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# 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Enhances Negative Selection of T Cells in the Thymus but Allows Autoreactive T Cells to Escape Deletion and Migrate to the Periphery

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## ABSTRACT

Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an environmental pollutant, has been shown to cause thymic atrophy and apoptosis. However, whether TCDD alters the process of T-cell selection in the thymus is not clear. To this end, we investigated the effects of TCDD in the context of the HY-T-cell receptor (TCR) transgenic (Tg) mouse model. We noted that negatively selecting male HY-TCR Tg mice were significantly more sensitive to the thymotoxic effects of TCDD relative to positively selecting female HY-TCR Tg mice, including increased reduction in cellularity and increased induction of apoptosis. TCDD exposure also altered the thymocyte subset composition in HY-TCR Tg male but not female mice. In addition, TCDD treatment resulted in increased extracellularly regulated kinase phosphorylation and lymphocyte-specific protein tyrosine kinase expression in thymocytes of HY-TCR Tg male but not female mice. The increase in proportion of $CD8^+$ mature thymocytes noted in HY-TCR Tg male mice was reflected in the periphery, with TCDD-exposed HY-TCR Tg male mice having increased numbers of $CD8^+$ T cells. Finally, we noted that the proliferative response of HY-TCR Tg male T cells to HY(self)-Ag was enhanced after exposure to TCDD, whereas that of HY-TCR Tg female mice was decreased. Taken together, these data suggest that TCDD alters the process of thymic selection, possibly by enhancing negative thymocyte selection, whereas at the same time allowing autoreactive T cells to escape deletion in the thymus and immigrate to the periphery.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin) is a persistent environmental contaminant and is considered one of the most toxic compounds ever created (Kerkvliet, 2002; Paustenbach, 2002). The toxic effects of TCDD are believed to be mediated largely by transcriptional regulation through the aryl hydrocarbon receptor (AhR) (Rowlands and Gustafsson, 1997). Upon binding of TCDD or a congener, the ligand-AhR complex translocates to the nucleus where it heterodimerizes with the aryl hydrocarbon nuclear translocator (ARNT). This complex (ligand-AhR-ARNT) then binds to dioxin-responsive elements with the consensus sequence 5′-GCGTGNN(A/T)NNN(C/G)-3′ located in the regulatory regions of dioxin-responsive genes, in which it acts as a transcription factor (Yao and Denison, 1992). Therefore, TCDD has the potential to directly alter the expression of a large number of genes. For example, TCDD exposure has been shown previously to induce the expression of several genes through the above mechanism, including cytochrome P4501A1/2, cytochrome P4501B1, glutathione *S*-transferase Ya, aldehyde dehydrogenase 3, and UDP-glucuronosyl transferase 1*06 (Nebert et al., 2000). One of the most reproducible toxic endpoints resulting from TCDD exposure is thymic involution (Vos et al., 1997; Grassman et al., 1998). Recent studies from our group have demonstrated that TCDD acts, in part, by inducing apoptosis in thymocytes and, further, that TCDD-induced apoptosis of thymocytes is partially me-

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**ABBREVIATIONS:** TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ERK, extracellularly regulated kinase; Lck, lymphocyte-specific protein tyrosine kinase; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DP thymocyte, $CD4^+/CD8^+$ double-positive thymocyte; DN thymocyte, $CD4^-CD8^-$ double-negative thymocyte; TCR, T cell receptor; Tg, transgenic; MHC, major histocompatibility complex; LIGHT (TNFSF14), lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; ConA, concanavalin A; APC, antigen-presenting cell; FCS, fetal calf serum; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; SP, single positive; PE, phycoerythrin; HY-Ag, histocompatibility Y antigen.

diated through Fas/Fas ligand interactions (Rhile et al., 1996; Kamath et al., 1999; Dearstyne and Kerkvliet, 2002; Fisher et al., 2004). In addition, previous studies have demonstrated that TCDD does not affect resting T cells, but that T cells undergoing activation in response to antigen are highly sensitive to TCDD-induced apoptosis (Camacho et al., 2001, 2002). However, the underlying causes of thymic atrophy and increased thymocyte apoptosis after TCDD exposure remain elusive.

The primary function of the thymus is the generation of a T-cell repertoire capable of MHC-restricted antigen recognition while eliminating T-cells that are potentially autoreactive through clonal deletion (Sebzda et al., 1999). We have suggested that exposure to TCDD may alter the dynamics of thymic selection in part through dysregulation of coreceptors and costimulatory molecules believed to play a role in thymic selection (Fisher et al., 2004). This hypothesis has important implications inasmuch as failures of thymic selection can lead to autoimmune disease when negative selection is inefficient or to a deficient T-cell repertoire if positive selection is blocked. Most current data support the affinity/avidity model of thymic selection, in which interactions between the rearranged T-cell receptor (TCR) expressed on $CD4^+CD8^+$ double-positive (DP) thymocytes and MHC molecules expressed by thymic epithelial or hematopoietic cells bearing peptides derived from proteolytic processing of self-antigen (MHC/self-peptide complexes) induce TCR-dependent signaling in DP thymocytes. The intensity of the TCR signal determines the fate of individual thymocytes. Most (~95%) DP thymocytes, after V(D)J rearrangement, express a TCR that is not able to interact with MHC/self-peptide complexes on thymic APC. These thymocytes undergo apoptosis because they are not rescued by TCR-mediated survival signals. Thymocytes bearing TCRs capable of productive interaction with MHC/self-peptide complexes are either positively or negatively selected. Weak engagement of TCR on DP thymocytes with MHC/self-peptide complexes on thymic antigen-presenting cells rescues <5% of thymocytes from death by neglect (positive selection) (Sprent and Kosaka, 1993; Jameson et al., 1995; Hogquist, 2001; Sprent and Kishimoto, 2002). Negative selection removes potentially autoreactive thymocytes, those bearing rearranged TCR having a strong affinity for MHC/self-peptide complexes, which account for ~1% of thymocytes. Much recent study has focused on the interactions involved in positive and negative selection and, specifically, how signals mediated through the TCR are able to trigger both survival and apoptosis. Current models of T-cell selection suggest that the affinity/avidity of TCR for MHC/self-peptide complex is central to T-cell fate during selection by affecting the duration of the interaction between T cells and antigen-presenting cells (Dave et al., 1999). In particular, the interactions of TCR with high affinity for MHC/self-peptide complex are more transient than the interactions between low-affinity TCR and MHC/self-peptide. Under the duration model, high-affinity TCR interactions result in brief but intense intracellular signaling via TCR, leading to negative selection, whereas the TCR signaling mediated by low-affinity interactions is sustained and weak, leading to positive selection (Mariathasan et al., 2000, 2001).

The strength of the interaction between T cells undergoing selection and APCs is dependent on a variety of factors in addition to the avidity of TCR for MHC/self-peptide. Previous studies have shown that the outcome of the thymic selection is sensitive to levels of TCR expression on thymocytes, as well as to the concentration of the selecting ligand (Ashton-Rickardt et al., 1994; Sebzda et al., 1994, 1996; Dave et al., 1999). In addition, the importance of costimulatory molecules in negative selection has been demonstrated in several recent studies. For example, studies have shown that CD30 is involved in negative selection inasmuch as $CD30^{-/-}$ mice have impaired negative selection, whereas negative selection is enhanced in CD30 overexpressing mice (Amakawa et al., 1996; Chiarle et al., 1999; DeYoung et al., 2000). Likewise, mice lacking tumor necrosis factor family member LIGHT experience reduced negative selection, whereas overexpression of LIGHT enhances negative selection (Wang et al., 2001; Granger and Rickert, 2003; Wang and Fu, 2003). Taken together, these data demonstrate that altered expression of molecules involved in thymic selection can change the outcome of the selective process.

We have speculated previously that TCDD might affect positive and/or negative selection. Previous studies have shown that exposure to TCDD results in increased TCR expression (Rhile et al., 1996). In addition, we have demonstrated recently that TCDD exposure results in the up-regulation of CD30 and LIGHT in the thymus (Fisher et al., 2004). The effects of TCDD on the thymus clearly suggest the possibility that TCDD exposure might impact thymic selection and therefore alter the T-cell repertoire. In the present study, we tested the hypothesis that TCDD exposure alters thymic selection using the HY-TCR transgenic model (Kisielow et al., 1988). The HY-TCR recognizes a Y-chromosomal peptide (Markiewicz et al., 1998) in the context of $H\text{-}2D^b$. In female mice bearing the HY-transgenic TCR, thymocytes are positively selected into the $CD8^+$ compartment (Teh et al., 1988). However, in male HY-TCR Tg mice, thymocytes are negatively selected, resulting in deletion of thymocytes at the $CD4^+/CD8^+$ DP stage (Kisielow et al., 1988). We find that male HY-TCR transgenic mice are more sensitive to TCDD-induced atrophy of the thymus relative to female mice. Furthermore, the proliferative response of thymocytes and peripheral T cells in response to T-cell mitogens is reduced in male HY-TCR mice after TCDD exposure but is unaffected in TCDD-treated female HY-TCR mice.

## Materials and Methods

**Mice and TCDD Treatment.** HY-TCR transgenic mice [formal designation: C57BL/10AiTac-TgN(TCRHY)] aged 4 to 8 weeks were purchased from Taconic Farms (Germantown, NY). C57BL/6 mice were purchased from the National Institutes of Health (Bethesda, MD). Mice were housed in the Virginia Commonwealth University animal facility in a manner consistent with Institutional Animal Care and Use Committee guidelines in animal rooms maintained at 24°C on a 12-h light/dark cycle and were given water and rodent chow ad libitum. TCDD was a generous gift of Dr. Stephen Safe (Texas A&M University, College Station, TX). TCDD was dissolved in acetone and suspended in corn oil to a concentration of 10 $\mu$g/ml. The solution was then heated to 57°C to allow the acetone to evaporate. Groups of two to four mice were administered a single dose of 50 $\mu$g/kg body weight TCDD or vehicle (corn oil) via intraperitoneal injection for 48 or 72 h before death.

**Media and Culture Conditions.** After execution, thymi or peripheral lymphoid organs were harvested and rendered into a single cell suspension in RPMI 1640 medium supplemented with 10% FCS, $5 \times 10^{-5}$ M $\beta$-mercaptoethanol, and 100 U/ml gentamycin. Total

cellularity for each organ was determined under a phase-contrast microscope using the Trypan blue exclusion method. Total numbers of cells/thymus or lymph node ± standard deviation were depicted. Before detection of apoptosis, thymocytes were cultured in vitro for 18 h as described previously (Kamath et al., 1997). This was derived from the observation that apoptotic T cells are rapidly cleared in vivo by phagocytic cells present in the thymus, thereby making it difficult to detect such cells immediately after their isolation, and therefore a subsequent in vitro culture facilitates their detection because of a lack of an effective phagocytic system in vitro (Kamath et al., 1997).

**Flow Cytometry.** Freshly isolated or cultured cells ($1–2 \times 10^6$) were prepared for fluorescence-activated cell sorter analysis by washing twice with cold phosphate-buffered saline/1% FCS/0.1% $NaN_3$. Cells were incubated for 30 min on ice with the following mAbs: PE-CD8, CyChrome-CD4, FITC-T3.70 (anti-HY-TCR), FITC-H-$2D^b$, and FITC-$\beta$-TCR. All antibodies were purchased from BD Pharmingen (San Diego, CA). Cells were then washed with phosphate-buffered saline/1% FCS/0.1% $NaN_3$ and fixed in 1% paraformaldehyde. Before cytosolic staining or detection of apoptosis by TUNEL, cells were permeabilized by incubation with Triton X-100 in 0.1% sodium citrate on ice for 2 min. Cells were then washed and labeled with FITC-dUTP during a 1-h incubation at 37°C or incubated with Lck or phosho-ERK mAbs, followed by incubation with FITC-anti-mouse IgG. fluorescence-activated cell sorter analysis was performed using a Coulter FC500 (Beckman Coulter, Fullerton, CA), and 50,000 cells were analyzed per sample. For assessment of the effect of TCDD on T-cell subsets, T cells were triple-stained with CyChrome-CD4, PE-CD8, and FITC-HY-TCR (T3.70) mAbs, and HY-TCR–deficient cells were excluded from the analysis so that cells expressing endogenous TCR would not skew results. However, in both HY-TCR male and female mice, >95% of T cells were HY-$TCR^+$ and therefore T cells expressing endogenous TCR were insignificant. To assess apoptosis in T-cell subsets, T cells were stained with CyChrome-CD4, PE-CD8, and FITC-dUTP, and the various T-cell populations were gated on and analyzed for TUNEL positivity.

**T Cell-Proliferative Response to Mitogens.** Thymocytes, splenocytes, or cells from the lymph node obtained from oil- or TCDD-treated mice were tested for the ability to respond to various mitogens in vitro. Cells ($0.5 \times 10^6$/well) were cultured in 96-well flat-bottomed plates with 0.2 ml of medium for 48 h at 37°C in 5% $CO_2$ and stimulated with ConA (2 $\mu$g/ml) or anti-CD3 (5 $\mu$g/ml) and were pulsed with 2 $\mu$Ci [$^3$H]thymidine during the final 8 h of incubation. Cells were harvested using an automated cell harvester (Skatron, Sterling, VA). The amount of radioactivity was determined using a scintillation counter and the mean cpm ± standard deviation of triplicate cultures was calculated.

**Response of Lymphocytes to HY-Ag.** Lymphocytes were harvested from the popliteal lymph nodes of vehicle or TCDD-exposed (50 $\mu$g/kg, 72 h) male and female HY-TCR Tg mice, and $5 \times 10^5$ cells from each treatment group were stimulated in triplicate cultures with $4 \times 10^5$ 2000R-irradiated stimulator cells isolated from spleens of male HY-TCR Tg mice to assay the T-cell response to male HY-Ag. Effector and stimulator cells were cultured together for 48 h and pulsed with 2 $\mu$Ci [$^3$H]thymidine during the final 8 h of incubation. Proliferation was then measured as described earlier.

**Calculation of Statistical Significance.** Experiments were repeated three times, with each treatment group consisting of two to four mice. Statistical differences between pooled samples were detected using Student's $t$ test or the $\chi^2$ goodness-of-fit test as appropriate. A $p$ value of $\leq 0.05$ was considered significant.

## Results

**Response of the Wild-Type (C57BL/6) Thymus to TCDD.** We first assessed the effect of TCDD on the thymus of male and female C57BL/6 wild-type (WT) mice to confirm previous observations regarding the effects of TCDD the normal murine thymus. To this end, C57BL/6 WT mice were exposed to 50 $\mu$g/kg body weight of TCDD for 48 to 72 h. As shown in Fig. 1A, exposure of WT mice to TCDD for 72 h resulted in an approximately 30% decrease in thymic cellularity, and it caused a significant increase in thymic apoptosis compared with the control mice (Fig. 1B). There was a significant increase in apoptosis in both $CD4^-CD8^-$ double-negative (DN) and DP subpopulations of thymocytes after TCDD exposure (Fig. 1B), whereas the single positive cells failed to show increased apoptosis (data not shown). The induction of thymic atrophy and apoptosis suggested that TCDD may alter the T-cell differentiation in the thymus, and to investigate this further, we used HY-TCR mice.

**Male and Female HY-TCR Mice Are Differentially Susceptible to the Thymic Atrophy Induced by TCDD.** When TCDD (50 $\mu$g/kg) was administered into HY-TCR mice, as shown in Fig. 2A, male HY-TCR mice showed an average 62.5% reduction in thymic cellularity, whereas female HY-TCR mice showed only a 23.7% reduction in thymic cellular-

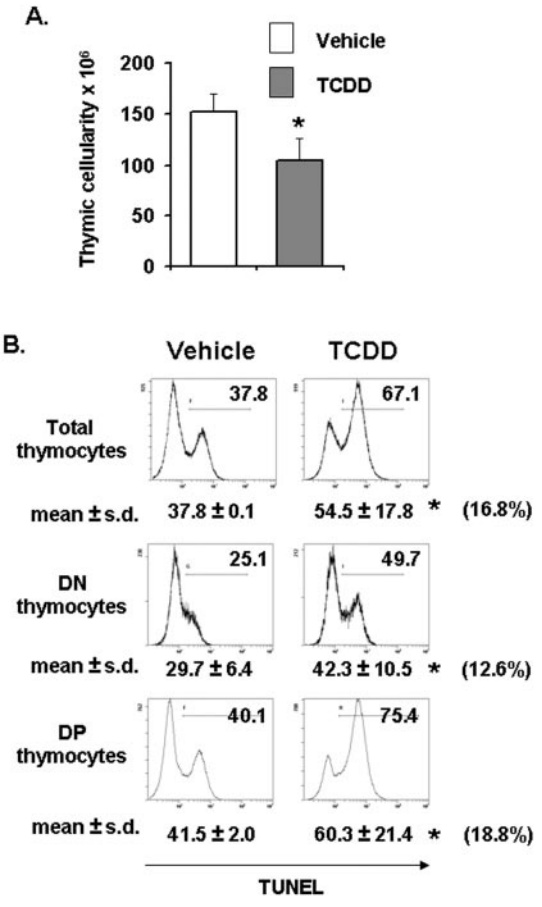


**Fig. 1.** Effects of TCDD exposure on the thymus of C57BL/6 WT mice. A, after exposure to either 50 $\mu$g/kg TCDD or vehicle for 72 h, thymi were harvested to determine cellularity. Data are expressed as mean cellularity/thymus ± S.D. Statistical significance ⋆, $p < 0.05$. B, thymocytes harvested as described above were assessed for apoptosis using TUNEL as well as stained with PE-anti-CD8 and CyChrome-anti-CD4 mAbs. Apoptosis in DN and DP thymocytes was determined by electronically gating those subpopulations before assessing FITC-dUTP labeling. Individual histograms show representative data with the percentage of apoptotic cells, and numbers below each histogram depict data from multiple experiments showing the mean percentage apoptosis ± S.D. The average percentage increase in apoptosis in TCDD-treated groups compared with the vehicle controls is indicated in parenthesis.

ity compared with the control mice. To determine whether the variation in the effects of TCDD on thymic atrophy between male and female HY-TCR mice might be caused by differential induction of apoptosis, thymocytes from male and female HY-TCR mice exposed to vehicle or TCDD were analyzed for apoptosis using TUNEL assay. As shown in Fig. 2B, TCDD treatment led to a significant increase in apoptosis in the whole thymus as well as in DN but not DP subpopulations compared with the control mice in male HY-TCR mice. It should be noted that the DP thymocytes isolated from HY-TCR male mice showed high levels of spontaneous apoptosis (>90%), which may be indicative of increased negative selection in male mice. This could be the reason why the TCDD-induced effect was not further apparent in male DP thymocytes. It is worth noting that although TCDD treatment did not cause a significant increase in the percentage of apoptosis in DP thymocytes from male mice, it did result in induction of apoptosis in almost the entire DP population. When apoptosis was similarly tested in female HY-TCR mice, TCDD also caused an increase in apoptosis in whole thymocytes as well as in both DP and DN thymocytes compared with the controls; however, this increase was not statistically significant. Overall, thymocytes from HY-TCR male mice seem to be more sensitive to TCDD-induced apoptosis than thymocytes from HY-TCR female mice. It is interesting that DN thymocytes from HY-TCR male mice seemed to be particularly more sensitive to TCDD-induced apoptosis relative to DN thymocytes from HY-TCR female mice. It should be noted that there are no known gender differences in the

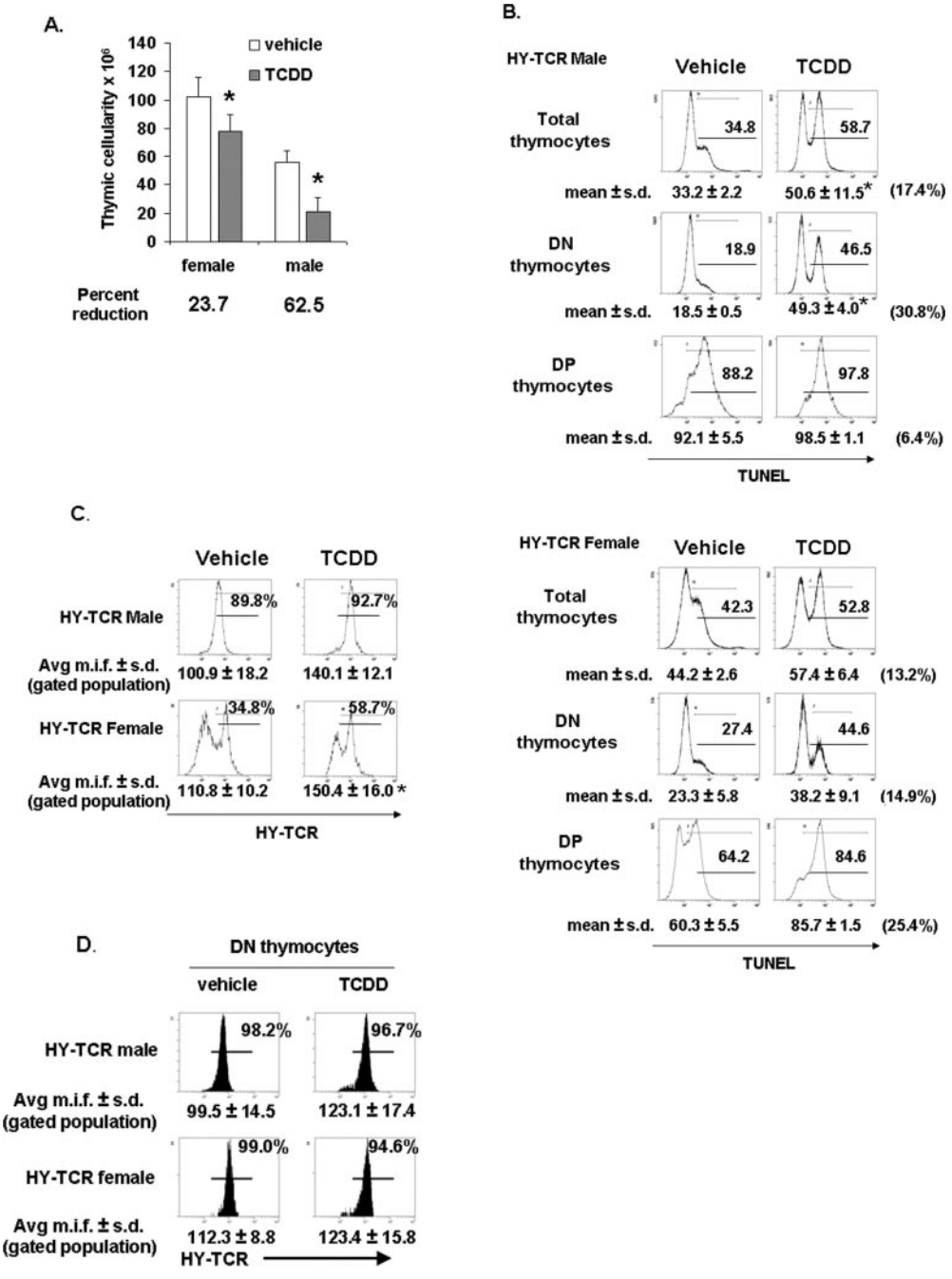


**Fig. 2.** Effects of TCDD exposure on the thymus of male and female HY-TCR Tg mice. A, after exposure to either 50 $\mu$g/kg TCDD or vehicle for 72 h, thymi were harvested to determine cellularity. Bars indicate the average cellularity/thymus ± S.D. for vehicle and TCDD-exposed mice, with four mice/group. The average percentage reduction in thymic cellularity after TCDD exposure compared with vehicle controls is depicted below the bar graph. ⋆, $p < 0.05$, statistical significance between treatments. B, apoptosis in total thymocytes as well as in DP or DN T cell subsets in male and female HY-TCR Tg mice was assessed as described in Fig. 1. Individual histograms show representative data with the percentage of apoptotic cells, and numbers below each histogram depict data from multiple experiments showing the mean percentage apoptosis ± S.D. The average percentage increase in apoptosis in TCDD-treated groups compared with the vehicle controls is indicated in parenthesis. C, cell-surface expression of HY-Ag–specific Tg TCR was assessed using an FITC-labeled monoclonal antibody (T3.70). Gated populations in histograms representing HY-TCR expression in female HY-TCR Tg mice are considered HY-TCR$^{HI}$. The percentage of HY-TCR$^{HI}$ thymocytes is indicated in the histogram. The mean intensity florescence (m.i.f.) ± S.D. of HY-TCR expression on HY-TCR$^{HI}$ thymocytes from multiple experiments is indicated below the histograms. ⋆, statistically significant differences in HY-TCR expression (m.i.f.) between TCDD- and vehicle-treated groups. D, HY-TCR expression on the CD4$^-$/CD8$^-$ (DN) thymocyte subpopulation. DN thymocytes were electronically gated and then analyzed for TCR expression. Percentage of TCR$^{HI}$ thymocytes (gated population) is indicated within each histogram, and the average m.i.f. ± S.D. of HY-TCR expression is indicated below each histogram.

immunotoxic effects of TCDD (Silverstone et al., 1994; Lai et al., 1998, 2000).

**TCDD Exposure Increases HY-TCR Expression.** To determine whether the increase in thymocyte apoptosis might be caused in part by increased TCR signaling, we first investigated whether TCDD altered HY-TCR expression levels on thymocytes. As shown in Fig. 2C, the mean intensity fluorescence of HY-TCR expression on thymocytes from HY-TCR male mice increased from 100.9 ± 18.2 to 140.1 ± 12.1 after 48-h exposure to 50 μg/kg TCDD. In female HY-TCR mice, the percentage of HY-TCR$^{HI}$ thymocytes increased from 34.8 to 58.7%, and the mean intensity fluorescence of this population increased significantly from 110.8 ± 10.2 to 150.4 ± 16.0. To specifically address the possibility that the differences in TCDD-induced apoptosis between HY-TCR male and female DN thymocyte subsets described in Fig. 2B might be caused by differences in TCR expression, we measured the levels of HY-TCR in the DN subpopulation. As shown in Fig. 2D, >94% of the DN thymocytes were HY-TCR$^{HI}$ in both male and female mice. Moreover, TCDD exposure caused a modest increase in the levels of HY-TCR in both male and female mice.

**Effect of TCDD on T-Cell Subsets in Male and Female HY-TCR Mice.** The data from a representative experiment are shown in Fig. 3A, and those from multiple experiments are summarized in Fig. 3B. Vehicle-treated HY-TCR male mice showed very poor differentiation of DP and CD8$^+$ T cells, which is expected because of negative selection of T cells bearing CD8 in these mice. In contrast, the vehicle-treated female mice had a large proportion of DP and CD8$^+$ T cells caused by enhanced positive selection. After TCDD treatment, the male mice had a statistically significant reduction in DN thymocytes, from 78.1 ± 6.1 to 58.2 ± 8.0%, whereas the percentage of DP and single positive T cells increased significantly. In contrast, the HY-TCR female mice showed no significant change in the percentage of DN thymocytes, as well as all other subpopulations, after TCDD exposure.

**TCDD Treatment Results in Increased ERK Phosphorylation and Lck Expression in Thymocytes.** To determine whether increased TCR expression might have functional consequences such as enhanced TCR signaling, we assessed levels of ERK phosphorylation and Lck expression in thymocytes after exposure to 50 μg/kg TCDD for 48 h. As shown in Fig. 4A, exposure to TCDD resulted in a dramatic enhancement of ERK phosphorylation in thymocytes from HY-TCR male mice, with the percentage of phospho-ERK$^{HI}$ thymocytes increasing from 8.9 ± 2.6 to 38.4 ± 15.5%. However, thymocytes from HY-TCR female mice showed a small increase in the number of phospho-ERK$^{HI}$ thymocytes after TCDD exposure (2.4 ± 0.5 to 9.9 ± 6.6%). Assessment of Lck levels revealed a similar pattern (Fig. 4B) inasmuch as TCDD exposure resulted in a dramatic increase in Lck levels in male HY-TCR thymocytes, with the Lck$^{HI}$ population increasing from 3.8 ± 1.6 to 31.1 ± 13.4%. In contrast, there was no significant increase in the Lck$^{HI}$ population after TCDD exposure in female HY-TCR thymocytes (1.3 ± 0.5 to 5.2 ± 2.55).

**Effect of TCDD on the Proliferative Response of HY-TCR Male and Female Thymocytes.** To determine whether TCDD altered the ability of male and female HY-TCR thymocytes to respond to T-cell mitogens, we tested the response of thymocytes to ConA or anti-CD3 mAbs. The ability of male HY-TCR thymocytes to respond to either ConA or anti-CD3 was dramatically reduced after exposure to TCDD (Fig. 5). TCDD reduced the male response to ConA 71.1% and the response to anti-CD3 78.5%. It is remarkable that the response of female HY-TCR thymocytes was essentially unaffected by exposure to TCDD (Fig. 5).

**TCDD Exposure Differentially Affects Peripheral T Cells in Male and Female HY-TCR Mice.** Having demon-

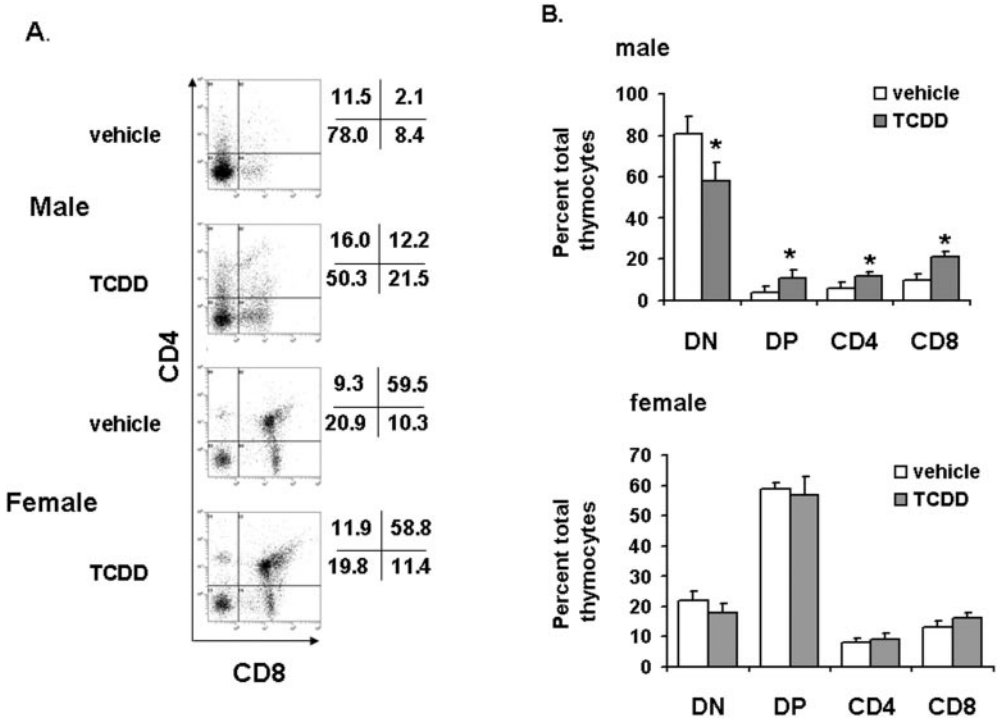


**Fig. 3.** Effects of TCDD exposure on thymocyte subpopulations in male and female HY-TCR Tg mice. After exposure to either 50 μg/kg TCDD or vehicle for 72 h, thymi were harvested and stained with mAbs against HY-TCR, CD8, and CD4. The expression of CD4 and CD8 was assessed after electronic exclusion of thymocytes not expressing the HY-TCR. The percentage of CD4$^+$CD8$^-$ SP, CD4$^-$CD8$^+$ SP, CD4$^+$CD8$^+$ DP, and CD4$^-$CD8$^-$ DN thymocytes is indicated in the appropriate quadrant of each dot plot. Representative dot plots of each treatment group are shown in A, and the data are summarized in B. ⋆, statistically significant differences between TCDD-treated and control groups. Results are representative of three independent experiments using two mice per treatment.

strated that the thymi of male and female HY-TCR mice are differentially affected by TCDD exposure, we then assessed the effects of TCDD on peripheral T cells in male and female HY-TCR mice. The data from a representative experiment are shown in Fig. 6A, and those from multiple experiments are summarized in Fig. 6B. TCDD exposure resulted in alterations in the proportion of $CD4^+$ and $CD8^+$ T cells in the spleen. In the spleens of HY-TCR male mice, TCDD exposure (50 $\mu$g/kg for 72 h) resulted in a significant increase in the percentage of $CD8^+$ T cells, from 11.4 ± 0.7 to 15.6 ± 2.2%, and a concomitant significant decrease in the percentage of $CD4^+$ T cells, from 10.7 ± 1.0 to 7.1 ± 0.8% (3.6% increase). These data suggested that more HY-TCR–bearing $CD8^+$ T cells had escaped deletion in the thymus and migrated to the periphery of TCDD-treated mice. In HY-TCR female mice, there was no change in the percentage of $CD4^+$ cells and a slight, although significant, increase in $CD8^+$ cells, from 5.9 ± 1.1 to 7.9 ± 0.4%. Likewise, findings were made in the lymph nodes (data not shown).

**TCDD Exposure Impairs the Response of HY-TCR Female Lymphocytes to Male HY-Ag but Enhances the Response of HY-TCR Male Lymphocytes to Male HY (Self)-Ag.** As shown in Fig. 7A, the response of lymphocytes isolated from male HY-TCR Tg mice to HY-Ag presented by irradiated APCs was significantly enhanced after exposure to 50 $\mu$g/kg TCDD for 72 h. In contrast, the response of lymphocytes isolated from HY-TCR female mice to male HY-Ag was dramatically decreased after TCDD exposure (Fig. 7A). It should be noted that vehicle-treated female mice showed a stronger response to HY-Ag stimulation than vehicle-exposed male mice. This is caused by the fact that T cells from male mice are tolerant HY-Ag because of constitutive expression of HY-Ag, whereas for female mice, the HY-Ag is recognized as a foreign Ag.

TCDD exposure also resulted in an increase in the cell-surface expression of the CD8 coreceptor on a subset of peripheral T cells from HY-TCR male mice. Male HY-TCR $CD8^+$ peripheral T cells ordinarily express abnormally low levels of CD8, and it has been suggested that these $CD8^+$ T cells evade deletion in the thymus because their reduced levels of CD8 impairs their ability to productively interact with MHC class I on APC (Kisielow et al., 1988). However, as summarized in Fig. 7B, almost 25% of $CD8^+$ peripheral T cells isolated from TCDD-exposed HY-TCR male mice expressed levels of CD8 commensurate with the lower limits of CD8 expression found on T cells isolated from HY-TCR female mice.

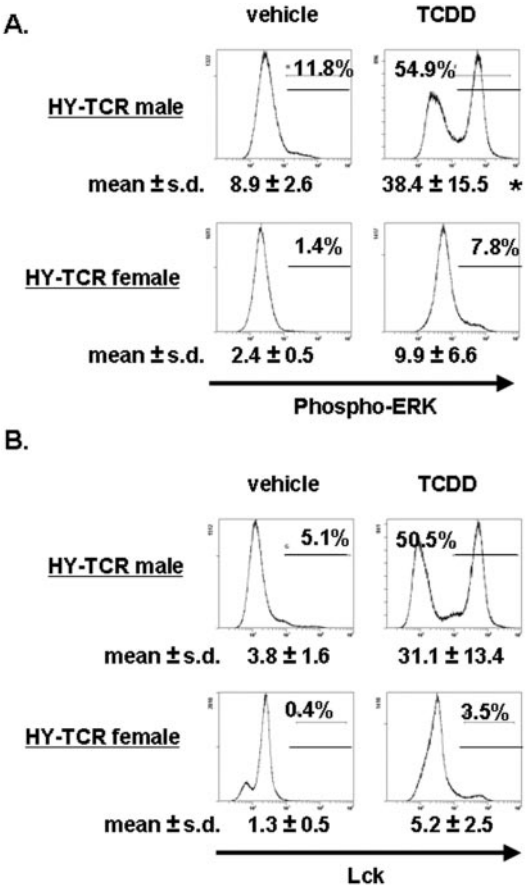


**Fig. 4.** TCDD exposure increases ERK1/2 phosphorylation and Lck expression in HY-TCR Tg thymocytes. After 48-h exposure to 50 $\mu$g/kg TCDD, thymocytes isolated from male and female HY-TCR Tg mice were fixed and permeabilized before intracellular staining with antibodies specific for the phosphorylated form of ERK (A) and for Lck (B). Numbers within histograms indicate the percentage of thymocytes phosphor-$ERK^{HI}$ and $Lck^{HI}$, and the average percentage of phosphor-$ERK^{HI}$ and $Lck^{HI}$ ± S.D. for four individual mice is indicated below the histograms.

## Discussion

In the present study, we tested the hypothesis that TCDD exposure can affect the process of thymocyte selection by assessing the relative susceptibility of negatively selecting male and positively selecting female HY-TCR Tg mice to the immunotoxic effects of TCDD. The hypothesis that TCDD may alter the dynamics of thymocyte selection is derived from our previous observation that exposure to TCDD increases the expression of TCR and accessory molecules such as CD30 and LIGHT, which have been shown to be involved in negative selection (Fisher et al., 2004), as well as the demonstration that TCDD exposure induces apoptosis in thymocytes and T cells through induction of Fas and Fas ligand (Kamath et al., 1997, 1999; Kerkvliet, 2002; Fisher et al., 2004). In the current study, we found that male HY-TCR

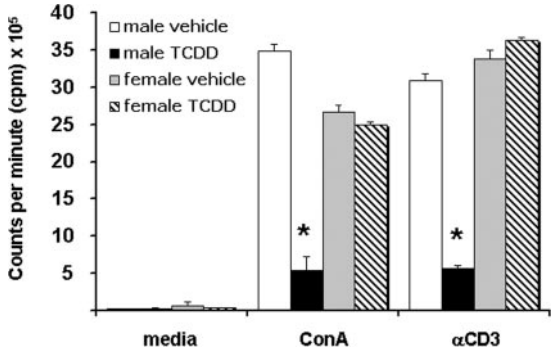


**Fig. 5.** Effect of TCDD on proliferative response of thymocytes from HY-TCR Tg male and female mice to T cell mitogens. Thymocytes were harvested from male and female HY-TCR Tg mice exposed to either TCDD (50 $\mu$k/kg) or vehicle for 72 h. After harvest, $5 \times 10^5$ thymocytes were cultured in vitro for 72 h with either 2 $\mu$g/ml ConA or 5 $\mu$g/ml anti-CD3 mAbs, or in 10% RPMI 1640 media alone. Thymocytes were pulsed with 2 $\mu$Ci of [$^3$H]thymidine during the final 8 h of culture. Data depicted are the mean cpm ± S.D. of three replicate cultures per each treatment group. ⋆, $p < 0.05$, statistically significant differences between treatment groups. Data are representative of two independent experiments using two mice per group.

mice were more sensitive to TCDD-induced atrophy of the thymus relative to both WT C57BL/6 mice and HY-TCR female mice. The hypothesis that TCDD acts to induce apoptosis of thymocytes through increased negative selection may partially explain the general observation that, although TCDD is highly toxic to thymocytes in vivo, attempts to induce apoptosis in thymocytes via in vitro culture, except for fetal thymic organ culture systems, have not been successful (Lai et al., 1998). In particular, our hypothesis may partially explain the inactivity of TCDD in vitro because the apoptotic signals induced during negative selection require interactions between T cells and APCs in the thymus. These observations suggest that TCDD acts to sensitize T cells to apoptotic signals, rather than inducing apoptosis through direct cytotoxic effects.

Consistent with studies of WT mice exposed to TCDD, which up-regulate CD3/TCR (Kamath et al., 1998), we noted that TCDD exposure led to increased expression of transgenic HY-TCR, especially in female mice. HY-TCR expression, as measured by mean intensity fluorescence, also increased on thymocytes from male HY-TCR mice after TCDD exposure; however, the increase was not statistically significant. This may be because thymocytes from male HY-TCR mice expressing high levels of the transgene are rapidly deleted through negative selection. Previous studies have demonstrated that quantitative differences in the expression of TCR can lead to quantitative changes in the efficiency of thymic selection. Using a clever model in which HY-TCR transgenic mice were crossed with AND TCR transgenic mice (termed dual TCR-expressing mice), which effectively resulted in the cell-surface dilution of both transgenic TCRs, Dave et al. demonstrated that reduction in TCR expression led to a reduction in the efficiency of negative selection. In particular, a significant population of DP thymocytes appeared in male dual TCR-expressing mice, so that even though these thymocytes expressed an HY-Ag–specific TCR, they were not negatively selected. The data presented here are consistent with this model and suggest that the increase in HY-TCR expression observed in thymocytes of male mice may have facilitated increased apoptosis and possibly enhanced negative selection. However, it should be noted that the increased apoptosis in thymocytes of male mice may also result from other mechanisms, including up-regulation of Fas, CD30, and LIGHT, as shown previously (Fisher et al., 2004). We also point out that up-regulation of TCR in female mice would not have any effect on negative selection because the TCR ligand (HY antigen) is missing in female mice. Nonetheless, there was increased apoptosis seen in thymocytes from female mice, thereby suggesting that TCDD may trigger other pathways of apoptosis, as discussed above.

It is interesting that in the current study, we found that the DN thymocytes from both male and female HY-TCR mice exposed to TCDD showed significant levels of apoptosis and that thymocytes from male mice showed higher levels of apoptosis compared with the female mice. The precise mechanism of apoptosis induced by TCDD in DN thymocytes is not clear. It has been suggested that because expression of HY-TCR is high in these mice, the DN thymocytes may audition for selection prematurely (Lacorazza et al., 2001). To test this further, we determined the levels of HY-TCR expression in the DN thymocytes and found that a majority of the DN thymocytes in both male and female mice were HY-TCR$^{HI}$ and expressed similar levels of TCR. Thus, the increased sensitivity of DN thymocytes from male mice to TCDD-induced apoptosis compared with similar cells from female mice may result from interactions between HY-TCR and endogenous HY ligand that is expressed only in male mice. However, the fact that DN thymocytes from TCDD-treated female mice undergo increased levels of apoptosis compared with vehicle-treated female mice also suggests that alternative mechanisms may be operating to facilitate apoptosis, including TCDD-induced up-regulation of costimulatory molecules and/or death receptor/ligand interactions, as shown previously (Kamath et al., 1997, 1999; Kerkvliet, 2002; Fisher et al., 2004). Thus, it seems possible that the increase in apoptosis in TCDD-exposed male HY-TCR DN thymocytes could be caused by premature negative selection, which could also be one of the contributing factors toward increased thymic atrophy seen in these mice.

In the current study, we noted that TCDD exposure resulted in a relative increase in the percentage of CD8$^+$ SP thymocytes in HY-TCR male mice, although not in HY-TCR female or C57BL/6 WT mice. Kronenberg et al. demonstrated that TCDD exposure could result in the appearance of mature CD8$^+$ SP thymocytes even in the absence of TCR interaction with MHC I/peptide. In particular, this group demonstrated that TCDD exposure resulted in the appearance of a significant population of CD8$^+$ SP thymocytes in fetal thymic organ cultures gener-

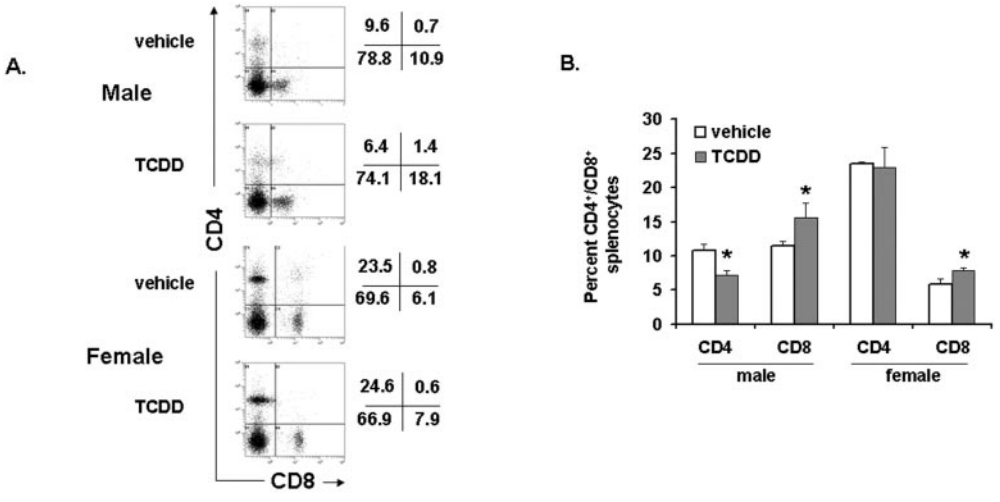


**Fig. 6.** Effect of TCDD on T-cell subpopulations in the periphery. Proportion of CD8$^+$ and CD4$^+$ T cells in the periphery of male and female HY-TCR Tg mice exposed to either 50 $\mu$k/kg TCDD or vehicle for 72 h. Spleens (A) were harvested from male and female HY-TCR Tg mice and stained with labeled mAbs against CD8 or CD4. Dot plots depicting representative data are shown in A, and the percentages of CD8$^+$ and CD4$^+$ T cells are indicated in the appropriate quadrant of each plot. The mean percentages of CD8$^+$ and CD4$^+$ splenic T cells from each treatment group from multiple experiments are shown in B, and statistically significant differences between treatments are indicated.

ated from $\beta_2$ microglobin-deficient mice, which lack functional MHC class I molecules. This effect is a possible explanation for the enhanced $CD8^+$ SP population we note in HY-TCR male mice exposed to TCDD given the efficiency with which the HY-TCR mediates negative selection in these mice. However, the above interpretation does not fully explain why there was no significant increase in $CD8^+$ SP cells in female mice and why there was an enhanced response to HY-Ag noted in TCDD-exposed HY-TCR male mice. We propose that TCDD allows some autoreactive $CD8^+$ SP cells to escape negative selection, which also explains our results in which HY-TCR male mice demonstrated an increase in $CD8^+$ T cells in the periphery and showed an enhanced proliferative response to self (HY) antigen after exposure to TCDD. These results have important implications because they suggest that TCDD exposure may result in autoimmune disease by allowing autoreactive thymic T cells to escape negative selection.

We examined the effect of TCDD exposure on $p56^{lck}$ (Lck) expression and ERK phosphorylation because these two molecules are central to positive/negative selection and lineage commitment during thymocyte maturation and are induced by TCR ligation (Mariathasan et al., 2000, 2001; Hogquist, 2001). Consistent with our model in which TCDD acts in part by increasing TCR expression, and therefore TCR signaling, both ERK phosphorylation and Lck expression were dramatically increased in HY-TCR Tg male mice after exposure to TCDD. As discussed briefly in the Introduction, it is currently believed that the level of ERK activation after TCR ligation is central to T-cell fate (Mariathasan et al., 2000, 2001). Strong, transient ERK activation results in negative selection of thymocytes, whereas weak, sustained ERK activation results in positive selection. Lck is critical in lineage commitment of thymocytes. Lck is typically believed to drive thymocytes toward a $CD4^+$ SP fate because Lck has much higher affinity for the CD4 cytoplasmic tail than that of the CD8 costimulatory molecule, and, in normal mice, ~40% of CD8 molecules are alternative splice variants that lack the cytoplasmic domain for Lck binding. However, Lck is unlikely to result in a $CD4^+$ SP fate in the HY-TCR model because the HY-TCR recognizes Ag in the context of MHC class I H2-$D^b$, and therefore, TCR/MHC/peptide interaction occurs only in the context of CD8 costimulatory molecules. Thus, it is more likely that increased TCR signaling in association with CD8 may cause the increase in Lck we note in this study. Likewise, the increase in ERK activation is also suggestive of increased TCR signaling. It is interesting that the increases in ERK activation and Lck expression in male HY-TCR mice are dramatic, suggesting a significant increase in TCR signaling, which in these mice would result in increased negative selection. However, in HY-TCR female mice, the increases in both ERK and Lck were relatively subtle, even though the increase in TCR expression in female mice was dramatic. This result can be explained by the fact that increased expression of TCR in female mice will not result in negative selection in the absence of a negatively selecting ligand. Because the link between increased TCR expression on thymocytes after TCDD exposure and increased ERK phosphorylation and Lck expression in HY-TCR Tg male mice was not formally tested in these studies, there are clearly alternative explanations for our data. Exposure to TCDD has been shown to alter mitogen-activated protein kinase signaling in a number of models (Lai et al., 1997; Jeon and Esser, 2000; Davis et al., 2001; Ramakrishna et al., 2002; Tsukumo et al., 2002; Kwon et al., 2003). For example, TCDD exposure resulted in increased ERK1/2 activation in murine lung tumors induced by *N*-nitrosodimethylamine, possibly as a result of increased raf-1 (Ramakrishna et al., 2002). In addition, in vitro exposure of human Jurkat T cells to TCDD has been shown to result in ERK1/2 activation (Kwon et al., 2003). Finally, studies using fetal thymic organ cultures implicated ERK1/2 activation after TCDD exposure in the skewing of immature thymocytes toward a $CD8^+$ fate (Tsukumo et al., 2002). Thus, altered expression of ERK and Lck in thymocytes after exposure to TCDD may occur through TCR-dependent and -independent mechanisms, and further studies are necessary to address this. Recent studies have also suggested that TCDD exposure acts largely through the induction of cell-cycle arrest in DN thymocytes (Laiosa et al., 2003). However, we note that if induction of cell-cycle arrest in DN thymocytes was the primary mechanism of TCDD-mediated thymic atrophy, we would expect similar responses to TCDD in the DN population of male and female HY-TCR mice, which is not the case. Rather, we noted that TCDD exposure induced significant apoptosis in female HY-TCR Tg DP thymocytes. This increase in apoptosis almost certainly was not caused by an alteration in cell fate from a positive to a negative outcome because, as noted previously, HY-TCR female mice lack a negatively selecting ligand. Neither is the increase probably caused by a failure of positive selection leading to death by neglect, because we have demonstrated that female HY-TCR thymocytes express high levels of HY-TCR, which would lead to increased positive selection. If nothing else, this observation highlights the complexity of the effects of TCDD on the thymus.

Our results clearly demonstrate that the effects of TCDD on the thymus of HY-TCR transgenic mice are mirrored in the periphery. One of the controversies surrounding TCDD-induced immunotoxicity is whether the thymotoxic effects of TCDD have functional consequences in the periphery. Certainly in the HY-TCR Tg system, the effects of TCDD on the HY-TCR Tg thymus are mirrored in the periphery. It is likely

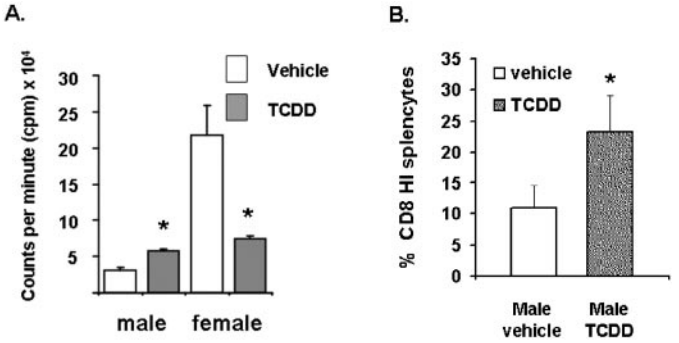


**Fig. 7.** TCDD exposure increases the response of male HY-TCR Tg lymphocytes in male HY-Ag. A, triplicate cultures of $5 \times 10^5$ splenocytes isolated from male and female HY-TCR Tg mice exposed to either 50 $\mu$k/kg TCDD or vehicle for 72 h were cultured in vitro with $4 \times 10^5$ irradiated adherent splenocytes (APCs) isolated from HY-TCR Tg male mice for 48 h. Cultures were pulsed with 2 $\mu$Ci [$^3$H]thymidine during the final 8 h of culture. Depicted is the mean cpm ± S.D. of each treatment group. B, proportion of $CD8^+$ T lymphocytes isolated from vehicle and TCDD-exposed HY-TCR Tg male mice expressing high cell-surface levels of the CD8 coreceptor (high expression defined as levels commensurate with those recorded in HY-TCR Tg female mice). ⋆, statistically significant differences between treatment groups.

that the effects of TCDD are magnified in the HY-TCR Tg system because these mice lack the TCR diversity of normal mice. However, these data certainly suggest that TCDD exposure has the ability to alter the TCR repertoire, although such a hypothesis will be difficult to test in WT mice. In summary, these data demonstrate that TCDD exposure may result in enhanced negative selection, and furthermore, the effects of TCDD on the thymus have functional consequences for peripheral immune function.

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