response to TCDD exposure (18, 21, 22). All of these features are reproduced in vitro by direct addition of TCDD to fetal thymus organ culture (FTOC) (19, 23, 24), indicating that the target cells responsible for the alterations are present in the thymus. The results of previous studies that have included histological examination have led to the hypothesis that thymic stromal cells, and not thymocytes themselves, are the direct targets of TCDD that induce thymus involution (25). This hypothesis was supported by a study showing that the stroma of a fetal thymus reaggregation culture treated with an AhR-binding halogenated aromatic hydrocarbon, not the thymocytes, induced thymus involution (24). By contrast, a recent study in which chimeric mice having AhR-deficient hemopoietic cells and wild-type stromal cells or vice versa were exposed to TCDD demonstrated that the AhR in the hemopoietic compartment, that is in the thymocytes or their precursor cells, is responsible for the TCDD-induced thymus involution (18).

The results of other studies have also shown or suggested that the AhR in T cells plays an essential role in TCDD-induced immunotoxicity. A recent study by Kerkvliet et al. (26) in a mouse graft-vs-host model injected with $AhR^{+/+}$ or $AhR^{-/-}$ T cells showed that AhR activation in T cells is critical to the suppression of CTL activity by TCDD. In our own study examining the effect of TCDD on OVA-specific Ab production in mice (27), TCDD exposure suppressed the increase in T cell number in the spleen and production of IL-2 and Th2-type cytokines before the inhibition of Ag-induced Ab production, suggesting that the AhR activation in T cells causes suppression of T cell activation and subsequent immune reactions leading to Ab production. However, it is difficult to determine the specific role of AhR activation in T cells alone in TCDD-exposed mice, because the AhR in all cell types, including B cells and APCs, is simultaneously activated, and indirect effects cannot be excluded. Chimeric mouse models and a T cell transfer system produced by using AhR-deficient mice or their cells are very useful tools for studying the primary cell target of TCDD, but they are inconvenient, because generation of chimeric mice and reconstitution by T cell transfer require highly specialized techniques. In addition, when chimeric mice are used, it must be borne in mind that their hemopoietic cells contain precursor cells not only for T cells but for B cells and APCs as well (28).

To investigate the role of AhR activation in TCDD-induced immunotoxicity, in the present study we generated transgenic (Tg) mice that specifically express a constitutively active mutant of AhR (CA-AhR) in T-lineage cells by expressing a CA-AhR with a minimal deletion in the PAS-B domain (29) under the regulation of a CD2 promoter. The AhR mutant constitutively localizes to the nucleus, heterodimerizes with ARNT, and activates transcription by binding XRE sequences in a ligand-independent manner (29, 30). The results of the present study demonstrate that AhR activation in T-lineage cells alone directly induces the thymocyte changes. They also show that the increase in number of splenocytes after immunization is suppressed in the Tg mice, whereas resting splenocytes in nonimmunized mice are much less affected, suggesting that the AhR plays a role in the growth of activated and proliferating T cells.

Materials and Methods

Generation of Tg mice

The CA-AhR expression construct (VA hCD2-CA-AhR) was generated by subcloning PAS B-domain-deleted mouse AhR cDNA with poly(A) signal (29) into the *EcoRI/BamHI* site of the VA human CD2 (hCD2) minigene, an improved version of a human CD2 minigene-based vector (31). Tg founder mice were obtained by microinjecting the transgene expression construct into C57BL/6J × DBA/2 eggs as described previously (32). In some lines, VA hCD2-GFP was coinjected with VA hCD2-CA-AhR. One line carrying both CA-AhR and GFP constructs (line A) and two lines with the CA-AhR construct alone (lines K and N) were chosen for further studies and subsequently were backcrossed into C57BL/6J mice. Founders and subsequent littermates were genotyped by PCR of tail DNA using primers for VA hCD2-CA-AhR (5′-GAACAGAGAGTTTGTCCAGC-3′, located in hCD2 promoter, and 5′-CTTCCAAAGGTAAGCATAAGAGTC-3′, located in N terminus of CA-AhR). Integrated CA-AhR copy number was determined by Southern blot analysis. Genomic DNA from a tail sample was digested with *EcoRI* and *PstI*, separated by agarose gel electrophoresis, blotted onto a Hybond filter (Amersham), and hybridized with a digoxigenin (DIG)-labeled probe. The DIG-labeled probe was synthesized from the *HincII* digestion fragment of pEB6CAG-CA-AhR-GFP (29) with a DIG-high prime DNA labeling and detection starter kit I (Roche Diagnostics) and was detected with CSPD as a substrate according to the manufacturer's instructions. Heterozygous ($CA\text{-}AhR^{+/-}$) mice were used for experiments after crossing into C57BL/6 mice for two to six generations. Their nontransgenic ($CA\text{-}AhR^{-/-}$) littermates (designated as wild type) were used as controls.

Cell preparation

Single cell suspensions of thymus and spleen were prepared by forcing cells in RPMI 1640 medium supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (complete medium) through a stainless-steel mesh. Spleen cells and bone marrow cells prepared from thigh bones were treated with ammonium chroride/EDTA solution (0.83% NH_4Cl, 0.1% $KHCO_3$, 0.37% EDTA (pH 7.4)) for 2 min at room temperature to eliminate RBCs and then were washed with PBS (33). Cells were counted with a hemocytometer after staining with trypan blue.

RT-PCR

Total RNA was isolated from cells or tissues with an RNeasy Mini kit (Qiagen). After checking the quality of the RNA by electrophoresis, RT-PCR was performed with an RNA LA PCR kit (AMV) ver1.1 (TaKaRa Biomedicals) according to the manufacturer's instructions. The amplification was conducted by heating at 94°C for 2 min, cycling at 94°C for 30 s, 60 or 66°C for 30 s, and 72°C for 30 s, and then extension at 72°C for 10 min after the final cycle. The primer sequences and annealing temperatures for each gene are shown in Table I. The primers for detecting mRNA expression of CA-AhR were designed to span the region coding for PAS B domain to distinguish PCR products between CA-AhR and wild-type AhR. The PCR products were separated with a 1.2% Synergel (Diversified Biotech) containing 0.5 μg/ml ethidium bromide, and the gel images were captured and visualized using an Electrophoresis Documentation and Analysis System 290 (Eastman Kodak).

Flow cytometry

Cells were stained with mAbs against lymphocyte surface markers or streptavidin-allophycocyanin (BD Pharmingen) for 20 min on ice. After staining, the cells were washed, treated with 7-aminoactinomycin D (7-AAMD; Sigma-Aldrich) to label dead cells, and measured with a FACS-Calibur (BD Biosciences). Live cells were gated and analyzed (22). The following mAbs, all purchased from BD Pharmingen, were used: PE-conjugated anti-CD4 (anti-CD4-PE, clone GK-1.5), FITC-conjugated anti-CD8

Table I. *List of primers used for RT-PCR*

Description	Primer Sequence (5′-3′)	Annealing Temperature (°C)	Product Size (bp)
CA-AhR	TTACCTGGGCTTTCAGCAGT AACTGGGGTGGAAAGAATCC	66	506
CYP1A1	CCATGACCGGGAACTGTGG TCTGGTGAGCATCCTGGACA	60	344
Adseverin	GTGCTTCTAAGCATTTCCCC GAGTGAATGGCATCCAAGTG	60	121
CD4	AAGGGCTCTCCCTGAGAGTC AAAGAGGAAAAAAGGGGAAGG	60	104
Spatial	GAAGGTGACAGCGAAAATCA AAGGCATTAGACAGGTTGGG	60	112
β-Actin	GAGGCCCAGAGCAAGAGAG GGCTGGGGTGTTGAAGGT	60	225
HPRT	GCTGGTGAAAAGGACCTCT CACAGGACTAGAACACCTGC	60	249

(anti-CD8-FITC, clone 53-6.7), biotinylated anti-CD8 (anti-CD8-biotin, clone 53-6.7), anti-CD3-PE (clone 145-2C11), anti-CD19-biotin (clone ID3), anti-B220-FITC (clone RA3-6B2), anti-CD127(IL-7Rα)-biotin (clone B12-1), and anti-CD61-PE (clone 2C9.G3). Biotinylated rat IgG2a was used as an isotype-matched control.

Immunization

OVA (albumin, chicken egg, grade VII) was purchased from Sigma-Aldrich. Alum-precipitated OVA (OVA/alum) was prepared as follows (27, 34). OVA (1 mg/ml) in PBS was mixed with an equal volume of 9% (w/v) $AlK(SO_4)_2$, and pH of the mixture was adjusted to 6.5 with KOH. The precipitate was washed three times with PBS and then resuspended in PBS at 0.5 mg/ml. Mice were i.p. immunized with the OVA/alum (100 μg OVA/mouse).

TCDD treatment

TCDD (50 μg/ml in nonane) purchased from Cambridge Isotope Laboratories was diluted with corn oil to adjust it to a dose volume of 10 μl/g body weight. TCDD was administered to the mice orally.

Fetal thymus organ culture

Line A heterozygous Tg mice backcrossed into C57BL/6J mice for five generations were mated, and homozygous CA-AhR$^{+/+}$ Tg mice were obtained. Male CA-AhR$^{+/+}$ mice were mated with female C57BL/6J mice, and thymuses were collected from fetuses on gestation day 16.5. One or two lobes of the thymuses were placed on a nitrocellulose filter (45-μm pore size) set in a 24-well culture plate with 1 ml of complete medium and were cultured for 4 days (35). To deplete them of thymocytes, the lobes were cultured in the presence of 1.35 mM 2-deoxyguanosine (Sigma-Aldrich) for 4 days (36, 37).

Results

Generation of T cell-specific CA-AhR Tg mice

We used the VA hCD2 vector to generate Tg mice expressing a CA-AhR mutant (Fig. 1A) specifically in T-lineage cells. We chose one line carrying both CA-AhR and GFP constructs (line A) and two lines with only the CA-AhR construct (lines K and N) functioning under the control of the VA hCD2 vector for the subsequent experiments. The transgene-positive mice were mated with C57BL/6 mice and maintained as heterozygotes. Heterozygous mice were used in all experiments unless otherwise specified, and their nontransgenic (CA-AhR$^{-/-}$) littermates (designated as wild type) were used as controls. Integrated CA-AhR copy numbers were determined by Southern blotting to be 2 for line A, 6–7 for line K, and 9–11 for line N. All of the lines were fertile, exhibited a normal sex ratio at birth, showed no increase in mortality after birth, and appeared healthy.

Fig. 1*B* shows CA-AhR mRNA expression in various organs in line A Tg mice. CA-AhR mRNA was detected in the thymus and spleen as expected, and was also found in the lung and, to a very minor extent, in the kidney. Expression of the AhR-responsive gene CYP1A1 was also detected in the thymus and spleen in the Tg mice in contrast with their wild-type littermate mice (Fig. 1*C*). In the lung, CYP1A1 mRNA was detected in the wild-type mice. The lung is reported to express the highest level of AhR mRNA among the tissues examined, including the thymus and spleen, in the mice (38). Recently, endogenous ligand was isolated from porcine lung (39). Thus, the lung may contain abundant AhR and endogenous ligand may activate the receptor and induce CYP1A1. The expression of CYP1A1 in the lung was further increased in the Tg mice (Fig. 1*C*). Expression of CA-AhR and CYP1A1 mRNA was also confirmed in the thymus and spleen of lines K and N (see Fig. 4).

Expression of the transgene in immune cells was measured by flow cytometry analysis of the GFP expression in line A Tg mice (Fig. 2). Thymocytes showed a broader peak of the GFP-positive population (Fig. 2*A*). CD4 and CD8 T cells in the spleen were confirmed to be GFP-positive, and B cells did not express GFP (Fig. 2*B*).

We then investigated whether the bone marrow cells of the CA-AhR Tg mice expressed the transgene and, as shown in Fig. 3, the CD3$^-$CD127$^+$ (IL-7Rα-expressing) lymphocyte progenitor fraction (40) was found to be faintly GFP-positive (Fig. 3*C*, R3). Although a previous study reported that the VA hCD2 vector functions in megakaryocytes as well as T-lineage cells (32), CD61$^+$ megakaryocytes (41) in the bone marrow did not express the transgene (Fig. 3*C*, R4 and R5).

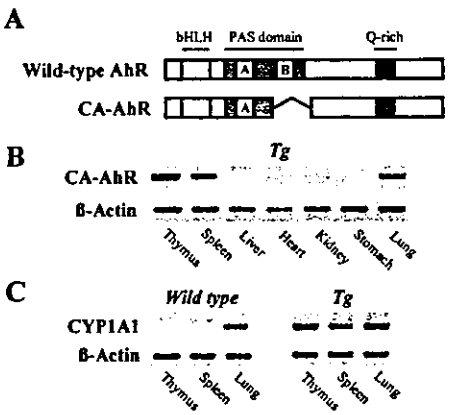

FIGURE 1. Generation of T cell-specific CA-AhR Tg mice. *A*, Schematic representation of the wild-type mouse AhR and the CA-AhR mutant lacking the minimal PAS B motif. *B*, Different tissues from line A heterozygous Tg mice were examined for CA-AhR mRNA expression by RT-PCR. C, Functional activation of the CA-AhR was confirmed by detection of CYP1A1 expression by RT-PCR.

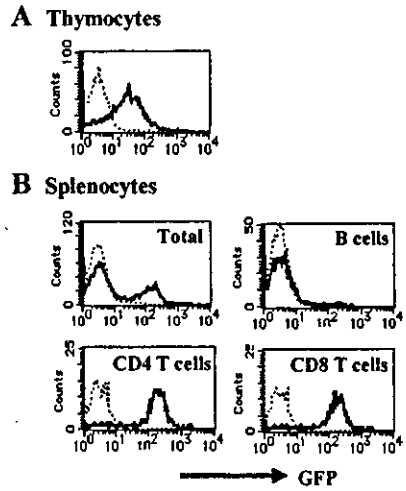

FIGURE 2. Transgene expression in thymocytes and splenocytes. *A*, Thymocytes prepared from line A heterozygous mice were stained with 7-AAMD and analyzed with a FACSCalibur flow cytometer. 7-AAMD-negative live cells were gated and expression of coinjected GFP was analyzed. *B*, Splenocytes from line A heterozygous mice were stained with a combination of anti-CD19-biotin/streptavidin-allophycocyanin and anti-CD3-PE or a combination of anti-CD8-biotin/streptavidin-allophycocyanin and anti-CD4-PE and then were stained with 7-AAMD and analyzed with a FACSCalibur. CD4 T cells, CD8 T cells, and CD19$^+$ B cells in the 7AAMD-negative live cells were gated, and GFP expression was analyzed. The staining obtained in Tg mice is represented by the bold line, and the staining in wild-type mice is represented by the dotted line.

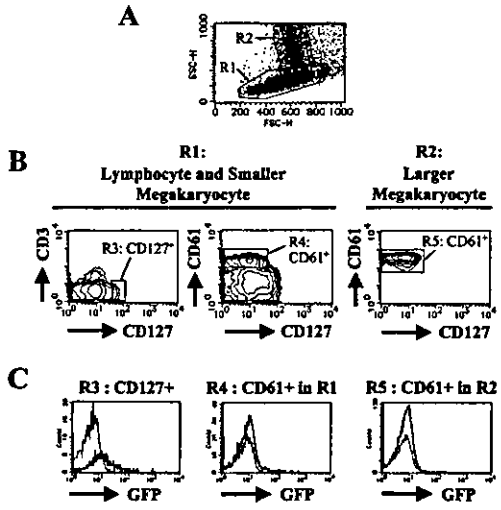

FIGURE 3. The transgene is faintly expressed in CD127$^+$ cells in the bone marrow. Bone marrow cells from line A heterozygous mice were stained with anti-CD127-biotin/streptavidin-allophycocyanin and either anti-CD3-PE or anti-CD61-PE and then were stained with 7-AAMD and analyzed with a FACSCalibur. *A*, Side scatter vs forward scatter of the cells analyzed. Region 1 (R1), including lymphocytes and smaller size megakaryocyte, and R2, including larger size megakaryocytes (41), in the 7-AAMD-negative live cells are indicated in the dot plot. *B*, R3, including CD127$^+$ cells, and R4, including CD61$^+$ cells in R1, and R5, including CD61$^+$ cells in R2, are indicated in the contour plot. *C*, Expression of GFP as analyzed by gating R3–R5 is shown in the histogram plot. The staining obtained in Tg mice is represented by the bold line, and the staining in wild-type mice is represented by the thin line.

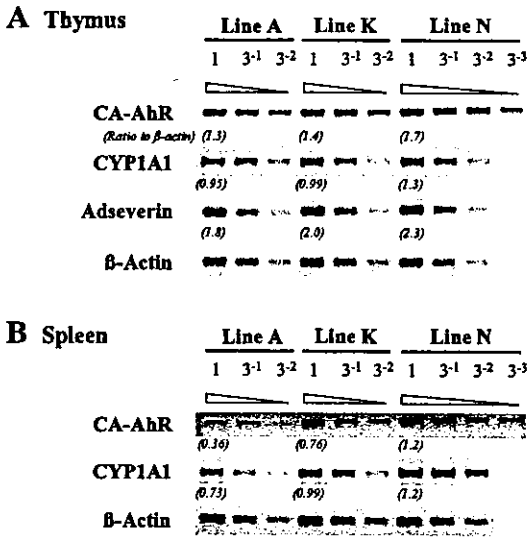

FIGURE 4. Comparison of CA-AhR and target gene expression in the thymus and spleen from line A, line K, and line N heterozygous mice. Total RNA was prepared from the thymus and spleen of the three lines with the RNeasy mini kit. cDNAs prepared from 20 ng of total RNA and serial dilutions (3^{-1}–3^{-3}) were amplified by PCR using primers for CA-AhR, CYP1A1, adseverin, or β-actin as a housekeeping gene. The expression of genes was quantified by densitometrically scanning gel images, and the values normalized to β-actin mRNA are indicated in parentheses. The numbers of PCR cycles for CA-AhR in thymus and spleen were 32 and 34, respectively, 26 and 32 for CYP1A1, and 20 for β-actin in both tissues. Mice were used after crossing into C57BL/6 mice for six generations in line A and for three generations in lines K and N.

CA-AhR expression induces target gene mRNA in both thymus and spleen, but reduces thymocyte number alone

Expression of the CA-AhR transgene and its target genes and phenotypic changes in the thymus and spleen were examined in the three lines. RT-PCR analyses showed that CA-AhR and CYP1A1 expression in the thymuses and spleens of lines A, K, and N were increased according to the integrated CA-AhR gene numbers (Fig. 4). Expression of adseverin, which was reported to be induced by TCDD in mice thymuses in an AhR-dependent manner (37), was also increased according to the transgene numbers.

The thymus weight was reduced in all three lines, by 36% in line A, 70% in line K, and 63% in line N (Fig. 5*A*). The thymocyte number was reduced by 49% in line A, 96% in line K, and 92% in line N (Fig. 5*A*). The thymocyte population defined by CD4 and CD8 expression was also affected in the Tg mice, with reduced percentages of CD4$^+$CD8$^+$ DP cells and increased percentages of CD8 SP and double negative (DN) cells (Table II). The ratios of CD4 SP/CD8 SP were significantly reduced in the Tg mice (Table II). The remarkable increases in the percentage of DN cells in line K and N were parallel to the large decreases in the total cell numbers.

By contrast, spleen weight was unaffected by the expression of CA-AhR (Fig. 5*B*). Splenocyte number was significantly reduced only in line N (by 40%), in which CA-AhR is most highly expressed (Fig. 5*B*), and the percentages of CD4 T cells, CD8 T cells, and B cells were unchanged, even in line N (data not shown). All of these findings are consistent with those observed in the thymuses exposed to TCDD (18, 21, 22).

We further confirmed that the CA-AhR is not expressed in the stromal cells and that CA-AhR expression in T-lineage cells alone is capable of inducing the thymus alteration in FTOC. mRNA expression was examined in whole thymus and stroma obtained by culturing thymus tissues in the presence of 2-deoxyguanosine to deplete it of thymocytes. As expected, CD4 mRNA was detected only in whole thymus, and Spatial mRNA, which is specifically expressed in thymic stromal cells (42), was detected in both the

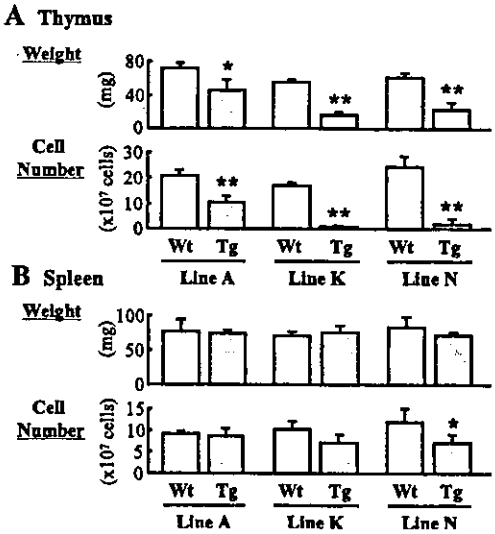

FIGURE 5. CA-AhR expression in T-lineage cells reduces thymus weight and cell number, but affects the spleen less. Thymus and spleen from female heterozygous Tg mice and nontransgenic littermate wild-type mice of line A (8 wk old, n = 4/each group), line K (10 wk old, n = 5), and line N (8–9 wk old, n = 5) were examined. Mice were used after crossing into C57BL/6 mice for two generations in line A and for three generations in lines K and N. The differences between Tg mice and wild-type mice were analyzed by Student's t test. The data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$.

Table II. *CA-AhR expression affects thymocyte population*[a]

	Line A (n = 4)		Line K (n = 5)		Line N (n = 5)	
	Wt	Tg	Wt	Tg	Wt	Tg
	(%)		(%)		(%)	
DN	4.7 ± 0.5[b]	6.4 ± 1.2	4.2 ± 1.1	52.6 ± 7.8	6.8 ± 2.8	27.9 ± 14.1
DP	82.2 ± 1.7	79.3 ± 1.4	87.2 ± 1.8	33.5 ± 7.6	83.2 ± 2.4	53.1 ± 15.9
CD4 SP	10.0 ± 1.5	9.5 ± 2.0	6.9 ± 0.7	8.7 ± 1.2	7.0 ± 0.8	10.2 ± 0.9
CD8 SP	3.1 ± 0.2	4.9 ± 0.4	1.8 ± 0.4	5.2 ± 0.5	3.1 ± 0.3	8.8 ± 1.2
CD4/CD8[c]	2.6 ± 1.1	2.0 ± 0.5	4.0 ± 0.7	1.8 ± 0.6**	2.3 ± 0.3	1.2 ± 0.1**

[a] Thymocytes from female heterozygous (CA-AhR$^{+/-}$) Tg mice and nontransgenic (CA-AhR$^{-/-}$) littermate mice (Wt) (8–10 wk old) were examined by flow cytometry.
[b] The data was expressed as means ± S.D. The differences between CD4/CD8 ratio in Tg mice and Wt mice were evaluated with Wilcoxon rank sum test. **, $p < 0.01$.
[c] Ratio of CD4 SP cells/CD8 SP cells.

whole thymus and the stroma (Fig. 6). CA-AhR was detected only in whole thymus, not in the stroma, in this system (Fig. 6). Thus, AhR activation in thymocytes alone was demonstrated to cause the cellular loss and population changes in the thymus.

CA-AhR suppresses the increase in spleen weight and splenocyte number caused by immunization

We previously reported finding that TCDD administration to mice immunized with OVA suppressed the immunization-induced increase in spleen weight and splenocyte number (27, 34). Consistent with these findings, the increase in spleen weight and splenocyte number observed in wild-type mice after immunization with OVA was suppressed in line A Tg mice (Fig. 7), although their spleen was unaffected when not immunized, as stated above (Fig. 5). Interestingly, increases of both CD4 T cells and B cells were significantly suppressed despite the specific expression of CA-AhR in T cells (Fig. 7). The number of CD8 T cells was also fewer in the Tg mice than in the wild-type mice, although the difference was not significant. Simultaneous suppression of the T and B cell increase was also observed in OVA-immunized and TCDD-exposed wild-type mice (27).

To estimate how much TCDD induces the corresponding level of AhR activation, CYP1A1 expression in the thymus and spleen of line A Tg mice and TCDD-exposed wild-type mice was compared. As shown in Fig. 8, the level of expression of CYP1A1 mRNA in the thymus of the Tg mice was slightly higher than its level of expression in wild-type mice exposed to a single dose of 20 μg/kg TCDD. CYP1A1 expression in the spleen of the Tg mice was less than in wild-type mice exposed to 20 μg/kg TCDD, which seems plausible because only T cells express CYP1A1 mRNA in the spleen of Tg mice, whereas both T and B cells express CYP1A1 mRNA in TCDD-exposed wild-type mice (43). These results show that the function of the activated AhR in line A heterozygous mice and AhR activated by 20 μg/kg TCDD are roughly comparable. In our previous study, 20 μg/kg TCDD suppressed the splenocyte number to 60% of the number in unexposed control mice 1 wk after immunization (27). The same dose of TCDD reduced the thymus weights to 40–60% (44). The fact that a similar extent of suppression was observed in the thymus and spleen of the Tg mice indicates that the major portion of the effect of TCDD in these organs is attributable to AhR activation in the T cells.

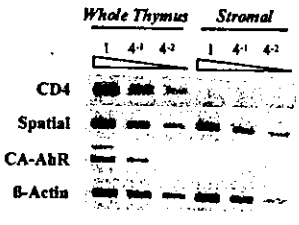

FIGURE 6. CA-AhR is expressed in the thymocytes, but not in the stromal cells, in the thymus. line A male CA-AhR$^{+/+}$ mice were mated with female C57BL/6J mice, and thymuses were collected from fetuses on gestational day 16.5. The thymuses were cultured for 4 days on a nitrocellulose filter floated on complete medium. To deplete them of thymocytes and obtain stromal cells, the lobes were cultured in the presence of 1.35 mM 2-deoxyguanosine. Fifteen or 16 lobes were pooled from each treatment group and were used to prepare RNA. cDNAs prepared from 20 ng of total RNA and serial dilutions (4^{-1}, 4^{-2}) were amplified by PCR

Discussion

To prove the role of activated AhR in T cells in TCDD-induced immune suppression, we generated Tg mice in which expression of CA-AhR cDNA is controlled by an improved version of hCD2 promoter and their T cells specifically express activated form of AhR. We obtained three lines bearing different copy numbers of the transgene, ranging from 2 to 10. All three lines showed expression of CA-AhR and an AhR-induced target gene in the thymus and spleen. In line A mice, which coexpress GFP in addition to CA-AhR under the control of the same CD2 promoter, the transgene was confirmed to be strongly expressed in the thymocytes, CD4, and CD8 T cells, but not in the thymus stromal cells or in the B cells. CD2 is principally expressed on T cells and NK cells in humans (45), but low expression has also been reported on subsets of other cell types, including B cell progenitors (46, 47). Consistent with the observation, faint expression of the transgene was detected on the CD3$^-$CD127$^+$ lymphocyte progenitor cells in our

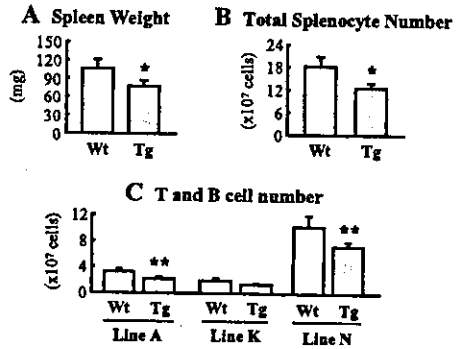

FIGURE 7. CA-AhR expression suppresses the increase in spleen weight and both CD4 T cell and B cell numbers after immunization. Line A female heterozygous Tg mice and littermate wild-type mice (8 wk old, n = 4) were immunized with OVA/alum, and their spleens were examined 7 days later. Mice were used after crossing into C57BL/6 mice for five generations. The differences between the Tg mice and wild-type mice were analyzed by Student's t test. The data are expressed as mean ± SD. *, $p < 0.05$; **, $p < 0.01$.

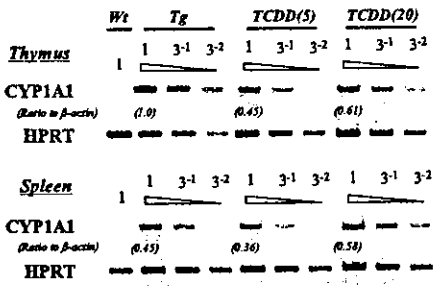

FIGURE 8. Comparison of CYP1A1 induction by CA-AhR in Tg mice and by TCDD exposure. Female C57BL/6 mice were given 5 or 20 μg/kg TCDD, and 3 days later total RNAs were prepared from thymocytes and splenocytes. CYP1A1 induction was compared with its induction in female line A heterozygous mice. cDNAs prepared from 20 ng of total RNA and serial dilutions (3^{-1}, 3^{-2}) were amplified by PCR using primers for CA-AhR or hypoxanthine phosphoribosyltransferase as a housekeeping gene. The expression of genes was quantified by densitometrically scanning gel images, and the values normalized to β-actin mRNA are indicated in parentheses. In both tissues, 30 PCR cycles were used for CYP1A1 and 20 for hypoxanthine phosphoribosyltransferase.

Tg mice. The transgene was also found to be expressed in the lung and, to a very minor extent, in the kidney in the Tg mice. Although the mechanism for the expression of CA-AhR in those tissues is unknown, presence of cells expressing CA-AhR in those tissues, such as T cells in the lung, may partly contribute to the transgene expression. Albeit the expression of the vector is found in other cell types, these CD2-based vectors, including the VA hCD2 vector, have been proved to be very useful to study specific functions of molecules in T cells. Likewise, the Tg mice we developed in the present study enable a new approach to explore the effect of AhR activation in T cells in the immune suppression. Lymphocyte progenitor cells have been reported to be affected by TCDD, and its effect was suggested to contribute to a loss of thymocytes (48, 49). Although the expression of CA-AhR in the lymphocyte progenitor cells was much fainter than that in the thymocytes and T cells (Figs. 2 and 3), the effects of low expression of CA-AhR may need to be considered.

All three lines of our Tg mice were characterized by thymus involution, including reduced thymocyte number and increased percentage of CD8 SP cells, the same as observed in TCDD-exposed mice. The fact that direct exposure of FTOC to TCDD reproduces the thymus involution induced by TCDD exposure in vivo (19, 24) shows that TCDD directly affects the thymus, in which the target cells are present. However, the results of previous studies have suggested that two types of cells in the thymus, thymocytes (18, 28) and stromal cells (24, 25), are the primary targets. The results of the present study in the Tg mice demonstrate that AhR activation in T-lineage cells alone can cause the thymus alterations, including loss of thymocytes and increase in percentage of CD8 SP thymocytes, without AhR activation in the stromal cells. Tomita et al. (50) recently produced T cell-specific ARNT-deficient mice in which the ARNT gene is disrupted under the control of T cell-specific $p56^{lck}$ proximal promoter, and showed that the thymus of the Tg mice is resistant to TCDD. Their results are consistent with our own showing that the AhR/ARNT heterodimer in the thymocytes, but not stromal cells, is essential for the occurrence of thymus involution.

Whereas thymus undergoes involution upon TCDD exposure, the splenocytes and splenic T cells of nonimmunized animals are unaffected by TCDD (18, 51). The same finding was observed in the spleen of our T cell-specific CA-AhR Tg mice, even though the CA-AhR was fully expressed in both the spleen T cells and the thymocytes. Although we examined the expression of CYP1A1 and adseverin as sensitive AhR-dependent target genes to estimate the extent of AhR activation, the genes responsible for the thymus involution remain to be identified. Previous studies have suggested suppression of thymocyte proliferation (24, 28) and induction of apoptosis (52) as the biological process involved in the thymus atrophy caused by TCDD. Our own recent study demonstrated that CA-AhR expression in Jurkat T cells inhibits cell growth by inducing both apoptosis and cell cycle arrest (29). Several genes in these CA-AhR-expressing Jurkat T cells that are related to apoptosis or cell cycle arrest, such as Fas, cyclin G_2, and growth arrest and DNA damage-inducible protein 34, were shown to be up-regulated in an XRE-mediated transcription-dependent manner (29), and these genes may be responsible for the loss of thymocytes.

In contrast with the nonimmunized mice in which splenocytes were less affected by AhR activation, as described above, the increase in splenocyte number after immunization was suppressed in the CA-AhR Tg mice, suggesting that the AhR/ARNT heterodimer inhibits cell growth in activated and proliferating T cells, but not in resting T cells. From this point of view, the effect of AhR activation in thymocytes may be also attributable to the effect on activated or proliferating cells: in thymus atrophy, the suppression of DN cell proliferation (28) and the loss of DP cells (52) are suggested to be responsible for cellular loss, DN thymocytes are vigorously proliferating cells, and DP cells receive an activation signal via their T cell receptors. Thus, activation state of the cell seems to affect the sensitivity of T-lineage cells to AhR activation.

The results of the present study also demonstrated that AhR activation in T cells alone suppresses the increase in both T and B cells in the spleen after OVA immunization. In terms of primary target cells of TCDD toxicity in immune reaction, Kerkvliet et al. (26) recently showed that AhR in both CD4 and CD8 T cells is necessary for full suppression of CTL response by TCDD in a mouse acute graft-vs-host model in which T cells, or CD4 or CD8 subsets, from $AhR^{+/+}$ and $AhR^{-/-}$ C57BL/6 mice were injected into C57BL/6 × DBA/2 F_1 host mice. Consistently, our results indicated that AhR activation in T cells is involved in changes in immune reaction. We previously reported that TCDD administration to OVA-immunized mice suppresses the growth of T and B cells and the production of Th2-type cytokines before suppression of Ab production (27, 34), which suggested that TCDD inhibits Ab production by suppressing T cell activation and the subsequent Th2-cell differentiation. The results of the present study strongly support the hypothesis that activation of the AhR directly inhibits cellular activation of the T cells and their subsequent proliferation and differentiation, leading to the suppression of T cell help on B cell proliferation. Alternatively, indirect effect of $CD4^+CD25^+$ regulatory T cells may be involved in the immune suppression. The regulatory T cells are positively selected in the thymus when their TCR receives a signal with intermediate strength (53). TCDD exposure to thymus is shown to affect thymocyte selection, possibly through up-regulation of Notch 1 (54) or activation of the ERK pathway (35) in the thymocytes, and these mechanism may alter the selection of $CD4^+CD25^+$ regulatory T cells. Further study of our Tg mice will clarify whether the suppression of T cell growth by AhR activation inhibits Th2-type cytokines and following Ab production by B cells, and it will also provide a clue for solving the mechanism of inhibition.

Andersson et al. (55) recently produced Tg mice expressing a CA-AhR mutant (30), which has a structure very similar to the one we used, under the control of an Ig H chain enhancer (Eμ), which promotes transgene expression in both B- and T-lineage cells (56). In addition to exhibiting thymus atrophy, their mice have a reduced life span and develop tumors in the glandular part of the stomach

(55). The Tg mice generated in our study, in contrast, are fertile and do not exhibit any overt phenotype differences except thymus atrophy, showing that AhR activation in T cells is not responsible for the stomach tumors.

Although T cells contain functional AhR and directly respond to TCDD (57), phenotypic changes caused by TCDD in T cells, such as changes in proliferation and differentiation, are difficult to detect in vitro, possibly because of optimized culture conditions that compensate for the effects of TCDD (6). The Tg mice expressing CA-AhR in T cells will be a useful model for investigating the role of activated AhR in the T cells. In particular, immunization of the Tg mice is expected to show suppression of various immune reactions, including Ab production and CTL activity, the same as observed in TCDD-exposed mice. Dioxins are persistent environmental contaminants and as such animals are continually exposed to them. TCDD maternally exposed is transferred to fetus and pups through the placenta or milk and activates their AhR (58, 59). Our Tg mice express CA-AhR mRNA in the fetal thymuses (data not shown) and the expression continues in the T-lineage cells after birth. Thus, these Tg mice will also be a useful model for clarifying the effect of persistent activation of AhR in T cells. Studies using our CA-AhR Tg mice should shed light on the role of the AhR in T cells in immune suppression by TCDD and also in physiological reactions.

Acknowledgments

We thank Y. Miyamoto, K. Ao, and M. Matsumoto for their excellent technical assistance, Drs. M. Kubo and R. Abe (Science University of Tokyo, Noda, Japan) for their useful discussions, and Drs. H. Toyoshiba and J. Yonemoto (National Institute for Environmental Studies, Tsukuba, Japan) for their valuable advice on statistics.

Disclosures

The authors have no financial conflict of interest.

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International Immunopharmacology 5 (2005) 331–343

International Immunopharmacology

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Search for the target genes involved in the suppression of antibody production by TCDD in C57BL/6 mice

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Received 2 September 2004; received in revised form 4 October 2004; accepted 7 October 2004

Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) suppresses antibody production through activation of a transcription factor, the aryl hydrocarbon receptor (AhR). To explore the genes that are involved in the suppression of antibody production by TCDD, we investigated TCDD-induced changes in gene expression in the CD4 T cells and B cells of C57BL/6 mice immunized with ovalbumin (OVA) plus alum as an adjuvant. Changes in gene expression were analyzed with Affymetrix oligonucleotide microarrays. The results showed that OVA-immunization alone up-regulated expression levels of many genes in the CD4 T cells as early as 3 h after immunization, with 55 up-regulated and 5 down-regulated. At 24 h, 42 genes were found to be up-regulated and 30 down-regulated. Fewer genes were affected in the B cells than in the CD4 T cells. In contrast to the up-regulation of genes induced by immunization in the CD4 T cells, administration of TCDD to mice 3 h prior to the immunization mainly caused down-regulation of genes in the CD4 T cells when compared with immunization alone, with 1 being up-regulated and 4 down-regulated at 3 h after immunization and 3 up-regulated and 34 down-regulated at 24 h. In particular, at 3 and 24 h, TCDD suppressed expression of three and seven genes, respectively, that were up-regulated by immunization. Another characteristic of the TCDD-induced changes in gene expression was the suppression of many genes encoding proteins that are involved in GTP-binding protein-linked signaling in CD4 T cells. These results suggest that the inhibition of immunization-induced gene expression and modulation of G-protein-linked signaling in CD4 T cells are responsible for the TCDD-induced suppression of antibody production.

Keywords: TCDD; Gene expression; CD4 T cell; B cell; Antibody production

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doi:10.1016/j.intimp.2004.10.001

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic halogenated aromatic hydrocarbon, induces a variety of adverse biological and physiological reactions in mammals [1–3], including immunotoxicity, such as thymus atrophy and suppression of antibody production and cytotoxic T cell activity [4,5]. Recent studies in aryl hydrocarbon receptor (AhR)-deficient mice have shown that the majority of these TCDD-induced toxicities are mediated through the AhR, a ligand-activated transcription factor [6–10]. Upon binding with TCDD, the AhR translocates from the cytoplasm to the nucleus and dimerizes with another transcription factor, the AhR nuclear translocator (Arnt). The AhR–Arnt complex binds with a *cis*-acting DNA enhancer sequence, the xenobiotic responsive element (XRE), located in the enhancer region of target genes, and modulates gene expression [11,12]. Comprehensive study of changes in expression of these genes has recently been made possible by the development of technologies, such as gene microarrays and serial analysis of gene expression technique [13–16]. However, the effects of TCDD greatly vary, depending on the type of species, tissues, cells, and the state of the cells, and genes and proteins responsible for the individual TCDD toxicities remain to be identified.

We previously investigated the effect of TCDD on T-cell-dependent antibody production in mice [17,18]. When mice were immunized with ovalbumin (OVA) plus alum as an adjuvant, antigen-specific IgM and IgG1 production was detected in the plasma on day 7 and day 10, respectively, after immunization [17]. In the series of reactions leading to antibody production, B cell expansion and differentiation into antibody-producing cells require the help of Th2 cells, the effector cells differentiated from naïve CD4 T cells and characterized by the production of cytokines, such as IL-4, IL-5, and IL-6 [19]. In our previous study, Th2-type cytokine production and an increase in spleen T cells and B cells following immunization were observed prior to antibody production [17], whereas simultaneous administration of TCDD at the time of immunization suppressed the Th2-derived cytokine production and the increase in T cells and B cells and resulted in inhibition of OVA-specific IgM and IgG1 production [18]. These results suggest that the suppression of T cell activation, differentiation to Th2 cells, and Th2-type cytokine production by TCDD adversely affect reactions involved in antibody production.

In the present study, we used Affymetrix oligonucleotide microarrays to investigate the gene expression profiles of CD4 T cells prepared from TCDD-exposed and OVA-immunized mice. We particularly focused on early gene expression, i.e., 3 and 24 h after immunization, to identify genes that are involved in the induction of TCDD-induced antibody suppression. Gene expression in B cells prepared at the same times was investigated for comparison. The results revealed characteristic TCDD-induced changes in gene expression in immunized CD4 T cells and B cells.

2. Materials and methods

2.1. Mice

Five-week-old female C57BL/6J mice were obtained from Clea Japan (Tokyo) and maintained in our animal facility under controlled conditions at a temperature of 24±1 °C, humidity of 50±10%, and a 12:12-h-light/dark cycle. Mice were allowed to acclimate for 1 week and were handled in a humane manner according to the NIES guidelines for animal experiments.

2.2. Experimental protocol

TCDD (50 μg/ml in nonane, 98% pure) was purchased from Cambridge Isotope Laboratories (Andover, MA). The TCDD in nonane solution was diluted with corn oil to obtain a dose volume of 10 μl/g body weight. Ovalbumin (OVA; chicken egg, grade VII) was purchased from Sigma (St. Louis, MO). Alum-precipitated OVA (OVA/alum) was prepared by mixing 1 mg/ml of OVA in PBS with an equal volume of 9% (w/v) $AlK(SO_4)_2$ and adjusting the pH of the mixture to 6.5 with KOH [17,18]. The precipitate was washed three times with PBS and then resuspended in PBS at 0.5 mg OVA/ml. Corn oil or TCDD (20 μg/kg) was orally administered to 6-week-old mice, and, 3 h later, they were intraperitoneally immunized with OVA/alum (100 μg OVA/mouse). The animals'

spleens were excised 3 or 24 h after immunization. The experimental protocol is summarized in Fig. 1.

2.3. Isolation of murine CD4 T cells and B cells

CD4 T cells and B cells were prepared from the spleens by positive selection with antibody-labeled magnetic particles. A single-cell suspension was prepared from pooled spleens from three mice per group as previously described [20]. The spleen cells were incubated with BD IMag Mouse CD4 Particles-DM or Mouse B220 Particles-DM (BD Biosciences), and the particle-labeled cells were isolated by applying a magnet according to the manufacturer's instructions. The purity of the isolated cells was assessed by flow cytometry as described below. The purity of the isolated CD4 T cells and B cells was within the 96–99% range.

2.4. Flow cytometry

The percentages of T cells and B cells in the spleen and the purity of the isolated cells were determined by flow cytometry. Cells were stained with monoclonal antibodies against lymphocyte surface markers or streptavidin-APC (Pharmingen, San Diego, CA) for

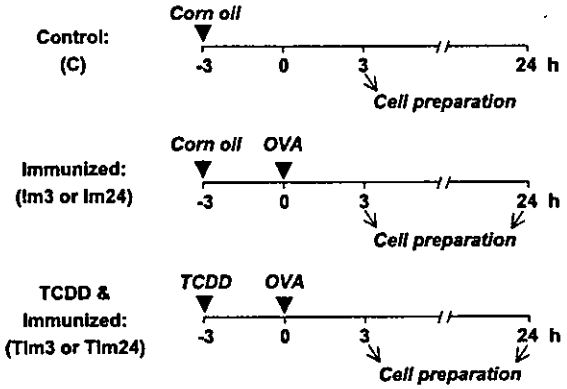


Fig. 1. Schematic diagram of the experimental protocol. Six-week-old mice (3 mice/group) were given corn oil or TCDD orally (20 μg/kg). In the control (C) group, CD4 T cells and B cells were prepared from the spleen 6 h after corn oil administration. In the immunized group, mice were intraperitoneally immunized with OVA/alum 3 h after corn oil administration, and CD4 T cells and B cells were prepared 3 (Im3) and 24 h (Im24) after immunization. In the TCDD group, the mice were intraperitoneally immunized with OVA/alum 3 h after TCDD-administration, and cells were prepared 3 (TIm3) and 24 h (TIm24) after immunization.

20 min on ice. After staining, the cells were washed, treated with 7-aminoactinomycin D (7-AAMD, Sigma) to label dead cells, and measured with a FACSCalibur flow cytometer (BD Biosciences). Live cells were gated and analyzed [21]. The following monoclonal antibodies, all purchased from Pharmingen, were used: fluorescein isothiocyanate-conjugated anti-CD8 (anti-CD8-FITC, clone 53-6.7), phycoerythrin-conjugated anti-CD4 (anti-CD4-PE, clone GK-1.5), anti-B220-PE (clone RA3-6B2), and biotinylated anti-CD3ε (anti-CD3ε-biotin, clone 145-2c11).

2.5. Affymetrix GeneChip analysis

Affymetrix GeneChip analysis was performed according to the Affymetrix expression analysis technical manual (Affymetrix, Santa Clara, CA), with some modifications. Total RNA was extracted from CD4 T cells and B cells with an ISOGEN RNA isolation kit (Nippon Gene, Toyama) and purified with an RNeasy Mini Kit (Qiagen, Chatsworth, CA). Double-stranded cDNA was synthesized from 1 μg of total RNA with SuperScript II reverse transcriptase (Invitrogen) and T7 oligo$(dT)_{24}$ primer (Affymetrix) and purified by phenol/chloroform extraction followed by ethanol precipitation. Biotin-labeled cRNA was prepared from the double-stranded cDNA by in vitro transcription with a Bioarray High-Yield RNA Transcript-labeling kit (Enzo Diagnostics, Farmingdale, NY) and purified with an RNeasy kit. A 15-μg sample of the biotin-labeled cRNA was fragmented and hybridized to a Mouse Expression Array 430A (Affymetrix). After being hybridized for 15 h, the array was washed, stained, and scanned. Data were analyzed with Affymetrix Microarray Suites 5.0 software. A comparison analysis was performed to select genes with two- or more fold increases or decreases in expression between two groups (target and reference), and gene expression that reproducibly changed two- or more fold in two independent experiments was judged to have changed significantly.

2.6. RT–PCR

To confirm the gene expression changes detected by the microarray analyses, we prepared two total RNA samples for each group independently of the samples for GeneChip analyses and investigated gene

Table 1
List of primers used for RT–PCR

Description	Left primer sequences (5′–3′)	Right primer sequences (5′–3′)	PCR cycle number	Annealing temperature (°C)	Product size (bp)
AhRR	cctggaccttgtgctctctc	tgtcctgcccttaggagvta	33	60	336
SLC15A2	gcggagaccagtttgaagag	cccattgcaaacacaacaag	33	60	211
CYP1A1	ccatgaccgggaactgtgg	tctggtgagcatcctggaca	29	67	344
RGS2	gcagaattcctctgctcctg	gaggacagtttttggggtga	23	60	243
Pleckstrin	actttggcaaacggatgttc	gatacaaagcccccaagtca	27	60	210
GPR 91	ttaaagaggaggagccagca	ctgttcagaaaggccagagg	30	67	221
GPR 35	accactgccttctcactgct	ggcagcacaggtattgaggt	28	60	237
GPR 43	cttcccggtgcagtacaagt	gctcttgggtgaagttctcg	26	60	179
Transglutaminase 2	aggacatcaacctgaccctg	cttgatttcgggattctcca	27	60	188
FGD2	ccagagagcactggacatga	aaccaggtagcgttccattg	27	67	208
Protein tyrosine phosphatase RO	acggacaggaaccttcattg	gcttcttcctcagccacatc	27	60	179
Protein phosphatase 1	cgagtcacggtcaagtacga	ctcctggacgaagtcctctg	28	67	225
RhoE	aaatatggccaagcagatcg	tcttcgctttgtcctttcgt	27	60	230
CD5 antigen-like	cttcggtcttgccttttgag	tcctccttttctccccagtt	27	60	188
Bcl11a	tcccatcgagatgaaaaagg	gctgctgggctcatctttac	28	60	244
CCL6	aggctggcctcatacaagaa	tcccctcctgctgataaaga	27	60	197
HPRT	gctggtgaaaaggacctct	cacaggactagaacacctgc	23	60	249

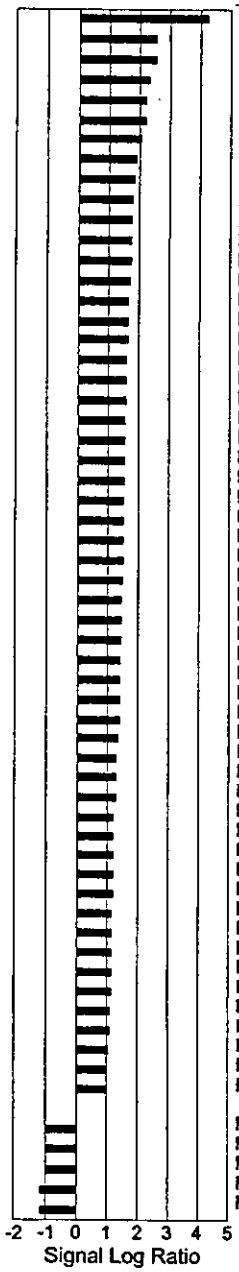
expression by RT–PCR as described previously [22]. Briefly, total RNAs were isolated from cells as described above, and RT–PCR was performed with an RNA LA PCR kit (AMV) ver1.1 (TaKaRa Biomedicals, Tokyo) according to the manufacturer's instructions. Amplification was carried out by heating at 94 °C for 2 min, cycling at 94 °C for 30 s, 60 or 67 °C for 30 s, and 72 °C for 30 s, and then extension at 72 °C for 10 min after the final cycle. The primer sequences, PCR cycle numbers, and annealing temperatures used for each gene are shown in Table 1.

3. Results

The CD4 T cells and B cells of immunized mice were investigated for TCDD-induced changes in gene expression with an Affymetrix GeneChip Mouse Expression Array 430A, which represents approximately 14,000 well-characterized mouse genes. The animal treatment protocol is summarized in Fig. 1. The CD4 T cells and B cells were prepared 6 h after corn oil administration [Control (C)]. Cell samples from immunized animals (Im3 or Im24) were prepared 3 or 24 h after OVA immunization from mice pretreated with corn oil. Cells from TCDD-exposed and immunized animals (TIm3 or TIm24) were prepared 3 or 24 h after OVA immunization from mice pretreated with TCDD. Gene expression of these cells were compared between C and Im3 and between C and Im24 in CD4 T cells and B cells, respectively, to detect immunization-dependent gene expressions. Differences between Im3 and TIm3 and between Im24 and TIm24 were examined to detect TCDD-dependent changes in expression in the immunized CD4 T cells and B cells, respectively. Two independent replicate experiments were performed for each pair, and, when two- or more fold changes in gene expression, either up or down, were reproducibly shown, expression of the gene was concluded to have "changed."

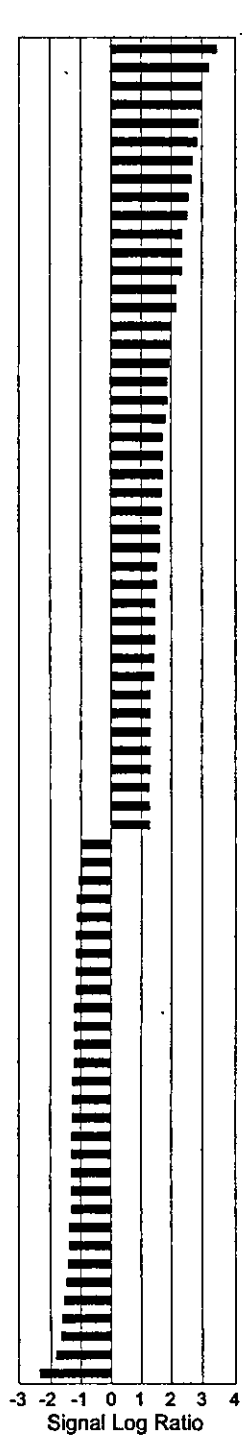
3.1. Changes in expression in CD4 T cells in response to OVA-immunization

Immunization-induced changes in gene expression in CD4 T cells at 3 and 24 h are shown in Figs. 2 and 3, respectively. At 3 h after immunization (Fig. 2), 55 genes in CD4 T cells were found to have been up-regulated by immunization, and five genes were found to have been down-regulated. The up-regulated genes included genes involved in cytokine signaling pathways, such as IL-4Rα, SOCS3, STAT2, IL-17R, IL-6 signal transducer (IL-6ST), and IL-1β, genes involved in growth regulation through apoptosis and/or cell cycle regulation, such as GADD45γ, immediate early response 3, and insulin-like growth factor binding



Unigene ID	Description	Changes in other comparisons[a]
Mm.281298	GADD45γ	
Mm.3064	properdin factor	Im24 vs. C:↑ Tim3 vs. Im3:↓
Mm.25613	immediate early response 3	
Mm.233802	IL-4Rα	
Mm.3468	SOCS3	Im24 vs. C:↑
Mm.259316	synaptotagmin 11	
Mm.87150	integrin β3	
Mm.1068	Bcl3	
Mm.1167	Jun-B	
Mm.293120	STAT2	
Mm.34867	Rab27A	
Mm.275071	Jun	
Mm.22506	DNA segment, Chr 17, human D6S56E 5	
Mm.4481	IL-17R	
Mm.37214	transferrin	Im24 vs. C:↑
Mm.370	C1qα	Im24 vs. C:↑ Tim3 vs. Im3:↓
Mm.30504	CYP4F16	Tim24 vs. Im24:↓
Mm.233799	IGFBP4	Im24 vs. C:↑
Mm.342555	RIKEN cDNA 2410129E14 gene	
Mm.279256	Ndr3	
Mm.34316	Mrf1	
Mm.29432	RIKEN cDNA 2510001I10 gene	Im24 vs. C:↑
Mm.199381	Interferon induced transmembrane protein 2	
Mm.271782	C-type lectin	
Mm.276405	FK506BP5	
Mm.218473	AIGP1	
Mm.3200	RIKEN cDNA 1300017K07 gene	
Mm.177194	AKT2	
Mm.175661	Interferon induced transmembrane protein 1	
Mm.2570	C1qβ	Im24 vs. C:↑
Mm.172346	TGF-βR2	
Mm.14627	phospholipid scramblase 1	
Mm.76649	VCAM1	Im24 vs. C:↑ Tim3 vs. Im3:↓
Mm.9277	phospholipase A2, group VII	Im24 vs. C:↑
Mm.271877	thioredoxin interacting protein	
Mm.16766	protein kinase, cAMP dependent, catalytic, β	Im24 vs. C:↑
Mm.314113	ELOVL family member 6	
Mm.4364	IL-6ST	Im24 vs. C:↑
Mm.266156	SLC41A1	
Mm.254134	GPI-anchored membrane protein 1	Im24 vs. C:↑
Mm.3453	C1qγ	Im24 vs. C:↑
Mm.3275	MAP3K8	
Mm.293517	Cab45	
Mm.2931	flotillin 1	
Mm.21642	Spi-C	
Mm.5181	Ring finger protein 19	
Mm.28733	Mitc 1	
Mm.276389	heme oxygenase 1	
Mm.222830	IL-1β	
Mm.50642	ELAC1 homolog	
Mm.330731	transglutaminase 2	Tim24 vs. Im24:↓
Mm.152780	GPR35	Tim24 vs. Im24:↓
Mm.292082	AIM1	
Mm.250605	Sel1 homolog	
Mm.209265	Gene trap locus 6	Im24 vs. C:↑
Mm.25709	inhibitor of growth family, member 1	
Mm.276276	—	
Mm.89979	GPR34	
Mm.302138	epsilon-N-trimethyllysine	
Mm.28262	regulator of G-protein signaling 2	Tim24 vs. Im24:↓

Fig. 2. Differential gene expression induced in CD4 T cells at 3 h after immunization. As shown in Fig. 1, gene expression in CD4 T cells prepared from two groups, the C group and Im3 group, was compared with Im3 as the target and C as the reference (Im3 vs. C). Two independent replicate experiments were performed, and genes that reproducibly showed two- or more fold change in expression were selected. [a]Changes observed in other comparisons in CD4 T cells.



Unigene ID	Description	Changes in other comparisons[a]
Mm.229107	RIKEN cDNA C530043G21 gene	
Mm.6424	Integrinβ5	
Mm.233813	YWHAG	
Mm.281124	glycoprotein galactosyltransferase α1, 3	
Mm.37214	transferrin	Im3 vs. C:↑
Mm.29432	RIKEN cDNA 2510001I10 gene	Im3 vs. C:↑
Mm.252776	protein kinase C, Nu	
Mm.3064	properdin factor	Im3 vs. C:↑ Tim3 vs. Im3:↓
Mm.216321	arginine-tRNA-protein transferase 1	
Mm.277376	ABCA1	
Mm.153230	ATP8A1	
Mm.6676	CD5 antigen like	Tim24 vs. Im24:↓
Mm.135581	RNA polymerase 1-4	
Mm.2570	C1qβ	Im3 vs. C:↑
Mm.87150	integrin β3	Im3 vs. C:↑
Mm.16766	protein kinase, cAMP dependent, catalytic, β	Im3 vs. C:↑
Mm.305152	apolipoprotein E	
Mm.370	C1qα	Im3 vs. C:↑ Tim3 vs. Im3:↓
Mm.254134	GPI-anchored membrane protein 1	Im3 vs. C:↑
Mm.3453	C1qγ	Im3 vs. C:↑
Mm.57726	serine hydroxymethyl transferase 1	
Mm.2180	HSP90	
Mm.4364	IL-6ST	Im3 vs. C:↑
Mm.342187	Ig heavy chain VJ558	Tim24 vs. Im24:↓
—	Ig κ chain V28	
Mm.244362	Ig κ light chain	Tim24 vs. Im24:↓
Mm.331784	utrophin	
Mm.28756	SLC40A1	
Mm.209265	gene trap locus 6	Im3 vs. C:↑
Mm.330428	CD44	
Mm.233799	IGFBP4	Im3 vs. C:↑
Mm.76649	VCAM1	Im3 vs. C:↑ Tim3 vs. Im3:↓
Mm.9277	phospholipase A2, group VII	Im3 vs. C:↑
Mm.46397	RIKEN cDNA 3100002B05 gene	
Mm.196110	hemoglobin α, adult chain 1	Tim24 vs. Im24:↓
Mm.291928	Catenin β	
Mm.3468	SOCS3	Im3 vs. C:↑
Mm.10747	allograft inflammatory factor 1	
Mm.193925	Gα 13	
Mm.345208	signal transducing adaptor molecule 2	
Mm.10510	ficolin A	
Mm.175403	Arl6ip2	
Mm.4128	AXL receptor tyrosine kinase	
Mm.261270	Interferon activated gene 203	
Mm.203556	RIKEN cDNA 2310011G23 gene	
Mm.24045	RIKEN cDNA 2510004L01 gene	
—	inactive X specific transcripts	
Mm.288565	RIKEN cDNA 2810441O16 gene	
Mm.197518	lysosomal-associated protein transmembrane 4B	
Mm.332474	G-rich RNA sequence binding factor 1	
Mm.293449	RIKEN cDNA 4930485D02 gene	
—	gene trap ROSA 26	
Mm.275354	RIKEN cDNA 4930470H18 gene	
Mm.276226	sestrin 1	
Mm.218423	RIO Kinase 2	
Mm.167298	RIKEN cDNA C030046M14 gene	
Mm.248876	Zinc Finger protein 326	
Mm.236795	mitochondrial ribosomal protein L13	
Mm.257403	RIKEN cDNA 4432412D15 gene	
Mm.182628	RAD21 homolog	
Mm.209568	sterol carrier protein 2	
Mm.277626	ubiquitin-activating enzyme E1C	
Mm.44069	Ring Finger protein 12	
Mm.41800	GTP-binding protein 4	
Mm.289584	B lymphoma Mo-MLV insertion region 1	
Mm.18472	proteasome 26S subunit	
Mm.195310	heterogeneous nuclear ribonucleoprotein D-like	
Mm.266611	G-proten signaling modulator 1	
Mm.6718	Interferon-induced protein with tetratricopeptide repeats 1	
Mm.195803	nicotinamide nucleotide transhydrogenase	
Mm.247542	peroxiredoxin 4	
Mm.261614	SLC12A6	
Mm.20477	Uty	

Fig. 3. Differences in gene expression in CD4 T cells 24 h after immunization. Gene expression in CD4 T cells prepared from the C group and Im24 group (Fig. 1) was compared with Im24 as the target and C as the reference (Im24 vs. C). Two independent replicate experiments were performed, and genes that reproducibly showed two- or more fold change in expression were selected. [a]Changes observed in other comparisons in CD4 T cells.

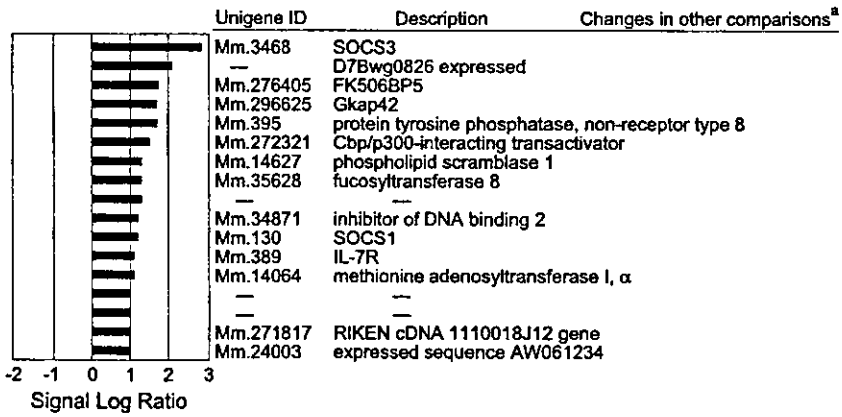


Fig. 4. Differential gene expression induced in B cells at 3 h after immunization. Gene expression of B cells prepared from the C group and Im3 group (Fig. 1) was compared with Im3 as the target and C as the reference (Im3 vs. C). Two independent replicate experiments were performed, and genes that reproducibly showed two- or more fold change in expression were selected. [a]Changes observed in other comparisons in B cells.

protein 4 (IGFBP4), and genes involved in G protein-linked signaling, such as Rab27A, transglutaminase 2, and a G-protein-associated protein GPR35. Genes encoding transcription factors (Bcl3, Jun-B, Jun, Spi-C, and Mitc 1) and adhesion molecules (integrin β3, C-type lectin, and VCAM1) were also shown to be up-regulated. Expression of IL-4Rα [23], SOCS3 [24,25], IL-17R [26], and Bcl3 [27] has previously been reported to be increased during T cell activation.

At 24 h after immunization (Fig. 3), 42 genes in CD4 T cells were up-regulated, and 30 were down-regulated. The up-regulated genes included cytokine signaling-pathway-related genes (IL-6ST and SOCS3), growth-regulation-related genes (IGFBP4), and genes encoding adhesion molecules (integrinβ5, integrinβ3, CD44, and VCAM1) and G protein (Gα13). Among the down-regulated genes were genes involved in GTP-binding protein-linked signaling (GTP-binding protein 4 and G protein signaling modulator 1) and in proteasome degradation (proteasome 26S subunit and ubiquitin-activating enzyme E1C).

As shown in Figs. 2 and 3, 15 genes were persistently up-regulated in the CD4 T cells at 3 and 24 h after immunization.

3.2. Expression changes in B cells in response to OVA immunization

The expression level of fewer genes was altered in the B cells 3 and 24 h after OVA-immunization than in the CD4 T cells; at 3 h, 16 genes in B cells were up-regulated (Fig. 4), and, at 24 h, 11 genes were up-

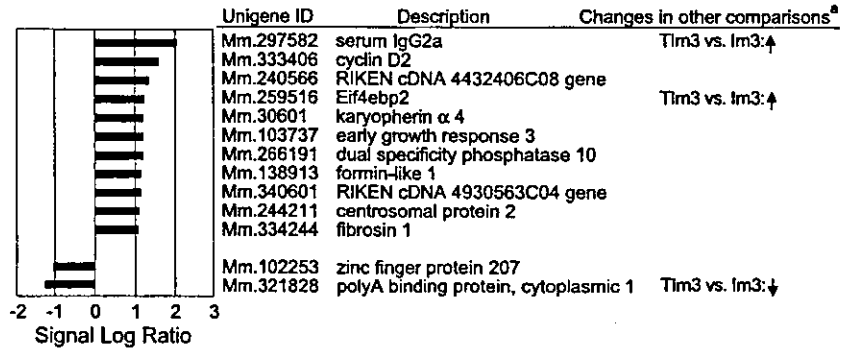


Fig. 5. Differences in gene expression in B cells 24 h after immunization. Gene expression in B cells prepared from the C group and Im24 group (Fig. 1) were compared with Im24 as the target and C as the reference (Im24 vs. C). Two independent replicate experiments were performed, and genes that reproducibly showed two- or more fold change in expression were selected. [a]Changes observed in other comparisons in B cells.

regulated and 2 genes were down-regulated (Fig. 5). Three genes that were up-regulated in CD4 T cells 3 and/or 24 h after immunization, SOCS3, FK506BP5, and phospholipid scramblase 1, were also up-regulated in B cells at 3 h after immunization (Fig. 4). One of the up-regulated genes at 24 h, cyclin D2, has previously been reported to be induced during B cell activation [28].

3.3. TCDD-induced changes in gene expression in immunized CD4 T cells

The TCDD-induced changes in gene expression in immunized CD4 T cells, which were detected by comparison between TCDD-exposed and OVA-immunized mice and OVA-immunized mice, are summarized in Fig. 6. The only up-regulated gene at 3 h was

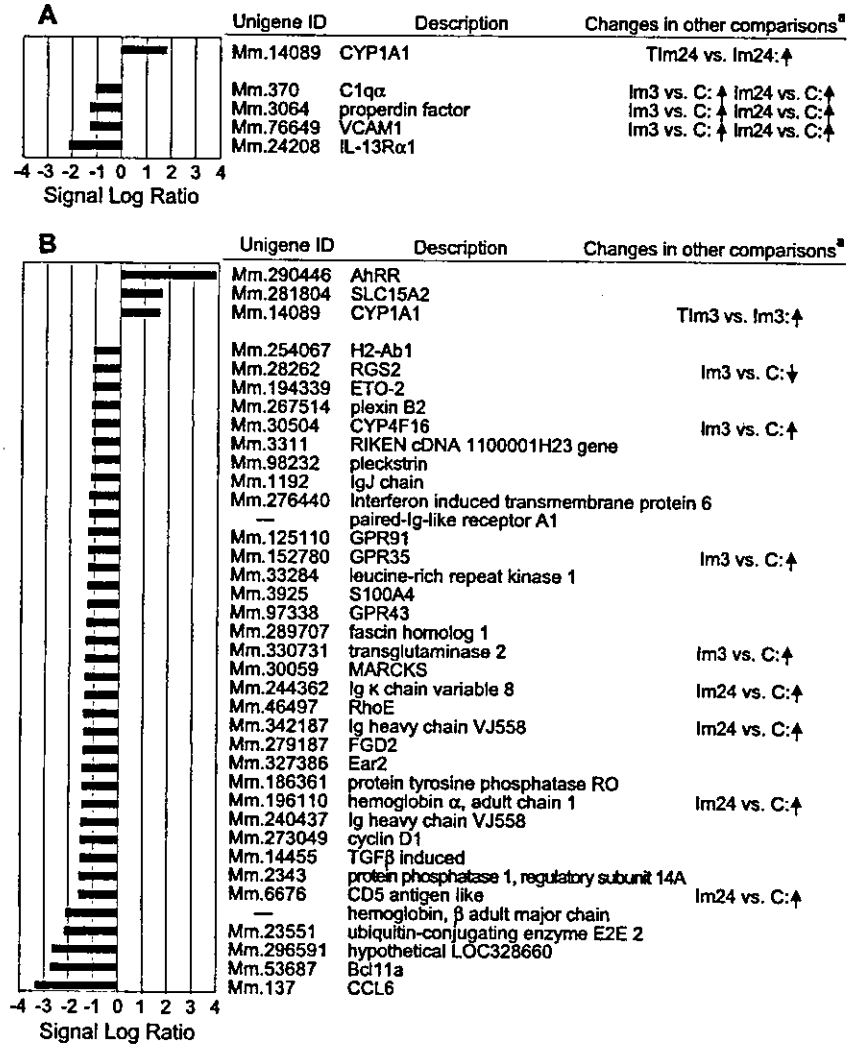


Fig. 6. TCDD-induced changes in gene expression in CD4 T cells 3 and 24 h after immunization. (A) Gene expression in CD4 T cells prepared from the Im3 and TIm3 groups (Fig. 1) was compared with TIm3 as the target and Im3 as the reference (TIm3 vs. Im3). (B) Gene expression of CD4 T cells prepared from the Im24 and TIm24 groups (Fig. 1) were compared setting TIm24 as the target and Im24 as the reference (TIm24 vs. Im24). Two independent replicate experiments were performed, and genes that reproducibly showed two- or more fold change in expression were selected. [a]Changes observed in other comparisons in CD4 T cells.

CYP1A1, one of the most sensitive targets whose expression is induced by binding of AhR-ARNT complex to the XRE sequences in their promoter regions [29] (Fig. 6A). Expression of four genes, complement component C1qα, properdin factor, VCAM1, and IL-13Rα1, was down-regulated by TCDD at 3 h (Fig. 6A). Interestingly, three of the down-regulated genes, C1qα, properdin factor, and VCAM1, were up-regulated by immunization both at 3 and 24 h (Figs. 2 and 3).

At 24 h, 3 genes and 34 genes in CD4 T cells were up-regulated and down-regulated, respectively, by TCDD exposure (Fig. 6B). Up-regulation of CYP1A1 was maintained at 24 h. In addition, expression of aryl hydrocarbon receptor repressor (AhRR), a target of XRE-dependent gene expression by the AhR [30], and a solute carrier family protein SLC15A2 were found to be increased at 24 h. The profile of down-regulated genes, on the other hand, was characterized by many genes involved in GTP-binding protein-linked signaling, such as regulator of G protein signaling 2 (RGS2), pleckstrin, GPR91, 35 and 43, transglutaminase 2, RhoE, and FGD2. The genes involved in immune functions, such as histocompatibility antigen H2-Ab1, CD5 antigen-like, Bcl11a and chemokine (C–C motif) ligand 6 (CCL6); kinases and phosphatases, such as leucine-rich repeat kinase 1, protein tyrosine phosphatase RO, and protein phosphatase 1; and PKC substrates, such as pleckstrin and MARKS, were also identified. Among the 34 genes down-regulated by TCDD at 24 h (Fig. 6), seven were up-regulated at 3 or 24 h after immunization alone (Figs. 2 and 3).

3.4. TCDD-induced changes in expression in immunized B cells

At 3 h after immunization, the level of expression of 15 genes in B cells was increased by TCDD, and expression of two genes was decreased (Fig. 7A). Expression of two of the up-regulated genes, Tiparp [31] and GADD45α [22], plus to CYP1A1, has been reported to be induced by TCDD in an AhR/XRE-dependent manner. Fos expression has also been reported to be induced by TCDD but via estrogen receptor activation by activated AhR not through XRE-binding [32]. Expression of the genes encoding two GTP-binding protein-linked proteins, receptor activity modifying protein 2 and regulator of G-

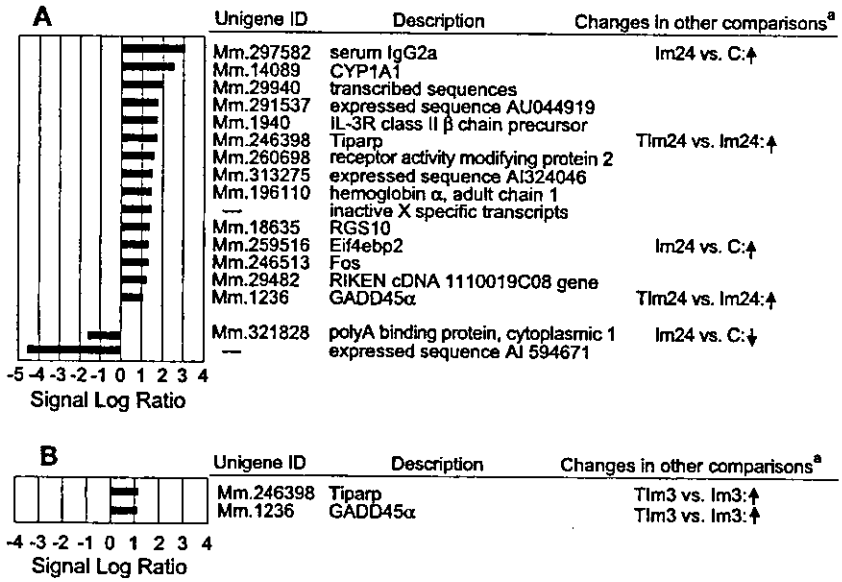


Fig. 7. TCDD-induced changes in gene expression in B cells 3 and 24 h after immunization. (A) Gene expression of B cells prepared from the Im3 and TIm3 groups (Fig. 1) were compared setting TIm3 as the target and Im3 as the reference (TIm3 vs. Im3). (B) Gene expression of B cells prepared from the Im24 and TIm24 groups (Fig. 1) were compared with TIm24 as the target and Im24 as the reference (TIm24 vs. Im24). Two independent replicate experiments were performed, and genes that reproducibly showed two- or more fold change in expression were selected. [a]Changes observed in other comparisons in B cells.

protein signaling 10, was also up-regulated. At 24 h after immunization, GADD45α and Tiparp were persistently up-regulated by TCDD (Fig. 7B).

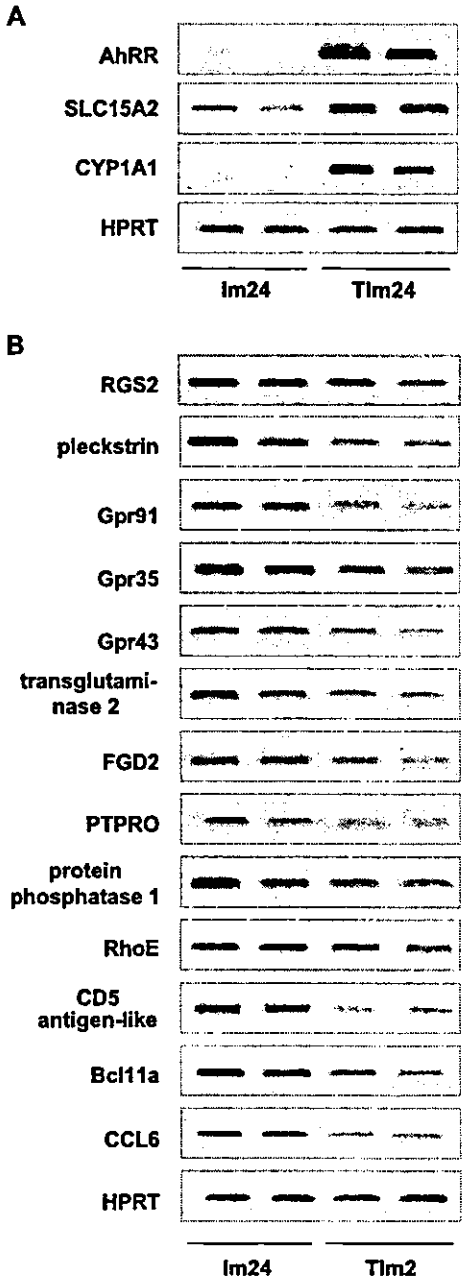


Fig. 8. Confirmation of gene expression changes by RT–PCR. Independently of the microarray analysis, total RNAs were prepared from two CD4 T cell samples, each from the Im24 and TIm24 groups, and after reverse transcription, their expression was measured by PCR.

3.5. Confirmation of the GeneChip results by RT–PCR

To verify the results of the microarray analyses, the levels of expression of TCDD-modulated genes in CD4 T cells identified by comparison between TIm24 and Im24 (Fig. 6) were investigated by RT–PCR. Independently of the microarray analysis, 24 h after immunization, total RNAs were prepared from two CD4 T cell samples, each isolated from corn-oil-treated mice and TCDD-administered mice (Im24 and TIm24). The mRNAs were then reverse-transcribed, and their expression levels were measured by PCR. As shown in Fig. 8A, the RT–PCR analysis showed a marked increase in expression of the three genes detected by GeneChip analysis, and thus corroborated the microarray data. The results of RT–PCR for GTP-binding protein-related genes (RGS2, pleckstrin, GPR91, GPR 35, GPR 43, transglutaminase 2, RhoE, and FGD2), immune function-related genes (CD5 antigen-like, Bcl11a, and CCL6) and phosphatases (protein tyrosine phosphatase RO and protein phosphatase 1) also corroborated their down-regulation.

4. Discussion

TCDD causes suppression of various immune reactions through activation of a ligand-dependent transcription factor, the AhR. We previously reported that TCDD exposure suppresses the antigen-specific antibody production of mice immunized with OVA plus alum as an adjuvant [17,18]. In the present study, we investigated TCDD-dependent changes in gene expression in the CD4 T cells and B cells of OVA-immunized mice to identify genes involved in the induction of antibody suppression by TCDD, focusing our attention on two early times after immunization, 3 and 24 h.

The results showed that OVA-immunization alone up-regulated many genes in the CD4 T cells as early as 3 h. They included genes involved in cytokine signaling pathway, growth regulation, transcription, GTP-binding protein-linked signaling, and cellular adhesion, as described in the results (Fig. 2). At 24 h after immunization, fewer genes were up-regulated by immunization in the CD4 T cells than at 3 h, but the number of down-regulated genes had increased (Fig.

3). The level of expression of 15 genes was persistently up-regulated at 3 and 24 h (Figs. 2 and 3). These findings seem to indicate that T cells are actively stimulated by antigen-presenting cells at these early time points in mice immunized with OVA plus alum as an adjuvant. Immunization also mainly enhanced expression of genes in the B cells at 3 and 24 h, but fewer genes were affected in B cells than in CD4 T cells (Figs. 4 and 5).

In contrast to the finding that immunization up-regulated a variety of genes in the CD4 T cells at 3 and 24 h, TCDD mainly caused down-regulation of genes in the CD4 T cells (Fig. 6). Expression of 4 genes in the CD4 T cells at 3 h and of 34 genes at 24 h was decreased by TCDD exposure. A characteristic feature of the expression profile is down-regulation by TCDD of the immunization-induced genes. Three of the four genes down-regulated by TCDD at 3 h were genes whose expression had been up-regulated by immunization. Likewise, seven genes that were down-regulated by TCDD at 24 h had been up-regulated by immunization. The suppression of immunization-induced gene expression by TCDD may be involved in the immunization-dependent effect of TCDD on splenocytes. Although TCDD does not affect splenocyte number in non-immunized mice [33], it suppresses the splenocyte increase in response to immunization [17].

Another characteristic of the TCDD-induced changes in expression in immunized CD4 T cells was the suppression of numerous genes involved in GTP-binding protein-linked signaling at 24 h after immunization. Among the genes down-regulated by TCDD at 24 h, RGS2 protein [34] belongs to a family of GTP-activating (GAP) factors for Gα termed "regulators of G protein signaling." GPR91 [35], GPR35 [36], and GPR43 [37] are seven-transmembrane G-protein-coupled receptors; FGD2 [38] and transglutaminase 2 [39] are guanine nucleotide exchange factors (GEFs); RhoE [40] belongs to the Rho GTPase family; and pleckstrin [41] is a molecule closely associated with G-protein-linked signaling. Many physiological factors, such as steroids, retinoids, thyroid hormones, and cyclic AMP, have been reported to modulate transcription of elements involved in GTP-binding protein-linked signaling [42]. Although it is unknown how TCDD suppresses the immunization-induced expression of genes observed in this study, some of the physiological changes induced by TCDD may indirectly affect the transcription of the elements in GTP-binding protein-related signaling. Some may also be directly affected by binding of the AhR to the XRE sequences in their enhancer regions. Actually, a search of the NCBI mouse genome database revealed that the RGS2, GPR35, FGD2, and RhoE genes contain the XRE consensus sequences (5′-TNGCGTG-3′ or 5′-CACGCNA-3′) between −1300 and their transcription start sites.

GTP-binding protein-linked signaling is involved in a wide variety of physiological reactions and signal transduction pathways, including activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) pathway [42–44]. Although previous studies have shown that TCDD activates the ERK pathway [45–49], the mechanism has not been clarified. The results of the present study may provide a clue to the linkage between TCDD and ERK pathway activation through changes in GTP-binding protein-linked signaling. The ERK pathway plays a crucial role in T cell activation and its effector function [50,51]. Therefore, provided the TCDD-induced modulation of the GTP-binding protein-linked signaling pathway affects the ERK pathway, it would inhibit normal T cell activation and its effector function, such as Th2-type cytokine production, leading to inhibition of antibody production. We also detected down-regulation of genes involved in immune reactions, kinases, and phosphatases, as described in the results, which would include genes taking a significant role in T activation and function. Further studies to ascertain if the changes of the genes detected in the present study are accompanied by alterations in the protein levels and functions using either in vivo or in vitro models should provide a valuable clue to identify the genes responsible for the immune suppression by TCDD.

On the other hand, fewer genes in B cells were affected by TCDD than in CD4 T cells, and TCDD affected the expression of these genes differently from the genes in CD4 T cells, mainly up-regulating them. The up-regulated genes included CYP1A1, Tiparp [31], and GADD45α [22] whose expression has been reported to be induced by TCDD in an AhR/XRE-dependent manner, and Fos, which has been reported to be up-regulated by TCDD via AhR/ER activation [32], provided evidence of TCDD-induced AhR activation

in B cells. Although we previously reported that expression of a constitutively active mutant of AhR induces GADD45α in Jurkat T cells [22], this gene was up-regulated in B cells rather than in CD4 T cells in the experimental system in the present study. Likewise, expression of XRE-containing genes, such as Tiparp and AhRR, was shown to be increased differently by TCDD in CD4 T cells and B cells. These findings seem to indicate the presence of factors that modulate AhR function in a cell-type-specific manner.

In summary, this study identified genes in CD4 T cells and B cells whose expression level is modulated by TCDD prior to the suppression of antigen-specific antibody production. A remarkable feature of the expression profile was that TCDD suppressed expression of many genes in CD4 T cells whose expression is induced by immunization. Another characteristic was the TCDD-induced down-regulation of numerous genes involved in GTP-binding protein-linked signaling pathway in CD4 T cells. These results provide a clue to new approaches to exploration of the mechanism of TCDD-induced immune suppression.

Acknowledgments

We gratefully acknowledge Drs. Richard E Peterson and Tien-Min Lin (University of Wisconsin) for technical advice on Affymetrix GeneChip analyses. We also thank Dr. Tomohiro Ito (NIES) for his useful discussions, Kazuhiro Shiizaki (NIES) for his technical advice, and Michiyo Matsumoto for her secretarial assistance.

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