

2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin Impairs Stable Establishment of Oral Tolerance in Mice

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The toxic environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent immunomodulatory chemical. TCDD activates the aryl hydrocarbon receptor (AhR) and suppresses peripheral humoral and cellular adaptive immune responses. Though the major route of uptake is via food, little is known until now on the immunotoxic effects of TCDD on the gut-associated lymphoid tissue. We show here that AhR is strongly expressed along the small intestine, especially in intestinal epithelial cells (IEC). The AhR marker gene *cyp1a1* is induced in IEC by oral TCDD exposure. We asked how TCDD affects oral tolerance, a unique function of mucosal immunity. C57BL/6 mice were injected with 10 µg/kg body weight TCDD and fed with ovalbumin (OVA) in a high-dose tolerization protocol. Mice were immunized and boosted with OVA on days 12, 23, and 55 after tolerization. Five of 14, 6 of 15, and 13 of 14 TCDD-treated mice generated OVA-specific immunoglobulin (Ig)G1 antibodies after the first, second, and third immunization with OVA, respectively. Only one mouse harbored anti-OVA IgG1 antibodies in the control group even after the third immunization with OVA. OVA-specific IgA in fecal samples of tolerized and TCDD-exposed mice could be detected at the levels of nontolerized mice, whereas completely absent in tolerant control mice. Correlated to this, we found in TCDD-treated mice an increase in interleukin-6 producing CD103⁺ dendritic cells (DC) present in the gut-draining mesenteric lymph nodes (MLN) and a small increase in the frequency of Th17 cells. Neither the frequencies nor the absolute numbers of immune cells in the lamina propria (LP) or in intraepithelial lymphocytes were changed by TCDD treatment. Our data not only have implications for food allergies in settings of environmental exposure but also raise concerns regarding the harmlessness of overdosing potential AhR agonist in food, which needs to be studied further.

Key Words: mucosal immunity; Th17; aryl hydrocarbon receptor; tolerance; dendritic cells.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other polyhalogenated hydrocarbons, including biphenyls and furans, are environmental pollutants with far-reaching toxic potential in humans and animals. They mediate toxicity via binding to and

activating the aryl hydrocarbon receptor (AhR), a transcription factor and chemical sensor present in many (but not all) cell types. Classically, it mediates gene transcription in complex with aryl hydrocarbon receptor nuclear translocator (ARNT), another per-arnt-sim basic helix-loop-helix protein, and targets promoter elements called “dioxin-responsive element” (Beischlag *et al.*, 2008). Recently, alternative signaling of AhR via association with RelA or RelB was described, resulting in transcription of nuclear factor κB-responsive genes (Tian, 2009).

Immunotoxicological studies in mice exposed to TCDD showed drastic changes in thymocyte lineage decisions and impaired functions of many immune cells, including changes in cytokine profiles (Kerkvliet, 2009; Lai *et al.*, 1997; Laiosa *et al.*, 2003). The correlated systemic effects are strong immunosuppression of the humoral, cellular, and innate immune responses (Kerkvliet, 2002, 2009; Majora *et al.*, 2005; Schecter *et al.*, 2006). A definite role for AhR in immunity emerged from studies on Th17 cells and dendritic cells (DC) (reviewed in Esser *et al.*, 2009), two cell types with high expression of AhR (Jux *et al.*, 2009; Platzer *et al.*, 2009; Veldhoen *et al.*, 2008).

In humans, approximately 90% of environmental exposure to TCDD is via food (Schecter *et al.*, 2006; van Leeuwen *et al.*, 2000). Moreover, abundant constituents of food, such as plant flavonoids and indoles, or bacterial tryptophan products are agonists of the AhR and may activate this pathway in the gut (Nguyen and Bradfield, 2008). The gut is a highly active immune site in its own right, with about one third of all T cells of the body found intraepithelially and in the gut-associated lymphoid tissue (GALT), i.e., lamina propria (LP), Peyer's patches (PP), and mesenteric lymph nodes (MLN). GALT differs from the peripheral immune system because it has to balance responsiveness between pathogenic and harmless antigens, including food constituents and the symbiotic gut flora. The gut is lined with a single layer of epithelia cells, connected by tight junctions, and interspersed with goblet and paneth cells, which provide mucus and antimicrobial peptides, respectively (Artis, 2008). Interspersed in the epithelium are intraepithelial lymphocytes (IEL), mostly CD8⁺ cells and, in particular, CD8αα⁺ T cell receptor

(TCR) $\gamma\delta^+$ T cells. The latter appear to strengthen the epithelial barrier function, e.g., by providing the lymphokine keratinocyte growth factor (Chen *et al.*, 2002). Immune cells in the LP include CD4⁺ T cells, plasma cells, DC, macrophages, natural killer cells, and mast cells.

It has been known for decades that feeding protein antigens prevents a subsequent immune response against this antigen, a phenomenon called “oral tolerance.” The dose of antigen might be decisive regarding underlying mechanism. Although it is a matter of continuing debate, current evidence suggests that low doses preferentially induce regulatory T cells and immunosuppressive cytokines interleukin (IL)-4, IL-10, and transforming growth factor β (TGF β), whereas high antigen doses lead to anergy, deletion, or increased susceptibility to apoptosis of antigen-specific T cells. The two forms are most likely not mutually exclusive (Faria and Weiner, 2005; Friedman and Weiner, 1994). Oral tolerance is maintained by specialized CD103⁺ DCs, which sample the gut lumen, migrate to the MLNs, and present antigen (Jaensson *et al.*, 2008; Worbs *et al.*, 2006). Although the role and effects of AhR and TCDD on the function of peripheral DCs and differentiation of T cells have received considerable interest (Esser *et al.*, 2009), surprisingly little is known on the effects of TCDD on cells of GALT, with only one study indicating an influence on oral tolerance (Kinoshita *et al.*, 2006). These authors reported that a single oral administration of low-dose TCDD led to systemic sensitization to oral antigens and decreased immunoglobulin (Ig)A secretion in the gut by impairing B-cell function. We here extend these studies and asked whether and how TCDD exposure affects the homeostasis of gut immune cells and induction and maintenance of oral tolerance against the harmless food antigen ovalbumin (OVA) in a high-dose oral tolerance model.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice aged 8–10 weeks at the onset of experiments were from Janvier (Le Genest-Saint-Isle, France) and bred under specific pathogen-free conditions, receiving standard chow and water *ad libitum*. The experiments have been approved by and done in accordance with relevant German animal welfare laws.

Chemicals and reagents. TCDD (Cambridge Isotope Laboratories; dissolved in dimethylsulfoxide [DMSO]) was diluted in olive oil and applied by gavage at 10 $\mu\text{g}/\text{kg}$ body weight (b.w.). Control mice received DMSO/olive oil only.

Feeding and immunization regimen. Mice were fed by gavage on days 0 and 2 and five times with 20 mg OVA (Sigma-Aldrich, Munich, Germany) in 100 μl PBS. This represents a “high-dose” oral tolerization scheme (Friedman and Weiner, 1994). Preliminary tests indicated that this protocol led to complete failure to produce peripheral anti-OVA-specific IgG1 antibodies after immunization. Lower repetitive doses or a single dose of 50 mg OVA per mouse led to incomplete suppression of antibody response in our hands, as also reported in the literature (Mowat *et al.*, 1982). Feeding was started 3 days after TCDD treatment, i.e., at a time point when TCDD is fully absorbed and distributed throughout the body. Mice were immunized with 10 μg OVA in 100 μl PBS/CFA emulsion (first immunization) or PBS/IFA emulsion (booster immunizations) on days 12, 23, and 55. To trigger OVA-specific antibody

secretion into the gut, mice were fed three times 1 mg OVA in 100 μl PBS by gavage on days 38, 41, and 43.

Sample preparation. Serum was prepared from coagulated blood and frozen at -20°C until further use. For fecal samples, two to five fecal pellets per mouse were collected and dried at 4°C for weighing (Vetvik *et al.*, 1998). A total of 100 mg of fecal matter was dissolved in 1 ml PBS/0.01% NaN₃ by shaking at room temperature (RT) for 3 h. Particulate matter was removed by centrifugation and samples stored at -20°C .

Enzyme-linked immunosorbent assay. Total and OVA-specific antibody concentrations were measured by ELISA. For detection of OVA-specific antibodies, ELISA plates were coated with 100 μg OVA/ml and blocked. Serum or fecal samples were diluted (1:10,000–1:80,000 or 1:8, respectively) in PBS/1% fetal calf serum and added to the wells. The plate was developed with biotinylated anti-IgG1 or IgA antibodies (SBA, Birmingham, AL). The amount of OVA-specific antibodies is given relative to our own standard sera and fecal samples acquired from immunized mice.

Total antibody concentration was determined using isotype-specific antibodies for coating. All antibodies were from Sigma (anti-IgA coating) or SBA. IgA and IgG1 standards (hybridomas 233.1.3 and N1 G9) were a kind gift of C. Uthoff-Hachenberg (University of Cologne).

RT-PCR. RNA and complementary DNA preparation was according to standard procedures. For RNA from complete intestine, about 1-cm² pieces were put on a shaker for 3 h at 37°C in TRIzol (Invitrogen) before chloroform extraction. RT-PCR was performed as previously published (Frericks *et al.*, 2007).

Single cell preparations of IEL and LP cells. The small intestine was carefully removed and stripped of mesenteric tissue. The inside was flushed with PBS, PPs were removed with a scalpel, and the intestine was opened lengthwise. Segments (approximately 1 cm in length) were placed in 5mM EDTA in PBS (pH7.2) and shaken vigorously in a water bath at 37°C for 40 min to release the intestinal epithelial cells (IEC) and IELs into the solution. IELs were purified further on a 75%/40%/25% Percoll (Biochrom) gradient. LP cells were obtained by digesting the remaining solid gut pieces with 100 U/ml collagenase VII from *Clostridium histolyticum* (Sigma) for 1.5 h at 37°C under constant stirring at 200 rpm (Westendorf *et al.*, 2009).

Flow cytometry. Single cell suspensions were incubated with Fc-block (anti-CD16/32) before staining with the indicated antibodies (10 min, 4°C). Antibodies used were anti-CD16/32 (Fc-Block, clone 2.4G2), anti-CD4^{APC} (clone RM4-5), anti-CD8a^{PerCP} (clone 53-6.7), anti-CD8b^{PE} (clone H35-17.2), anti-CD19^{FITC or APC} (clone 1D3), anti-TCR $\gamma\delta$ ^{FITC} (clone GL3), and anti-CD69^{PE} (clone H1.2F3), all from BD Pharmingen (Heidelberg, Germany). Anti-CD11c^{FITC} (clone N418), anti-MHCII^{APC} (clone M5/114.15.2), and anti-CD103^{PE} (clone 2E7) antibodies for surface staining were from eBioscience (Frankfurt, Germany). The FoxP3^{FITC} staining kit (antibody clone FJK-16s) from eBioscience was used for staining regulatory T cells. A total of 5×10^6 cells/ml from MLNs or PPs were stimulated *in vitro* with phorbol myristate acetate (5 ng/ml) and ionomycin (250 ng/ml). BrefeldinA (1 $\mu\text{l}/\text{ml}$; Golgi Plug, BD Pharmingen) was added 5 h prior to staining to inhibit cytokine secretion. After surface staining, cells were fixed in 2% paraformaldehyde in PBS for 20 min at RT and permeabilized with 0.5% saponin. Cells were then incubated for 20 min at RT with the indicated intracellular antibodies in PBS/0.5% saponin and analyzed as above (Janke *et al.*, 2010). Anti-IL-6^{PE}, IL-17^{PE}, interferon-(IFN) γ ^{APC}, and IL-10^{PE} antibodies were from BD Pharmingen. At least 50,000 cells were collected in list mode on a fluorescence activated cell sorter (FACS) Calibur and analyzed with FlowJo software.

Cell sorting. Mesenteric DC were enriched using CD11c magnetic beads (Miltenyi, Bergisch Gladbach, Germany) and CD11c⁺ MHCII⁺ CD103⁺ triple-positive cells were sorted on a FACS to purity greater 95%. CD4⁺ and CD8⁺ T cells were sorted with the magnetic cell sorter (MACS) (Miltenyi) to purity greater 80%. IECs were identified based on their scatter prior to Percoll centrifugation and obtained more than 95% pure.

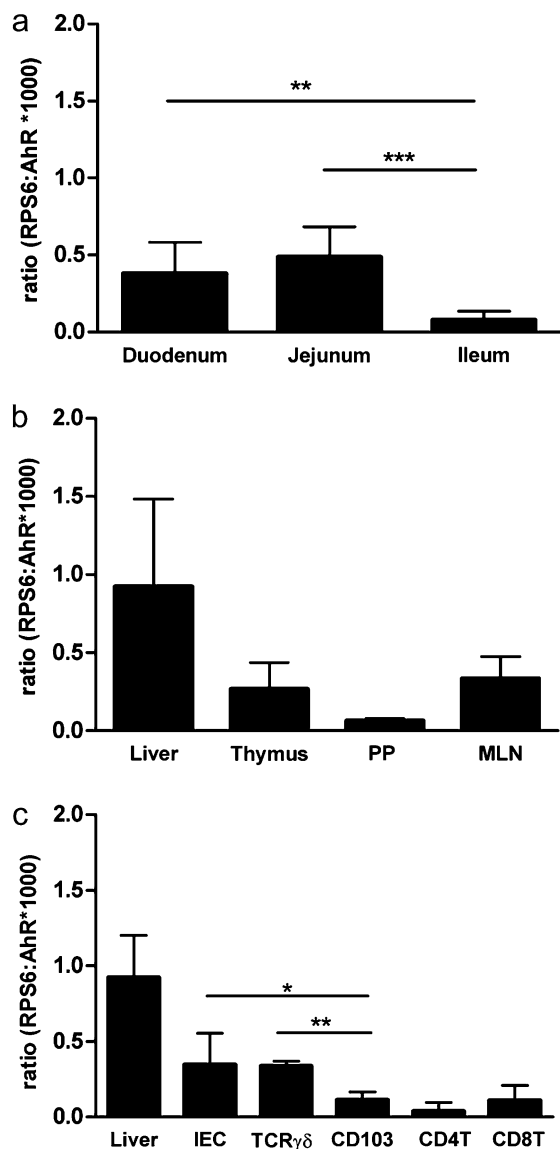


FIG. 1. Messenger RNA levels of AhR in gut, gut-associated immune cells, and liver. Expression levels of AhR were determined by real-time PCR in relation to housekeeping gene Rps6. (a) Total duodenal, jejunal, and ileal sections. (b) PPs and MLNs compared with liver and thymus (organs of high-AhR expression). (c) IEC, CD8 $\alpha\alpha^+$ TCR $\gamma\delta^+$ (TCR $\gamma\delta$), and CD103 $^+$ DC (CD103DC) were FACS sorted to purity of more than 95% from IEL or MLN. CD4 and CD8 T cells were sorted via MACS from MLN to purity greater 80%. Graphs show that data are from $n = 3-6$ mice and assayed in two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Statistics. Data were analyzed with GraphPad Prism software. Results are expressed as mean value $\pm$ SD (unless indicated otherwise). p values < 0.05 as determined with Student's t -test or ANOVA were considered significant.

RESULTS

GALT and Gut Epithelial Cells Express Functional AhR

AhR is expressed abundantly in some organs, such as liver and lung, but absent in, e.g., testis and muscle (Frericks *et al.*,

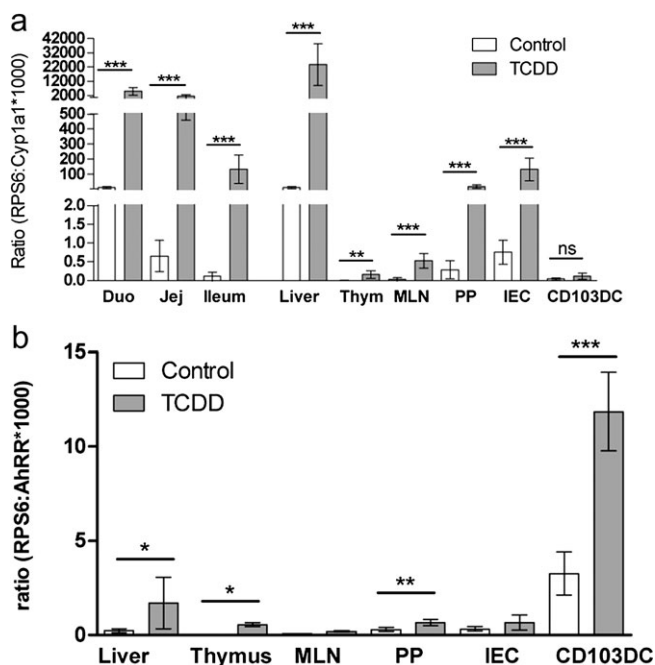


FIG. 2. Induction of the AhR target genes *cypl1* and *ahrr* in IECs, GALT, and immune cell subsets of TCDD-treated mice. C57BL/6 mice were fed with 10 $\mu\text{g/kg}$ b.w. TCDD in olive oil. Three days later, gut was removed and *cypl1* (a) and *ahrr* (b) messenger RNA measured by real-time PCR. White bars represent tissue or cells from mice fed with solvent only. Gray bars represent tissue or cells from mice fed with TCDD. For comparison with strongly AhR-expressing tissues, data of liver and thymus are included. Data shown are the expression ratio compared with the housekeeping gene *rps6*; ($n = 3-6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2007). With respect to the immune system, analysis of sorted subsets has revealed differential and specific AhR expression in only some immune cell subsets, in particular differentiated Th17 cells and CCR6 $^+$ $\gamma\delta$ T cells, Langerhans cells, and other DC subsets (Jux *et al.*, 2009; Martin *et al.*, 2009; Veldhoen *et al.*, 2008). We tested AhR expression in gut cells by semiquantitative PCR. AhR transcripts were detectable in the duodenal, jejunal, and ileal gut segments (Fig. 1a). MLNs expressed AhR at levels similar to those of the thymus (an organ with known AhR abundance). In PPs, AhR transcripts were barely detectable (Fig. 1b). IECs and CD8 $\alpha\alpha^+$ TCR $\gamma\delta^+$ IEL, a specialized T-cell type probably responsible for epithelial integrity and immune surveillance (Hayday and Spencer, 2009), also expressed AhR strongly (Fig. 1c). In contrast, CD103 $^+$ DC in MLN expressed far less AhR.

To test for functionality of the AhR in gut tissue, we treated C57BL/6 mice with 10 $\mu\text{g/kg}$ b.w. TCDD, a moderate dose (Kerkvliet and Brauner, 1990), and analyzed induction of *cypl1*, considered to be a biomarker of AhR activation. As shown in Figure 2a, basal as well as inducible *cypl1* expression decreased in the jejunum and ileum compared with the more proximal intestine sections. Expression of another target gene and thus marker for AhR activity, AhR repressor (*ahrr*), increased from low levels several fold by TCDD.

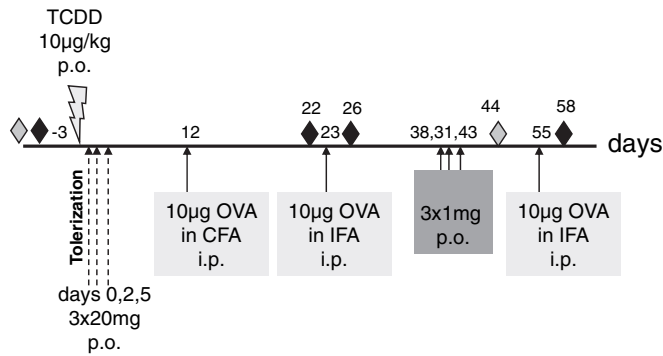


FIG. 3. Time scheme of TCDD feeding (flash arrow), tolerization (dashed arrows), and immunizations (black arrows). Numbers refer to the respective days after first feeding of OVA (= day 1). Diamonds denote time points of serum (?) and fecal sampling (?).

A spatial expression gradient could not be observed (data not shown). Taken together, AhR is functionally expressed in the gut, with *cypl1* gene induction by TCDD found decreasing in a gradient from proximal to distal in the small intestine.

AhRR is known to repress AhR activity. We found that *cypl1* induction in the different GALT tissues and cell types was inversely related to *ahrr* expression (cf. Figs. 2a and 2b). Thus, in MLN and PP (low in *ahrr* expression), *cypl1* transcripts increased strongly by TCDD. Similarly, *cypl1* RNA increased 100-fold in IEC in which *ahrr* transcripts were almost absent. In contrast, CD103⁺DC expressed AhRR at considerably levels, and *cypl1* was barely induced by TCDD. Note that TCDD exposure of mice led to an even higher expression of *ahrr* in CD103⁺DC.

TCDD and Oral Tolerance

Oral tolerance is defined as unresponsiveness of the immune system against foreign antigens, which are taken up through the gut after feeding or eating them. We fed C57/BL6 mice with 10 µg/kg b.w. TCDD in olive oil 3 days before starting a standard tolerization protocol of feeding OVA, followed by parenteral immunization against OVA. Serum samples were taken at the indicated time points and analyzed for total and OVA-specific IgG1. To detect OVA-specific IgA in fecal samples, mice were fed again with OVA after the end of the immunization scheme.

Figure 3 shows the scheme of immunizations and serum/fecal sampling, which allows looking at the influence of TCDD on the IgG1 immune response against OVA in immunized versus tolerized animals. First, TCDD treatment suppressed the humoral immune response after ip immunization with OVA. However, TCDD suppression was abrogated by a second immunization and IgG1 titers reached levels of control animals (PBS treatment) (Fig. 4, white bars). In summary, TCDD alone induced immunosuppression in the nontolerized mice, and the effect was abrogated after the second immunization.

Second, regarding tolerance, TCDD treatment prior to tolerization was permissive for IgG1 production at levels

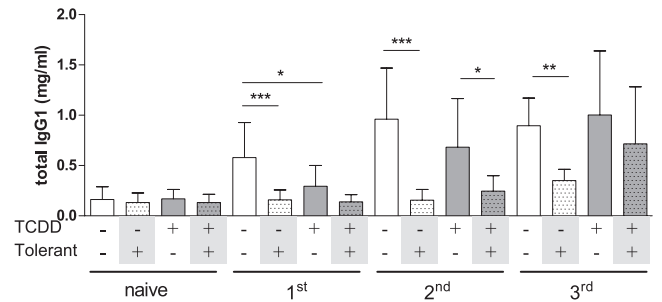


FIG. 4. Serum concentrations of IgG1 in micrograms per milliliter serum. Samples were taken 10 days after the first immunization and 3 days after each booster injection and measured via ELISA ($n > 12$, two independent experiments). Results are shown as mean $\pm$ SD. Significant differences were calculated using ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

similar to those of nontolerized mice, albeit only after two booster immunization (Fig. 4, dotted gray bars). The kinetics of OVA-specific IgG1 titer paralleled the total IgG1 titer (cf. Figs. 4 and 5a).

The results of sera taken after the third immunization are shown in Figure 5b. The control groups behaved as expected: anti-OVA IgG1 titers in the nontolerized group increased with each booster immunization, whereas OVA-specific IgG1 was always below the detection limit in the tolerized mice. In contrast, TCDD-treated/OVA-tolerized mice produced significant amounts of OVA-specific IgG1 antibodies, albeit levels remained below those of the TCDD- or PBS-treated control mice. With each booster injection, more mice in the TCDD-treated group displayed break of tolerance (Table 1). Five of 14 animals had high OVA-specific IgG1 in the serum after the first, and six after the second immunization, whereas after the third immunization, 13 of 14 TCDD-treated mice produced anti-OVA-specific antibodies (Table 1). In contrast, in the absence of TCDD, only 1 of 13 mice produced anti-OVA-specific antibodies after three OVA challenges. Thus, TCDD exposure prevented the stable establishment of oral tolerance.

IgA Secretion into the Gut Lumen by TCDD Treatment

Oral tolerance also prevents production of antigen-specific IgA into the gut lumen (Kato *et al.*, 2001). We therefore measured OVA-specific IgA in fecal samples of tolerized and TCDD-exposed mice (see scheme given in Fig. 3). In OVA-tolerized mice, OVA-specific IgA was not detectable in the fecal samples. In contrast, after TCDD treatment and tolerization, OVA-specific IgA antibodies were detected at the levels of nontolerized mice (Fig. 6, white bars), affirming that TCDD breaks tolerance not only regarding peripheral antibody production but also gut antibodies.

Immune Cell Distribution Is Only Altered in MLN by TCDD

To exclude selective toxicity of 10 µg TCDD/kg b.w. on IEL and LP lymphocytes, we tested composition of immune cell

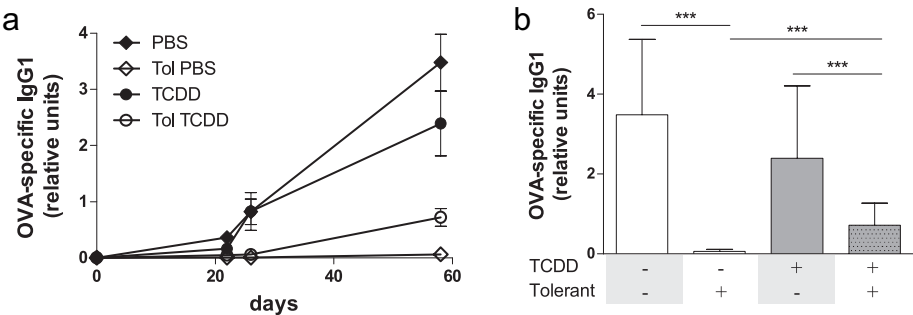


FIG. 5. Amount of OVA-specific IgG1 is given as relative units compared with a standard serum measured in ELISA. (a) Samples were taken 10 days after the first immunization and 3 days after each booster injection. TCDD-induced immunosuppression was abrogated after the second immunization (TCDD). Tolerant mice (Tol-PBS) do not mount an immune response, whereas pretreatment with TCDD (Tol-TCDD) led to significant OVA-specific IgG1 production. Results are shown as mean $\pm$ SEM. (b) OVA-specific IgG1 3 days after the third immunization. Results derived of two independent experiments and presented as mean $\pm$ SD; $n > 12$ per group of mice. Significant differences were calculated using ANOVA ($***p < 0.001$).

subsets 10 days after exposure. IEL yield was approximately 1.5×10^6 cells per small intestine in both TCDD-treated and control animals. Moreover, the immune cell subset frequencies did not differ between TCDD-exposed and control mice (~44% CD8 $\alpha\alpha$ TCR $\gamma\delta$, 20% CD8 $\alpha\alpha$ TCR $\alpha\beta$, 29% CD8 $\alpha\beta$ TCR $\alpha\beta$, 2% CD8 $\alpha\beta$ TCR $\gamma\delta$, and 4% CD4). Similarly, neither the absolute number of cells nor frequencies of CD4 $^+$, CD8 $^+$, CD19 $^+$, and CD103 $^+$ MHCII $^+$ CD11c $^+$, subsets in PP or LP cells were affected by 10 μ g/kg b.w. TCDD exposure. The data are summarized in Tables 2 and 3. Thus, break of tolerance by TCDD, as described above, is not because of toxic elimination of any or all effector subsets of immune cells in GALT.

Antigen-sampling CD103 $^+$ DC, which migrate to MLN, is critical for induction of regulatory T cells against gut antigens (Jaensson *et al.*, 2008). TCDD might affect DC frequencies in the MLN or their tolerogenic capacities. We determined the frequencies of DC in MLN 3, 10, and 14 days after feeding TCDD. Confirming literature results (Coombes *et al.*, 2007), we found about 2–3% MHCII $^+$ CD11c $^+$ DC in MLN, of which 40% were CD103 $^+$. At all time points after feeding TCDD, the percentage of total DC in MLN was unchanged. However, on days 10 and 14 after TCDD exposure, CD103 $^+$ DC frequency

was significantly increased from 47.9% to $61.2\% \pm 6.2\%$ (see Table 2). Unfortunately, whether this correlates to significant changes in absolute number could not be determined because of the small numbers and biological variation between mice.

TCDD Affects the Regulatory T-cell/Th17 Balance in MLN

The balance between regulatory T cells (Treg) and proinflammatory T-cell subsets, in particular Th17 cells, can shift and is relevant in tolerance as well as in inflammatory disease (Duarte *et al.*, 2010; Izcue *et al.*, 2009; Kroetz and Deepe, 2010). We determined the frequencies of CD4 $^+$ T-cell subsets in MLN of TCDD-treated and control mice, 3 days after tolerization. We detected a shift in the balance of Treg (FoxP3 $^+$) versus Th17 (IL-17 $^+$) cells in MLN, as Th17 cells doubled (Fig. 7a). No changes were detected in

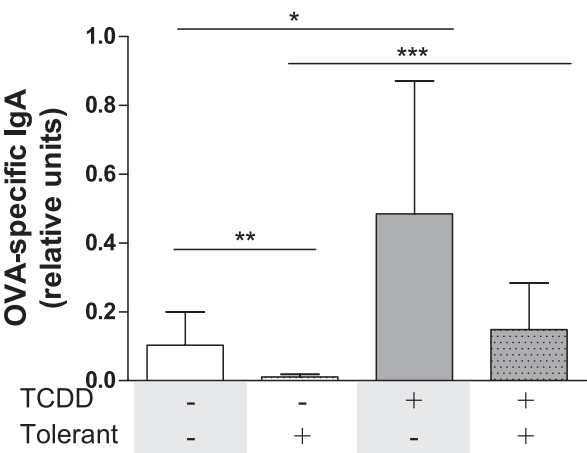


FIG. 6. OVA-specific IgA concentration was measured in fecal samples via ELISA ($n > 12$, two independent experiments) and is given as relative units compared with a standard sample. Samples were collected 1 day after oral challenge with OVA on days 38, 41, and 43 (see Fig. 3), dried, and dissolved in PBS/NaN $_3$ at a concentration of 100 mg/ml. Significant differences were calculated using ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

TABLE 1
Number of Mice Producing Anti-OVA-Specific IgG1 Antibodies above Background after Oral Tolerization Against OVA and up to Three Immunizations with OVA

Treatment before oral tolerization ^a	First immunization ^b	Second immunization ^b	Third immunization ^b
Solvent ($N = 16$)	0/13	0/13	1/13
TCDD ($N = 14$)	5/14	6/14	13/14

^aFor treatment scheme, see Material and Methods section and Figure 3.
^bBlood was taken for ELISA measurements 10 days after first immunization or 3 days after the second and third booster immunizations.

TABLE 2
Lymphocyte Numbers and Frequencies in LP, MLNs, and PPs in mice treated with TCDD for 10 days^a

	LP		MLNs		PPs	
	Control	TCDD	Control	TCDD	Control	TCDD
Absolute numbers × 10 ⁶	2.8 ± 0.9	3 ± 0.7	10 ± 2.8 ^a	12 ± 4.2	0.4 ± 0.2	0.34 ± 0.18
Percentages						
CD4 ⁺	20.2 ± 7.9	17.8 ± 3.8	40.0 ± 7.4	39.6 ± 8.0	17.6 ± 6.8	16.0 ± 6.2
CD69 ^{+b}			16.2 ± 2.3	17.2 ± 2.8	50.3 ± 7.6	53.2 ± 6.4
CD8 ⁺	4.3 ± 1.5	4.1 ± 1.9	29.5 ± 4.4	30.1 ± 5.2	7.0 ± 3.1	5.9 ± 1.9
CD69 ^{+b}			6.4 ± 3.6	6.9 ± 2.8	13.3 ± 4.5	15.2 ± 6.0
CD19 ⁺	29.5 ± 9.9	32.2 ± 15	21.5 ± 6.4	20.3 ± 6.5	69.0 ± 6.8	65.8 ± 7.6
DC ^c	3.4 ± 1.6	3.0 ± 1.7	2.3 ± 0.8	2.0 ± 0.7	2.8 ± 1.4	3.0 ± 1.4
CD103 ^{+b}	51.5 ± 3.9	55.0 ± 4.1	47.9 ± 4.2 46.7 ± 9.4 ^d	61.2 ± 6.2 ^{***} 45.3 ± 5.6 ^d	38.8 ± 8.9	36.1 ± 7.4

^aThe indicated organs were isolated, single cell suspensions prepared, counted, and analyzed flow cytometrically. The percentages shown refer to life-gated cells expressing each marker as assessed by flow cytometry. The means ± SD of at least three independent experiments with three mice per group are shown. Mice were treated with 10 µg/kg b.w. TCDD 10 days prior to cell isolation and staining.

^bFrequencies of cells expressing subset markers (CD69 and CD103) are given as percentages of the correspondent population, i.e., CD4, CD8, or DC cells.

^cDC were defined as CD11c⁺ MHCII⁺.

^dFrequencies for CD103⁺DC on day 3 after TCDD.

****p* < 0.001, comparison control animals with TCDD-treated animals. Significant values are highlighted in bold.

frequencies of IL-10 or IFNγ-positive cells of MLN or PP. Note that in PP, Th17 cell frequencies remained unchanged by TCDD treatment. Our data fit with recent reports that AhR is important for Th17 differentiation (Veldhoen *et al.*, 2008) and of Treg/Th17 ratio shifts in, e.g., models of oral tolerance protection against collagen-induced arthritis (Duarte *et al.*, 2010). The frequency of Th17 cells is low compared with Treg cells. DC produces IL-6, a known target of the AhR in the presence of lipopolysaccharide (Bankoti *et al.*, 2010). IL-6 instructs T-cell differentiation (Kimura and Kishimoto, 2010; Stockinger *et al.*, 2007). Therefore, we analyzed IL-6 expression in MLN DC. As shown in Figures 7d

and 7e, TCDD-exposed tolerized mice had a significantly higher frequency of IL-6 producing CD11c⁺MHCII⁺ DC in their MLN, whereas no differences were detected in PP cells (Fig. 7f).

DISCUSSION

It has been known for decades that the environmental pollutant TCDD suppresses the peripheral immune system via AhR activation. We show here that AhR expression is high in the gut and that TCDD affects immunity of the gut as well. A single injection of a moderate dose of TCDD led to loss of orally induced tolerance on subsequent systemic antigen challenges. The precise mechanisms of oral tolerance are controversial but might depend on antigen dose (high dose vs. low dose of antigen) or on the feeding scheme (single vs. multiple) (Faria and Weiner, 2005; Friedman and Weiner, 1994; Mowat *et al.*, 1982). In particular, evidence suggests that high-dose antigen feeding preferentially leads to anergy or deletion of T cells, whereas low-dose antigen induces regulatory T cells and is associated with inhibitory cytokine production of IL-4, IL-10, or TGFβ. The two forms are not mutually exclusive and may overlap (Faria and Weiner, 2005; Smith *et al.*, 2000). In general, tests of oral tolerance reported in the literature do not go beyond one or two challenge immunizations. It is a novel and interesting finding that we could not break high dose-induced tolerance even after a third booster immunization, i.e., 12 of 13 control mice remained tolerant. This finding argues against a simple deletion of

TABLE 3
IEL Frequencies^a

	IEL	
	Control (%)	TCDD (%)
CD4 ⁺	4.0 ± 2.5	4.0 ± 2.1
CD8α ⁺ TCRγδ ⁺	43.4 ± 10.0	44.9 ± 9.2
CD8α ⁺ TCRαβ ⁺	20.6 ± 8.6	20.5 ± 5.8
CD8αβ ⁺ TCRγδ ⁺	2.6 ± 2.5	2.3 ± 1.3
CD8αβ ⁺ TCRαβ ⁺	29.0 ± 12.5	28.2 ± 12.3

^aIELs were prepared from the small intestine as described in Material and Methods section. The data shown are the percentages of life-gated cells expressing each cell surface marker as assessed by flow cytometry. Shown are the means ± SD of at least three independent experiments with three mice per group. Mice were treated with 10 µg/kg b.w. TCDD 10 days prior to cell isolation and staining.

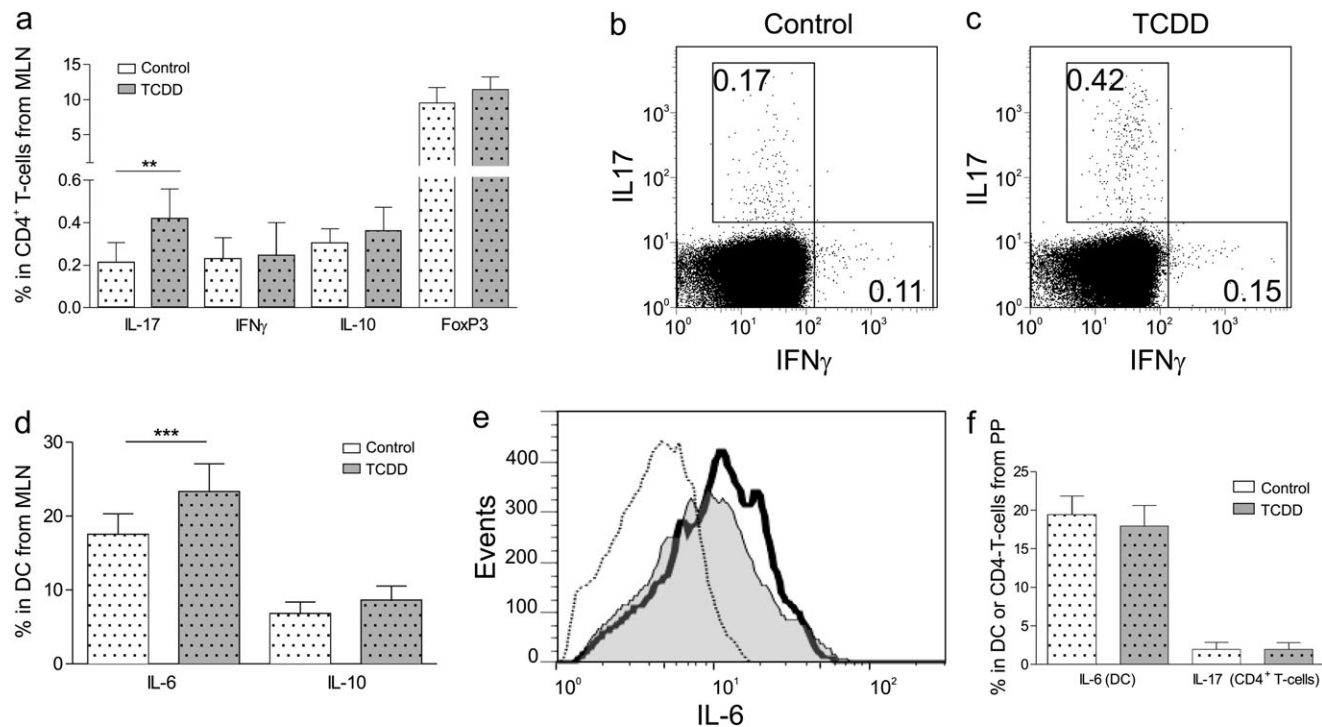


FIG. 7. Mice were treated with TCDD, tolerized as shown in Figure 3, and MLN or PP analyzed 1 day later. Controls: white dotted bars and TCDD-treated animals: gray dotted bars. (a) Frequencies of CD4⁺ T-cell subsets, which produce the indicated cytokines versus Treg cells. (b and c) Intracellular cytokine staining of IL-17 and IFN γ in CD4⁺ T cells from MLN in control mice and TCDD-treated mice. (d) Frequencies of IL-6 and IL-10 cytokine producing DC in MLN. (e) Histogram overlays of IL-6 producing DC from MLN from control mice (shaded) and TCDD-treated mice (black line). (f) Frequencies of IL-6 producing DC and IL-17 producing CD4⁺ T cells in PP. Results are either shown as mean $\pm$ SD from three independent experiments, $n = 6-9$ (bar graphs) or as a representative flow cytometry graph. $**p < 0.01$, $***p < 0.001$.

antigen-specific T cells, as one would expect OVA-specific T cells to be replaced with time from new thymic emigrants. The obvious explanation would be that regulatory T cells are still present in the mice. Also, it is possible to consider infectious tolerance by DC and T cells alike, a concept shown for nickel-fed mice. In this model, very few cells were capable of spreading the tolerance in serial adoptive transfers (Roelofs-Haarhuis *et al.*, 2004). It will be interesting to test for this by, e.g., adoptive transfer experiments.

Rather than abrogating oral tolerance completely, our data indicate that the persistence of oral tolerance is impaired by TCDD. Interestingly, Kinoshita *et al.* (2006) reported that a dose of only 1 μg TCDD/kg b.w. was permissive for generation of antibodies in OVA-tolerized mice on two challenges (both given in CFA). At the late time point, these authors analyzed that TCDD would have been eliminated from the system. Thus, we think that TCDD affects cells responsible for tolerance induction and memory early after treatment, and TCDD presence, once the disruption is set, is no longer needed. Whether or not TCDD can break an already established tolerance remains to be shown.

We confirmed literature data that TCDD treatment ("sine" tolerization) suppresses the humoral response (Kerkvliet *et al.*, 1990), here against OVA. Moreover, we show for the first time

that suppression does not persist after booster immunizations. Most likely, this is not because of an insufficient TCDD concentration in the mice at the time point measured, despite the fact that TCDD is eliminated much faster in mice than in humans (half-life of 12 days vs. a half-life of 7 years) (Van den *et al.*, 1994). The estimated body burden at the time of the second immunization is 2.6 $\mu\text{g}/\text{kg}$ b.w., and immunosuppression was already described at doses as low as 1 $\mu\text{g}/\text{kg}$ b.w. (Vorderstrasse *et al.*, 2003). Adoptive transfer experiments with OVA-specific TCR-transgenic cells suggested that the TCDD-impaired humoral response was related to impaired CD4 help (Shepherd *et al.*, 2000). Our results challenge this view as suppression can be overcome with booster immunizations. Our results can explain why findings regarding the humoral immune response in humans after dioxin, furan, or biphenyl exposure yielded ambiguous results regarding suppression of antibody responses, i.e., often normal antibody titers were observed in exposed persons (Esser, 2005).

Recently, Ishikawa (2009) analyzed IgA secretion into the gut lumen in mice after low-dose TCDD treatment via nursing and reported an AhR-dependent decrease in fecal IgA. We were not able to confirm this result in our adult mice but that might be because of experimental differences. However, similar to our results, Ishikawa *et al.* did not observe any

TCDD-dependent gross changes in MLN cellularity or immune cell subset distribution of CD4, CD8, B cells, or CD11c⁺CD11b⁺ cells in MLN and PP. As they found an impaired migration of B1 B cells (a major source for intestinal IgA) toward B-cell chemokines and reduced chemokine receptor expression, they suggest that TCDD-reduced fecal IgA is because of suppression of an appropriate B1 B-cell response.

How could TCDD affect induction of oral tolerance? The underlying mechanism is currently unclear. Our tolerizing protocol of 3×20 mg OVA does not necessarily imply that anergy/deletion of OVA-specific T cells is the only possibly underlying mechanism. Very recent findings, which take into account the timing of antigen feedings, suggest an alternative or complementary mechanism. Plasmacytoid dendritic cells (pDCs) in the liver were shown to take up antigen and rapidly delete antigen-specific T cells in a first line of tolerization. Only later, regulatory T cells are induced via migratory DC. Oral tolerance could be transferred by pDC, and was abrogated, if pDC were missing (Goubier *et al.*, 2008). Thus, multiple feedings over a time of 7–10 days could activate different mechanisms than a single feeding. TCDD is known to damage liver, but no information exists about TCDD effects on pDC, and further experiments must consider this. We looked at DC migrated into MLN from the LP and at T cells in the gut-draining MLN. We did not find fewer DC in the gut or MLN, ruling out DC-specific cell death or a failure of DC to migrate to MLN. Also, we did not observe deletion of gut-specific T cells or B cells by TCDD, i.e., not a directly toxic effect. Gut-derived CD103⁺DC became known as “tolerogenic DC” (Coombes *et al.*, 2005) because of their capacity to shift the balance between Treg and Th17 cells. We found a small but significant increase in IL-6 producing DC in MLN. IL-6 is a regulator for Th17/Treg (Kimura and Kishimoto, 2010). Nonetheless, CD103⁺DC expressed very low amounts of AhR, and because of high-*ahrr* expression, the classical AhR-ARNT signaling pathway is possibly blocked similar to the situation in Langerhans cells (Jux *et al.*, 2009). As AhR can bind to RelA or RelB, TCDD could trigger IL-6 via this pathway (Tian, 2009). Possibly, abundant AhRR in CD103⁺DC is a control mechanism to push DC into preference of the NFκB pathway, if AhR ligands are present, and thus involved in microbiota immune surveillance. Concomitant to the increase in IL-6 producing DC, we found a doubling in IL-17 expressing CD4⁺ T cells in MLN. AhR activation is involved in deciding Th17 and Treg cell differentiation fates (Marshall and Kerkvliet, 2010; Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). It is therefore tempting to speculate that in the presence of TCDD MLN, DC become less tolerogenic and that Th17 cells are induced, albeit further experiments are needed to address this. The increase in Th17 cell frequency was small, but recent literature reports shift in the ratio of Treg/Th17 cells associated with disease severity in a model of protective oral tolerization against

collagen-induced arthritis and lung inflammation (Duarte *et al.*, 2010; Kroetz and Deepe, 2010; Tong *et al.*, 2010). It will be interesting to determine antigen specificity of these Th17 cells and the (much more abundant) Treg cells present in the MLN. The manipulation of Treg and T-cell subsets in general is of high therapeutic interest for the management of inflammatory and autoimmune disorders. Our data show that stability of mucosal tolerance can be changed by environmental AhR agonists encountered via the oral route. Further experiments will be needed to study the kinetics of DC changes, the breakage of an existing tolerance, or the role of Th17 and Treg cells.

IECs transfer signals from nutrition and the bacterial flora of the gut lumen and control whether gut DC will become tolerogenic (Rimoldi *et al.*, 2005). AhR was conspicuously abundant in IEC, conceivably it is related to the xenobiotic metabolism in IEC. IEC form a physical and biological barrier against the gut lumen. Recent evidence has revealed a role for IEC as an immunological barrier. They instruct CD103⁺DC to become tolerogenic or inflammatory (Shale and Ghosh, 2009), e.g., by secretion of granulocyte macrophage colony stimulating factor, TGFβ, indoleamin-2,3-dioxygenase (IDO), or thymic stromal lymphopoietin. Moreover, induction of toll-like receptors signaling led to increased turnover and faster migration of CD103⁺DC into the MLN (Schulz *et al.*, 2009). It is currently not known whether AhR participates in the immunological functions of IEC.

Although gut barrier function is not the focus in our study here, we note that IL-22 production by Th17 cells requires AhR (Veldhoen *et al.*, 2008) and that we observed increased IL-22 expression in MLN cells of TCDD-treated mice (data not shown). It is not known whether other IL-22 producing cells of the gut, such as NKp46⁺ cells (Sanos *et al.*, 2009), require AhR as well, and thus, AhR might be an important positive factor for gut barrier function. IL22^{−/−} mice display increased epithelial damage, systemic bacterial burden, and mortality (Zheng *et al.*, 2008).

Food contains many potential AhR agonists, such as flavonoids, bacterial tryptophan breakdown products, or bilirubins (Ciolino *et al.*, 1999; Heath-Pagliuso *et al.*, 1998). Beyond their recognized antioxidative health benefit, they might be important as regulators of AhR. However, some dietary supplements with flavonoids can peak AhR agonist concentration in the gut in an unprecedented way and thus could overturn beneficial effects. Further studies must explore, whether “too much” of AhR agonists in natural food constituents abrogates oral tolerance, as shown here for TCDD.

In conclusion, we have shown that a single moderate dose of TCDD prevents the stable induction of oral tolerance, affecting DC and CD4⁺ immune cell subset frequencies in MLN but not in PP. Our data not only have implications for food allergies in settings of environmental exposure but also raise concerns regarding the harmlessness of overdosing potential AhR agonists in food.

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