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2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) enhances negative selection of T cells in the thymus while allowing auto-reactive T cells to escape deletion and migrate to the periphery[1]

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Running title: Effects of TCDD on thymic selection

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ERK, *e*xtracellularly *r*egulated *k*inase; Lck, *l*ymphocyte-specific protein tyrosine *k*inase; AhR, *a*ryl *h*ydrocarbon *r*eceptor; ARNT *a*ryl hydrocarbon *r*eceptor *n*uclear *t*ranslocator; FasL, Fas *l*igand; DP thymocyte, $CD4^+/CD8^+$ *d*ouble *p*ositive thymocyte; DN thymocyte, $CD4^-/CD8^-$ *d*ouble *n*egative thymocyte; TCR, *T c*ell *r*eceptor; MHC, *M*ajor *H*istocompatibility *C*omplex; LIGHT (TNFSF14), *l*ymphotoxin-like, exhibits *i*nducible expression, and competes with HSV *g*lycoprotein D (gD) for *H*VEM, a receptor expressed by *T* lymphocytes; FACS, *f*luorescence *a*ctivated *c*ell *s*orter; TUNEL, *T*erminal deoxynucleotidyl Transferase Mediated d*U*TP *N*ick-*e*nd *L*abelling; Con A, *c*oncanavalin *A*; APC, *A*ntigen *P*resenting *C*ell; FTOC, *f*etal *t*hymic *o*rgan *c*ulture;

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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-TCR transgenic 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 males but not female mice. In addition, TCDD treatment resulted in increased ERK phosphorylation and Lck 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 males was reflected in the periphery, with TCDD-exposed HY-TCR Tg males 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 following exposure to TCDD, while that of HY-TCR Tg females was decreased. Taken together, these data suggest that TCDD alters the process of thymic selection, possibly by enhancing negative thymocyte selection, while at the same time allowing auto-reactive T cells to escape deletion in the thymus and emigrate to the periphery.

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Introduction

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 thought 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 (DREs) with the consensus sequence 5'-GCGTGNN(A/T)NNN(C/G)-3' located in the regulatory regions of dioxin responsive genes, where 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 previously been shown 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 (Grassman et al., 1998; Vos et al., 1997). 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 mediated through Fas/Fas ligand (FasL) interactions (Dearstyne and Kerkvliet, 2002; Fisher et al., 2004; Kamath et al., 1999; Rhile et al., 1996). 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; Camacho et al., 2002). However, the underlying causes of thymic atrophy and increased thymocyte apoptosis following 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 auto-reactive 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 co-receptors and co-stimulatory 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 auto-immune 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, following V(D)J re-arrangement, 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 TCR 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

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thymocytes from death by neglect (positive selection) (Hogquist, 2001; Jameson et al., 1995; Sprent and Kishimoto, 2002; Sprent and Kosaka, 1993). Negative selection removes potentially auto-reactive thymocytes, those bearing rearranged TCR having 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). Specifically, 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, while the TCR signaling mediated by low affinity interactions is sustained and weak, leading to positive selection (Mariathasan et al., 2000; Mariathasan et al., 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 concentration of selecting ligand (Ashton-Rickardt et al., 1994; Dave et al., 1999; Sebzda et al., 1996; Sebzda et al., 1994). In addition, the importance of co-stimulatory 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

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selection, while negative selection is enhanced in CD30 over-expressing mice (Amakawa et al., 1996; Chiarle et al., 1999; DeYoung et al., 2000). Similarly, mice lacking TNF family member LIGHT experience reduced negative selection, while over-expression of LIGHT enhances negative selection (Granger and Rickert, 2003; Wang et al., 2001; Wang and Fu, 2003). Taken together, these data demonstrate altered expression of molecules involved in thymic selection can change the outcome of the selective process. We have previously speculated 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 recently demonstrated that TCDD exposure results in the up-regulation of CD30 and LIGHT in the thymus (Fisher et al., 2004). Clearly, the effects of TCDD on the thymus 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-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^{+} double-positive (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 females. Furthermore, the proliferative response of thymocytes and peripheral T-cells in response to T-cell mitogens is reduced in male HY-TCR mice following TCDD exposure, but unaffected in TCDD-treated female HY-TCR mice.

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Materials and Methods

Mice and TCDD treatment

HY-TCR transgenic mice (formal designation: C57BL/10AiTac- TgN(TCRHY)) 4-8 weeks of age 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 VCU animal facility in a manner consistent with IACUC guidelines in animal rooms maintained at 24°C on a 12 hr light/dark cycle and were given water and rodent chow *ad libitum*. TCDD was a generous gift of Dr. Stephen Safe. TCDD was dissolved in acetone and suspended in corn oil to a concentration of 10 µg/ml. The solution was then heated to 57°C to allow the acetone to evaporate. Groups of 2-4 mice were administered a single dose of 50 µg/kg body weight TCDD or vehicle (corn oil) via i.p. injection for 48 or 72 hours prior to sacrifice.

Media and culture conditions

Following sacrifice, thymi or peripheral lymphoid organs were harvested and rendered into a single cell suspension in RPMI-1640 medium supplemented with 10% FCS, 5 x 10^{-5} M ß-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 (LN) ± standard deviation were depicted. Prior to detection of apoptosis, thymocytes were cultured *in vitro* for 18 hrs as previously described (Kamath et al., 1997). This was based on 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 following their isolation, and therefore a

subsequent *in vitro* culture facilitates their detection due to lack of an effective phagocytic system in vitro (Kamath et al., 1997).

Flow cytometry

Freshly isolated or cultured cells (1-2 x 10^6) were prepared for FACs analysis by washing 2X with cold PBS/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-2 D^b, FITC-ß-TCR. All antibodies were purchased from BD Pharmingen. Cells were then washed with PBS/1% FCS/0.1% NaN_3 and fixed in 1% paraformaldehyde. Prior to 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-hour incubation at 37° C, or incubated with Lck or phosho-ERK mAbs, followed by incubation with FITC-anti mouse IgG. FACs analysis was performed using a Coulter FC500 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 males and females, >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.

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T-cell proliferative response to mitogens

Thymocytes, splenocytes, or cells from the lymph node (LN) obtained from oil- or TCDD-treated mice were tested for the ability to respond to various mitogens *in vitro*. Cells (0.5×10^6/well) were cultured in 96-well flat bottomed plates with 0.2 ml medium for 48 hr at 37° C in 5% CO_2, and stimulated with ConA (2 μg/ml) or anti-CD3 (5 μg/ml) and were pulsed with 2 μCi ^{3}H-thymidine during the final 8 hrs 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 c.p.m. ± 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 μg/kg, 72 hrs.) male and female HY-TCR Tg mice and 5×10^5 cells from each treatment group were stimulated in triplicate cultures with 4×10^5 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 hrs, and pulsed with 2 μCi ^{3}H-thymidine during the final 8 hrs of incubation. Proliferation was then measured as described earlier.

Calculation of statistical significance

Experiments were repeated three times, with each treatment group consisting of 2-4 mice. Statistical differences between pooled samples were detected using Student's *t* test or the

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Chi-Square good-of-fit test as appropriate. A p value of ≤ 0.05 was considered significant.

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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 µg/kg body weight of TCDD for 48 to 72 hrs. As shown in Fig. 1A, exposure of WT mice to TCDD for 72 hr resulted in an approximately 30% decrease in thymic cellularity, as well as caused a significant increase in thymic apoptosis when compared to the controls (Fig. 1B). There was a significant increase in apoptosis in both DN and DP subpopulations of thymocytes following TCDD exposure (Fig. 1B), while 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 µg/kg) was administered into HY-TCR mice, as shown in figure 2A, male HY-TCR mice showed an average 62.5% reduction in thymic cellularity while female HY-TCR mice showed only a 23.7% reduction in thymic cellularity when compared to the controls. To determine if the variation in the effects of TCDD on thymic atrophy between male and female HY-TCR mice might be due to 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 figure 2B, TCDD

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treatment led to a significant increase in apoptosis in the whole thymus as well as in DN but not DP subpopulations when compared to the controls in male HY-TCR mice. It should be noted that the DP thymocytes isolated from HY-TCR males 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-treament 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 when compared to the controls; however, this increase was not statistically significant. Overall, thymocytes from HY-TCR male mice appear to be more sensitive to TCDD-induced apoptosis than thymocytes from HY-TCR females. Interestingly, DN thymocytes from HY-TCR males seemed to be particularly more sensitive to TCDD-induced apoptosis relative to DN thymocytes from HY-TCR females. It should be noted that there are no known gender differences in the immunotoxic effects of TCDD (Lai et al., 1998; Lai et al., 2000; Silverstone et al., 1994).

TCDD exposure increases HY-TCR expression

To determine if the increase in thymocyte apoptosis might be due in part to 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 males increased from 100.9 ± 18.2 to 140.1 ±

12.1 following 48 hr 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 due to differences in TCR expression, we measured the levels of HY-TCR in the DN sub-population. 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 have been shown in Fig. 3A, and those from multiple experiments have been summarized in Fig. 3B. Vehicle-treated HY-TCR male mice showed very poor differentiation of DP and CD8$^+$ T cells, which is expected due to 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 due to enhanced positive selection. Following TCDD treatment, the male mice had a statistically significant reduction in CD4$^-$CD8$^-$ (DN) thymocytes, from 78.1 ± 6.1% to 58.2 ± 8.0%, while the percentage of DP and single positive T cells increased significantly. In contrast, the HY-TCR females showed no significant change in the percentage of DN thymocytes as well as all other subpopulations, following TCDD exposure.

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TCDD treatment results in increased ERK phosphorylation and Lck expression in thymocytes

To determine if increased TCR expression might have functional consequences such as enhanced TCR signaling, we assessed levels of ERK phosphorylation and Lck expression in thymocytes following exposure to 50 µg/kg TCDD for 48 hours. As shown in Fig. 4A, exposure to TCDD resulted in a dramatic enhancement of ERK phosphorylation in thymocytes from HY-TCR males, with the percentage of phospho-ERK^{HI} thymocytes increasing from 8.9±2.6 to 38.4±15.5%. However, thymocytes from HY-TCR females showed a small increase in the number of phospho-ERK^{HI} thymocytes following 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 following 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 if 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 following exposure to TCDD (Fig. 5). TCDD reduced the male response to ConA 71.1% and the response to anti-CD3 78.5%. Remarkably, the response of female HY-TCR thymocytes was essentially unaffected by exposure to TCDD (Fig. 5).

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TCDD exposure differentially affects peripheral T-cells in male and female HY-TCR mice

Having demonstrated 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 have been shown in Fig. 6A, and those from multiple experiments have been summarized in Fig. 6B. TCDD exposure resulted in alterations in the proportion of $CD4^+$ and $CD8^+$ T-cells in the spleen. In the spleen of HY-TCR males, TCDD exposure (50 μg/kg, 72 hrs) resulted in a significant increase in the percentage of $CD8^+$ T-cells, from $11.4 \pm 0.7\%$ to $15.6 \pm 2.2\%$, and a concomitant significant decrease in the percentage of $CD4^+$ T-cells, from $10.7 \pm 1.0\%$ to $7.1 \pm 0.8\%$ (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 females, there was no change in the percentage of $CD4^+$ cells, and a slight, though significant, increase in $CD8^+$ cells, from $5.9 \pm 1.1\%$ to $7.9 \pm 0.4\%$. Similarly, 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 following exposure to 50 μg/kg TCDD for 72 hrs. In contrast, the response of lymphocytes isolated from HY-TCR female mice to male HY-Ag was dramatically decreased following TCDD

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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 due to the fact that T cells from male mice are tolerant HY Ag due to constitutive expression of HY Ag, while 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 co-receptor on a subset of peripheral T cells from HY-TCR males. 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 females.

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 based on 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 FasL (Fisher et al., 2004; Kamath et al., 1999; Kamath et al., 1997; Kerkvliet, 2002). In the current study, we found that male HY-TCR 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, while 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). Specifically, 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 following 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 or DTE 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. Specifically, a significant population of DP thymocytes appeared in male DTE mice, so that even though these thymocytes expressed a 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 seen 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 wish to 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,

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there was increased apoptosis seen in thymocytes from female mice thereby suggesting that TCDD may trigger other pathways of apoptosis as discussed above.

Interestingly, 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 when compared 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 when compared to 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 when compared to vehicle-treated female mice, also suggests that alternative mechanisms may be operating to facilitate apoptosis, including TCDD-induced up regulation of co-stimulatory molecules and/or death receptor/ligand interactions as shown previously(Fisher et al., 2004; Kamath et al., 1999; Kamath et al., 1997; Kerkvliet, 2002). Thus, it seems possible that the increase in apoptosis in TCDD-exposed male HY-TCR DN thymocytes could be due to premature negative selection, which could also be one of the contributing factors towards increased thymic atrophy seen in these mice.

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In the current study, we noted that TCDD exposure resulted in a relative increase in the percentage of $CD8^+$ SP thymocytes in HY-TCR males, though not in HY-TCR females 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. Specifically, this group demonstrated that TCDD exposure resulted in the appearance of a significant population of $CD8^+$ SP thymocytes in fetal thymic organ cultures (FTOC) generated from $ß_2$ 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 males 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 males. We propose that TCDD allows some auto-reactive $CD8^+$ SP cells to escape negative selection, which also explains our results in which HY-TCR males demonstrated an increase in $CD8^+$ T-cells in the periphery and showed an enhanced proliferative response to self (HY) antigen following exposure to TCDD. These results have important implications as they suggest that TCDD exposure may result in autoimmune disease by allowing auto-reactive 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 (Hogquist, 2001; Mariathasan et al., 2000; Mariathasan et al., 2001). Consistent with our

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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 following exposure to TCDD. As briefly discussed in the introduction, it is currently believed that the level of ERK activation following TCR ligation is central to T-cell fate (Mariathasan et al., 2000; Mariathasan et al., 2001). Strong, transient ERK activation results in negative selection of thymocytes, while weak, sustained ERK activation results in positive selection. Lck is critical in lineage commitment of thymocytes. Lck is typically thought to drive thymocytes towards a $CD4^+$ SP fate as Lck has much higher affinity for the CD4 cytoplasmic tail than that of the CD8 co-stimulatory 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 co-stimulatory molecules. Thus, it is more likely that the increase in Lck we note in this study may be due to increased TCR signaling in association with CD8. Similarly, the increase in ERK activation is also suggestive of increased TCR signaling. Interestingly, 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 females, the increases in both ERK and Lck were relatively subtle, even though the increase in TCR expression in females was dramatic. This result can be explained by the fact that increased expression of TCR in females will not result in negative selection in the absence of a negatively selecting ligand. Because the link

between increased TCR expression on thymocytes following TCDD exposure and increased ERK phosphorylation and Lck expression in HY-TCR Tg males was not formally tested in these studies, there are clearly alternative explanations for our data. Exposure to TCDD has been shown to alter MAP kinase signaling in a number of models (Davis et al., 2001; Jeon and Esser, 2000; Kwon et al., 2003; Lai et al., 1997; Ramakrishna et al., 2002; Tsukumo et al., 2002). For example, TCDD exposure resulted in increased ERK1/2 activation in murine lung tumors induced by *N*-Nitrosodimethylamine (NDMA), 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 ERK 1/2 activation (Kwon et al., 2003). Finally, studies using fetal thymic organ culture (FTOC) implicated ERK1/2 activation following 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 following 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 induction of cell-cycle arrest in DN thymocytes (Laiosa et al., 2003). However, we would note that if induction of cell cycle arrest in DN thymocytes were 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 due to an alteration in cell fate from a positive to a negative outcome, because, as noted previously, HY-TCR females lack a negatively selecting ligand. Neither is the increase

likely due to a failure of positive selection leading to death-by-neglect, as 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 to 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 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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Legends for Figures

FIGURE 1. Effects of TCDD exposure on the thymus of C57BL/6 WT mice. (*A*) Following exposure to either 50 μg/kg TCDD or vehicle for 72 hours, thymi were harvested to determine cellularity. Data are expressed as mean cellularity/thymus +/- standard deviation. Statistical significance ($p < 0.05$) is indicated by an asterisk. (*B*) Thymocytes harvested as described above were assessed for apoptosis using the 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 sub-populations prior to assessing FITC-dUTP labeling. Individual histograms show representative data with % apoptotic cells and numbers below each histogram depict data from multiple experiments showing the mean % apoptosis ± standard deviation. The average percent increase in apoptosis in TCDD treated groups when compared to the vehicle controls has been indicated in parenthesis.

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FIGURE 2. Effects of TCDD exposure on the thymus of male and female HY-TCR Tg mice. (*A*) Following exposure to either 50 μg/kg TCDD or vehicle for 72 hours, thymi were harvested to determine cellularity. Bars indicate the average cellularity/thymus +/- standard deviation for vehicle and TCDD exposed mice, with 4 mice/group. The average percent reduction in thymic cellularity following TCDD exposure when compared with vehicle controls is depicted below the bar graph. Statistical significance between treatments ($p < 0.05$) is indicated by an asterisk. (*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 % apoptotic cells and numbers below each histogram depict data from multiple experiments showing the mean % apoptosis ± standard deviation. The average percent increase in apoptosis in TCDD treated groups when compared to the vehicle controls has been indicated in parenthesis. (*C*) Cell-surface expression of HY-Ag specific Tg TCR was assessed using a 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 % HY-TCR^{HI} thymocytes are indicated in the histogram. The mean intensity florescence (m.i.f.) ± standard deviation 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 versus vehicle-treated groups are indicated by an asterisk. (*D*) HY-TCR expression on the CD4^{-}/CD8^{-} (DN) thymocyte sub-population. DN thymocytes were electronically gated and then analyzed for TCR expression. Percent TCR^{HI} thymocytes (gated population) are

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indicated within each histogram and the average m.i.f. ± standard deviation of HY-TCR expression is indicated below each histogram.

FIGURE 3. Effects of TCDD exposure on thymocyte sub-populations in male and female HY-TCR Tg mice. Following exposure to either 50 μg/kg TCDD or vehicle for 72 hours, thymi were harvested and stained with mAbs against HY-TCR, CD8, and CD4. The expression of CD4 and CD8 was assessed following 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 is summarized in (*B*). Asterisks indicate statistically significant differences between TCDD-treated and control groups. Results are representative of three independent experiments using two mice per treatment.

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FIGURE 4. TCDD-exposure increases ERK1/2 phosphorylation and Lck expression in HY-TCR Tg thymocytes. Following 48 hrs exposure to 50 μg/kg TCDD, thymocytes isolated from male and female HY-TCR Tg mice were fixed and permeabilized prior to intracellular staining with antibodies specific for the phosphorylated form of ERK (A) and for Lck (B). Numbers within histograms indicate the percent thymocytes phosphor-ERK-Hi and Lck-Hi and the average percent phosphor-ERK^{HI} and Lck^{HI} ± standard deviation for four individuals is indicated below the histograms..

FIGURE 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 μk/kg) or vehicle for 72 hours. Following harvest, 5 x 10^5 thymocytes were cultured *in vitro* for 72 hours with either 2 μg/ml ConA or 5 μg/ml anti-CD3 mAbs, or in 10% RPMI media alone. Thymocytes were pulsed with 2 μCi of ^{3}H-thymidine during the final 8 hours of culture. Data depicted are the mean c.p.m. ± standard deviation of three replicate cultures per each treatment group. Statistically significant differences ($p < 0.05$) between treatment groups are indicated by asterisks. Data is representative of two independent experiments using two mice per group.

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FIGURE 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 μk/kg TCDD or vehicle for 72 hours. 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 percent $CD8^+$ 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.

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FIGURE 7. TCDD exposure increases the response of male HY-TCR Tg lymphocytes male HY-Ag. (*A*) Triplicate cultures of 5 x 10^5 splenocytes isolated from male and female HY-TCR Tg mice exposed to either 50 μk/kg TCDD or vehicle for 72 hours were cultured *in vitro* with 4 x 10^5 irradiated adherent splenocytes (APCs) isolated from HY-TCR Tg male mice, for 48 hours. Cultures were pulsed with 2 μCi of ^{3}H-thymidine during the final 8 hours of culture. Depicted is the mean c.p.m. ± standard deviation of each treatment group. (*B*) Proportion of $CD8^+$ T lymphocytes isolated from vehicle and TCDD-exposed HY-TCR Tg males expressing high cell surface levels of the CD8 co-receptor (high expression defined as levels commensurate with those recorded in HY-TCR Tg females). In *A* and *B*, asterisks indicate statistically significant differences between treatment groups.

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Fig 1

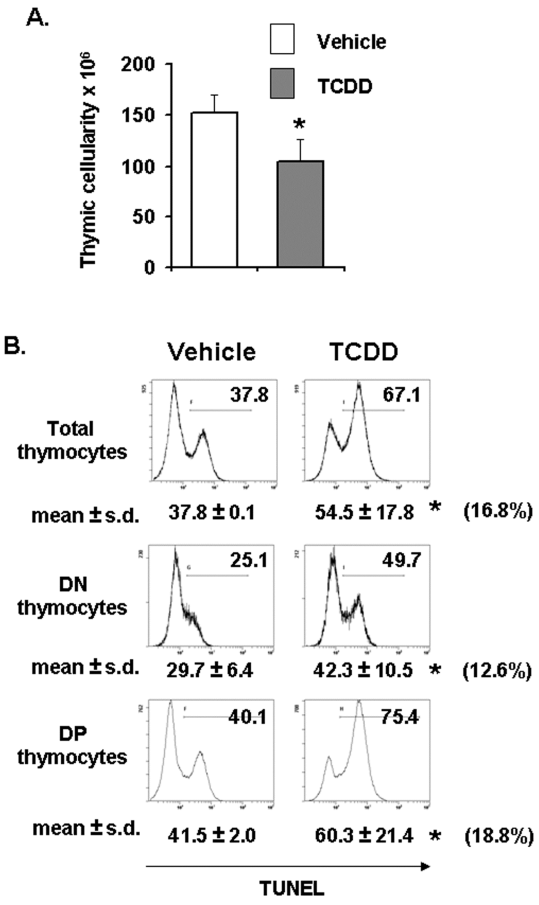

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Fig 2

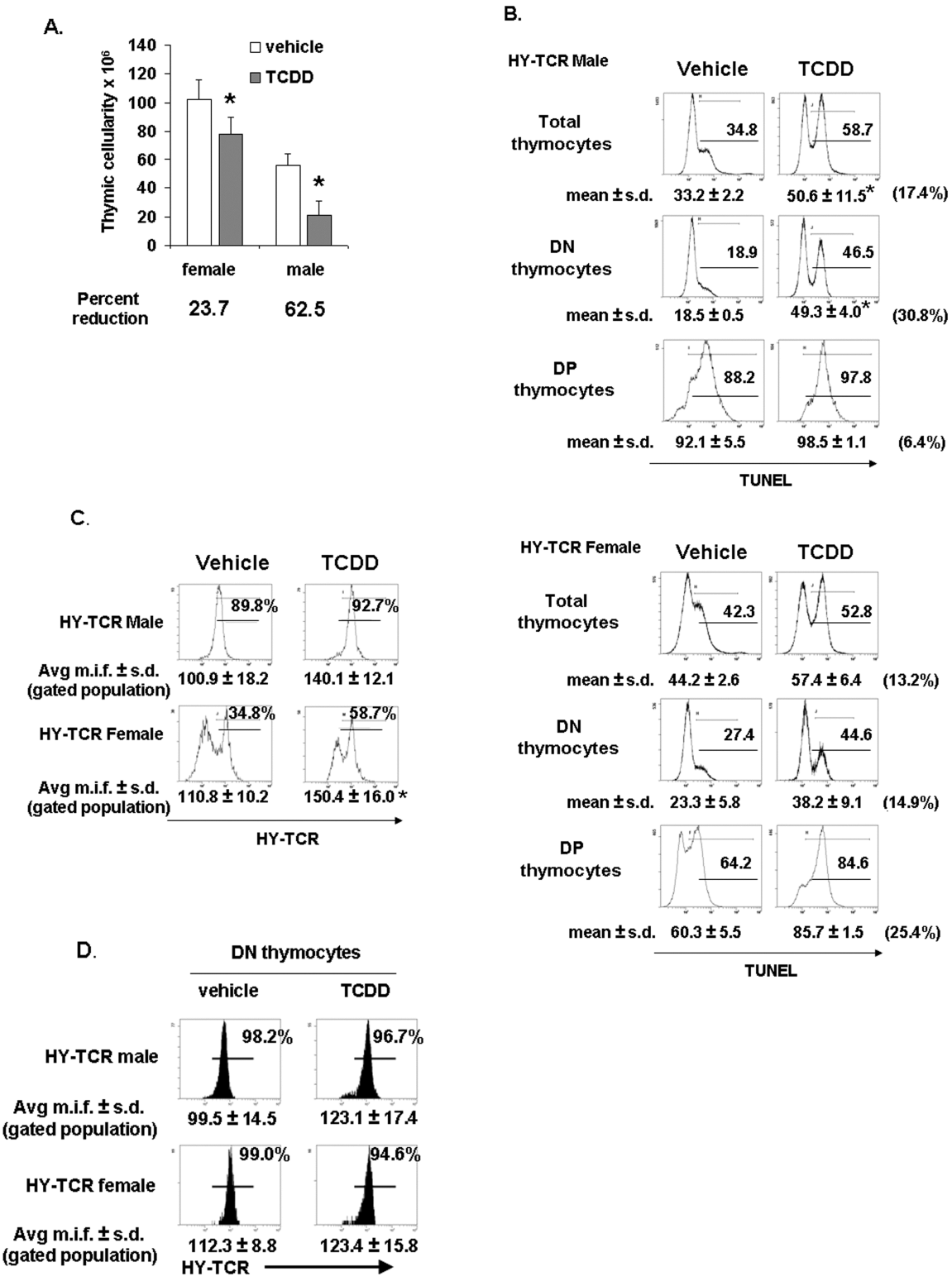

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Fig 3

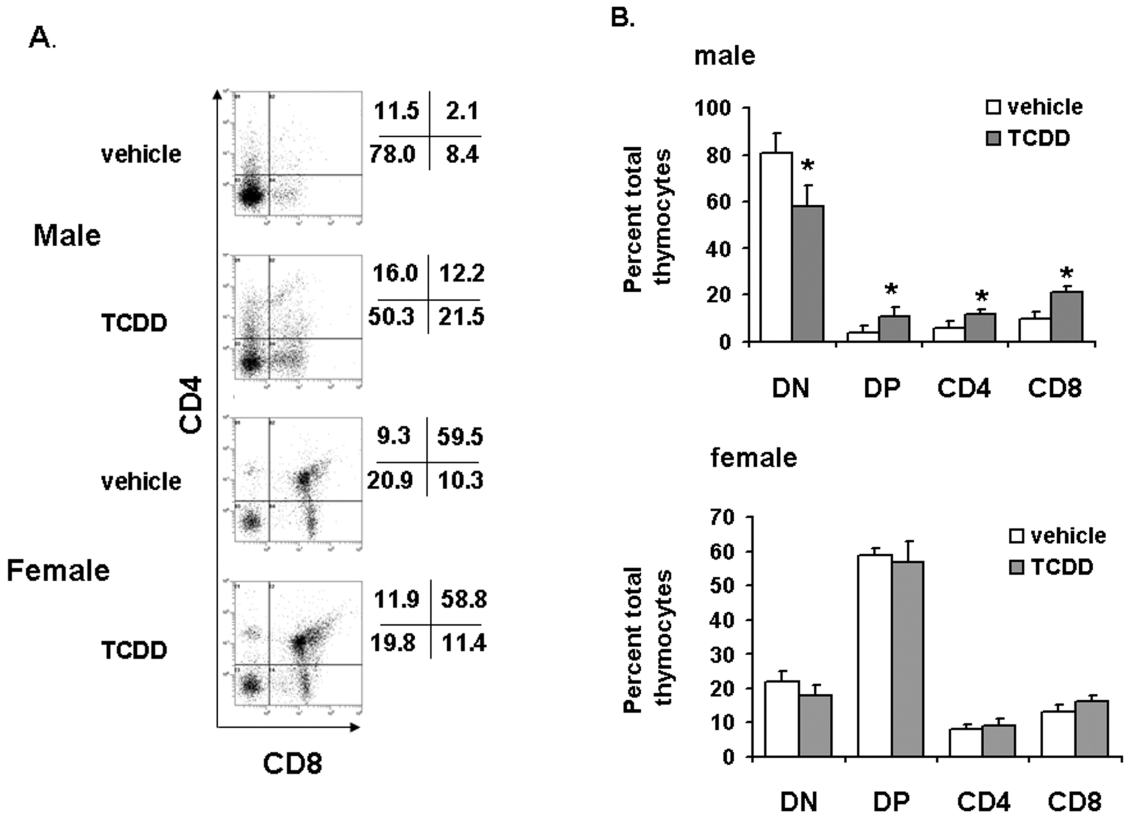

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Fig 4

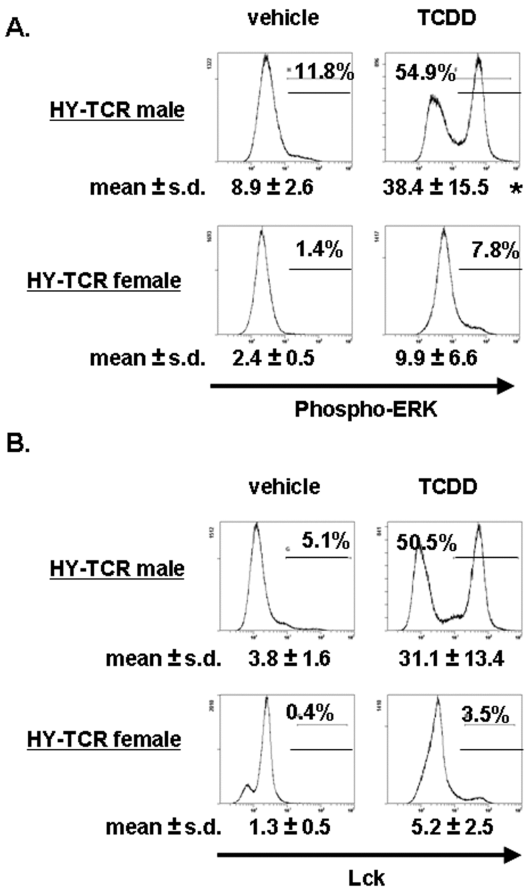

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Fig 5

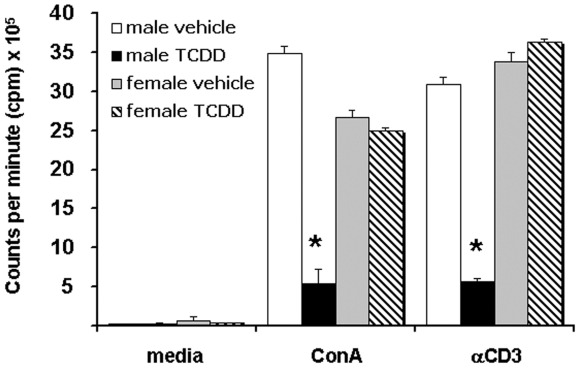

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Fig 6

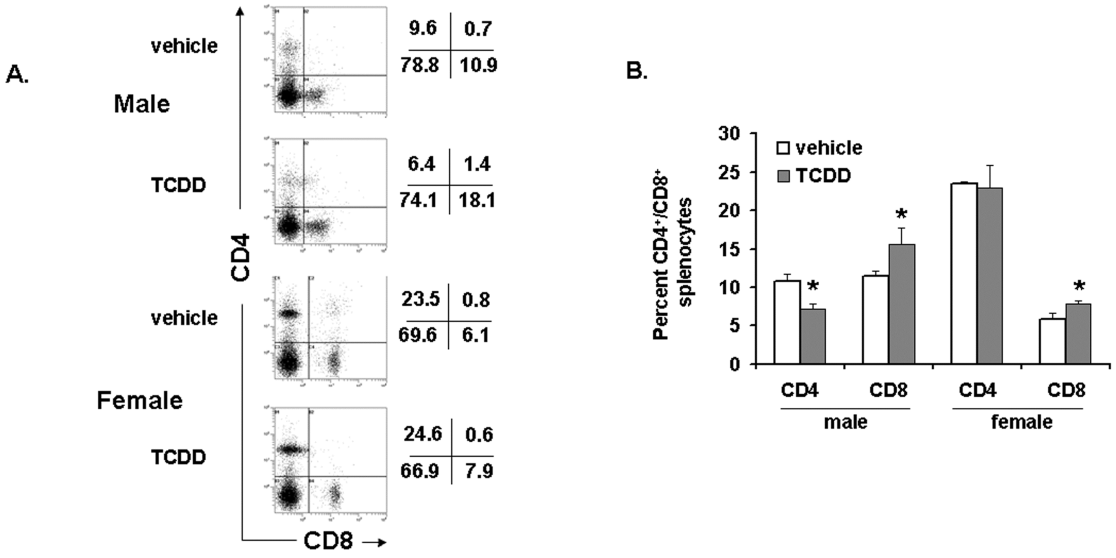

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Fig 7