2,3,7,8-Tetrachlorodibenzo-p-Dioxin Regulates the Expression of Aryl Hydrocarbon Receptor-Related Factors and Cytokines in Peripheral Blood Mononuclear Cells and CD4+ T cells from Patients with Atopic Dermatitis and Psoriasis

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Background: Aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, is important for xenobiotic metabolism and binds to various endogenous and exogenous ligands in the skin. However, the functional role of AhR in patients with psoriasis (PS) and atopic dermatitis (AD) remains unclear. Objective: We aimed to determine whether AhR-regulated factors (AhR, CYP1A1, interleukin [IL]-17, IL-22) were affected by AhR ligands (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) in chronic inflammatory skin diseases such as PS and AD. Methods: The expression levels of AhR-related factors were determined by quantitative PCR, western blotting, and immunocytochemistry. Specific siRNA targeting AhR was used to inhibit gene expression in human peripheral blood mononuclear cells (PBMC). Cytokine assays were performed to determine the protein production of CD4+ T cells. Results: In comparison with healthy controls, TCDD-treated PBMCs and CD4+ T cells from patients with PS and AD showed an increase in AhR gene levels as well as significantly increased expression of AhR-related factors (such as AhR, CYP1A1, IL-17, and IL-22). In contrast, 6-formyl indolo [3,2-b] carbazole (FICZ) inversely affected the differentiation of CD4+ T cells and their cytokine expression levels as compared with TCDD. CD4+ T cells from patients with AD and PS showed higher expression levels of AhR, CYP1A1, IL-17, and IL-22. Conclusion: Our results suggest that TCDD-induced AhR-related factor upregulation in AD and PS patients may increase the expression of AhR-regulatory genes, thereby contributing to the development of AD and PS. (Ann Dermatol 32(5) 360 ~ 369, 2020)

Keywords: 6-formyl indolo [3,2-b] carbazole (FICZ) psoriasis, Dermatitis, atopic, Polychlorinated dibenzodioxins, Psoriasis, Receptor, aryl hydrocarbon

INTRODUCTION

Environmental pollution is known to contribute to the increased prevalence of inflammatory skin diseases such as atopic dermatitis (AD) and psoriasis (PS)\(^1\). The incidence of AD has greatly increased in the last few decades in western countries\(^2\) and AD prevalence is higher in developed countries and urban areas than in developing countries and rural areas\(^3\). While the causes underlying PS are not completely understood, environmental risk factors\(^4\),
including tobacco smoking, are known to be associated with this pathology.\textsuperscript{5,6} The biological response to many environmental pollutants involves their direct interactions with a receptor of xenobiotics, aryl hydrocarbon receptor (AhR), which binds to several exogenous ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as well as endogenous ligands such as 6-formyl indolo [3,2-b] carbazole (FICZ).\textsuperscript{1} TCDD is a major environmental pollutant in automobile exhaust, cigarette smoke, various foods, and industrial wastes.\textsuperscript{1,7} FICZ is formed from tryptophan in the presence of riboflavin and ultraviolet (UV) light, which explains the reported UV-dependent activation of CYP1 enzymes in human skin.\textsuperscript{8} Although evidence suggests a relationship between AhR ligands and cutaneous inflammatory disorders, the precise underlying mechanisms remain unclarified.\textsuperscript{9} AhR has been recently recognized as a major transcription factor involved in the development of Th17 and Th22 cells\textsuperscript{7,10,11} and is thought to be essential for the production of interleukin (IL)-22.\textsuperscript{12} In the present study, we hypothesized that the stimulation of AhR with exogenous and endogenous AhR ligands could be associated with the pathogenesis of chronic inflammatory skin diseases. Therefore, we aimed to determine whether AhR-regulated genes (AhR, CYP1A1, IL-17, IL-22) are affected by AhR ligands (TCDD) in chronic inflammatory skin diseases such as PS and AD.

### MATERIALS AND METHODS

#### Subjects

We recruited 18 patients of each category, AD, PS, and healthy volunteers, who visited the clinic between January 2012 and January 2016. The clinician’s diagnosis of AD was carried out according to the criteria proposed by Hanifin and Rajka.\textsuperscript{13} The diagnosis of PS was performed based on observations of the skin, and microscopic examinations of biopsied samples.\textsuperscript{10} The clinical severity of AD was assessed by the Eczema Area and Severity Index (EASI) (Table 1).\textsuperscript{10} The severity of PS was assessed according to the Psoriasis Area and Severity Index (PASI) (Table 1).\textsuperscript{11} The study protocol was approved by the Institutional Review Board (2012-05-45) and all subjects were required to sign informed consent forms.

#### 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

A total of $1 \times 10^4$ cells in 0.1 ml of culture medium were seeded into 96 well and cultured for 24 hours. These cells were incubated with medium alone, medium plus TCDD at various concentrations (0, 0.1, 1, 10, and 100 nmol/L), or medium plus FICZ at various concentrations (0, 0.1, 1, 10, and 100 nmol/L) for another 12, 24, or 48 hours. Subsequently, 20 $\mu$l of MTT (2 mg/ml; Duchefa, Haarlem, The Netherlands) was added to each well and incubation was continued for 4 hours at 37°C. Absorbance was determined using a THERMOmax microplate reader (Molecular Devices, Oxnard, CA, USA) at 540 nm. Cell viability percentage was calculated as follows: Viability percentage (%) = (absorption value of TCDD- or PCB-treated group)/(absorption value of the control group) x 100.

#### PBMC culture

As there is still controversy regarding the effect of AhR ligands on particular cell subsets (naïve CD4+ T cells, T cells during cytokine induced differentiation, etc.), Peripheral blood mononuclear cells (PBMC) were evaluated with respect to the effects of TCDD in an environment where all these cell types were represented and could interact. Blood from each subject, four AD, four PS patients, and four healthy controls, was drawn and prepared in an ethylenediaminetetraacetic acid tube. PBMC were isolated using Ficoll-Paque\textsuperscript{TM} PREMIUM (GE Healthcare, Uppsala, Sweden) by the density gradient centrifugation method. Cells were dispensed into a 96-well plate at $1 \times 10^5$ cells/ml and cultivated for one day in RPMI 1640 medium (Biowest, USA).

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**Table 1.** Severity of disease in patients participating in the study

| Sample | Psoriasis (PASI)* | Atopic dermatitis (EASI) † |
|--------|-------------------|-----------------------------|
| PBMC   | 1 10.3 18.8       | 2 9.9 13                     |
|        | 3 8.1 15.2       | 4 19.2 21.6                 |
| CD4+ T cell | 1 4.4 10.6 | 2 10.3 11.5                     |
|        | 3 5.7 15.6       | 4 3.2 10.7                  |
|        | 5 3.2 37         | 6 9.8 28.9                 |
|        | 7 14.4 25.5      | 8 13.8 18.8               |
|        | 9 13.2 13        | 10 13.2 37                 |
|        | 11 12.1 29.1     | 12 12 28.9                |
|        | 13 11.5 23.7     | 14 10.3 22.4               |

PBMC: peripheral blood mononuclear cells, PASI: Psoriasis Area and Severity Index, EASI: Eczema Area and Severity Index. *The severity of psoriasis was assessed according to the PASI. †The clinical severity of atopic dermatitis was assessed by the EASI.
Kansas, MO, USA) containing 10% fetal bovine serum (FBS; Biowest) and 1% penicillin/streptomycin (Biowest). To compare the expressions of AhR-related genes, cells were treated with the appropriate dilutions of TCDD (Sigma, Bellefonte, PA, USA). Cells were then cultivated for two days.

**CD4+ T cell culture**

Blood from each subject, 14 AD and 14 PS patients and 14 healthy controls, was drawn and CD4 + T cells were isolated and divided into a control group, TCDD-treated group (10 nmol/L of TCDD, seven patients from each group), and a FICZ-treated group (0.3 μmol/L of FICZ, seven patients from each group). After isolation of PBMC by density gradient centrifugation using Ficoll-Paque™ PREMIUM (GE Healthcare), isolated PBMC were mixed with anti-human CD4+ T cell microbeads (Miltenyi Biotec, Auburn, CA, USA) and incubated at 4°C for 15 minutes. The samples washed with magnetic activated cell sorting (MACS) buffer, and then CD4 + T cells were isolated using a MACS separator. Isolated CD4+ T cells were washed with HBSS (Gibco, Grand Island, NY, USA) and cultured in RPMI 1640 medium (Gibco) containing inactivated 10% FBS (Gibco) and 1% antibiotic-antimycotic (Gibco). Anti-CD3 at 0.5 μg/ml (R&D Systems, Minneapolis, MN, USA) and anti-CD28 (R&D Systems) at 1 μg/ml were added to activate CD4+ T cells (24-well plate, 1×10⁵/well and 6-well plate, 1×10⁶/well, respectively). TCDD and FICZ were diluted to achieve a final concentration of 10 nM. Two days after incubation, both recombinant human IL-23 (PeproTech, Inc., Rocky Hills, NJ, USA) and recombinant human IL-2 (PeproTech) were diluted to achieve a final concentration of 10 nM. At day seven, cell differentiation was analyzed using fluorescence-activated cell sorting, and cytotoxicity was assessed by performing enzyme-linked immunosorbent assay (ELISA) on cell supernatants.

**CD4+ T cell cytokine assay**

To measure the concentration of cytokines such as tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), IL-4, IL-13, IL-17, and IL-22, a cytokine ELISA Immunoassay kit (R&D Systems) was used. The absorbance of the reacted solution was quantified with DTX880 Multimode (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm.

**Immunocytochemistry**

Using Cytopro® Cytocentrifuge (ELITech, Logan, UT, USA), CD4+ T cells were attached to a slide glass. Methanol-fixed cells were washed with phosphate buffered saline (PBS), and treated with Triton X-100 for 10 minutes to detect intracellular materials. To prevent nonspecific antibody binding, pretreatment with bovin serum albumin (BSA) was performed for 30 minutes. Then samples were treated with 1:50 anti-IL-17 (Abcam, Cambridge, MA, USA) and anti-IL-22 (Abcam) for one hour at room temperature. After washing three times with PBS, samples were stained with 1:100 anti-mouse Alexa Fluor 594 and 1:100 anti-rabbit Alexa Fluor 488 for one hour at room temperature. After washing five times using PBS, VECTASHIELD Mounting Medium (Vector, Burlingame, CA, USA) including 4′,6-diamidino-2-phenylindole, dihydrochloride was added. Cells were observed by a Cell® Illuminator system (Olympus, Tokyo, Japan).

**Western blot**

We used radioimmunoprecipitation assay buffer lysis buffer (Biosesang, Seoul, Korea) to extract proteins. Cells were treated with the following primary antibodies: beta-tubulin (Millipore, Billerica, MA, USA), IL-17 (Santa Cruz, Santa Cruz, CA, USA), and IL-22 (Abcam). For horseradish peroxidase (HRP) detection, a chemiluminescent substrate (Thermo, Waltham, MA, USA) was used along with an X-ray film. The protein bands were determined by ImageJ program.

**AhR sequence-specific interfering RNA (siRNA) transfection**

Specific siRNA targeting AhR (Santa Cruz) was used to inhibit gene expression in human PBMC. After one day of cell culture in serum free media, the following processes for transfection were performed using a siRNA reagent system (Santa Cruz). Mixed siRNA for AhR and transfection reagent were reacted for 45 minutes at room temperature, diluted in transfection media, and applied to cells without culture media. After five hours in a CO₂ incubator at 37°C, samples were cultured for one day in media containing FBS and a twofold greater quantity of antibiotics than previously added. Thereafter, culture media was removed, replenished with new media, and cultured for an additional day.

**Quantitative PCR**

Quantitative PCR (qPCR) was performed as previously described. Briefly, mRNA was isolated by using an RNasy Mini Kit (QiAGEN, Hilden, Germany) according to the manufacturer’s instructions and the RNA density and purity were measured using a DU 730 UV/Vis spectrophotometer (Beckman Coulter, St. Charles, IL, USA). Reverse transcription was performed using 400 ng RNA and a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA, USA). cDNA was synthesized in a Veriti™ 96-well Thermal Cycler (Applied
Biosystems). After mixing 2.5 μl synthesized cDNA with a probe and reagent supplied by Quantifast Probe Assay (QiAGEN), qPCR was performed using a Light Cycler 480 II (Roche, Rotkreuz, Switzerland) programmed to 95°C, 30 seconds for denaturation, 62°C, 30 seconds for annealing, and 72°C, ten second for extension for 30 cycles.

**Statistical analysis**

Results were obtained from at least three independent experiments. For statistical analysis, results are shown as mean±standard error of the mean. Using Prism software for Windows (GraphPad Software Inc., La Jolla, CA, USA), statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) for factorial comparisons and by the Tukey’s multiple comparison tests for multiple comparisons. Results of immunohistochemical staining were analyzed using the Mann–Whitney test. The differences in the positivity rates were evaluated with Fisher’s exact test. Statistical analyses were carried out using the SPSS statistical software (ver. 12.0; SPSS Inc., Chicago, IL, USA).

**RESULTS**

**Effect of TCDD on AhR gene expression in PBMC of patients with PS and AD**

Results showed that compared to the levels of AhR in healthy controls, the levels of AhR were higher in patients with PS and lower in those with AD (Fig. 1A). To investigate the effect of TCDD on AhR expression, we performed qPCR, accordingly. These results showed that expression of AhR was higher in TCDD-treated PBMC in PS than in healthy controls (Fig. 1B). These results indicated that TCDD-treated PBMC significantly induced AhR activation in PS compared to healthy controls. To investigate the effect of TCDD on AhR-related factors (CYP1A1,
Effect of silencing AhR on TCDD-treated PBMC of patients with PS and AD

To further confirm the effect of AhR, PBMC were transfected with control siRNA or AhR-siRNA. The results showed that AhR-siRNA significantly inhibited TCDD-induced AhR expression compared with control siRNA (Fig. 2). These results suggest that AhR plays a regulating role in patients with AD and PS. AhR-siRNA significantly inhibited TCDD-induced AhR-related factors (AhR, CYP1A1, IL-17, and IL-22) in patients with AD and PS (Fig. 2B).

Effect of TCDD on AhR gene expression in CD4+ T cells of patients with PS and AD

To investigate the effect of TCDD on AhR expression in CD4+ T cells, we performed qPCR accordingly. Our results showed that the expression of genes coding for AhR-related factors (AhR, CYP1A1, IL-17, and IL-22) was significantly upregulated in TCDD-treated CD4+ T cells of patients with AD and PS than in healthy controls (Fig. 3).

Effect of TCDD on cytokine expression levels in CD4+ T cells

To confirm the effects of AhR activation on the polarization of different T cells, we analyzed the production of IFN-γ, IL-4 and IL-13, IL-17, and TNF-α and IL-22. These results showed that compared to healthy controls, patients with PS had significantly higher levels of TNF-α, IFN-γ, IL-17, and IL-22, and patients with AD had significantly higher levels of IL-4, IL-13, IL-17, and IL-22 (Fig. 4A). TCDD treatment of CD4+ T cells resulted in increased levels of TNF-α, IL-4, IL-13, IL-17, and IL-22, but decreased levels of IFN-γ in healthy controls. The production of

![Fig. 2. Effect of silencing aryl hydrocarbon receptor (AhR) with AhR-specific small interference RNA as determined by quantitative PCR. Cells were transfected with specific interfering control (siControl) or specific interfering AhR (siAhR) for 24 hours and stimulated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for 24 hours. IL: interleukin. Values are presented as mean±standard error of mean. *p<0.05 vs. siControl+TCDD; **p<0.01 vs. siControl+TCDD; †p<0.05 vs. siAhR+TCDD.](image)
TCDD Regulates AhR-Related Factors in AD and PS

**Fig. 3.** mRNA expression levels of aryl hydrocarbon receptor (AhR), CYP1A1, interleukin (IL)-17, and IL-22 in CD4+ T cells from patients with atopic dermatitis (AD), psoriasis (PS), and healthy controls (HC) were examined by quantitative PCR. **p < 0.01 vs. control, ***p < 0.001 vs. control.

TNF-α, IFN-γ, IL-17, and IL-22, but not IL-17, significantly increased in patients with PS. Among TCDD-treated groups, patients with PS showed higher levels of TNF-α, IFN-γ, IL-17, and IL-22, and those with AD showed significantly higher levels of IL-17 than healthy controls (Fig. 4B). Furthermore, the levels of IL-4, IL-13, IL-17, and IL-22 increased among patients with AD, and a significant increase in the production of IL-4 and IL-13 was reported (Fig. 4C). The levels of IL-17 and IL-22 were higher in patients with PS and AD than in healthy controls (Fig. 4D). Production of IL-17 and IL-22 increased in all three groups when CD4+ T cells were treated with TCDD. IL-17 expression was usually detected inside the cells, while IL-22 expression was observed on the cell surface. In CD4+ T cells treated with TCDD, the levels of IL-17 noticeably increased in patients with PS and IL-22 levels prominently increased in patients with AD. However, no differences were observed after day seven (Fig. 4D). Western blot analysis revealed an increase only in IL-17 levels in patients with PS and AD after treatment of CD4+ T cells with TCDD. On the other hand, no bands were observed for IL-22 (Fig. 4E). These results suggest that TCDD significantly regulates the expression of AhR-related factors in CD4+ T cells of PS and AD.

**Effect of FICZ on cytokine expression levels in CD4+ T cells**

In the control group, the levels of TNF-α, IFN-γ, IL-17, and IL-22 were higher in patients with PS, and IL-4, IL-13, IL-17, and IL-22 were higher in patients with AD than in healthy controls (Fig. 5A). After treatment with FICZ, the levels of TNF-α, IFN-γ, IL-4, IL-13, IL-17, and IL-22 decreased in healthy controls; these differences were statistically significant except for IL-17 and IL-22. In patients with PS, the decreases in IFN-γ, IL-17, and IL-22 levels with FICZ treatment were statistically significant, except for TNF-α. Lastly, in patients with AD, the decreases in IL-17 and IL-22 levels with FICZ treatment were statistically significant, except for IL-4 and IL-13 (Fig. 5B). Furthermore, the level of IL-17 was significantly higher in patients with PS than in healthy controls and patients with AD after FICZ treatment (Fig. 5C). These results show that TCDD
Fig. 4. (A–C) Cytokine expression levels of CD4+ T cells after treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Cytokine levels were assessed in the supernatants at day seven of culture. (A) Significantly higher levels of tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-17, and IL-22 were observed among patients with psoriasis (PS) compared to healthy controls (HC). (B) In TCDD(+) groups, the levels of TNF-α, IFN-γ, IL-17, and IL-22 were significantly higher in patients with PS and the level of IL-13 was significantly higher in patients with atopic dermatitis (AD) than in HC. (C) Among TCDD-treated groups, the levels of TNF-α, IFN-γ, and IL-22 were significantly higher in patients with PS and those of IL-4 and IL-13 were significantly increased in patients with AD compared to HC. (D) Intracellular levels of IL-17 and IL-22 in CD4+ T cells from patients with AD, PS, and HC after 24 and 48 hours of treatment with TCDD. After 24 hours treatment of CD4+ T cells with TCDD, the production of IL-17 noticeably increased in patients with PS, and IL-22 levels prominently increased in patients with AD. Scale bar = 25 μm. (E) Western blot results of IL-17 in CD4+ T cells from patients with AD, PS, and HC after one, two, and seven days of treatment with TCDD. After treatment of CD4+ T cells with TCDD, the production of IL-17 noticeably increased in patients with PS and AD. *Statistically significant (p < 0.05).
and FICZ inversely regulated the production of cytokines in CD4+ T cells.

**DISCUSSION**

Our previous study demonstrated higher expression levels of AhR-related factors in lesional skin from patients with PS and AD than in samples from the control group. We investigated the role of AhR and its ligands in PBMC from AD and PS, and analyzed the polarization of CD4+ T cells. The TCDD treatment of PBMC from patients with PS resulted in an increase in the expression levels of AhR-related factors, IL-22 and IL-17, and the same findings were observed for IL-22 in PBMC from patients with AD. Activated PBMC from patients with PS and AD showed higher expression levels of AhR-related factors and cytokines such as IL-22 and IL-17 than those from healthy controls. After TCDD treatment, the levels of AhR-related factors, IL-22 and IL-17, showed greater differences in patients with PS and AD than in healthy subjects. To confirm the effects of AhR activation on the polarization of different T cells, we analyzed the production of IFN-γ, IL-4 and IL-13, IL-17, and TNF-α and IL-22. ELISA revealed that treatment with TCDD induced an increase in the production of TNF-α, IFN-γ, and IL-22 by CD4+ T cells in patients with PS, and IL-4 and IL-13 by CD4+ T cells in patients with AD. Notably, the production of IL-22 and IL-4 increased in both patient types. On the other hand, FICZ treatment decreased the levels of IFN-γ, IL-17, and IL-22 in patients with PS, and IL-17 and IL-22 in patients with AD. These results show that TCDD and FICZ reciprocally regulated the production of cytokines in CD4+ T cells. PS is sustained by pro-inflammatory CD4+ T helper cells mainly belonging to the Th1, Th17, and Th22 lineages. In AD, Th22 cells play a role in skin barrier impairment through IL-22, and AD is often consid-
ered as a Th2/Th22-dominant allergic disease. While PS is emerging as an IL-23/Th17-skewed disease and AD is considered as a Th2-centered disease, both share a common Th22 component. In AD, the importance of Th17 appears to differ depending on race. Recent data suggest that AhR is mainly involved in the development of Th17 and Th22 subsets. Lack of AhR was shown to cause hyper-inflammation, whereas AhR activation with FICZ improved the inflammatory profile in both human PS skin samples and a mouse model of PS skin inflammation. These results are consistent with our study results with FICZ, which reduced the production of circulating inflammatory cytokines, and TCDD induced the differentiation of T cells into Th22 cells and increased the production of IL-22. The role of AhR ligands present in cigarette smoke on immune activation and pathogenesis of PS and AD has been revealed. Tobacco smoke extract induced Th17 generation from central memory T cells in vitro and increased IL-17 and IL-22 expression, resulting in PS exacerbation. Although AhR has been consistently involved in IL-22 production by T cells, its role in IL-17 production is still controversial. For instance, TCDD and FICZ have been reported to induce IL-17 production in mice but inhibit IL-17 level in humans. According to a previous study that generated transgenic mice constitutively expressing AhR in keratinocyte, severe skin lesions with itching resemble typical AD developed postnatally in mice. More AhR studies should be done in AD patients in the future. Our conclusion is that TCDD-induced AhR gene induction in AD and PS patients may increase the expression of AhR-regulatory genes, thereby contributing to the development of AD and PS.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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