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*J Immunol* 2009; 182:6576-6586; doi: 10.4049/jimmunol.0802289
http://www.jimmunol.org/content/182/10/6576

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Neonatal Exposure to Low-Dose 2,3,7,8-Tetrachlorodibenzo-p-Dioxin Causes Autoimmunity Due to the Disruption of T Cell Tolerance

Naozumi Ishimaru,* Atsuya Takagi, † Masayuki Kohashi,* Akiko Yamada,* Yosuke Arakaki,* Jun Kanno, ‡ and Yoshio Hayashi**

Although 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been shown to influence immune responses, the effects of low-dose TCDD on the development of autoimmunity are unclear. In this study, using NFS/sld mice as a model for human Sjögren’s syndrome, in which the lesions are induced by the thymectomy on day 3 after birth, the autoimmune lesions in the salivary glands, and in later phase, inflammatory cell infiltrations in the other organs were developed by neonatal exposure to nonapoptotic dosage of TCDD without thymectomy on day 3 after birth. We found disruption of thymic selection, but not thymic atrophy, in TCDD-administered mice. The endogenous expression of aryl hydrocarbon receptor in the neonatal thymus was significantly higher than that in the adult thymus, suggesting that the neonatal thymus may be much more sensitive to TCDD compared with the adult thymus. In addition, the production of Th1 cytokines such as IL-2 and IFN-γ from splenic CD4+ T cells and the autoantibodies relevant for Sjögren’s syndrome in the sera from TCDD-exposed mice were significantly increased compared with those in control mice. These results suggest that TCDD/aryl hydrocarbon receptor signaling in the neonatal thymus plays an important role in the early thymic differentiation related to autoimmunity. The Journal of Immunology, 2009, 182: 6576–6586.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

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Received for publication July 14, 2008. Accepted for publication March 17, 2009.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802289

The toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the environmental contaminant, has been shown to influence various biological responses such as immunological, reproductive, and neurobehavioral (1–3). It has been reported that TCDD induces thymic atrophy and suppresses a variety of T cell-dependent immune responses, including delayed-type and contact hypersensitivity responses and the activity of CTL itself (4–7). However, TCDD has been shown to enhance the proliferation and cytokine production of mitogen- or Ag-stimulated T cells (8). In this context, when a DO11.10 transgenic T cell model was used to investigate the effects of TCDD on the activation of Ag-specific CD4+ T cells by transfer of CD4+ T cells into TCDD-treated recipient mice, the exposure to TCDD had little effect on the initial activation, but on day 3 after OVA-peptide injection the T cell proliferation of TCDD-treated recipients was enhanced compared with that of control recipients (9). Thus, the effect of TCDD seems to be dependent on the developmental state and active state of the T cells. As for the effects of TCDD on B cells, it was reported that TCDD inhibited B cell proliferation triggered by LPS, surface Ig cross-linking, or PMA/solynomycin (10, 11). Moreover, the in vivo suppressive effect of TCDD on T cell-dependent Ab response to sheep RBC (SRBC) was found as an immunotoxicity of TCDD (12). However, the effects of TCDD on autoimmunity or on autoantibody production in autoimmune animal models have not been demonstrated.

A combination of immunologic, genetic, and environmental factors may play a key role on the development of autoimmune disease, which is induced by the breakdown of central or peripheral tolerance (13–15). Sjögren’s syndrome (SS) is generally considered to be a T cell-mediated autoimmune disorder characterized by lymphocytic infiltrates and destruction of the exocrine glands, particularly of the salivary glands, and systemic production of autoantibodies against the ribonucleoprotein particles SS-A/Ro and SS-B/La (16–18). We have established and analyzed an animal model for SS in NFS/sld mutant mouse thymectomized 3 days after birth (3d-Tx) (19–21). It is well established that 3d-Tx in a certain strain of mice results in spontaneous development of inflammatory lesions similar to human autoimmune diseases in the thyroid, ovary, kidney, testis, and stomach, but little is known about the mechanisms leading to the induction of autoimmunity (22–25). From the findings in 3d-Tx autoimmune models, the initiation of autoreactivity is thought to be due to the retardation of regulatory T (Treg) cell differentiation together with lymphopenia caused by neonatal thymectomy. In other words, the impairment of T cell differentiation and/or maturation in the neonatal thymus may cause the initiation of T cell self-reactivity. Although the perinatal exposure to TCDD has been shown to induce the suppression of cell-mediated immunity to a more severe degree than those in adult exposure, the association of neonatal exposure to TCDD with the development of autoimmunity remains unclear (5, 6).
FIGURE 1. Inflammatory lesions induced by neonatal exposure to low-dose TCDD. A, Histology of salivary glands in female and male mice (6 mo) treated with 0 and 10 ng of TCDD were shown. H&E staining was performed using paraffin-embedded sections. Photos are representative of five to seven mice in each group. B, Histological score of the salivary glands at 6 mo of age was evaluated using the sections stained with H&E. Results are shown as the mean ± SD in the five to seven mice in each group. C, The change of inflammatory lesions from 1 to 6 mo of age in female and male mice treated with low-dose TCDD. Results are shown as the mean ± SD in the five to seven mice in each group. *, p < 0.05; **, p < 0.005. D, Immune cells in the inflammatory lesions of salivary glands from TCDD-treated mice at 6 mo of age were analyzed by immunofluorescence staining using anti-CD4, CD8, B220, and CD11c mAbs with Alexa Fluor 568-conjugated rat IgG (H+L) as the secondary Abs. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Photos are representative of three to five sections in each group. E, Inflammatory lesions of liver, lung, and kidney induced by TCDD treatment. The sections from TCDD-treated mice at 6 mo of age were stained with H&E. Photos are representative of five to seven mice in each group.

One mechanism of TCDD action is binding and activation of the aryl hydrocarbon receptor (AhR) (1, 26). The AhR is a cytosolic transcription factor of the basic helix-loop-helix family. The activated receptor heterodimerizes with the AhR nuclear translocator (ARNT) in the nucleus and binds the xenobiotic response elements (XREs), also known as dioxin responsive elements (DREs), and alters the expressions of various genes such as cytochrome P450 1A1 (CYP1A1). TCDD, via the AhR, has been shown to have a variety of effects on T cell development and function, including decreasing the number of thymocytes by apoptosis and altering the effector functions of mature Th and T killer cells (27–30). Although a variety of studies have been performed to determine how high-dose TCDD is influencing T cells and the thymus (29–31), the mechanism and targets of its actions are still unclear. In addition, TCDD also induced the binding of several NF-κB proteins to a κB site, one of which overlapped with a DRE site (32). It has been uncertain whether the neonatal exposure to low-dose TCDD in vivo influences the TCDD signaling including AhR, CYP1A1, or NF-κB of immune cells.

In this study, we evaluated whether the immunotoxicity of non-apoptotic and low-dose TCDD during neonatal period influences the development of autoimmune disease in the murine SS-susceptible strain. Moreover, the correlation between the TCDD-induced signaling pathway in neonatal T cells and the initiation of self-reactivity in vivo was analyzed.

Materials and Methods

Mice

NFS/N strain carrying the mutant gene sld was reared in our specific pathogen-free mouse colony, and given food and water ad libitum. Experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of the National Institute of Health Sciences, Tokyo, Japan and the University of Tokushima, Tokushima, Japan.

Neonatal administration of TCDD

Intraperitoneal injection of 10 μl of corn oil including TCDD (0, 0.1, 1, or 10 ng/mouse) with neonatal mice was performed on day 0, 1, and 2 after birth. Treatment of TCDD and TCDD-injected mice followed the rules of the National Institute of Health Sciences.

Histology

All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2), and prepared for histologic examination. Formalin-fixed tissue sections were subjected to H&E staining, and three pathologists independently evaluated the histology without being informed of the condition of each individual mouse. Histological changes were scored according to the method proposed by White and Casarett (33), as follows: 1 = 1–5 foci composed of >20 mononuclear cells per focus; 2 = >5 such foci, but without significant parenchymal destruction; 3 = degeneration of parenchymal tissue; 4 = extensive infiltration of the glands with mononuclear cells and extensive parenchymal destruction. Histological evaluation was performed in a blinded manner, and one tissue section from each salivary and lacrimal gland was examined.

Confocal microscopic analysis

Frozen sections were stained with 1 μg/ml primary Abs against CD4, CD8, B220, and CD11b/c (eBioscience) for 1 h. After three washes in PBS, the sections were stained with Alexa Fluor 568 donkey anti-rat IgG (H+L) (Molecular Probes) as the second Abs for 30 min and washed with PBS. The nuclei was stained with 4′,6-diamidino-2-phenylindole. The sections were visualized with a laser scanning confocal microscope (Carl Zeiss). A 63 × 1.4 oil differential interference contrast objective lens was used. Quick Operation Version 3.2 (Carl Zeiss) for imaging acquisition and Adobe Photoshop CS2 (Adobe System) for image processing was used.
Flow cytometric analysis

Surface markers were identified by mAbs with BD FACSCant flow cytometer (BD Biosciences). Rat mAbs to FITC-, PE-, or PE-Cy5-conjugated anti-B220, Thy1.2, CD4, CD8, CD25, and CD44 mAbs (eBioscience) were used. Intracellular Foxp3 expression was analyzed with an intracellular Foxp3 detection kit (eBioscience) according to the manufacturer’s instructions. For intracellular AhR expression, cells were stained with PE-Cy5.5-conjugated anti-CD4, PE-conjugated anti-CD8, PE-Cy7-conjugated anti-CD44, allophycocyanin-conjugated anti-CD25 mAbs, and then fixed in fixation/permeabilization solution (eBioscience) for 18 h at 4°C. After washing twice with the permeabilization buffer (eBioscience), the cells were blocked with Fc block for 40 min on ice, and incubated in rabbit anti-AhR polyclonal Ab (BIOMOL) for 2 h at 4°C. After washing with the permeabilization buffer, the cells were stained with FITC-conjugated anti-rabbit IgG for 30 min at 4°C for flow cytometric analysis of multicolors. The data were analyzed with FlowJo FACS Analysis software (Tree Star).

Proliferation assay

Cell proliferation was evaluated by counting of divisions by CFSE (Molecular Probes) dilution of labeled cells. After stimulation by anti-CD3 and

![Figure 2](http://www.jimmunol.org/)
anti-CD28 mAbs, or LPS for 72 h, cell division of CD4^{+} or B220^{+}- gated spleen cells was analyzed by flow cytometry.

**ELISA**

The JS-1-, SS-A/Ro, SS-B/La, or ss-DNA-specific Abs of sera from mice were measured by an ELISA reader (model 680; Bio-Rad) with a spectrophotometer reading at 490 nm. Igs (IgG2a and IgG1) in sera were determined by using the mouse immunoglobulins ELISA quantitation kit (Beckman Coulter). Abs (IgG2a and IgG1) in sera were determined by using the mouse immunoglobulins ELISA quantitation kit (Beckman Coulter).

**Real-time quantitative RT-PCR**

Total RNA was extracted from thymus, spleen, and cultured thymocytes in NFS/1 or NFS/sid mice using Iogen (Wako Pure Chemical), and reverse transcribed. Transcript levels of T-bet, GATA-3, AhR, Cyclin A1, Bcl-xL, TNF-α, IFN-γ, mRNA levels of cytokines as previously described (34).

**NF-κB transcription activity assay**

The transcriptional activity of NF-κB of the nuclear extracts from thymocytes was analyzed with NF-κB transcription factor colorimetric assay kit (Millipore) (Millipore). Nuclear extracts were incubated with biotinylated double-strand oligonucleotide probe containing the consensus sequence for NF-κB on a streptavidin-coated plate. Captured complexes, including active NF-κB protein, were incubated with the primary Abs for p50 and RelA and HRP-conjugated secondary Ab and tetramethylbenzidine substrate. The absorbance of the samples was measured with a microplate reader at 450 nm.
Statistical test

The Student t test was used for statistical analysis. Values of $p > 0.05$ were considered as significant.

Results

Induction of inflammatory lesions by neonatal administration of low-dose TCDD

To elucidate whether inflammatory lesions are induced by neonatal administration of low-dose TCDD into NFS/sld mice, i.p. injection of 0, 0.1, 1, and 10 ng/mouse TCDD was performed on day 0, 1, and 2 after birth. At 1, 2, and 6 mo of age, all the organs of treated mice were histopathologically analyzed. The inflammatory lesions in salivary glands of TCDD-injected mice, similar to those of thymectomized NFS/sld mice, were found whereas no lesion was observed in the salivary glands of vehicle-treated mice. The lesions of female mice were more severe than those of male mice (Fig. 1, A–C). Lymphocyte infiltration around ducts with destruction of acinar cells was observed in the TCDD-induced lesions (Fig. 1A). Severity of the inflammatory lesions was increased in a dose-dependent manner of TCDD (Fig. 1, B and C). In addition, more severe lesions developed with aging, and observed mainly in female mice (Fig. 1C). To characterize the infiltrating immune cells in the inflammatory lesions of salivary glands, the frozen sections were analyzed using the markers of T cells, B cells, and dendritic cells by immunofluorescence staining. CD4$^+$ T cells were mainly infiltrated in the inflammatory lesions of salivary glands from TCDD-treated mice, whereas a small population of CD8$^+$ T cells, B cells, and CD11c$^+$ dendritic cells were seen in the lesions (Fig. 1D).

In contrast, the inflammatory lesions of lung, liver, or kidney were also observed in the mice treated with TCDD (Fig. 1E). The incidence of the lesions is shown in Table I. At 6 mo of age, slight inflammatory lesions in the liver of 30–50% male mice by 0.1 or 1 ng of TCDD injection were observed. The inflammatory lesions of liver were found in 30–70% of male mice and 50% of the female mice treated with 10 ng of TCDD at 6 mo of age. Also, the inflammatory lesions of lung with a small number of lymphocyte infiltrates around bronchus or blood vessels were observed in both 100% female and male mice treated with 10 ng of TCDD at 6 mo of age. In addition, the slight inflammation in the kidney from 100% male mice treated with 10 ng of TCDD was observed at 6 mo of age. As for female mice, the renal lesions were found in ~50% of the mice treated with 10 ng of TCDD at 6 mo of age. Induction of inflammatory lesions by TCDD might be dependent on the sex or the sensitivity of each organ, although its precise mechanism is unclear.
Influence of in vivo low-dose TCDD injection on T cell phenotypes

To examine the influence of neonatal exposure to low-dose TCDD on T cell phenotypes, flow cytometric analysis of the expressions of surface T cell markers was performed on female mice at 6 mo of age (Fig. 2). There was no significant difference in the expression profile of CD4 and CD8 on the spleen cells by treatment of TCDD (Fig. 2A). A significantly increased population of memory phenotype, CD44<sup>hi</sup>CD4<sup>+</sup>CD8<sup>+</sup> T cell, was observed in the female mice treated with TCDD (Fig. 2B). As for thymic maturation, the CD4<sup>+</sup>CD8<sup>+</sup> double-negative (DN) cells were considerably reduced by 10 ng of TCDD while double-positive (DP) cells significantly decreased by 10 ng of TCDD injection. By contrast, both CD4 single-positive (SP) and CD8SP cells were significantly increased by TCDD injection (Fig. 2, C and D). Furthermore, when the increased DN cells were analyzed using differentiation markers such as CD25 and CD44, CD44<sup>hi</sup>CD25<sup>+</sup> (DN1) and CD44<sup>+</sup>CD25<sup>+</sup> (DN4) cells were significantly reduced by in vivo treatment of 10 ng of TCDD, but significantly increased populations of CD44<sup>+</sup>CD25<sup>+</sup> (DN2) and CD44<sup>+</sup>CD25<sup>+</sup> (DN3) cells were observed (Fig. 2, E and F). These results suggested that neonatal exposure to TCDD might influence thymic differentiation including negative or positive selection of T cells.

The influence of low-dose TCDD on peripheral T cell functions

To know the effect of TCDD on T cell functions in the periphery, cytokine secretions from splenic T cells activated by plate-coated anti-CD3 mAb were analyzed using the culture supernatants by ELISA. T<sub>H1</sub> cytokine production, including IL-2 and IFN-γ from activated T cells of TCDD-treated mice, was significantly increased compared with that of control mice (Fig. 3A). By contrast, there was no influence on T<sub>H2</sub> cytokine secretion such as IL-4 and IL-10 by in vivo TCDD injection (Fig. 3A). Moreover, proliferative response of splenic T cells stimulated with anti-CD3 and CD28 mAbs was analyzed using CFSE dilutions during 3 days. The cell divisions during the late stage were significantly enhanced by in vivo TCDD injection compared with those of control mice (Fig. 3B). In contrast, there was no difference in Foxp3<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup> T cells, classical Treg cells, by neonatal TCDD exposure (Fig. 3C). It has been known that T-bet for T<sub>H1</sub> and GATA-3 for T<sub>H2</sub> are prime candidates for key transcription factors of each cytokine production of T<sub>H</sub> cells (35). T-bet mRNA expression of purified T cells from spleen in TCDD-treated mice was higher than that in control mice. However, there was no change of the GATA-3 mRNA expression by TCDD injection (Fig. 3D). In addition, to examine the role of IL-17 in the pathogenesis for TCDD-induced autoimmunity, no change was observed in IL-17 production from anti-CD3 mAb-stimulated T cells of TCDD-treated mice (Fig. 3E). These findings show that the neonatal exposure to low-dose TCDD influences on T cell activation or proliferation through enhanced secretion of T<sub>H1</sub> cytokines in the periphery.

Direct influences of TCDD on neonatal thymus

When adult NFS/sld mice at 2 mo of age were injected with low-dose TCDD, no inflammatory lesion in any organ was observed until 6 mo of age (our unpublished data). Neonatal exposure to low-dose TCDD may affect the induction of inflammatory lesions in salivary glands resembling the SS model. To evaluate whether
the exposure to TCDD has much more influence on neonatal thymus compared with adult thymus, the expression of AhR was analyzed by quantitative RT-PCR. Interestingly, in contrast to adult thymus, the expression of AhR mRNA of neonatal thymus from NFS/sld mice was much higher (8- to 9-fold) (Fig. 4A). The expression was reduced with aging (<3 mo of age). The expression of AhR mRNA in neonatal spleen was higher (2-fold) than that in adult spleen (Fig. 4A). Next, to clarify the direct effects of TCDD on thymocytes, neonatal and adult thymocytes were incubated with 0, 100, and 1000 nM TCDD for 3 h to analyze AhR expression. More increased expression of AhR in neonatal thymocytes was observed by TCDD stimulation compared with that in adult thymocytes (Fig. 4B). In addition, mRNA expression of CYP1A1, one of target genes for TCDD/AhR/XRE (36), in neonatal thymocytes was much enhanced by TCDD incubation, whereas there was no change in mRNA expression of CYP1A1 in adult thymocytes by TCDD stimulation (Fig. 4C). In contrast, there were no significant changes in mRNA expressions of AhR and CYP1A1 of the spleen cells in the response to TCDD between neonatal and adult mice (data not shown). To understand association between TCR and TCDD signaling in thymocytes of neonatal and adult NFS/sld mice, plate-coated anti-TCR mAb was used for the stimulation of thymocytes with or without TCDD. Up-regulated mRNA expression of AhR by TCDD in both neonatal and adult thymocytes was clearly reduced by stimulation of anti-TCRβ mAb (Fig. 4D). In addition, TCDD-induced CYP1A1 mRNA expression in neonatal thymocytes was reduced to the level of non-stimulation by anti-TCRβ mAb (Fig. 4E). These findings suggest that neonatal thymocytes may be sensitive to TCDD through highly expressed AhR in NFS/sld mice, and that neonatal injection of TCDD might influence thymic differentiation to induce breakdown of tolerance.

Expression of AhR in neonatal thymus

To confirm the higher expression of AhR as a protein in neonatal thymus tissues of NFS/sld mice, Western blot analysis was performed. The highest expression of AhR was observed on day 2 after birth, and the expression on day 3 was relatively decreased. The expression in adult (3 mo of age) was lower than that of neonatal thymus (Fig. 5A). Next, we tried to detect the intracellular AhR expression in subpopulation of thymocytes by flow cytometric analysis. Flow cytometric analysis showed that most thymocytes clearly expressed AhR in adult (3 mo of age) C57BL/6 mice, and the fluorescence intensity of AhR expression was increased depending on the dose of anti-AhR Ab (Fig. 5B). When compared, the AhR expressions at each stage including DN, DP, CD4SP, and CD8SP cells in neonatal thymus of NFS/sld mice from day 0 to day 3 after birth with those in adult thymus (3 mo), a significantly increased AhR expression of neonatal (days 0, 1, 2, and 3) DN T cells was observed than that of adult DN cells. In particular, much more AhR expression of DN cells on day 2 was detected during neonatal stage. AhR expression of DP T cells on days 2 and 3 was significantly higher than that of adult DP cells. In contrast, although AhR expression of CD4SP cells on day 2 was significantly higher than that of adult CD4SP cells, there was no change in the expression of AhR of CD8SP cells between neonatal and adult thymus (Fig. 5, C and D). Furthermore, when the AhR expression of each differentiation stage of DN such as CD44+CD25− (DN1), CD44+CD25+ (DN2), CD44−CD25+ (DN3), and CD44−CD25−

FIGURE 6. Influence of TCDD on central tolerance in thymus. A, Transcription activity of NF-κB in thymocytes stimulated with anti-TCR mAb in the presence or absence of TCDD was evaluated. Results are shown as mean ± SD of three samples. *, p < 0.05. B and C, In vivo effect of TCDD on target genes of NF-κB was evaluated to detect the mRNA expressions. The mRNA expressions of NF-κB-regulated genes in thymus tissues from TCDD-treated mice were analyzed by real-time PCR. Results are shown as mean ± SD of four to six mice per each group. *, p < 0.05. D, AIRE mRNA expression of thymus from TCDD-treated mice. E, The mRNA expressions of salivary protein-1 and GAD67 in thymus from TCDD-treated mice. The expressions of thymus from TCDD-treated mice were detected by real-time PCR. *, p < 0.05; **, p < 0.005.
The influence of exposure to low-dose TCDD on central tolerance

Because higher AhR expression of T cells in neonatal thymus of NFS/sld mice was observed (Fig. 5), the cell signal pathway to regulate central tolerance in thymus via TCDD/AhR was analyzed. We focused on NF-κB, one of the responsive factors for TCDD/AhR/XRE signaling (37), which is known to be a key transcription factor for regulation of T cell differentiation, development, and activation (38). When the transcriptional activity of NF-κB in between neonatal and adult thymocytes stimulated with anti-TCR mAb in the presence of TCDD was compared, the neonatal activity was significantly increased relative to that of adult thymocytes (Fig. 6A). Also, the NF-κB activity of neonatal thymocytes stimulated with anti-TCR mAb was largely enhanced by the addition of TCDD, whereas the increased activity of adult thymocytes was not observed (Fig. 6A). Next, to understand the in vivo cell signaling through NF-κB and TCDD/AhR in thymus, the mRNA levels of NF-κB target genes were analyzed by real-time PCR using the thymus tissues from neonatal TCDD-treated mice. Among them, Bcl-xL and TNF-α mRNAs in thymus tissues from TCDD-treated mice were significantly increased in the dose-dependent manner compared with control mice (Fig. 6B). There were no changes to the mRNA expressions of IRF-1, GADD45, IL-1β (Fig. 6C), IL-6, inducible NO synthase, and Fas ligand (data not shown) which are target genes of NF-κB for controlling T cell signal.

In contrast, AIRE, an essential transcription factor for the expression of tissue-specific autoantigen in thymic epithelial cells (TECs), is well known to play a key role in T cell differentiation and development related with autoimmunity (39). When AIRE mRNA level in thymus tissues, including TECs from neonatal TCDD-treated NFS/sld mice, was analyzed, the expression in 10 ng of TCDD-treated mice was significantly decreased compared with that in control mice (Fig. 6D). Moreover, salivary protein-1 and GAD67 are known to be representative for the tissue-specific Ag in salivary gland and pancreas respectively (40). Interestingly, both salivary protein-1 and GAD67 mRNA expressions of the thymus tissues from neonatal TCDD-treated NFS/sld mice were significantly reduced relative to those from control mice (Fig. 6E). These findings show that neonatal exposure to low-dose TCDD in NFS/sld mice might influence the impairment of central tolerance in thymus, resulting in the induction of autoimmune disease.

The influences of low-dose TCDD exposure on B cells

The effects of neonatal exposure to low-dose TCDD on B cell phenotype and function were analyzed (Fig. 7). There was no difference in the number of B220+ B cells from spleen between TCDD-treated and control mice (Fig. 7A). Furthermore, no change was observed in the proliferative response of splenic B cells to LPS from TCDD-treated mice compared with that from control mice (Fig. 7B). The serum titers of autoantibodies that are associated with SS, including anti-SSA/Ro, anti-SSB/La, and anti-ssDNA, were examined (17, 18). In this study, serum titers of anti-SSA/Ro and anti-SSB/La autoantibodies were significantly increased in TCDD-treated mice at 6 mo of age compared with those in control mice (Fig. 7C). It has been reported that thymectomized NFS/sld mice and human SS patients have high titers of serum autoantibody against α-fodrin (20, 34). The higher titers of anti-α-fodrin autoantibody in the sera from TCDD-treated mice were also detected from control mice at 6 mo of age (Fig. 7D). No significant change for anti-ssDNA was observed between TCDD-treated and control mice (data not shown). In addition, when the ratio of IgG2a and IgG1 that is associated with Th1 and Th17 or cellular and humoral immune responses was analyzed using sera from TCDD-injected mice, the ratio from TCDD-injected mice was significantly higher than that from control mice at 6 mo of age (Fig. 7E).
Discussion

TCDD is a widespread environmental contaminant that influences several basic homeostatic control mechanisms in the body via AhR (3). T cells are a possible direct target for TCDD, as evidenced by the presence of the AhR in T cells, and inhibition of T cell growth by the expression of a constitutively active AhR mutant in AhR-null Jurkat T cells or following TCDD treatment (1, 41). It has been demonstrated that expression of AhR in both CD4+ and CD8+ T cells is required for a full suppression of an allospecific CTL response by TCDD, indicating a direct role for AhR in these TCDD-induced immunosuppressive effects (1, 42). However, the relationship between in vivo TCDD exposure and breakdown in T cell tolerance has not been well defined.

In this study, we demonstrated that neonatal exposure to low-dose TCDD could induce autoimmunity in the salivary glands using a NFS/sld strain associated with disease-susceptible autoantibody production, such as anti-SSA/La, anti-SSB/Ro, and anti-ß-fodrin Abs. It has been reported that TCDD causes extensive damage to the thymus to suppress T cell-dependent immune responses in vivo, including delayed-type and contact hypersensitivity responses and the generation of CTL (4, 43, 44). By contrast, neonatal exposure to TCDD had little influence on thymic atrophy in our experiment in which low-dose (0.0486 ± 0.0088 to 8.37 ± 0.7 µg/kg) TCDD was administered into neonatal mice on days 0, 1, and 2 (body weight: 1.2 ± 0.1 to 2.1 ± 0.35 g) after birth. The dosage of TCDD was considerably lower than that in the experiments in which thymic atrophy or apoptosis was induced by in vivo exposure to TCDD (30–50 µg/kg) (31, 32). For instance, it was reported that 60% apoptotic cells of thymus were observed in normal mice injected with 50 µg/kg TCDD, whereas 20–30% apoptotic cells of thymus were observed in vehicle-injected mice. In addition, although the loss of mitochondrial membrane potential related to apoptosis of thymocytes was not detected in ~10 µg/kg TCDD-treated mice, the loss was observed in 10–50 µg/kg TCDD-treated mice (31). Thus, the exposure to low dosage under 10 µg/kg TCDD may have an influence on neonatal thymic differentiation or selection in NFS/sld mice, but not atrophy or apoptosis, to induce autoimmune disease as the late effect. Moreover, T cell proliferation by anti-CD3 and anti-CD28 mAbs and Th1-type cytokine production, such as IL-2 and IFN-γ from splenic CD4+ T cells, were significantly more enhanced by neonatal TCDD treatment than those in control mice. These findings were consistent with the reports that TCDD enhances proliferation and cytokine production of mitogen- or Ag-stimulated T cells or T cell clones (1, 8). Because there were alterations in the percentage and number of DN cells in TCDD-treated mice, we analyzed this population using CD44 and CD25 markers. After TCDD treatment in this set, there was a decrease in the percentage of CD44+/CD25− cells (DN1) and CD44+/CD25+ cells (DN4), and a relative increase in the percentage of CD44+/CD25− cells (DN2) and CD44+/CD25+ cells (DN3). Analysis of the actual numbers of cells in each population compared with controls suggested that thymic maturation or negative selection at DN2 or DN3 might be affected by neonatal exposure to the low-dose TCDD. These results suggest that TCDD is interfering with the development and/or the proliferation of DN cells. Furthermore, the early stage of DN and the late stage into CD4SP or CD8SP in the thymic differentiation were disturbed by low-dose TCDD treatment, indicating that the immunotoxicity of TCDD on neonatal thymus might lead to the development of T cell-dependent autoimmunity. The inflammatory lesions were observed in the organs other than salivary gland including kidney, lung, and liver in TCDD-treated mice. Although the inflammatory lesions in liver, lung, and kidney from female mice treated with 10 ng of TCDD at 2 mo of age were observed, the severity of extraglandular lesions was lower than that of salivary gland, and the onset was later compared with that in salivary gland. Most of the extraglandular lesions of both female and male mice were developed with aging. We have previously demonstrated that the extraglandular lesion such as autoimmune arthritis in 3d-Tx NFS/sld mice was observed with aging (45, 46). Therefore, it is possible that TCDD may enhance any age-related reaction in the organs to induce autoimmunity.

In this study, there were no changes in the B cell number and proliferation in spleen from low-dose TCDD-treated mice, although the suppressive effect of the T cell-dependent Ab response to sheep RBC was reported in C57BL/6 mice injected with TCDD (47). In addition, TCDD selectively inhibited terminal B cell differentiation into plasma cells in response to trinitrophenol-LPS without altering early events in B cell activation or proliferation (48). By contrast, in our study significantly increased autoantibody productions such as anti-SSA/La, anti-SSB/Ro, and anti-ß-fodrin were observed by neonatal exposure to low-dose TCDD. Although it is still unclear whether the direct or indirect effect of TCDD on B cells influences autoantibody production, this new finding may be a key to understand the association of TCDD immunotoxicity with the development of autoimmunity.

AhR is a cytoplasmic receptor protein and has been described as a ligand-activated transcription factor that mediates induction of xenobiotic metabolizing enzymes (27, 28, 49). Upon ligand binding, the AhR translocates into the nucleus and dimerizes with ARNT. The AhR/ARNT complex binds to specific gene promoter elements (50). In this study, significantly increased expressions of AhR mRNA and protein in neonatal thymus were observed compared with those in adult thymus. This suggests that neonatal exposure to low-dose TCDD may affect thymic differentiation and/or maturation through AhR by disrupting the T cell tolerance more intensively than those in adult thymus. If negative or positive selection in the neonatal thymus is disrupted by low-dose TCDD exposure, autoreactive T cells may be released to the periphery and expand in response to any autoantigen leading to induce autoimmunity. Increased expression of AhR mRNA and the disrupted thymic differentiation in the neonatal thymus by low-dose TCDD exposure may support this hypothesis.

CYP1A1 is known to have pivotal roles in cell growth and apoptosis (51, 52). In the present study, CYP1A1 mRNA of neonatal thymocytes was readily up-regulated by TCDD, whereas the expression of adult thymocytes was constant in the response to TCDD. Neonatal exposure to low-dose TCDD may influence proliferation, differentiation, or apoptosis of thymocytes through CYP1A1 at early stages, such as DN. Namely, it is possible that negative selection leading cell apoptosis at DN3 might be disturbed by neonatal exposure to TCDD. As a result, autoreactive T cells leaking from thymic selection might survive and proliferate in response to any autoantigen in the periphery, leading to the induction of autoimmune lesions. The activation of AhR by TCDD results in an increased binding activity to NF-κB subunit RelB of AhR itself to form AhR/RelB complex, which was associated with an increased mRNA level of multiple inflammatory genes (53). Overexpression of AhR and RelB led to an increased level of CCL1 and IRF-3 in control as well as TCDD-stimulated cells supporting the role of RelB and AhR for the transcriptional regulation of these genes (54). In the present study, TCDD enhanced TCR-mediated classical NF-κB activation of neonatal thymocytes from NFS/sld mice more than adult thymocytes. In addition, some NF-κB-target genes such as Bcl-xL and TNF-α in thymus were up-regulated by in vivo TCDD injection. Our data demonstrate that...
TCDD/AhR signal may influence the differentiation or development of T cells in the neonatal thymus associated with autoimmunity. However, the precise mechanism of how the NF-kB activation, including classical and nonclassical pathway, interacts with TCDD/AhR signal is still unclear.

It has been well known that autoimmune lesions of multiple organs such as lacrimal glands, salivary glands, pancreas, and liver are observed in AIRE gene-deficient mice (39). AIRE was reported to play a pivotal role in the expression of tissue-specific autoantigens such as salivary protein-1, GAD67, insulin, or other self-proteins in the TECs that express the MHC class II on the cell surface and function as APCs to immature T cells for the immunological selection of central tolerance in the thymus (55). In this study, mRNA expressions of AIRE and tissue-specific autoantigens such as salivary protein-1 and GAD67 in the thymus were reduced by the in vivo neonatal exposure to low-dose TCDD in NFS/ild mice. The finding indicated that TCDD might influence the selection of autoreactive T cells in the thymus through AIRE. There may be any complex molecular mechanisms related to the avidity of TCR, haplotype of MHC class II, Ag-specificity, T cell apoptosis, interaction with TEC, or TCDD signal.

The AhR has been shown to mediate various immunotoxic responses induced by environmental pollutants like TCDD (56). Although our results show that activation of the AhR by TCDD effects T cell development, the receptor does not seem to play a key role in the establishment of a normal T cell compartment. The AhR has been shown to play important roles in regulating the expression of several cytokines. For example, exposure of rats to TCDD led to up-regulation of IL-1β and TNF-α in the liver (57, 58). Interestingly, although TCDD suppressed the production of IFN-γ by mediastinal lymph node cells, there was a 10-fold increase in the IFN-γ level in the lungs of TCDD-treated mice (59). Autoimmune disease is caused by heterogeneous etiology, involving interplay between predisposing genes and triggering environmental factors. Although a lot of studies have demonstrated the immunotoxicity of TCDD, this study is the first to induce autoimmunity by neonatal low-dose TCDD treatment. Recently it has been reported that AhR links Tgβ17 cell-mediated experimental autoimmune encephalomyelitis to environmental toxins through altering the differentiation of Treg cells (60, 61). The low-dose TCDD exposure in our model had little influence on the number of Treg cells in splenocytes that express the MHC class II on the cell surface and function as APCs to immature T cells for the immunological selection of central tolerance in the thymus. However, the precise mechanism of how the NF-κB signal may influence the differentiation or development of T cells in the neonatal thymus associated with autoimmunity, and the function of TH17 cells, such as IL-17 production in our model had little influence on the number of Treg cells in splenocytes that express the MHC class II on the cell surface and function as APCs to immature T cells for the immunological selection of central tolerance in the thymus.

T cells in the neonatal thymus associated with autoimmunity, including classical and nonclassical pathway, interacts with TCDD/AhR signal is still unclear.

Acknowledgments
We thank Ai Nagaoka, Noriko Kino, Risa Okada, Ritsuuko Oura, and Satoko Yoshida for technical assistance.

Disclosures
The authors have no financial conflict of interest.

References


