Suppression of Experimental Autoimmune Uveoretinitis by Inducing Differentiation of Regulatory T Cells via Activation of Aryl Hydrocarbon Receptor

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PURPOSE. Aryl hydrocarbon receptor (AHR) has been identified as a regulator of CD25+CD4+ regulatory T-cell (Treg) and Th17 cell differentiation in mice, and activation of AHR by its ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces functional Treg cells. In this study, the authors examined whether the AHR-mediated effect of TCDD suppresses mouse experimental autoimmune uveitis (EAU) by inducing Treg cell differentiation.

METHODS. C57BL/6 mice were injected with TCDD 1 day before immunization with human interphotoreceptor retinoid-binding protein peptide 1–20 (hIRBP-p), and the severity of EAU was assessed clinically and histopathologically. Immunologic responses of draining lymph node cells and splenocytes to hIRBP-p and anti–CD3 monoclonal antibody (mAb) were assessed by T-cell proliferation and cytokine production. In addition, differentiation of Foxp3+ T cells and their immunosuppressive roles in TCDD-injected mice were evaluated.

RESULTS. TCDD injection increased Foxp3+ T cells in the lymph nodes and in the spleen. Development of EAU was completely suppressed by TCDD injection, and suppression was abolished by treatment with anti-CD25 mAb before TCDD injection. Both lymphocytes and splenocytes obtained from TCDD-injected mice immunized with hIRBP-p failed to produce IFN-γ and IL-17 on stimulation with hIRBP-p, and the failure of IL-17 production was observed even when stimulated with anti–CD3 mAb. However, this protocol did not interfere with IL-10 production and T-cell proliferation response when assessed on stimulation with anti–CD3 mAb.

CONCLUSIONS. Activation of AHR by TCDD markedly suppressed autoimmune uveoretinitis through mechanisms that expand CD25+Foxp3+ Treg cells and interfere with the activation of Th1 and Th17 cells.

Experimental autoimmune uveitis (EAU) in mice serves as a model for posterior uveitis of suspected autoimmune etiology in humans.1–4 EAU is induced by immunization with retinal antigens such as arrestin (S-Ag) and the interphotoreceptor retinoid-binding protein (IRBP)5,6 or by adoptive transfer of retinal antigen-specific CD4+ T cells between syngeneic donors.7–9 Patients with uveitis have serum autoantibodies to retinal antigens, including S-Ag and IRBP,5,6 suggesting their primary or secondary involvement in the disease process. In immunologically normal mice, EAU is a cell-mediated, Th1-dependent disease that targets the neural retina, leading to irreversible destruction of photoreceptor cells.

In healthy persons, pathogenic autoimmunity is controlled by a special subset of T cells named CD25+CD4+ regulatory T cells (Treg).11 Treg cell differentiation and function are driven by the transcription factor Foxp3.12–15 Treg cells have been shown to play a major role in regulating autoimmune responses in mice, and these cells have also been identified in humans.14–18 We have previously reported that Treg cell–deleted mice develop uveoretinitis through spontaneous activation of S-Ag and IRBP-specific T cells.19 Furthermore, Treg cells have the ability to inhibit the activation of IRBP-reactive T cells that have already been activated in vivo; adoptive transfer of these cells suppresses EAU even in the effector phase.20 Treg and interleukin (IL)-17–producing T cells (Th17) are known to be reciprocally related cell populations with opposite roles in the immune response.21 Transforming growth factor (TGF)-β1 induces the differentiation of Treg cells,22 whereas TGF-β1 in combination with IL-6 or IL-21 results in the differentiation of Th17 cells.23–24 Th17 cells express the transcription factor ROR-γt, which participates in the control of extracellular pathogens and plays an important role in human and experimental autoimmunity.25,26 Although a Th1-dominant response and Th1 effector cells are critical for EAU development, a recent study has demonstrated that IL-17 is related to the pathogenesis of EAU and that the Th17 response is necessary for EAU induction.27 Therefore, identification of the pathways that control Treg and Th17 cell differentiation is important in the treatment of autoimmune uveoretinitis.

Recently, several studies have demonstrated that the aryl hydrocarbon receptor (AHR) regulates the generation of Treg and Th17 cells in mice.25,28–30 AHR activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces Treg cells that suppress experimental autoimmune encephalomyelitis by a TGF-β1–dependent mechanism, whereas AHR activation by 6-formylindolo[3,2-b] carbazole interferes with Treg cell differentiation, boosts Th17 cell differentiation, and worsens encephalomyelitis. Thus, AHR regulates Treg and Th17 cell differentiation in a ligand-specific manner.
In the present study, we examined whether the administration of TCDD suppresses EAU in mice induced by immunization with human IRBP peptide 1–20 (hIRBP-p) and, if so, whether the regulation of Treg and Th17 cell differentiation is involved in the suppressive mechanisms of EAU.

**MATERIALS AND METHODS**

**Mice**

Six- to 8-week-old female C57BL/6 mice were obtained from JapanCLEA Inc. (Shizuoka, Japan). All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were performed under anesthesia with sodium pentobarbital or a mixture of ketamine and xylazine.

**Reagents**

hIRBP-p (GPTHLFQPSLVLDMAKVLLD) was purchased from TakaraBio Inc. (Shiga, Japan). Purified *Bordetella pertussis* toxin (PTX) and TCDD were purchased from Sigma Chemical (St. Louis, MO). Complete Freund’s adjuvant (CFA) and *Mycobacterium tuberculosis* strain H37Ra were obtained from Difco (Detroit, MI).

**Induction and Scoring of EAU**

C57BL/6 mice were injected intraperitoneally with 1 μg TCDD dissolved in 0.2 mL olive oil or an equivalent volume of olive oil and were immunized 24 hours later with 0.2 mL emulsion of 200 μg hIRBP-p in CFA (1:1 wt/vol) containing 5 mg/mL *M. tuberculosis* H37Ra. Concurrent with immunization, 1 μg PTX was injected intraperitoneally. On days 12, 18, and 21 after immunization, tropicamide 0.5% and phenylephrine HCL 0.5% (Santen Pharmaceutical Co., Osaka, Japan) were applied to the eyes of mice, and funduscopic examinations were performed. Three ophthalmologists performed the clinical assessments in a masked fashion. Vascular dilatation, white focal vascular lesions, white linear vascular lesions, retinal hemorrhage, and retinal detachment were evaluated. According to the severity of these findings, the EAU clinical scores were graded on a scale of 0 to 4 in half-point increments, according to a semiquantitative system described previously. Briefly, the minimal criterion to score an animal as EAU-positive by histopathology was the presence of inflammatory infiltration of the ciliary body, choroids, or retina. Progressively higher grades were assigned for the presence of discrete lesions in ocular tissues, such as vasculitis, granuloma, retinal folding or detachment (or both), and photoreceptor damage. The grading system takes into account lesion type, size, and number.

**Lymphocyte Proliferation Responses**

Regional lymph nodes near the eye (cervical and submandibular lymph nodes) and the spleen were collected from mice injected with 1 μg TCDD or an equivalent volume of olive oil and from those mice immunized with hIRBP-p (day 21 postimmunization). Each group consisted of five mice, and the cells collected from each group of five mice were pooled. Suspended cells were cultured in flat-bottom, 96-well microculture plates at a density of 5 × 10⁵ cells/well in 200 μL RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and antibiotics (culture medium) in the presence or absence of the indicated amounts of hIRBP-p or anti–CD3 monoclonal antibody (mAb). The cells were incubated for 72 hours at 37°C in 5% CO₂ in air, pulsed with [³H]-thymidine (1.0 μCi/10 μL/well) during the last 18 hours of incubation, and harvested onto glass filters using an automated cell harvester (Tomtec, Orange, CT). Radioactivity was assessed by liquid scintillation spectrometry, and the amount was expressed as cpm.

**Cytokine Production Assay**

Cervical and submandibular lymph node cells or splenocytes, prepared as described, were cultured in flat-bottom, 96-well microculture plates at a concentration of 5 × 10⁶ cells/well in 0.2 mL culture medium, in the presence or absence of the indicated amounts of hIRBP-p or anti–CD3 mAb. Supernatants were collected after 72 hours, and the amounts of IL-10 and IFN-γ were measured by cytometric bead array immunoassay (CBA Flex set; BD Biosciences, San Diego, CA), and the amount of IL-17 was measured by ELISA (Ready-SET-Go kit; eBioScience, San Diego, CA) according to the protocols recommended by the manufacturers.
Flow Cytometric Analysis

Cervical and submandibular lymph node cells or splenocytes (1 × 10⁶), prepared as described, were preincubated with unlabeled anti-CD16/32 mAb (2.4G2; eBioscience) to avoid nonspecific binding of antibodies to FcR and then incubated with FITC-labeled anti–mouse CD4 mAb (RM4–5; eBioscience) and APC-conjugated anti–mouse CD25 mAb (PC61.5; eBioscience). After two PBS washes, the cells were fixed and permeabilized with the Foxp3 staining buffer set. Then the cells were incubated with PE-labeled anti–mouse Foxp3 mAb (eBio7979; eBioscience). After two PBS washes, the stained cells were analyzed (FACSCalibur; BD Biosciences) and data were processed (Cell Quest; BD Biosciences).

Induction of EAU in Mice Depleted of CD25⁺ T Cells

To create CD25⁺ cell-depleted mice, healthy 6-week-old C57BL/6 mice received intraperitoneal injections of 50 mg rat anti-mouse IL-2R mAb (PC61; BD PharMingen, San Diego, CA) or the equivalent volume of isotype control antibodies three times a week for 2 weeks.³² A preliminary study confirmed that the 14-day treatment regimen with anti-CD25 antibody depleted the function of natural CD25⁺ Treg cells at the time of hIRBP-p immunization. After antibody treatment, mice were injected intraperitoneally with 1 μg TCDD dissolved in 0.2 mL olive oil or an equivalent volume of olive oil and were immunized 24 hours later with hIRBP-p. Data are compiled from two separate experiments. (A) Eyes were evaluated clinically and scored on days 12, 18, and 21 after immunization. Data are expressed as mean ± SD of each group. (B) On day 21, mice were killed, and eyes were examined histopathologically and scored. Each point represents one mouse (average of both eyes). Horizontal lines: average EAU score of the group. Clinical findings of EAU observed in a representative mouse injected with olive oil alone (C) and in a representative mouse injected with TCDD (D). (E, F) Histopathologic findings of the control mouse shown in (C) and of the TCDD-injected mouse shown in (D), respectively.

Effector T-Cell Suppression Assay

CD4⁺ T cells were prepared from lymph nodes of mice immunized with hIRBP-p (day 21 postimmunization) using CD4⁺ T-cell enrichment columns (R&D systems, Minneapolis, MN). CD4⁺ T cells at a concentration of 2 × 10⁶ cells/well in 0.2 mL culture medium were cocultured with various ratios of CD25⁺ CD4⁺ T cells, as described, in the presence of 5 μg/mL hIRBP-p. Supernatants were collected after 72 hours, and the concentrations of IL-17 and IFN-γ were measured by ELISA (Ready-SET-Go kit; eBioscience) according to the protocols recommended by the manufacturers.
RESULTS

Effect of TCDD on Differentiation of Foxp3+ T Cells

TCDD has been reported to interfere with Th17 cell differentiation and to promote the generation of Foxp3+CD4+ Treg cells.28 Therefore, we first analyzed Foxp3 expression in CD4+ cells obtained from lymph node cells and splenocytes of TCDD-injected mice using FACS. Representative results are shown in Figure 1. Percentages of Foxp3+CD4+ cells in lymph node cells were similar between olive oil-injected mice and TCDD-injected mice (15.57% and 17.35%, respectively), but those of splenocytes were apparently greater in TCDD-injected mice than in olive oil-injected mice (25.28% and 17.01%; Fig. 1A). In addition, Foxp3 expression in lymph node cells was promoted in TCDD-injected mice but not in olive oil-injected mice by immunization with hIRBP-p (24.5% and 17.69%; Fig. 1B).

Effect of TCDD Administration on the Development of EAU

We subsequently examined the in vivo effect of TCDD on EAU development. Twelve mice were injected intraperitoneally with 1 μg TCDD dissolved in 1 mL olive oil or an equivalent volume of olive oil and were immunized 24 hours later with hIRBP-p. All control mice injected with olive oil alone exhibited clinical signs characteristic of EAU on day 18 after injection, which progressed gradually until day 21 (Fig. 2A). Representative clinical signs of EAU observed in control mice included papillitis optica, arteriole dilatation, white focal lesions near the disc, and white linear vascular lesions (Fig. 2C). Administration of TCDD completely inhibited clinical development of EAU, and no mice injected with TCDD developed clinical signs of EAU (Figs. 2A, 2D). As were the results of clinical examination, EAU was confirmed histopathologically in all control mice injected with olive oil alone (mean SD of pathologic score; 2.25 ± 0.5), whereas pathologic findings of EAU were not found in mice injected with TCDD (Fig. 2B). In control mice, retinal vasculitis and the infiltration of inflammatory cells into the vitreous, retina, and optic nerve were observed, and the anatomic structure of retinal layers was par-

The Mann-Whitney U test. P < 0.05 was considered significant (denoted by asterisks in the figures).
tially destroyed (Fig. 2E). In mice injected with TCDD, the retina was intact, and no inflammatory sign was observed (Fig. 2F).

**Cytokine Production by T Cells Derived from Mice Injected with TCDD**

Because EAU is a T-cell–mediated disease, lymph node cells or splenocytes were collected from mice injected with olive oil alone or TCDD with or without immunization with hIRBP-p, and cytokine productions on stimulation with hIRBP-p were measured. Figure 3 shows the representative results of IFN-γ, IL-17, and IL-10 production by lymph node cells and splenocytes obtained from olive oil–injected or TCDD-injected mice with or without hIRBP-p immunization when they were stimulated in vitro with hIRBP-p. Lymph node cells derived from olive oil–injected hIRBP-p–immunized mice produced IFN-γ, IL-17, and IL-10 in a dose-dependent manner, which was significantly inhibited in TCDD-injected hIRBP-p–immunized mice (Figs. 3A–C). Similar results were observed in the splenic cells of mice injected with TCDD. Splenocytes of TCDD-injected hIRBP-p–immunized mice failed to produce IFN-γ, IL-17, or IL-10, and lymph node cells compared with those of olive oil–injected hIRBP-p–immunized mice when they were stimulated with hIRBP-p (Figs. 3D–F). Subsequently, to examine whether systemic immune responses were affected by TCDD injection, cytokine production on stimulation with anti-CD3 mAb was also measured. Figure 4 shows the representative results of IFN-γ, IL-17, and IL-10 production by lymph node cells and splenocytes obtained from olive oil–injected or TCDD-injected mice, with or without hIRBP-p immunization, when they were stimulated with anti-CD3 mAb. IFN-γ production by lymph node cells was greater in immunized mice than in nonimmunized mice, whereas that of splenocytes was greater in nonimmunized mice than in immunized mice. Although IFN-γ production by lymph node cells and splenocytes in TCDD-injected mice with or without hIRBP-p immunization was lower than in olive oil–injected mice, there was no significant difference (Figs. 4A, 4B). However, both lymph node cells and splenocytes in TCDD-injected hIRBP-p–immunized mice produced significantly less IL-17 but more IL-10 than in olive oil–injected hIRBP-p–immunized mice (Figs. 4C–F).
T-Cell Proliferation Responses in Mice Injected with TCDD

Figure 5 shows the representative results of proliferation responses by lymph node cells and splenocytes obtained from olive oil–injected or TCDD-injected mice, with or without immunization by hIRBP-p, when they were stimulated with hIRBP-p or anti–CD3 mAb. Compatible with the results of IFN-γ and IL-17 production, proliferation responses of lymph node cells were significantly lower in TCDD-injected hIRBP-p–immunized mice than in olive oil–injected hIRBP-p–immunized mice at all the concentrations of hIRBP-p tested (Fig. 5A), though dose-dependent proliferation responses were not observed in splenocytes from any groups (Fig. 5B). However, when stimulated with anti–CD3 mAb, proliferation responses of both lymph node cells and splenocytes were not reduced and were, rather, increased in TCDD-injected mice compared with those of olive oil-injected mice. Proliferation responses of lymph node cells in TCDD-injected hIRBP-p–immunized mice were lower than those of olive oil-injected hIRBP-p–immunized mice (Figs. 5C, 5D).

Inhibitory Effects of TCDD on EAU in Mice Depleted of CD25+ T Cells

Based on these results, it is reasonable to inquire whether the Foxp3+CD25+CD4+ Treg cells induced in TCDD-treated mice suppress the development of EAU and whether EAU can be suppressed in mice depleted of Foxp3+CD25+CD4+ Treg cells. To determine whether Foxp3+CD25+CD4+ Treg cells are responsible for the suppression of EAU, naive C57BL/6 mice were treated intraperitoneally with anti–CD25 mAb three times a week for 2 weeks using a regimen that profoundly depletes the number of CD25+CD4+ T cells in peripheral blood.32 Figure 6 shows representative results of Foxp3 and CD25 expression in cervical and submandibular lymph node cells of mice before and after treatment with control rat IgG or anti–CD25 mAb.32 Numbers indicate percentages within the quadrants.
FIGURE 7. TCDD is unable to inhibit EAU developed in CD25+/ regulatory T cell-depleted mice. C57BL/6 mice were injected intraperitoneally with 50 μg anti-CD25 mAb (PC61) or an equivalent volume of control rat IgG every other day six times (2 weeks). Then the mice were injected intraperitoneally with 1 μg TCDD dissolved in 1 mL olive oil or an equivalent volume of olive oil alone and were immunized 24 hours later with hIRBP-p. (A) Eyes of rat IgG-treated mice injected with olive oil (○), rat IgG-treated mice injected with TCDD (●), anti-CD25 mAb-treated mice injected with olive oil (△), and anti-CD25 mAb-treated mice injected with TCDD (◆) were evaluated clinically and scored on days 12, 18, and 21 after immunization. (B) On day 21, mice were killed and eyes were examined histopathologically and scored. Each point represents one mouse (average of both eyes). *P < 0.05, anti-CD25 mAb-treated mice injected with TCDD versus rat IgG-treated mice injected with TCDD.

Results of AHR stimulation in Treg cells.

The present study demonstrated that the administration of TCDD remarkably suppresses autoimmune uveoretinitis through mechanisms that involve the expansion of CD25+Foxp3+ Treg cells and interference with the activation of Th1 and Th17 cells.

AHR is a ligand-dependent transcription factor that mediates a range of critical cellular events in response to halogenated aromatic hydrocarbons and nonhalogenated polycyclic aromatic hydrocarbons such as TCDD.53 Although TCDD is a classical AHR ligand used in the toxicologic studies to analyze the effects of AHR activation, TCDD is clearly not a natural ligand, and pollutants such as TCDD are unlikely to have provided the evolutionary pressure for the function. The most relevant physiological ligand for AHR is a topic of ongoing debate in the field of toxicology.54 Future studies focusing on other physiological ligands of AHR that can be metabolized by AHR-regulated cytochrome P4501 (CYP1) enzymes and therefore induce only transient AHR activation may provide further insight into the consequences of AHR stimulation in Treg cells.

Characteristics of AHR, recently identified as a regulator of Treg and Th17 cell differentiation in a ligand-specific manner, resemble those of TGF-β in this aspect. Indeed AHR and TGF-β signaling pathways are known to cross-regulate each other in a cell-specific manner.55-56 Thus, it is possible that AHR and TGF-β are part of a signaling pathway that drives a common cell precursor toward the Treg or the Th17 cell differentiation program. AHR activation by TCDD boosted Treg cell differentiation and interfered with Th17 cell development.28,50,56 Our present results indicate that TCDD drastically inhibited IL-17 production by sensitized T cells obtained from either lymph node cells or splenocytes (Fig. 3). The inhibition of IL-17 was also observed even when it was stimulated with anti-CD3 mAb (Fig. 4). It is possible that the AHR-mediated effect of TCDD acts preferentially on effector T-cell lineages with a propensity to develop into Th17 cells and converts them to
CD25^+Foxp3^+ Treg cells. These results are compatible with previous reports.

Proliferation responses and IFN-γ or IL-17 production were inhibited in lymph node cells of TCDD-treated mice immunized with hIRBP-p, even when stimulated with anti-CD3 mAb, which is capable of stimulating most T cells, including IRBP-specific T cells (Figs. 3–5). One of the possible explanations is that hIRBP-p reactive T cells expanded to the major population and the main source of IFN-γ and IL-17 production in hIRBP-p-immunized mice, which were completely inhibited by TCDD-injected mice. Another is that an inhibitory function of Foxp3^+ Treg cells proliferated from naturally occurring Treg cells or converted from Th17 cells by the administration of TCDD. However, IL-10 production by lymph node cells and spleen cells on stimulation with anti-CD3 mAb was not inhibited and was, rather, increased in TCDD-injected mice immunized with hIRBP-p (Figs. 4E, 4F). Moreover, proliferation responses by lymph node cells and spleen cells of TCDD-injected mice without hIRBP-p immunization were not inhibited compared with those of olive oil-injected mice on stimulation with anti-CD3 mAb. Therefore, it is unlikely that the administration of TCDD in this protocol interferes with systemic cellular immune function. Increased IL-10 production in TCDD-injected mice immunized with hIRBP-p on stimulation with anti-CD3 mAb would be a relative substitutional reaction by the inhibited IL-17 production.

TCDD administration increased the number of Foxp3^+CD4^+ T cells in the spleen, and the increase in Foxp3^+CD4^+ T cells was comparable in nonimmunized mice and hIRBP-p-immunized mice (Figs. 1A, 1B). On the other hand, TCDD administration increased CD4^+Foxp3^+ T cells in the cervical and submandibular lymph nodes only in hIRBP-p-immunized mice. Given that the depletion of CD25^+T cells by administration with anti-CD25 mAb abolished the ability of TCDD-injected mice to inhibit EAU development, the CD25^+Foxp3^+ Treg cells in TCDD-injected mice were necessary for suppressing the development of EAU (Fig. 7). It is conceivable that TCDD promotes the proliferation of naturally occurring Treg cells in the spleen and induces the conversion of sensitized T cells to Treg cells in the lymph nodes, though further study should be conducted.

The effects of AHR stimulation on the immune system have been studied using TCDD as a ligand because of its toxicologic relevance. Although the adverse effects of TCDD on immune responses are well documented,29,30 no direct measurements of AHR expression on highly purified polarized subsets of CD4^+ T cells have been reported. TCDD, which cannot be metabolized and therefore stimulates AHR continuously in many cells of the body, is known to induce profound suppression of immune responses, but, despite decades of research, the underlying mechanisms for this profound toxicity remain unclear. In addition, in vitro stimulation of AHR by TCDD selectively affects T cells that have already been activated in vivo and does not interfere with naïve T cells. Therefore, it is conceivable that a single administration of TCDD in the active phase of autoimmune diseases suppresses the pathgenic T cells selectively and continuously without causing severe systemic immune deficiency, which may be a useful treatment strategy for patients with autoimmune uveoretinitis.

References