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

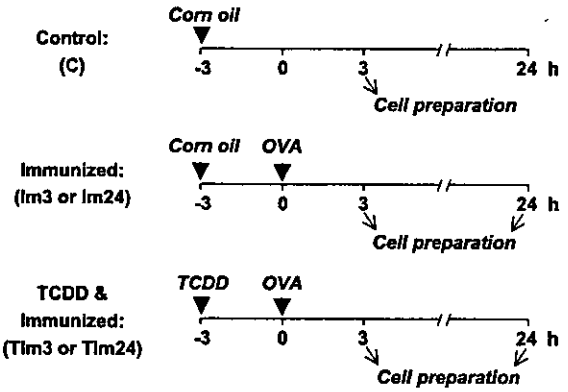


Fig. 1. Schematic diagram of the experimental protocol. Six-week-old mice (3 mice/group) were given corn oil or TCDD orally (20 μg/kg). In the control (C) group, CD4 T cells and B cells were prepared from the spleen 6 h after corn oil administration. In the immunized group, mice were intraperitoneally 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	tcccctectgctgataaaga	27	60	197
HPRT	gctggtgaaaaggacctct	cacaggactagaacacctgc	23	60	249

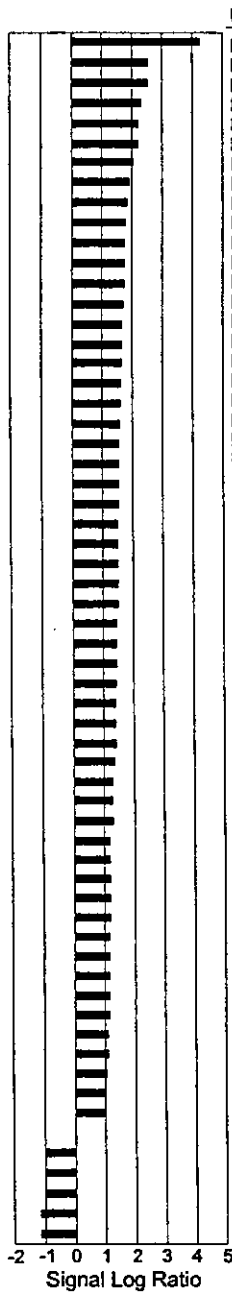
expression by RT–PCR as described previously [22]. Briefly, total RNAs were isolated from cells as described above, and RT–PCR was performed with an RNA LA PCR kit (AMV) ver1.1 (TaKaRa Biomedicals, Tokyo) according to the manufacturer's instructions. Amplification was carried out by heating at 94 °C 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."

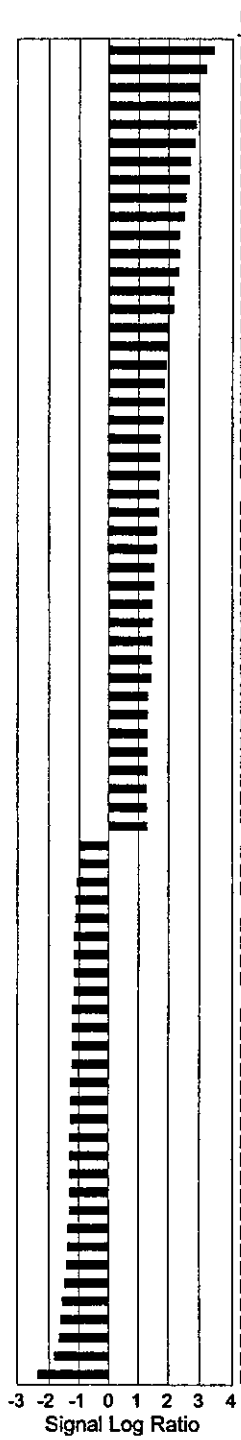
3.1. Changes in expression in CD4 T cells in response to OVA-immunization

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.

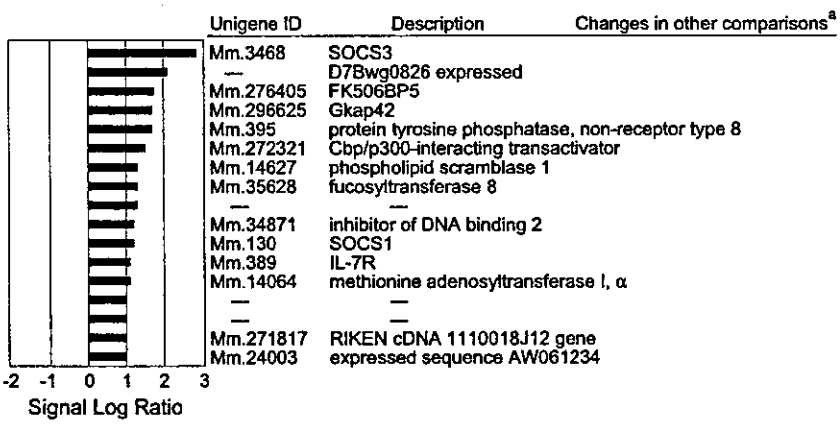


Fig. 4. Differential gene expression induced in B cells at 3 h after immunization. Gene expression of B cells prepared from the C group and Im3 group (Fig. 1) was compared with Im3 as the target and C as the reference (Im3 vs. C). Two independent replicate experiments were performed, and genes that reproducibly 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-

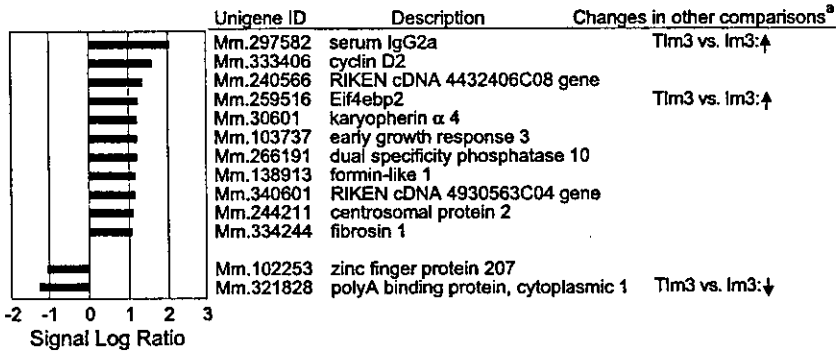


Fig. 5. Differences in gene expression in B cells 24 h after immunization. Gene expression in B cells prepared from the C group and Im24 group (Fig. 1) were compared with Im24 as the target and C as the reference (Im24 vs. C). Two independent replicate experiments were performed, and genes that reproducibly 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

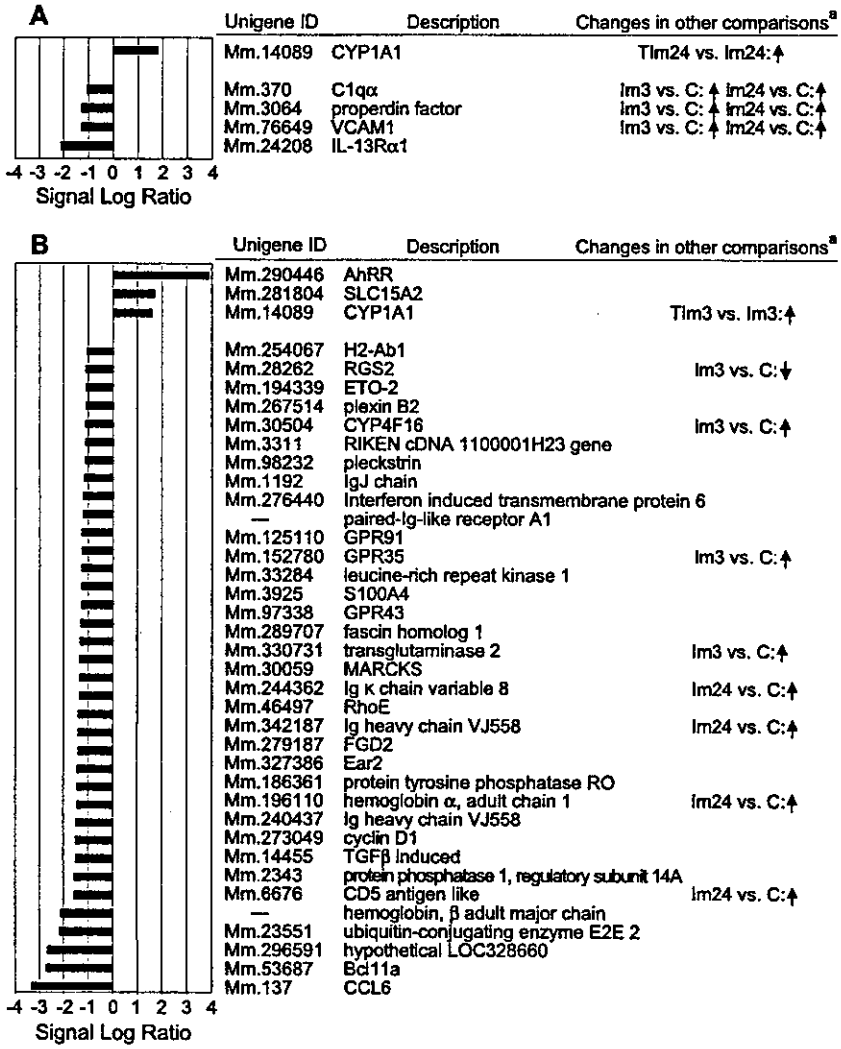


Fig. 6. TCDD-induced changes in gene expression in CD4 T cells 3 and 24 h after immunization. (A) Gene expression in CD4 T cells prepared from the Im3 and TIm3 groups (Fig. 1) was compared with TIm3 as the target and Im3 as the reference (TIm3 vs. Im3). (B) Gene expression of CD4 T cells prepared from 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-

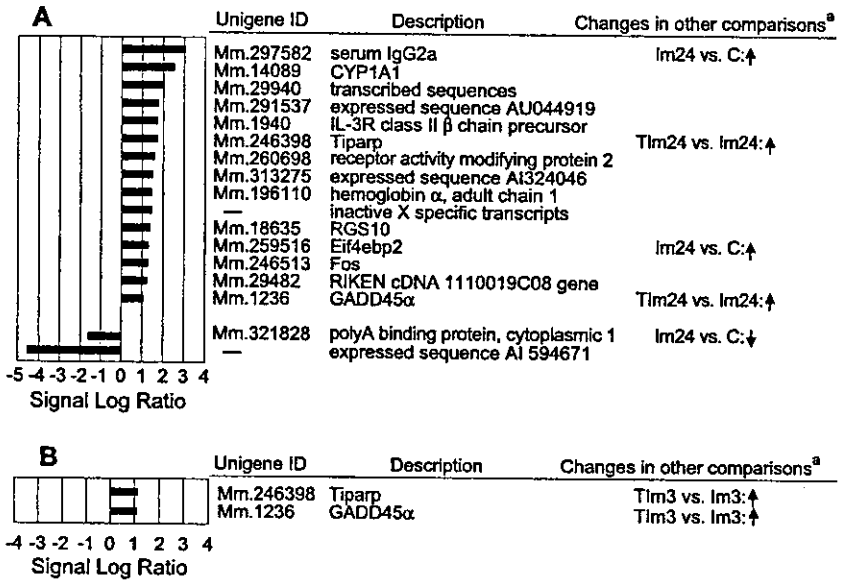


Fig. 7. TCDD-induced changes in gene expression in B cells 3 and 24 h after immunization. (A) Gene expression of B cells prepared from the Im3 and TIm3 groups (Fig. 1) were compared setting TIm3 as the target and Im3 as the reference (TIm3 vs. Im3). (B) Gene expression of B cells prepared from the Im24 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).

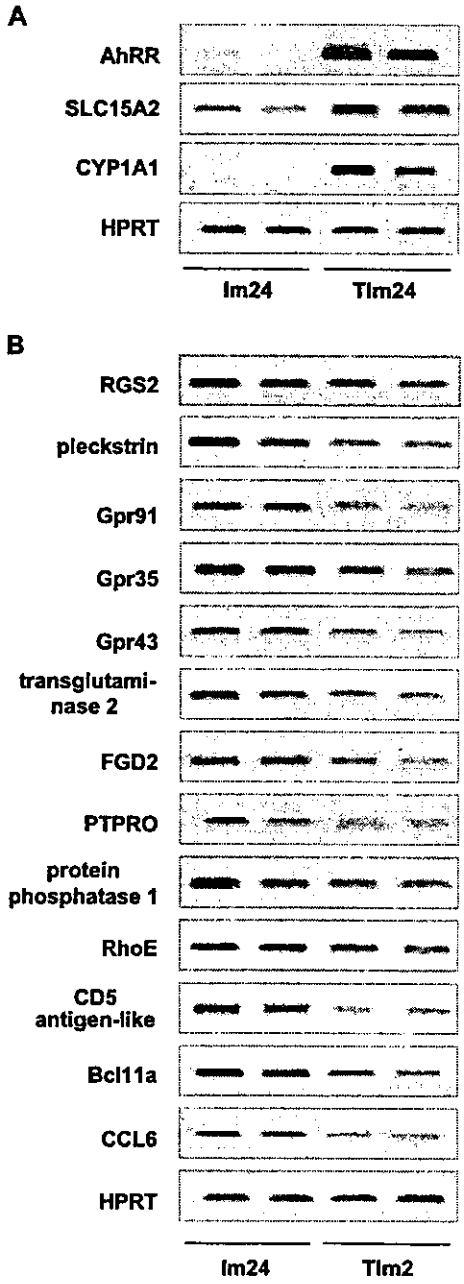


Fig. 8. Confirmation of gene expression changes by RT–PCR. Independently of the microarray analysis, total RNAs were prepared from two CD4 T cell samples, each from the Im24 and TIm24 groups, and after reverse transcription, their expression was measured by PCR.

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.

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Identification of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-inducible and -suppressive Genes in the Rat Placenta: Induction of Interferon-regulated Genes with Possible Inhibitory Roles for Angiogenesis in the Placenta

TETSUYA MIZUTANI, MIKI YOSHINO, TOMOKO SATAKE, MIYUKI NAKAGAWA, RYUTA ISHIMURA*, CHIHARU TOHYAMA*, KOICHI KOKAME**, KENJI KANGAWA** AND KAORU MIYAMOTO

Department of Biochemistry, Faculty of Medical Sciences, University of Fukui, Matsuoka, Fukui 910-1193, Japan; CREST, JST, Kawaguchi 332-0012, Japan

**Environmental Health Sciences Division, and Endocrine Disruptors and Dioxin Research Project, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba 305-8506, Japan; CREST, JST, Kawaguchi 332-0012, Japan*

***National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan*

Abstract. Exposure to a low dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) results in a variety of toxic manifestations, including fetal death. In order to evaluate the effects of low dose TCDD on placental function, pregnant Holtzman rats were given a single oral dose of 1600 ng TCDD/kg body wt or an equivalent volume of vehicle (control) on gestation day 15 (GD15), and changes in the gene expression in the placenta on GD20 were analyzed by two comprehensive methods, representational difference analysis (RDA) and DNA microarray technology. Candidates of TCDD-inducible and -suppressive genes were selected. Quantitative real-time PCR analysis was then performed to verify the induction or suppression levels of the candidate genes. Finally, we identified 81 TCDD-inducible and 21 TCDD-suppressive genes from the placenta of TCDD-treated Holtzman rats on GD20. One of the remarkable profiles of the gene expression was that glucose transporters were strongly up-regulated by the TCDD treatment. Furthermore, many interferon-inducible genes were also up-regulated by the treatment. They included several cytokines such as IP-10 known as a potent angiogenesis inhibitor. In addition, interferon molecules are known to suppress angiogenesis. The above observations suggest that activation of the interferon signaling pathway and the induction of anti-angiogenic factors by TCDD might have a role in causing the inhibition of neovascularization, resulting in the hypoxic state of placenta and increased incidence of fetal death.

Key words: TCDD, Placenta, Gene expression, Real-time PCR, DNA microarray

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is known to be the most toxic congener among the dioxin and related compounds found in the environment. Exposure to TCDD causes a diverse spectrum of toxicities in humans and laboratory animals [1–4]. The fetus is one of the most sensitive targets of TCDD and exhibits a wide range of biological responses at low TCDD levels that have no detectable effects on maternal side (usually the levels were ten to hundred times lower than those of LD50). One of the most severe adverse effects of TCDD is intrauterine fetal death [1, 5–7]. The incidence has been shown in all species studied to date, including the monkey, hamster, rat, and mouse. Although fetal death is an important aspect of TCDD toxicity, its precise mechanism is not well understood. Placenta plays a crucial role in maintaining normal fetal growth such as exchange of oxygen and carbohydrate nutrients. In previous studies Ishimura *et al.*

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Correspondence to: Dr. Kaoru MIYAMOTO, Department of Biochemistry, Faculty of Medical Sciences, University of Fukui, Matsuoka, Fukui 910-1193, Japan

demonstrated that exposure of pregnant rats to 800 or 1600 ng TCDD/kg on gestational day 15 (GD15) resulted in an increased incidence of fetal death on GD20 [8, 9]. In their experimental protocol, the placenta showed several abnormalities that led to increased risk for fetal death. Exposure to TCDD altered the glucose kinetics in placenta [8] and caused reduced blood flow and placental hypoxia [9], leading them to hypothesize that the increased incidence of the fetal death may be due to reduced blood flow into the placenta [8, 9]. In order to clarify what kind of genes are involved in these placental abnormalities or what other aspects of TCDD toxicities exist in the placenta, we conducted a comprehensive analysis of gene expression in placenta of pregnant Holtzman rats treated with TCDD using representational difference analysis (RDA) and DNA microarray technology. RDA is a subtraction cloning method developed by Pastorian *et al.* [10]. The RDA and the DNA microarray are very powerful and comprehensive methods to identify inducible or suppressive genes by given hormonal or pharmacological treatments. Previously we have reported many gonadotropin-inducible genes in the rat ovary by using those methods [11–13]. In this study, many candidates for placental TCDD-inducible or -suppressive genes were selected by those methods, and the induction or suppression was verified by quantitative real-time PCR.

Profiling analysis of the verified genes revealed that, in addition to the genes involved in the glucose uptake, those involved in the interferon signaling pathway were strongly induced by the TCDD treatment. Based on the results obtained, the molecular mechanisms of placental disorders by low dose TCDD will be discussed.

Materials and Methods

Reagents

2,3,7,8-TCDD was obtained from Cambridge Isotope Laboratory (Andover, MA). Rat cDNA glass array (Gene Chip Rat Expression Array 230A) was from Affymetrix, Inc., Santa Clara, CA.

Animals

Holtzman rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The animals were maintained in a controlled environment with 12/12 h light/dark cycles, and given free access to laboratory rat chow and water. Female rats were allowed to mate on proestrus overnight, and, if vaginal plugs were observed in the morning, the day was designated GD0. Six pregnant rats were treated with TCDD, and a total of 10 dead fetuses was observed among 83 fetuses. The pregnant rats were housed individually until exercised. All animal experiments were performed according to the guidelines for animal welfare of the National Institute for Environmental Studies [8, 9].

TCDD treatment

The pregnant animals were exposed to TCDD as described previously [8, 9]. TCDD was dissolved in nonane at a concentration of 20 μg/ml, and the solution was diluted with corn oil. On GD15 the pregnant rats were given a single oral dose of 1600 ng TCDD/kg body wt or an equivalent volume of mixture of nonane and corn oil solution (control). Placentas were collected from the TCDD treated or control rats on GD20. The placental samples were immediately frozen in liquid nitrogen and maintained at −80°C until used.

Representational difference analysis

For isolating TCDD-inducible and -suppressive genes from the placenta, representational difference analysis (RDA) was performed according to the procedure reported by Pastorian *et al.* [10]. Briefly, total RNA was extracted from each placenta. Each five placental RNA samples were mixed and used for further analysis. After Oligo-dT latex beads treatment, mRNA preparations were then converted to respective double-stranded cDNAs. Tester TCDD-treated placental cDNAs and driver control placental cDNAs were digested with a restriction enzyme DpnII. The digested tester cDNA fragments were ligated with first-round adaptor oligonucleotide molecules, and then mixed with the excess amount of the enzyme digested driver cDNAs. The mixture was denatured and then incubated at 67°C for 16 hours for hybridization. After the hybridization, only the tester specific cDNA fragments were amplified by PCR reaction using primers complementary to the adaptor sequence. For the second cycle RDA, the resulting PCR products, in which the tester specific cDNA fragments were enriched, were digested again with DpnII, and then followed by the second adaptor

ligation and hybridization in order to further enrich tester specific cDNA fragments. The PCR products after the second RDA reaction were ligated in to pGEM T-vector, and individual clones were analyzed to identify TCDD-sensitive genes.

DNA microarray

The microarray method was carried out according to the manufacturer's instruction.

Briefly, total RNA was extracted from the TCDD-treated and control placentas described above. Double stranded cDNA libraries were constructed from the mRNA of TCDD-treated and the control placentas using an oligo-dT primer with a T7-promoter sequence at the 5'-end. Biotin-labeled complementary RNA was *in vitro* transcribed by T7 polymerase using the cDNA libraries as template. The biotin-labeled RNA was fragmented and hybridized to the Rat cDNA glass array for 16 hr at 45°C and then washed and stained using the GeneChip Fluidics. The array was scanned by a GeneArray scanner, and hybridization patterns were detected as fluorescent light from reporter groups that have been incorporated into the target genes. The average difference measurements computed in the Affymetrix Microarray Analysis Suite 4.0 serve as a relative indicator of the level of expression.

Quantitative Real-Time PCR

Messenger RNA was extracted using an RNA extraction solution (Trizol) and oligo-dT latex beads as described previously [11–13]. Five micrograms of mRNA preparations were reverse-transcribed, and then converted to double stranded cDNA molecules. Complementary DNA was quantified by UV absorption measurement, and 1 ng was subjected to the PCR reaction as template. As an internal standard, TATA binding protein (TBP) was used instead of GAPDH, since GAPDH gene expression was affected by the TCDD treatment (data not shown). PCR reaction involved template cDNA samples, Advantage Taq Plus DNA polymerase (Clontech), dNTP, and Syber Green. Serial dilutions of the templates were used to create a concentration curve, and relative expression levels were calculated for each sample [14]. Abundance of each gene was referred to as a Ct (cycle threshold) value in this system.

Results

Exposure of pregnant Holtzman rats to 1600 ng TCDD/kg on GD15 resulted in an increased incidence of fetal death on GD20 [8, 9]. In these studies, each placenta from the TCDD-treated and control rats was prepared by the same exposure protocol as previous studies [8, 9], and gene expression in the placenta was analyzed by RDA and DNA microarray methods. Generally the RDA method is more sensitive than the DNA microarray, while the latter covers more comprehensive genes than the RDA method. A total of 2536 clones were picked-up and characterized from the RDA-subtracted cDNA libraries as candidate genes. In addition, the same tissues were used for the DNA-microarray, and among 13000 genes spotted on the array, 43 TCDD-inducible and 18 TCDD-suppressive candidate genes (cut-off values of 2.0 as inducible and 0.5 as suppressive genes) were also picked-up. All of these candidate genes picked-up by both methods were verified by using real-time PCR analysis, and genes that showed expression ratios (TCDD-treated/control) of more than 2.0 or less than 0.5 were finally identified as TCDD-sensitive. TCDD-inducible and -suppressive genes in the rat placenta identified by RDA and DNA microarray methods were summarized in Table 1 and Table 2, respectively.

TCDD-inducible genes in the placenta

As listed in Table 1, 81 genes were identified as TCDD-inducible genes in the placenta, 22 genes of which were from the results of DNA microarray analysis. Eleven genes were reported only on EST databases, and one gene showed no significant similarity to any gene on DNA databases. Other genes were all annotated as shown in Table 1 including those homologues of human or mouse. They were categorized into several groups. Enzyme genes include CYP1A1 and CYP1B1 that are known as the typical TCDD target genes [15, 16]. Inducible genes in placenta include some of the major blood proteins, such as prealbumin, apolipoproteins, transferrin, retinol-binding protein, prothrombin, and fibrinogens. This suggests that the placental production of the blood proteins was stimulated by the TCDD treatment. In addition, two placental specific genes, alpha-fetoprotein and pregnancy-specific glycoprotein (rnCGM3), were also induced by the TCDD treatment.

Table 1. TCDD-inducible genes in the rat placenta

Molecular function	Description	Array ratio	Real Time PCR ratio	Ct	Accession
Enzyme	(LMW) K-kininogen	3.32	8.14	30.06	M11884
	(LMW) T-kininogen I	ND	4.43	30.53	M11883
	Aldolase B	ND	2.72	34.15	M10149
	Alpha-1-protease inhibitor	ND	3.37	32.17	D00675
	Alpha-2 antiplasmin	ND	3.38	30.03	AY216659
	Alpha-fibrinogen	5.77	5.05	33.13	M35601
	Brain 4.1(L)	ND	2.24	29.48	AB019257
	CYP1A1	ND	425.81	26.13	K02246
	CYP1B1	ND	3.90	34.60	X83867
	Cathepsin B	ND	2.11	19.33	X82396
	Creatine kinase-B	ND	2.62	29.12	M57664
	Cytosolic NADP-dependent isocitrate dehydrogenase	ND	3.77	24.51	L35317
	Fibrinogen B-beta-chain	5.87	7.95	30.89	M27220
	Glutathione S-transferase Ya subunit	ND	4.27	31.73	M26874
	NADH dehydrogenase (ubiquinone) Fe-S protein 7	ND	2.99	26.11	BC013503
	NC1 protein (nc1 gene)	ND	2.25	28.63	AJ250730
	Peroxisomal enoyl hydratase-like protein (PXEL)	ND	2.01	22.38	U08976
	Protein C	3.91	4.10	29.36	X64336
	Prothrombin precursor (F2 gene)	4.38	5.92	24.44	X52835
	RASP1	ND	2.52	31.93	U55765
	Stearoyl-CoA desaturase 2	ND	2.31	29.98	AB032243
	Tissue factor protein	1.93	2.29	25.09	U07619
Transcription Factor	Zinc finger homeodomain enhancer-binding protein-1 (Zfhep-1)	ND	2.36	32.92	U51583
Immunity Protein	Da1-24	3.17	2.64	—	AY325253
	Interleukin-12 p40 precursor	ND	1.96	35.40	AF133197
	MHC class I antigen (RT1-E gene)	ND	2.42	—	AJ306619
	MHC class I protein	ND	6.46	—	L26224
	Pregnancy-specific glycoprotein (rnCGM3)	ND	2.17	—	U09815
Signal Transducer	Beta ig-h3	ND	2.73	31.93	AF305713
	CXC chemokine LIX	ND	2.60	31.90	U90448
	Inhibin alpha-subunit	ND	1.98	27.41	M36453
	Interferon beta	ND	5.07	36.92	D87919
	Interferon inducible protein 10 (IP-10)	5.03	9.48	25.38	U22520
	Macrophage inflammatory protein-2	ND	6.29	32.48	AB060092
	Monokine induced by interferon gamma (Mig)	ND	3.28	33.97	AF537208
	Proliferin-related protein	1.94	1.82	24.23	AF139809
Transporter	Alpha-fetoprotein (AFP)	ND	5.47	27.57	X02361
	ApolipoproteinA-I	ND	6.26	26.66	M00001
	Apolipoprotein A-IV	5.07	4.24	21.71	M00002
	Apolipoprotein B	3.36	7.07	28.26	M27440
	Beta-2 glycoprotein I	5.10	6.28	30.95	X15551
	Beta-globin	ND	3.71	13.83	X16417
	GLUT2	ND	7.39	31.47	J03145
	GLUT4	ND	2.84	31.49	D28561
	Retinol-binding protein (RBP)	2.75	5.39	32.55	M10934
	Transferrin	5.89	4.12	22.82	X77158
	Transferrin-like	ND	4.08	26.01	AF476964
Structural Protein and Other Groups	Angiopoietin-related protein 3	ND	8.90	30.40	*CB581433
	Claudin 2	2.29	3.27	26.96	*BM392116
	Collagen alpha 1 type XI	ND	2.60	33.72	AJ005396
	Collagen type XXVII proalpha 1 chain (col27a1)	ND	1.98	28.20	AY232999

Table 1. (continued)

Molecular function	Description	Array ratio	Real Time PCR ratio	Ct	Accession
	Decorin	ND	2.07	24.20	X59859
	Ficolin-B	ND	2.33	29.23	AB036792
	Glucocorticoid-attenuated response gene 16	ND	92.29	27.14	AJ276893
	Glucocorticoid-attenuated response gene 39	ND	5.54	28.07	*CB719539
	Hypothetical protein RMT-7	ND	2.09	23.13	AF465614
	Interferon-inducible protein homologue	ND	6.15	24.96	*CB610262
	Interferon, alpha-inducible protein (G1p2)	ND	2.76	23.17	*CB790136
	Matrix metalloproteinase inhibitor (TIMP-1)	2.15	2.11	25.20	L31883
	Mx1	4.51	6.41	26.00	X52711
	Mx2	3.80	7.27	25.71	X52712
	Mx3	ND	3.06	26.70	X52713
	Prealbumin	2.04	4.76	27.03	K03252
	Pro alpha 1 collagen type III	ND	2.56	26.78	X70369
	Proteasome subunit R-RING12	ND	2.92	27.78	D10757
	Ribosomal protein L5	ND	2.34	20.59	X06148
	Similar to PC-LKC gene product	1.92	2.06	27.53	*BI291884
	Similar to vinculin	ND	1.99	27.73	*CB757866
	Spp-24 precursor	3.03	6.13	25.56	U19485
	TORID	ND	2.53	25.27	AF370882
	Thrombospondin	ND	2.30	28.72	*AB113080
	Type VI collagen alpha3 subunit	1.92	1.94	25.49	*AI176126
Function Unknown	AB113071	ND	2.37	31.85	*AB113071
	Clone nrhy5-00111-g2	ND	3.17	27.96	*CB546450
	DRNBNB02	ND	2.06	24.98	*BG671212
	EST196998	1.98	2.16	30.78	*AA893195
	LRRGT00077	ND	2.29	30.17	*AY387063
	UI-R-BJ0p-afw-e-10-0-UI.r1	ND	2.14	27.92	*BF566943
	UI-R-C4-akz-c-07-0-UI.s1	3.22	2.99	24.70	*AW531805
	UI-R-E0-bq-f-06-0-UI.r1	ND	5.64	30.34	*BF550478
	Unknown	ND	2.05	26.30	ND

ND: Not determined, Ct: cycle threshold, *: Rat EST database

Two characteristic profiles of the induction were (1) glucose transporters and (2) interferon-related genes, details of which will be described elsewhere.

TCDD-suppressive genes in the placenta

As listed in Table 2, 21 genes were identified as TCDD-suppressive genes in the placenta, 10 genes of which were from the results of the DNA microarray analysis. Six genes were reported only on EST databases, and three genes showed no significant similarity to any gene on existing DNA databases. Other genes were all annotated as shown in Table 2 including those homologues of human or mouse.

Induction of glucose transporter family genes

As shown in Fig. 1, expressions of four major glucose transporter genes were examined. Quantitative real-time PCR revealed that GLUT2 and GLUT4 were strongly induced by the TCDD treatment in the placenta. Gene expression of GLUT3 was also marginally increased by the treatment (1.58-fold), but that of GLUT1 was less affected (1.24-fold). In the control placenta, GLUT3 was abundantly expressed (Ct26.57), while expression levels of the other three transporter genes were relatively low (Ct32.73 for GLUT1, Ct31.47 for GLUT2, and Ct31.49 for GLUT4). Therefore, in addition to the remarkable induction of GLUT2 and GLUT4 genes, a small but significant increase in GLUT3 gene expression may have a major physiolog-

Table 2. TCDD-suppressive genes in the rat placenta

Molecular function	Description	Array ratio	Real Time PCR ratio	Ct	Accession
Enzyme	Urokinase-type plasminogen activator	0.48	0.45	27.99	X63434
	LASP-1	ND	0.19	25.46	AF242187
Signal Transducer	Hepatic product spot 14	0.43	0.33	31.78	K01934
	Prepronociceptin	0.59	0.63	25.84	X97375
	Prolactin-like protein H	0.50	0.41	27.74	AB009889
Transcription Factor	Transcription factor GATA-1	0.55	0.47	31.50	D13518
	Zinc finger protein 52	ND	0.16	34.17	*CK470357
Transporter	mBLVR	ND	0.54	25.50	*CB557112
Structural Protein and Other Groups	Talin	ND	0.35	26.13	*CK366133
Function Unknown	Ab2-225	0.56	0.60	27.78	AY325197
	BB857172	ND	0.16	29.50	*BB857172
	Clone mrpe4-00034-a12	ND	0.16	32.27	*CB760299
	Eker rat-associated intracisternal-A	ND	0.43	32.27	U23776
	EST224029	0.59	0.62	24.96	*AI180286
	FAD104	ND	0.49	25.21	*CB702955
	Nuclear-localized inactive X-specific transcript (Xist)	0.27	0.33	27.18	*AI228978
	UI-R-BJ1-auz-g-11-0-UI.s1	ND	0.31	25.56	*BE111117
	UI-R-CX0s-cct-f-09-0-UI.s1	0.43	0.54	27.16	*BI284907
	Unknown mRNA	0.43	0.48	26.53	AF152002
	Unknown	ND	0.45	31.31	ND
	Unknown	ND	0.51	24.21	ND

ND: Not determined, Ct: Cycle threshold, *: Rat EST database

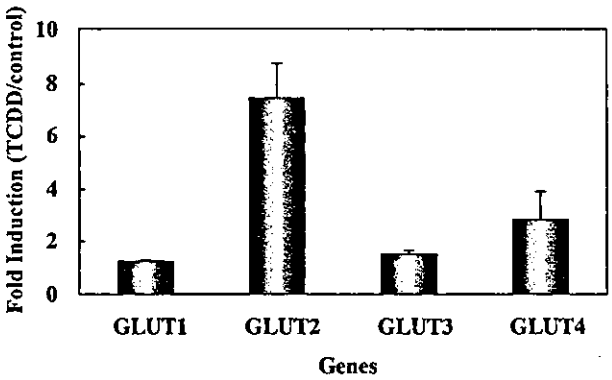

Fig. 1. Induction of glucose transporter genes in the rat placenta by TCDD.
Expression levels of major 4 glucose transporter genes were determined by quantitative real-time PCR, and changes in the expression levels were compared. Values were expressed as fold induction (TCDD/control). Serial dilutions of five points for each sample were used to make each dilution curve. TATA binding protein (TBP) was used as an internal standard to ensure equal loading of template cDNA molecules.

ical significance of the glucose-uptake in the TCDD-treated placenta.

Induction of interferon-related genes

One of the most remarkable findings of the pesent experiment was that many interferon-regulated genes were induced in the placenta of TCDD-treated animals. As shown in Fig. 2, 15 out of 18 interferon-inducible genes examined were strongly up-regulated by the TCDD treatment. Among them were interferon-inducible cytokines, such as IP-10 [17, 18], macrophage inflammatory protein-2, monokine induced by gamma interferon (Mig) [19] and CXC chemokine LIX [20]. Of these, IP-10 was reported to be involved in the interferon-mediated inhibition of angiogenesis [17, 18]. Since many interferon-inducible genes were up-regulated by the TCDD-treatment, we examined the levels of interferon family genes. At the transcription levels, the interferon beta gene was up-regulated (5.07-fold induction) in the TCDD-treated placenta (Table. 1), but we were not able to detect interferon-alpha and -gamma genes in our system (data not shown).

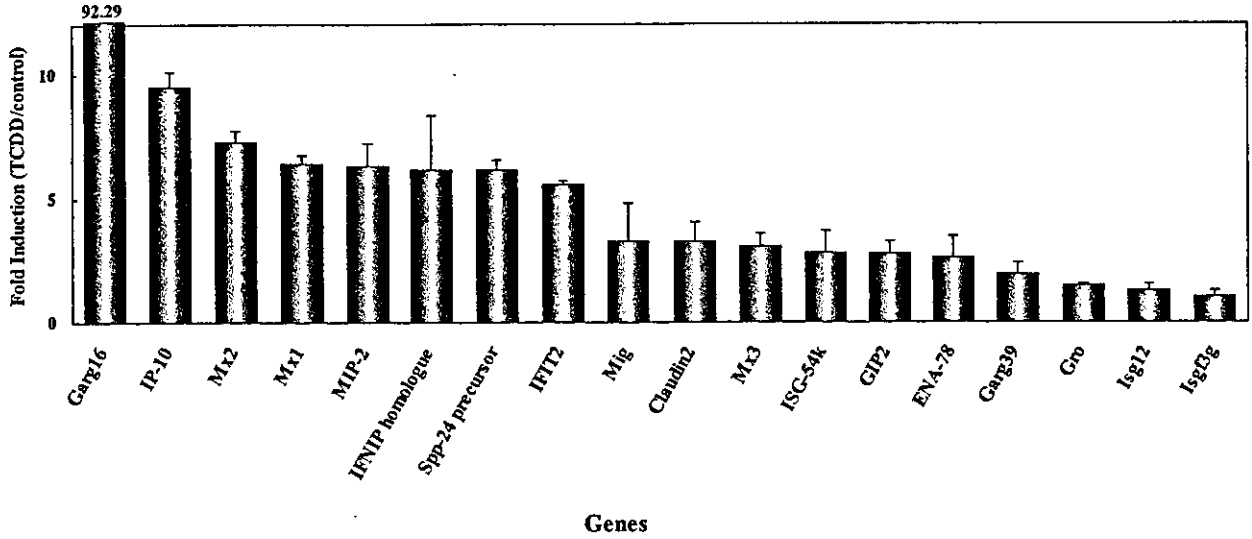

Fig. 2. Induction of interferon regulated genes in the rat placenta by TCDD.
Expression levels of 18 interferon regulated genes were determined by quantitative real-time PCR, and changes in the expression levels were compared. Values were expressed as fold induction (TCDD/control). Serial dilutions of five points for each sample were used to make each dilution curve. TATA binding protein (TBP) was used as an internal standard to ensure equal loading of template cDNA molecules.

Discussion

Previous studies have shown that a very low dose of TCDD results in the increased incidence of fetal death [8, 9]. Histological and protein profiling analysis revealed that the fetal death after the TCDD treatment may be due to the placental hypoxia [9]. In order to evaluate further the mechanisms of the induction of such the adverse effect, comprehensive analysis was done to examine changes in the placental gene expression. As clearly shown in Fig. 1, glucose transporter genes were all induced after the TCDD treatment. Ishimura *et al.* previously reported the histopathological changes in glycogen cells and the elevated levels of glucose content and GLUT3 mRNA expression in the placenta of the TCDD-exposed rats in comparison to those of the control rats [9]. In addition to GLUT3 mRNA, we showed here that GLUT2 and GLUT4 mRNAs were strongly induced by the TCDD-treatment, and that GLUT1 mRNA was less affected by the treatment, indicating that most of major glucose transporter genes were up-regulated by TCDD, which may lead to glycogen accumulation in the placenta. In our quantitative real-time PCR system, values of cycle threshold (Ct) represent expression levels of genes. The Ct value of GLUT3 mRNA was much lower than those of other GLUT family genes, suggesting that the major glucose transporter working in the placenta may be GLUT3, though strong induction of GLUT2 and GLUT4 genes may also play significant roles in the glucose accumulation in the TCDD-treated placenta. As far as we know, this is the first report describing that GLUT2 and GLUT4 genes were actually expressed in the placenta under specific conditions such as the TCDD treatment. It is likely that a tissue-specific responsive mechanism of TCDD exists for the induction of glucose transporters in the placenta, since those transporter genes were not up-regulated in other tissues such as the ovary from the same TCDD-treated animals (data not shown). Histological studies by Ishimura *et al.* showed that blood vessel formation in the placenta was severely impaired by the TCDD treatment (unpublished data), indicating that the angiogenesis was inhibited in the placentas of TCDD-treated animals on GD20. Therefore, the TCDD treatment may cause hypoxic conditions in placenta. It was reported that the expression of GLUT-1 and GLUT-3 mRNAs was up-regulated under hypoxic conditions [21].

Most striking observation of this experiment was that many interferon-inducible genes were up-regulated in the placenta of TCDD-treated animals. As shown in Fig. 2, 15 out of 18 interferon-inducible genes examined were induced by the TCDD treatment, which strongly suggests that interferon signaling pathway

[22] was activated by the TCDD treatment. We also examined the expression levels of interferon-alpha, -beta, and -gamma by the real-time PCR analysis. Only interferon-beta gene expression was detected, and the level was strongly up-regulated by the TCDD treatment. Although the data suggest that interferon-beta is the key factor, we must take into consideration of the fact that the interferon signaling pathway is activated by many other signaling molecules [23]. The typical interferon signaling pathway is activated through the JAK-STAT system [22]. It is well known that pituitary hormone GH and prolactin also activate the JAK-STAT system [23], and that several prolactin-like molecules including placental lactogen are abundantly produced in the placenta [24]. We can not exclude the possibility that, in addition to interferon-beta, the prolactin-like molecules produced in the placenta may also play significant roles in the induction of interferon-regulated genes.

It is noteworthy that interferon is known to be involved in the regulation of angiogenesis. Interferon-alpha and -gamma were reported to inhibit endothelial cell proliferation [25]. IP-10, one of the interferon-inducible genes, was also strongly induced in the TCDD-treated rat placenta. IP-10 is a member of the alpha-chemokine family, inhibits bone marrow colony formation, has anti-tumor activity *in vivo*, is chemoattractant for monocytes and T cells, and promotes T cell adhesion to endothelial cells. In addition to the above functions, IP-10 has been reported to be a potent inhibitor of angiogenesis *in vivo* [17, 18]. Furthermore, thrombospondin [26] and tissue inhibitor of metalloproteinase 1 (TIMP-1) [27] were also induced by the TCDD treatment, both of which are known to inhibit endothelial cell proliferation. Considering the above observations, the neovascularization in the placenta on GD20 may be impaired by the TCDD treatment through the induction of anti-angiogenic factors.

It is reasonable to speculate that the hypoxic state in the placentas of TCDD-treated rats may be due to the impairment of angiogenesis in the placenta, which may be caused by the activation of the interferon signaling pathway including the production of angiogenesis-inhibitory cytokine IP-10 as well as the production of anti-angiogenic factors such as thrombospondin and TIMP-1.

In conclusion, we identified 81 dioxin-inducible genes and 21 dioxin-suppressive genes from the placenta of TCDD-treated Holtzman rats on GD20. The present study revealed that glucose transporters were strongly up-regulated by the TCDD treatment.

In addition, many interferon-inducible genes were also up-regulated by the TCDD treatment, including IP-10, a potent angiogenesis-inhibitory cytokine. Activation of the interferon signaling pathway and the induction of anti-angiogenic factors may result in the hypoxic state of placenta, which may lead to the increased incidence of fetal death.

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