated significant apoptosis when stained with FITC-dUTP, whereas, thymocytes from *lpr* and *gld* mice failed to exhibit apoptosis even at 50 μg/kg body weight. The fact that thymocytes from *lpr* and *gld* mice fail to undergo apoptosis following TCDD-treatment was also corroborated by the observation that such cells failed to exhibit significant phenotypic changes characteristic of apoptotic cells as seen in wild-type mice (Kamath *et al*., 1998).

It is well established that many toxic manifestations of TCDD are mediated by the binding of TCDD to the AhR and subsequent changes in the gene expression in the responsive cells. TCDD is known to be a potent transcriptional regulator of several genes in a variety of tissues including the cytochrome P4501A1, TGF-α and nuclear inhibitor-2 (Poland and Knutson, 1982, Choi *et al*., 1991, Sutter *et al*., 1991). The dioxin responsive elements (DREs) are found in the regulatory regions of several genes including many cytokines and growth factors (Lai *et al*., 1996, Dohr *et al*., 1994). Thus, binding of TCDD to the AhR may up-regulate the expression of FasL which is considered to be a cytokine.

The presence of DREs in the promoter region of FasL is yet unknown. However, recent studies have suggested that TCDD up-regulates the binding of nuclear protein to activator protein-1 (AP-1) response element (Ashida and Matsumura, 1998). Furthermore, AP-1 was shown to induce the expression of FasL (Kasibhatla *et al*., 1998). Thus, TCDD may activate FasL through AP-1 response element.

Fas ligand is considered to be a cytokine belonging to the TNF family (Nagata, 1997). Furthermore, FasL is also produced in soluble form (Sato *et al*., 1996, Nagata, 1997). The soluble form of FasL may be responsible for inducing systemic toxicity. Although some studies have suggested that soluble form of FasL is less toxic than membrane-bound FasL, the former is implicated in many diseases including hepatitis, AIDS and

cancer. Sato *et al*. (1996) reported a case of nasal lymphoma accompanied by liver damage and pancytopenia. This was correlated with high levels of soluble FasL. After chemotherapy, the liver damage improved along with a decrease in serum soluble FasL. The soluble FasL may induce apoptosis in tissues and organs that constitutively express high levels of Fas. Fas is expressed at higher levels in the thymus, lungs, liver and heart and at lower levels in the spleen, lymph nodes and small intestine (Suda *et al*., 1995). This may be the reason why the thymus is more sensitive than peripheral T cells for apoptosis induction by TCDD. It should be noted that Fas is also produced in soluble form by alternative splicing of Fas mRNA (Hughes *et al*., 1995).

In addition to the soluble form of FasL, if the cells express both Fas and FasL, they may die through an autocrine pathway. Initial antigen stimulation of lymphocytes can lead to cell activation and growth. However, subsequent T cell receptor engagement can lead to apoptosis in activated cells. This process has been termed as propriocidal regulation (Critchfield *et al*., 1994). To this end, we noted in an earlier study that T cells activated *in vivo* with anti-CD3 mAbs from TCDD-treated mice underwent rapid apoptosis upon re-stimulation, whereas, naïve T cells failed to undergo apoptosis (Pryputniewicz *et al*., 1998). This may be because activated T cells express increased levels of Fas and therefore such cells may become more susceptible to FasL- based apoptosis mediated by TCDD. This may also explain why Fas-deficient peripheral T cells from *lpr* mice are more resistant to TCDD-mediated immunotoxicity (Rhile *et al*., 1996).

The apoptotic cell death required activation of caspases. The direct pathway leading to activation of caspases is triggered by the members of the TNF receptor superfamily which includes Fas. Fas and TNF have been shown to activate caspase 8 and/or caspase 2 to initiate the apoptotic pathway (Froelich *et al*., 1998). These caspases process caspase 3 which in turn, activates other "downstream" caspases such as 7, 4, 5, 6 and 9. Several studies have demonstrated that caspase-inhibitors can prevent induction of apoptosis *in vitro* and *in vivo* (Livingston, 1997). In the current study, we noted that

YVAD-CMK which inhibits caspases 1, 4, 3 and 7 and DEVD-CHO which inhibits caspase 3, could block the TCDD-induced apoptosis. These data suggested that TCDD-induced apoptosis involves production of caspases.

In an earlier study, we noted that TCDD-treatment caused significant alterations in the expression of surface molecules by thymocytes (Kamath *et al*., 1998). TCDD-treated thymocytes expressed higher density of CD3, αβTCR, IL-2R, and CD44 and decreased levels of CD4, CD8 and J11d. These alterations were identical to the phenotypic changes seen in thymocytes undergoing spontaneous apoptosis in culture (Kishimoto *et al*., 1995). These data together suggested that alterations in the density of expression of surface markers may serve as a useful marker for apoptosis induction *in vivo*. In the current study we noted that thymocytes from *lpr* and *gld* mice failed to exhibit apoptosis even at high concentrations of TCDD such as 50 μg/kg body weight. It was striking that the thymocytes from these mice also exhibited minimal phenotypic alterations when compared to the thymocytes from wild-type mice. These studies further corroborated our finding that thymocytes from *lpr* and *gld* mice were more resistant to TCDD-induced apoptosis and immunotoxicity, thereby implicating Fas and/or FasL. Together, the current study demonstrates that TCDD may mediate its toxicity on thymocytes by up-regulating the expression of FasL and inducing apoptosis in Fas^+ cells. Recent studies have shown that in the thymus, only the stromal cells but not the T cells express FasL (French *et al*., 1997). Inasmuch as, T cells interact closely with the stromal cells during differentiation in the thymus, up-regulation of FasL on stromal cells induced by TCDD may in turn trigger apoptosis in Fas^+ cells that come in contact with the stromal cells.