



FICZ, a Tryptophan photoproduct, suppresses pulmonary eosinophilia and Th2-type cytokine production in a mouse model of ovalbumin-induced allergic asthma

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ABSTRACT

Most studies about functions of aryl hydrocarbon receptor (AhR) in the pathogenesis of asthma have been carried out with non-physiological industrial by-products such as 2,3,7,8-tetrachlorodibenzo-p-dioxin and benzo(a)pyrene. In the present study, effects of 6-formylindolo[3,2-b]carbazole (FICZ), a tryptophan photoproduct postulated as a candidate physiological ligand of AhR, on the pathogenesis of asthma were examined and then underlying mechanisms of its immunomodulatory effects were investigated. FICZ significantly reduced pulmonary eosinophilia and Th2 cytokine expression in the lungs. Flow cytometric analysis of mediastinal lymph nodes showed that IL-4 producing cells decreased in FICZ-treated mice compared with PBS control. Next, effects of FICZ on *in vitro* Th2 differentiation and expression of the Th2 transcription factor GATA-3 were examined. CD4⁺ T cells were isolated from the spleen and incubated under the Th2 differentiation conditions. FICZ inhibited both Th2 differentiation and the expression of GATA-3. Finally, activation of STAT6, which is necessary for Th2 differentiation, was inhibited by FICZ.

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1. Introduction

The AhR is a ligand-activated transcription factor that belongs to the basic region-helix-loop-helix (bHLH) superfamily of DNA binding proteins [1]. It contains two highly conserved PAS (Period clock, Aryl hydrocarbon nuclear transporter, and Single-minded) domains, which confer gene target specificity via cofactor association [2]. Unbound AhR is found in the cellular cytoplasm chaperoned by heat shock protein 90, p23 and AhR-interacting protein [3,4]. Once a ligand binds to the AhR, the ligand-receptor complex is translocated to the nucleus where it forms a heterodimeric complex with a second bHLH transcription factor, the Ah receptor nuclear translocator (ARNT) [5]. This complex binds to aromatic hydrocarbon-responsive elements (AhRes, also called XREs or DREs) in the 5' flanking region of target genes such as CYP1A1, glutathione S-transferase and aldehyde dehydrogenase and acts as a transcriptional activator [6,7].

AhR is a cytosolic sensor of small synthetic compounds and natural chemicals, regulating cell growth and differentiation. Many studies about AhR have been performed with halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo(a)pyrene (B(a)P), a class of structurally related compounds that are widespread environmental contaminants. TCDD is the most

potent congener of the HAHs, causing a broad spectrum of toxic effects including immune, reproductive, and developmental toxicity [8]. In mice, TCDD has been shown to induce immune dysfunction characterized by thymus atrophy and suppression of specific effector functions associated with helper T cells, cytotoxic T cells and B cells [9,10]. Although TCDD is widely used as a surrogate ligand for AhR, interpretation of results studied with TCDD needs caution because it is not quickly metabolized in the body [11]. Its half life appears to be correlated to body weight, about 2 weeks in mice and several years in humans. AhR knockout mice exhibit hepatic defects, skin abnormalities, lower life expectancy, and immunological abnormalities without exposure to exogenous chemicals, suggesting important roles in many physiological functions [12–14]. A number of low-molecular weight, structurally diverse chemicals, including indoles, tetrapyroles, archidonic acid metabolites, and tryptophan metabolites, have been identified as naturally occurring exogenous and endogenous AhR ligands by analyzing their binding affinity to AhR and activation of AhR [15,16]. However, it remains to be determined which of these are actually relevant in a physiological sense.

Asthma, which is characterized by the infiltration of the airways with various cell types including eosinophils, mast cells, and T lymphocytes leading to airway obstruction and airway remodeling, is mainly developed by T helper (Th) 2 lymphocytes secreting IL-4 and IL-5 [17–20]. The incidence of allergic diseases in the developed world has increased over the last few decades and chemicals of low molecular weight (<1000 Da) may be an important contributor to this phenomenon. TCDD was shown to suppress allergic immune responses in mice [21,22]. Curcumin, an AhR ligand rich in Indian

Abbreviations: FICZ, 6-formylindolo[3,2-b]carbazole; OVA, ovalbumin.

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spice, attenuated ovalbumin-induced airway inflammation [23], providing evidence for the involvement of AhR ligands in the pathogenesis of allergy. However, there is no report on roles of AhR physiological ligands in allergic diseases.

FICZ is generated from tryptophan by ultraviolet B irradiation [24,25], and its metabolite has been identified in human urine [26]. FICZ has a higher AhR binding affinity than TCDD and activates AhR, inducing CYP1A1 mRNA expression [24,25]. Interestingly, AhR ligands are differentially involved in CD4+ T cell differentiation [27,28]. TCDD induces regulatory T cells, whereas FICZ stimulates Th17 differentiation. Although effects of UV irradiation on asthma in humans remain controversial [29], UV irradiation inhibited asthmatic phenotypes in a mouse model of asthma, reducing effector CD4+ T cells in the airways [30,31]. In the present study, we examined therapeutic effects of FICZ on OVA-induced allergic asthma in mice and investigated mechanisms underlying the anti-allergic function of FICZ.

2. Materials and methods

2.1. Mice

BALB/c mice, 6–12 weeks of age, were purchased from the Korean Institute for Chemistry (Taejeon, Korea). The animals were housed 5 mice per cage in a laminar air flow room maintained at $22 \pm 2^\circ\text{C}$ with relative humidity of $55 \pm 5\%$. Mice were cared and treated in accordance with the guidelines established by the Changwon National University public health service policy on the use of laboratory animals. The animal study was performed in the immunology laboratory, Department of Biology, Changwon national University.

2.2. Chemicals and reagents

FICZ and IL-4 were purchased from Enzo Life Sciences (USA) and from R & D (USA), respectively.

2.3. Antigen sensitization, challenge and administration of FICZ

Sensitization and challenge with OVA was previously described [32]. On day 0, mice were sensitized with an intraperitoneal (i.p.) injection of $15\ \mu\text{g}$ of OVA and $2\ \text{mg}$ of alum dissolved in $0.2\ \text{ml}$ PBS. The animals received a booster injection of this alum-OVA mixture 5 days later. Twelve days after the first sensitization, the mice were exposed for 1 h to aerosolized OVA (0.5% dissolved in 0.9% saline) which was produced by an ultrasonic nebulizer at a flow rate of $6\ \text{l/min}$. At the 13th day, mice were challenged again with OVA as performed on day 12. Mice were sacrificed at day 14. FICZ ($0.3 - 30\ \mu\text{g/kg}$ body weight) in $25\ \mu\text{l}$ DMSO was i.p. injected at day 0.

2.4. Bronchoalveolar lavage fluid (BALF) preparation

The tracheas of mice were cannulated and lavaged with two $0.8\ \text{ml}$ aliquots of cold PBS. Number of cells in the BALF was counted and used for cytospin and staining.

2.5. Cytospin preparations and Giemsa staining

Cells (4×10^4 cells) were centrifuged in $100\ \mu\text{l}$ PBS with 3% BSA onto a microscope slide using a CellSpin (Hanil Science, Korea). Cells were fixed in methanol, and stained with 'Hemacolor for microscopy' and identified under the microscope according to the criteria described in the protocol (Merck, USA).

2.6. Measurement of total and OVA-specific IgE in plasma

The concentration of IgE was determined by a sandwich ELISA, using a combination of a captured antibody to mouse IgE and a biotinylated

antibody to mouse IgE. For total IgE assay, a combination of affinity-purified rat mAb to mouse IgE (clone 23 G3, eBioscience, USA) and biotin-conjugated rat mAb to mouse IgE (clone RME-1, Biolegend, USA). Briefly, microtiter plates were coated overnight with $1\ \mu\text{g/ml}$ of anti-mouse IgE in $0.1\ \text{M}$ carbonate buffer (pH 9.6) at 4°C . Nonspecific binding was blocked with 3% bovine serum albumin for 1 h at room temperature. After incubating with test sera for 2 h, plates were incubated with biotin-anti-mouse IgE for 1 h and subsequently with streptavidin-HRP for 1 h. The reaction was developed using 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Triplicate samples were diluted 1:10–1:100. Ig levels in each sample were measured from optical density readings at 405 nm, and Ig concentrations were calculated from a standard curve generated using purified IgE. For OVA-specific IgE titration, plates were coated with OVA ($20\ \mu\text{g/ml}$) and biotin-conjugated rat mAb to mouse IgE (clone RME-1) was used as detection antibodies. Levels of OVA-specific antibodies were compared with IgE standards with predetermined concentrations ($\text{IgE} = 1\ \mu\text{g/ml}$). The concentration of standard serum was arbitrarily assigned as 1 ELISA unit (1 EU).

2.7. RNA preparation, RT-PCR, and real-time PCR

Total cellular RNA was extracted from cells using the RNeasy method (TEL-TEST, INC., USA). For PCR analysis, RNA was used after contaminating DNA was completely removed by DNase I treatment. RT-PCR analysis was performed using pairs of oligonucleotide primers. The PCR products were confirmed to correspond to their original sequence by DNA sequencing. Gene specific primers, number of cycles of amplification, annealing temperature, and expected size of PCR product are listed in Table 1. Real-Time PCR was performed to quantify PCR products. Power SYBR green PCR Master Mix and Real-time PCR system (7300, Applied Biosystems, USA) were used.

2.8. Western blotting

Cells or tissues were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), $50\ \mu\text{M}$ sodium vanadate, 20 mM p-nitrophenylphosphate, 50 mM sodium fluoride, leupeptin ($0.5\ \mu\text{g/ml}$), aprotinin ($10\ \mu\text{g/ml}$), and soybean trypsin inhibitor ($10\ \mu\text{g/ml}$). Proteins size-fractionated on SDS/PAGE were transferred to PVDF membranes, and the blots were blocked with 3% bovine serum albumin in TBS buffer (20 mM Tris-HCl pH 7.5/137 mM NaCl). The blots were sequentially treated with primary and secondary antibodies in TBST (20 mM Tris-HCl pH 7.5/137 mM NaCl/0.1% Tween 20) with intermittent washing with TBST. Immunodetection was performed with the ECL-plus kit (Amersham Pharmacia Biotech, USA). Antibodies used in the present study are: rabbit anti-human phosphorylated Tyr 641 of STAT6 polyclonal Ab and rabbit anti-human phosphorylated Tyr 694 of STAT5a/b polyclonal Ab (Santa Cruz Biotechnology (USA)), rabbit anti-STAT6 polyclonal Ab and rabbit anti-STAT5a/b polyclonal Ab (Cell Signaling Technology (USA)). HRP-

Table 1
Primers used in RT-PCR and real-time PCR.

Name	Nucleotide sequence	Annealing temperature ($^\circ\text{C}$)	Cycles ^a	Size ^b (bp)
mHGPRTfw	5'-gctggtgaaaaggacctctc-3'	60	25	248
mHGPRTrv	5'-cacaggactagaacacctgc-3'			
mgata3fw	atgcctgcggactctacat	60	30	289
mgata3rv	cgcgtcatgcacctttttgc			
mAhRfw	5'-ggccaagagctcttttgatg-3'	59	30	305
mAhRrv	5'-ctgggttttagagccacgtga-3'			
mCyp1A1-fw	5'-caccatccccacagcac-3'	60	35	125
mCyp1A1-rv	5'-tcgtttgggtcaccacacag-3'			

^a Indicates the number of cycles of amplification.

^b Indicates the expected size of PCR products.

linked anti-rabbit IgG (1:1000) was from Cell Signaling Technology (USA).

2.9. Helper T cell differentiation *in vitro*

Naïve CD4⁺CD62L⁺ Th cells were prepared from murine spleen using the CD4⁺CD62L⁺ T cell isolation kit II (Milteyi Biotec, Germany). Briefly, non-Th cells as well as regulatory T cells and TCR γ/δ ⁺ T cells are depleted by indirect magnetic labeling using a cocktail of lineage-specific biotin conjugated antibodies against CD8a, CD45R, CD49b, CD11b, and Ter-119, as well as antibodies against CD25 and TCR γ/δ in combination with anti-biotin microbeads. Subsequently, CD4⁺CD62L⁺ T cells are positively selected from the enriched CD4⁺ Th cell fraction with CD62L microbeads. For Th differentiation, 1–6 × 10⁵ naïve T cells in 0.4 ml culture medium were seeded into a well of the 48-well plate pre-coated with anti-CD3 (3 μ g/ml) and anti-CD28 (2 μ g/ml), stimulated in the presence of : anti-IL-12 (10 μ g/ml) and anti-IFN- γ (10 μ g/ml) (Th0); anti-IL-4 (10 μ g/ml) and IL-12 (10 ng/ml) (Th1); anti-IL-12 (10 μ g/ml), anti-IFN- γ (10 μ g/ml), IL-4 (10 ng/ml), and IL-2 (5 ng/ml) (Th2); anti-IL-4 (2 μ g/ml), anti-IFN- γ (2 μ g/ml), IL-6 (30 ng/ml), and TGF- β (2.5 ng/ml) (Th17); TGF- β (10 ng/ml) and IL-2 (5 ng/ml) (Treg), for 3 days (RNA analysis), and stimulated for 2 more days after being supplemented with 0.4 ml differentiation medium (FACS analysis).

2.10. Intracellular staining and FACS analysis

Intracellular IL-4/ IFN- γ staining was carried out using BD Cytofix/ Cytoperm Fixation/Permeabilization Kit (BD Biosciences, USA). Briefly, cells are first fixed and permeabilized with Fixation/Permeabilization solution and then stained with fluorochrome-conjugated anti-IL-4 or -IFN- γ antibodies.

2.11. Statistical analysis

Data were analyzed with the paired Student's *t* test. The level of significance was between $p < 0.05$ and < 0.001 .

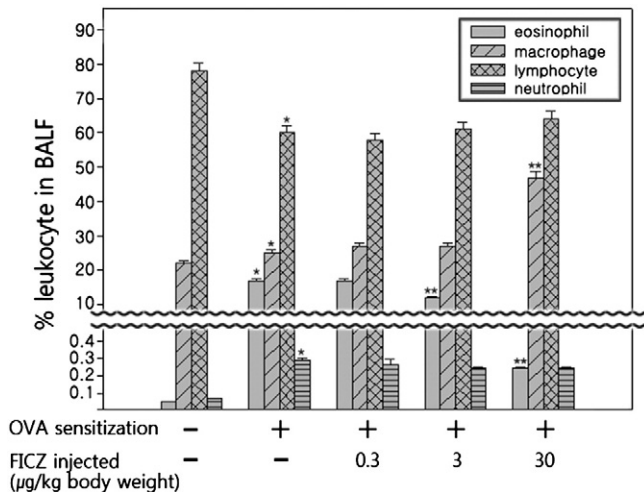


Fig. 1. FICZ reduced eosinophil infiltration into the lungs. Mice were sensitized with alum-OVA mixture on days 0 and 5, and challenged on days 12 and 13. On day 14, mice were sacrificed for collection of BALF. FICZ (0.3–30 μ g/kg body weight) was i.p. injected once at day 0. Cells were fixed in methanol, and stained with 'Hemacolor for microscopy' and identified under the microscope according to the criteria described in the protocol (Merck, USA). Results are presented as mean $\pm$ SD ($n = 3$ –5 mice per group) and are representative of two separate experiments. Statistical significance was analyzed using the Student's *t*-test. * $p < 0.05$ compared with unsensitized control. ** $p < 0.05$ compared with OVA-sensitized without FICZ injection.

Table 2

Number of cells and eosinophils recovered from BALF.

Group		Total cells $\times 10^5$	Eosinophiles
OVA sensitization	FICZ(μ g/kg body weight)		
-	-	1.56 $\pm$ 0.25	78 $\pm$ 12
+	0	1.87 $\pm$ 0.31	28050 $\pm$ 212
+	0.3	1.88 $\pm$ 0.27	28032 $\pm$ 324
+	3	1.73 $\pm$ 0.25	22049 $\pm$ 251
+	30	1.72 $\pm$ 0.29	860 $\pm$ 11

Mice were sensitized and challenged as described in Fig. 1 legend. Results are presented as mean $\pm$ SD ($n = 3$ –5 mice per group) and are representative of two separate experiments.

3. Results

3.1. Reduced pulmonary eosinophilia in BALB/c mice treated with FICZ

Mice were sensitized with alum-precipitated OVA and challenged with aerosolized OVA. 24 hrs after the last challenge of OVA, mice were sacrificed to collect BALFs. First, proportions of components of white blood cells were examined. In BALF samples from OVA-sensitized mice, proportions of eosinophils and neutrophils significantly increased, while lymphocyte proportion slightly decreased (Fig. 1). When FICZ was i.p. administered at concentrations ranging from 0.3 to 30 μ g/kg body weight, percentages of eosinophils dose-dependently decreased whereas macrophage proportions slightly increased. Then total cells as well as eosinophils were counted in BALF. As shown in Table 2, total number of cells in BALF was increased in OVA-sensitized mice (1.87×10^5) compared with unsensitized control mice (1.56×10^5). When FICZ was i.p. administered at concentrations ranging from 0.3 to 30 μ g/kg body weight, total number of cells slightly decreased. Eosinophil accumulation in BALF markedly increased in OVA-sensitized mice compared with unsensitized control mice. FICZ was very effective in preventing pulmonary eosinophilia, greatly reducing the number of infiltrating eosinophils.

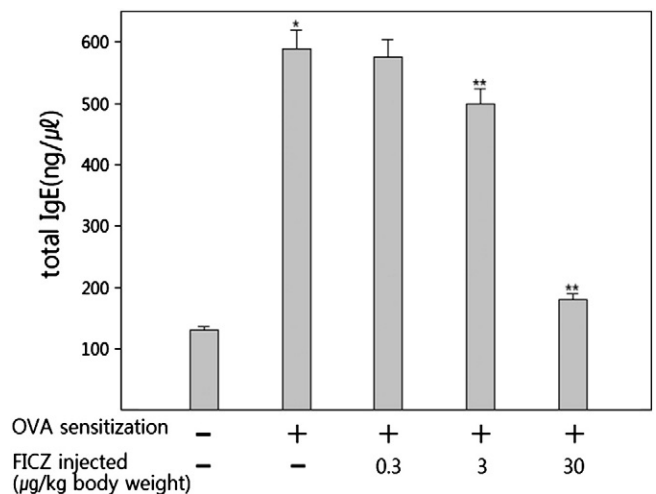


Fig. 2. FICZ reduced the plasma level of total IgE in OVA-sensitized mice. Mice were sensitized with alum-OVA mixture on days 0 and 5, and challenged on days 12 and 13. On day 14, mice were sacrificed for serum harvest. FICZ (0.3–30 μ g/kg body weight) was i.p. injected once at day 0. Sera were prepared and assayed for total IgE by ELISA. Results are presented as mean $\pm$ SD ($n = 3$ –5 mice per group) and are representative of two separate experiments. Statistical significance was analyzed using the Student's *t*-test. * $p < 0.05$ compared with unsensitized control. ** $p < 0.05$ compared with OVA-sensitized without FICZ injection.

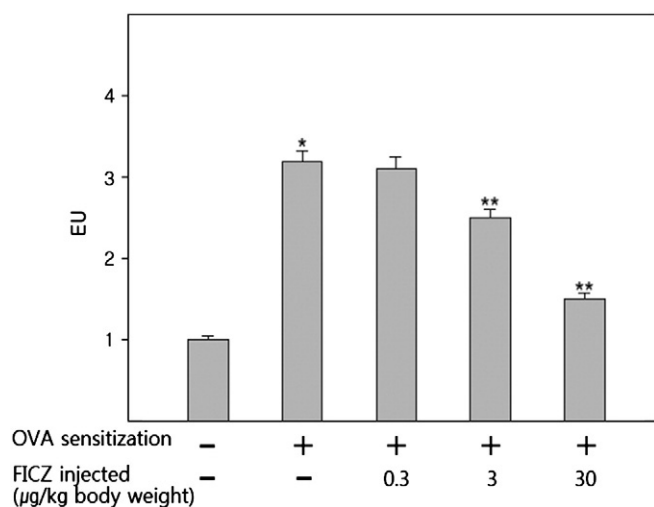


Fig. 3. FICZ reduced the plasma level of OVA-specific IgE in OVA-sensitized mice. Mice were sensitized with alum-OVA mixture on days 0 and 5, and challenged on days 12 and 13. On day 14, mice were sacrificed for serum harvest. FICZ (0.3–30 µg/kg body weight) was i.p. injected once at day 0. Sera were prepared and assayed for OVA-specific IgE by ELISA. Results are presented as mean ± SD ($n=3-5$ mice per group) and are representative of two separate experiments. Statistical significance was analyzed using the Student's *t*-test. * $p<0.05$ compared with unsensitized control. ** $p<0.05$ compared with OVA-sensitized without FICZ injection.

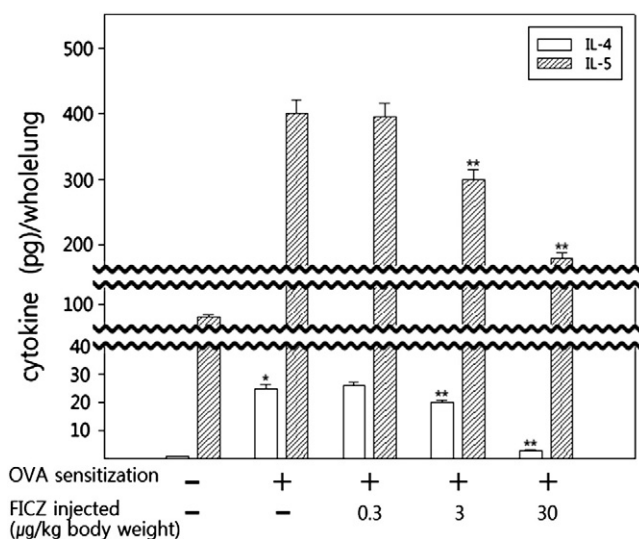


Fig. 5. IL-4 and IL-5 generation in lung tissue. Mice were sensitized with alum-OVA mixture on days 0 and 5, and challenged on days 12 and 13. FICZ (0.3–30 µg/kg body weight) was i.p. injected once at day 0. On day 14, mice were sacrificed, and lung extracts were prepared and assayed by ELISA. Three mice were chosen for each group and results are presented as mean ± SD of two independent experiments. Statistical significance was analyzed using the Student's *t*-test. * $p<0.05$ compared with unsensitized control. ** $p<0.05$ compared with OVA-sensitized without FICZ injection.

3.2. Reduced total and OVA-specific IgE concentration in mice treated with FICZ

Next, we measured serum levels of total and OVA-specific IgE in mice which were sensitized with alum-precipitated OVA and challenged with aerosolized OVA. The serum levels of IgE antibodies were determined 24 hrs after the last challenge of OVA. Unsensitized BALB/c mice produced lower levels of total IgE (about 130 ng/ml of serum) whereas the OVA group produced significantly increased levels of total IgE (about 590 ng/ml of serum) (Fig. 2). Levels of total IgE dose-dependently decreased when FICZ was i.p. administered at concentrations ranging from 0.3 to 30 µg/kg body weight. Next, OVA-specific IgE levels were examined. In OVA-sensitized mice the level of OVA-specific IgE, which increased about three-fold over unsensitized controls, dose-dependently decreased upon FICZ administration (Fig. 3).

3.3. Expression of IL-4 and IL-5 is suppressed in mice injected with FICZ

As cytokines including IL-4 and IL-5 are closely associated with the pathogenesis of asthma, we next examined expression of IL-4 and IL-5 and TNF-α in the lung. Twenty four hrs after the last challenge of OVA, lungs from the unsensitized, OVA-sensitized, or OVA-sensitized and FICZ-injected groups were harvested for RT-PCR analysis. Three mice from each group were randomly selected and their lungs were assayed for cytokine expression (Fig. 4). All three mice of the OVA-sensitized group expressed significantly increased levels of IL-4 and IL-5 mRNAs over the unsensitized group. When FICZ was injected to OVA-sensitized mice, the levels of the cytokine mRNAs decreased in a dose-dependent manner. In contrast, expression of IFN-γ, a Th1 type cytokine, of which production is stimulated by IFN-γ, was also increased in the OVA-sensitized group compared to the unsensitized group. However, FICZ injection was not able to

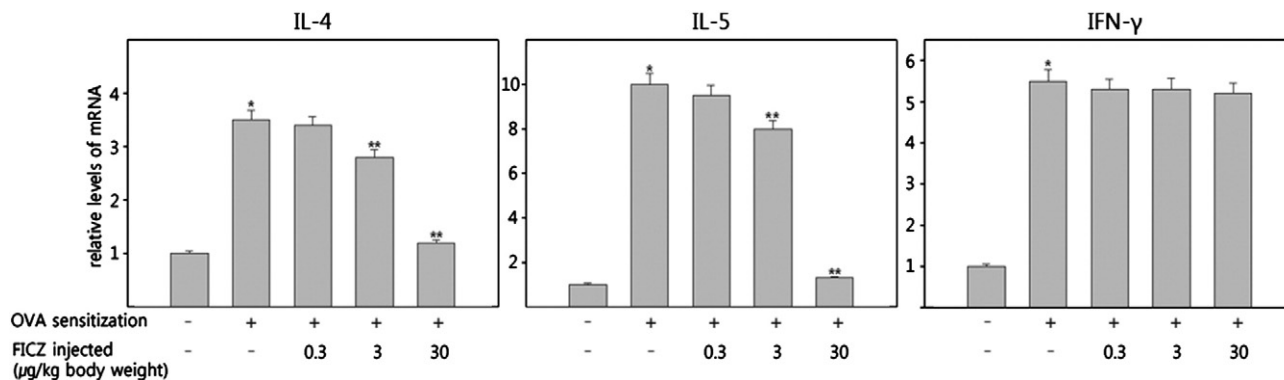


Fig. 4. Effects of FICZ on the expression of cytokines in the lung. Mice were sensitized with alum-OVA mixture on days 0 and 5, and challenged on days 12 and 13. On day 14, mice were sacrificed for lung tissue. FICZ (0.3–30 µg/kg body weight) was i.p. injected once at day 0. RNA was prepared from the lungs and examined for gene expression by real-time PCR. Three mice were chosen for each group and results are presented as mean ± SD of two independent experiments. Hypoxanthine guanine phosphoribosyl transferase (HGPRT) was used as an internal control. Statistical significance was analyzed using the Student's *t*-test. * $p<0.05$ compared with OVA-sensitized but not injected with FICZ.

revert the increased level of IFN- γ to that of the unsensitized group. Next, the amount of IL-4 and IL-5 protein was directly measured in the lung to confirm the RNA results. Protein levels of IL-4 and IL-5, which increased upon OVA-sensitization, dose-dependently reduced by FICZ administration (Fig. 5).

3.4. FICZ reduces the number of IL-4-producing T cells in mediastinal lymph nodes

To ask whether FICZ specifically skewed Th2 response *in vivo*, Th polarization was analyzed. Twenty four hrs after the last challenge of OVA, mediastinal lymph nodes were harvested and proportions of IL-4 producing cells in them were flow-cytometrically analyzed after being light-scatter gated for lymphocytes. The population of IL-4 producing CD4 T cells increased from 0.32 to 0.57% in OVA-sensitized animals (Fig. 6a). FICZ reduced Th2 population in a dose-dependent manner (Fig. 6b).

3.5. FICZ suppresses Th2 differentiation *in vitro*

Next, we asked whether FICZ modulates Th2 differentiation *in vitro*. Naïve CD4⁺CD62L⁺ T cells were cultured with FICZ under Th2 differentiation conditions for 5 days and proportions of IL-4-producing cells were flow-cytometrically analyzed after being light-scatter gated for lymphocytes. Proportions of IL-4-producing cells increased from 0.03 to 1.24% when naïve T cells were induced to differentiate into Th2 cells (Fig. 7a). FICZ dose-dependently suppressed the Th2 differentiation (Fig. 7b). Next, expression of GATA-3, a transcription factor for Th2 differentiation, was examined. GATA-3 expression, which was increased upon Th2 differentiation (Fig. 8a), was dose-dependently inhibited by FICZ treatment (Fig. 8b).

3.6. Activation of STAT6 is inhibited by FICZ

Activation of STAT5a and STAT6 is associated with IL-4-mediated Th2 differentiation [33]. Thus, we examined if the inhibition of Th2

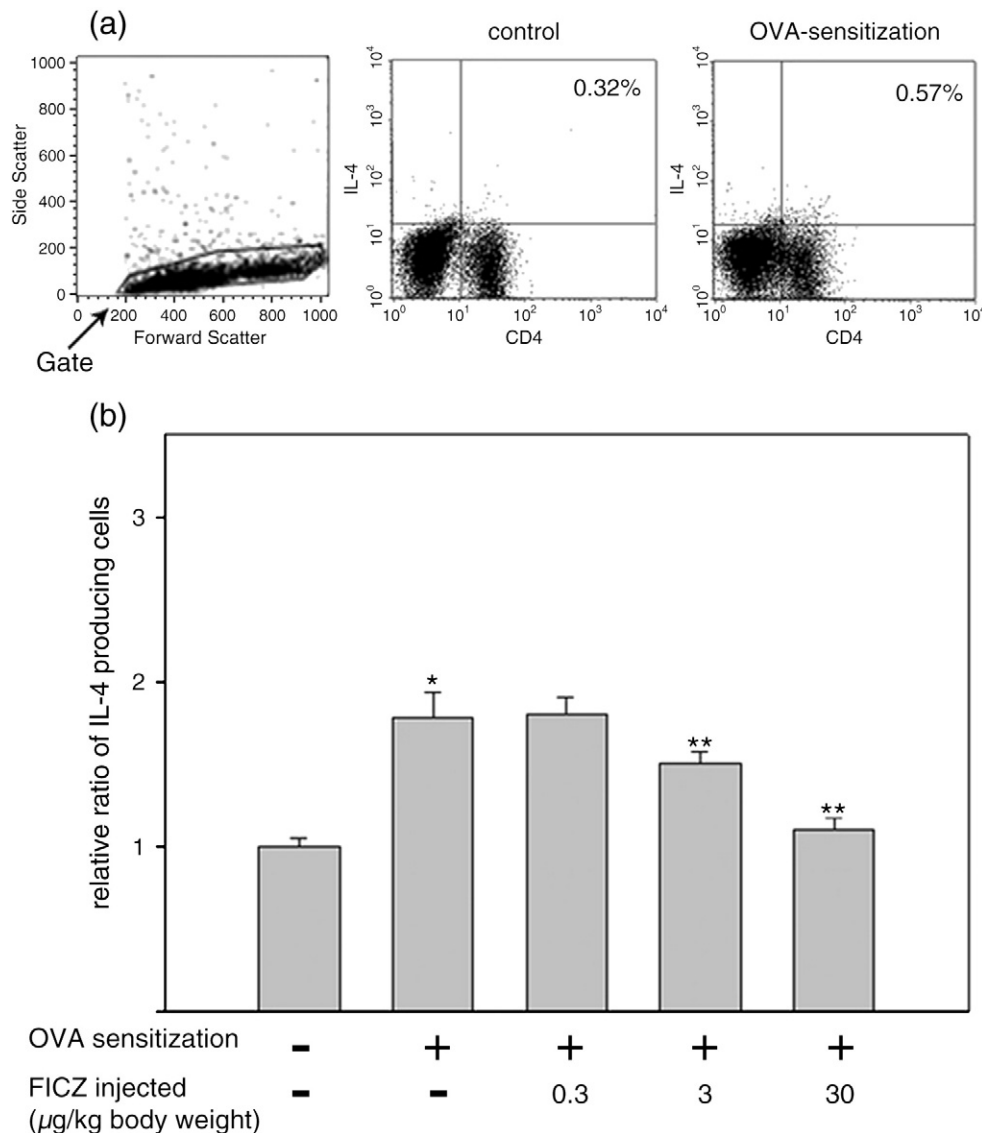


Fig. 6. Increase of IL-4 producing cells in the mediastinal lymph nodes upon OVA sensitization was repressed by FICZ. Mice were sensitized with OVA as described in Fig. 1 and mediastinal lymph nodes were collected on day 4, stimulated with phorbol-12,13-dibutyrate (500 ng/ml) and ionomycin (500 ng/ml) in the presence of Golgi Stop protein for 6 h, stained with FITC-anti-CD4 and PE-anti-IL-4, and flow-cytometrically analyzed after being light-scatter gated for lymphocytes (a). FICZ (0.3–30 $\mu\text{g/kg}$ body weight) was i.p. injected once at day 0 (b). Three mice were chosen for each group and results are presented as mean $\pm$ SD of two independent experiments. Statistical significance was analyzed using the Student's *t*-test. * $p < 0.05$ compared with unsensitized control. ** $p < 0.05$ compared with OVA-sensitized without FICZ injection.

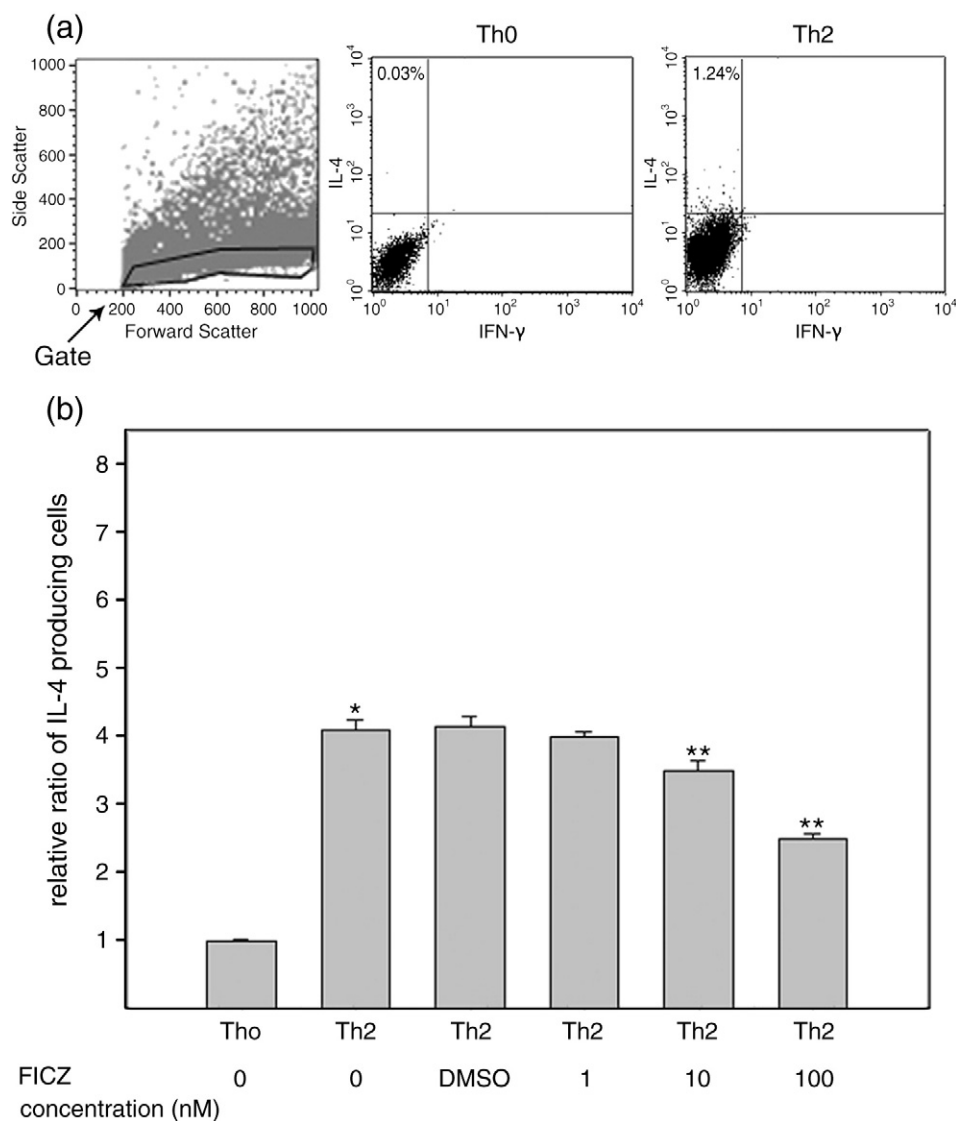


Fig. 7. FICZ inhibits in vitro Th2 differentiation. 1×10^5 naive CD4⁺ T cells in 0.4 ml culture medium were seeded into a well of the 48-well plate pre-coated with anti-CD3 (3 μ g/ml) and anti-CD28 (2 μ g/ml), stimulated in the presence of anti-IL-12 (10 μ g/ml), anti-IFN- γ (10 μ g/ml), IL-4 (10 ng/ml), and IL-2 (5 ng/ml) for first 3 days and two more days after being supplemented with IL-2 (10 ng/ml), IL-4 (10 ng/ml) and anti-IFN- γ (10 μ g/ml) in 0.4 ml culture medium. For Th0, anti-IFN- γ (10 μ g/ml) and anti-IL-4 (10 μ g/ml) were supplemented. Then, cells were harvested, stimulated with phorbol-12,13-dibutyrate (500 ng/ml) and ionomycin (500 ng/ml) in the presence of Golgi Stop protein for 6 h, intracellularly stained with FITC-anti-IFN- γ and PE-anti-IL-4, and flow-cytometrically analyzed after being light-scatter gated for lymphocytes (a). FICZ was added to the culture at the beginning of differentiation in the range of 1–100 nM and 0.01% DMSO was added as vector control (b). Results are presented as mean $\pm$ SD of three independent experiments. * $p < 0.05$, compared with Th0, ** $p < 0.05$, compared with DMSO.

differentiation by FICZ results from improper signaling transduction pathways. Naïve CD4⁺CD62L⁺ T cells were cultured under Th2 differentiation conditions in the presence or absence of 100 nM FICZ for 30 min. Then, protein extracts were examined for phosphorylation of STAT5a and STAT6, indicative of activation of both STATs, by western blotting. FICZ inhibited activation of STAT6, but not of STAT5a (Fig. 9).

3.7. AhR functions in Th2 cells

AhR is differentially expressed in T cells: highly in Th17 and Treg cells but little in Th1 and Th2 cells [27,28,34]. But there is no report whether AhR is functional under the Th2 differentiation condition. Naïve CD4⁺CD62L⁺ T cells were cultured with FICZ under Th differentiation conditions for 3 days and expression of AhR was examined by RT-PCR. The level of AhR mRNA was highest in Th17 cells followed by Treg cells (Fig. 10a, b). When AhR mRNA was quantitated by real-

time PCR, there was a 2–3-fold increase in Th1 and Th2 cells compared with Th0 cells. Then, up-regulated expression of Cyp1A1, indicative of functional AhR, was observed when Th2 cells were exposed to 100 nM FICZ (Fig. 10c, d), suggesting that AhR functions in Th2 cells.

4. Discussion

In this study, we demonstrated that FICZ, a tryptophan photoproduct postulated as a candidate physiological ligand of AhR, significantly reduced pulmonary eosinophilia, serum total and OVA-specific IgE concentration, and Th2 cytokine expression in the lungs of mice sensitized and challenged by OVA, and that number of IL-4 producing T cells in mediastinal lymph nodes was decreased in FICZ-injected mice. In Th differentiation experiments in vitro, FICZ inhibited Th2 differentiation, regulating GATA-3 expression and STAT6 activation. We also demonstrated that AhR was functional under the Th2 differentiation condition, inducing Cyp1A1 expression.

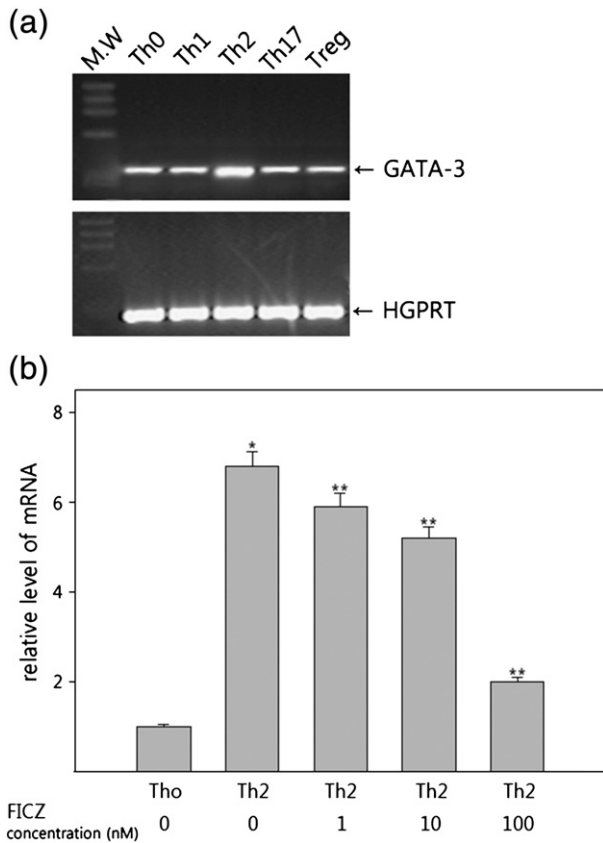


Fig. 8. GATA-3 expression was inhibited by FICZ. Naive CD4⁺ T cells purified from the spleen were differentiated into different lineages under specific conditions appropriate for each lineage as described in the *Materials & Methods*. 2×10^5 cells in 0.5 ml RPMI 1640 culture medium containing 10% FBS and 50 μ M β -mercaptoethanol were seeded into a well of the 48-well plate pre-coated with anti-CD3 (3 μ g/ml) and stimulated in the presence of soluble anti-CD28 (1 μ g/ml) for 3 days under lineage-specific conditions. Then RNA was isolated and analyzed (a). FICZ was added to the culture at the beginning of differentiation in the range of 1–100 nM and 0.01% DMSO was added as vector control (b). Results are presented as mean $\pm$ SD of three independent experiments. * $p < 0.05$, compared with Th0, ** $p < 0.05$, compared with DMSO.

There have been a few studies that AhR ligands have protective effects against allergic asthma. TCDD, and curcumin and quercetin, which are present in vegetables and medicinal plants, were shown to suppress allergic immune responses in mice [21–23,35]. AhR ligands

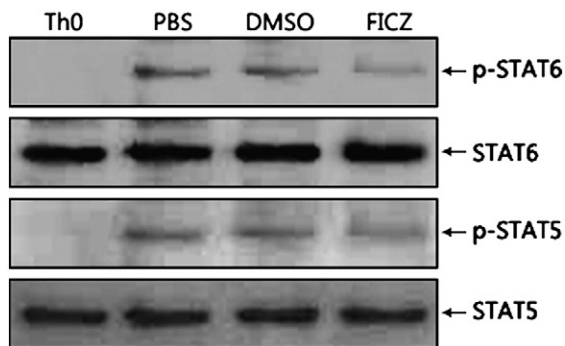


Fig. 9. Activation of STAT6 is inhibited by FICZ. Naive CD4⁺ T cells purified from the spleen were differentiated into Th2 lineage as described in the *Materials & Methods*. 2×10^5 cells in 0.5 ml RPMI 1640 culture medium containing 10% FBS and 50 μ M β -mercaptoethanol were seeded into a well of the 48-well plate pre-coated with anti-CD3 (3 μ g/ml) and stimulated in the presence of soluble anti-CD28 (1 μ g/ml) for 2 days and then cultured for 30 min under the lineage-specific conditions: for Th2, anti-IL-12 (10 μ g/ml), anti-IFN- γ (10 μ g/ml), IL-4 (10 ng/ml), and IL-2 (5 ng/ml); and for Th0, anti-IFN- γ (10 μ g/ml) and anti-IL-4 (10 μ g/ml). Then protein was isolated and subjected to western blotting analysis using antibodies against STAT5a, p-STAT5a, STAT6, and p-STAT6.

have been shown to play different roles in Th differentiation. Quercetin reduced IL-4 production and expression of GATA3, a Th2 transcription factor, in lung tissues of ovalbumin-sensitized and -challenged mice while increasing IFN- γ production and expression of T-bet, a Th1 transcription factor [35]. Effects of curcumin remain controversial. In a mouse model of asthma, curcumin inhibited recruitment of eosinophils into the lung airways, airway hyper-responsiveness, and expression of IL-4 [23] whereas curcumin regulated shift from Th1 to Th2 in trinitrobenzene sulphonic acid-induced chronic colitis [36]. TCDD induced regulatory T cells suppressing experimental allergic encephalomyelitis (EAE) in mice whereas FICZ interfered with the development of regulatory T cells but boosted Th17 differentiation aggravating EAE [27]. However, in a study of in vitro T cell differentiation, TCDD and FICZ both showed common effects, enhancing the differentiation of Th17 and regulatory T cells [34]. In the present study, FICZ was shown to inhibit in vivo and in vitro Th2 differentiation by repressing activation of STAT6 and expression of GATA3, providing more evidence that AhR ligands exert differential effects on Th differentiation.

UV irradiation to the skin is well known to modulate the immune system, inducing inflammation at higher doses while suppressing immunity and inducing synthesis of mediators including vitamin D at doses much lower than those required to cause inflammation [29,37,38]. So far, most studies about effects of UV radiation on the immune system have been centered on vitamin D, because the level of vitamin D is drastically increased upon UV radiation. 1,25-dihydroxyvitamin D3, the most active vitamin D metabolite, increases number and function of regulatory T cells while inhibiting differentiation of dendritic cells as well as Th1 and Th17 differentiation. Besides vitamin D, production of immune mediators including cisurocanic acid, nitric oxide, reactive oxygen species, prostaglandin E2, and IL-10 is also increased [37]. In animal studies, UV radiation inhibited allergic airways disease [30,31]. UVB irradiation of skin significantly suppressed airway hyperresponsiveness to methacholine- and ovalbumin-specific responses in the lung-draining lymph nodes and in the lung compartment by reducing effector CD4⁺ T cells [30,31]. In addition, UV irradiation of immunized mice induced type 1 regulatory T cells that suppressed tumor antigen specific cytotoxic T lymphocyte responses [39]. However, mediators associated with the immunosuppression were not identified.

FICZ is formed upon exposure of solutions of tryptophan [24], cell culture medium [25], or cells [40] to ultraviolet radiation [24,25], and its metabolites have been identified in human urine [26]. FICZ activates AhR transiently inducing CYP1A1 mRNA expression in human keratinocytes [24,25,41,42]. In addition, UVB exposure of human skin induced CYP1A1 and CYP1B1 expression, raising a possibility that tryptophan photoproducts including FICZ are involved in AhR activation [43].

The rate of conversion from tryptophan to FICZ is about 0.0001% in cells and *ex vitro* [40,44] and the turnover rate is high with a decrease by 81% after 24 h of incubation [44]. At present, it remains to be seen how much of FICZ is produced in the skin exposed to UV and how relevant the results in this study is to asthma incidence in humans, because of differences in tryptophan metabolism among mammalian species [45,46].

Although AhR mainly exerts its function being complexed with ARNT, accumulating evidence suggests that AhR have been shown to directly or indirectly interact with transcriptional activators or repressors [see references in 35]. In co-transfection experiments, AhR have been shown to directly bind to basal transcription factors, including TFIIB, TBP, TFIIF, to transcription factor Sp1, and to HAT coactivators such as CBP, SRC-1, RIP-140, RB, and Myb-binding protein 1a. In other set of experiments analyzing endogenous AhR/ARNT-responsive genes using chromatin immunoprecipitation (ChIP) assay, RNA interference, and real-time PCR, AhR and ARNT were shown to bind various transcription factors. P160 HAT coactivators SRC-1, NCoA-2, and p/CIP all associate with the mouse Cyp1a1 enhancer

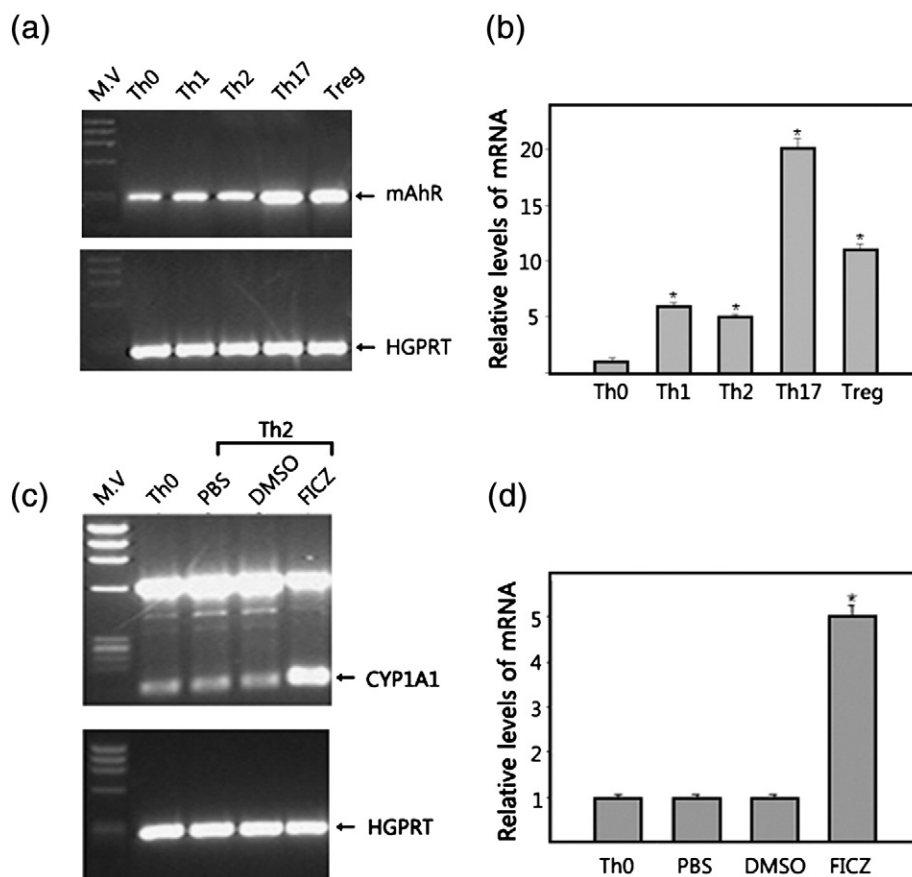


Fig. 10. AhR is functional Th2 cells. Naive CD4⁺ T cells purified from the spleen were differentiated into different lineages under specific conditions appropriate for each lineage as described in the Materials & Methods. 2×10^5 cells in 0.5 ml RPMI 1640 culture medium containing 10% FBS and 50 μ M β -mercaptoethanol were seeded into a well of the 48-well plate pre-coated with anti-CD3 (3 μ g/ml) and stimulated in the presence of soluble anti-CD28 (1 μ g/ml) for 3 days under lineage-specific conditions. Then RNA was isolated and analyzed by RT-PCR (a,c) and quantitated by real-time PCR (b,d). Results are presented as mean $\pm$ SD of three independent experiments. * $p < 0.05$, compared with Th0.

region after treatment of Hepa-1 cells with TCDD [47,48]. Brahma-related gene (Brg)-1, an ATPase-dependent chromatin modifier, also was shown to associate with the enhancer region of the mouse Cyp1a1 gene in vivo in a TCDD- and ARNT-dependent manner and to interact with the transcriptional activation domain of AhR [49]. Endogenous AhR in Hepa cells was also shown to interact with mediator subunits Med130 and Med220 in a TCDD-dependent manner [50]. In addition, AhR interacts with STAT1 and STAT5 under the Th17 differentiation conditions, regulating activation of STAT1 [34]. It remains to be seen whether AhR interacts with STAT6 in the Th2 differentiation conditions and if so, whether FICZ is necessary for the interaction.

In the present study FICZ was shown to inhibit activation of STAT6 while displaying little effect on STAT5a activation, suggesting that AhR ligands may exert differential effects on STAT activation. TCDD stimulates Treg differentiation, whereas FICZ stimulates Th17 differentiation [27,28], suggesting that different AhR ligands exert differential effects on helper T cell differentiation, possibly by modulating STAT activation in ligand-specific or Th lineage-specific ways. In preliminary studies performed in our lab, FICZ was shown to exert opposing effects on STAT activation, stimulating STAT3 activation under the Th17 differentiation condition while inhibiting STAT5 activation under the Treg differentiation condition (manuscript in preparation).

FICZ shows a 4–7-fold higher binding affinity to AhR than the prototypical AhR ligand TCDD [51]. FICZ is rapidly degraded through a NADPH-dependent metabolism in vitro and five major metabolites produced by several enzymes including Cyp1A1, Cyp1A2, and epoxide hydrolase have been detected and chemically identified [52], suggesting that FICZ fits into a model in which the ligand-activated AhR signaling is autoregulated by the induced metabolic enzymes.

Accumulating evidence indicates that there are various endogenous AhR ligands including indoles, tetrapyrroles, and other arachidonic metabolites, which are more physiologically relevant than prototypical AhR ligands such as TCDD and benzo(a)pyrene [53]. It remains to be seen what effects these endogenous ligands have on Th differentiation.

In conclusion, the results obtained in this study suggest that FICZ, which is synthesized in the skin exposed to UV radiation, has anti-asthmatic effects, inhibiting Th2 cytokine production leading to reduced pulmonary eosinophilia and IgE serum levels. In addition, FICZ also interferes with Th2 differentiation, likely by inhibiting activation of STAT-6 and expression of GATA3. The results presented in this study support epidemiological studies and animal studies that UV radiation may help protect against asthma [29–31,37,54], although the relationship between the prevalence of asthma and geographical latitude is still controversial: both positive [54,55] and negative associations [56,57] between them have been reported in the published literature. So far, most studies about effects of AhR ligands on the immune system have been performed with environmental contaminants such as TCDD, which is rarely metabolized. To understand inherent functions of AhR in the immune system, studies using physiological ligands such as FICZ should be performed along with prototypical ligands like TCDD.

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The authors declare that there are no conflicts of interest.

References

- [1] Burbach KM, Poland A, Bradfield CA. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc Natl Acad Sci U S A* 1992;89:8185–9.
- [2] Gu YZ, Hogenesch JB, Bradfield CA. The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol* 2000;40:519–61.
- [3] Chen HS, Perdew G. Subunit composition of the heteromeric cytosolic aryl hydrocarbon receptor complex. *J Biol Chem* 1994;269:27554–8.
- [4] Ma Q, Whitlock JP. A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Biol Chem* 1997;272:8878–84.
- [5] Swanson HI, Bradfield CA. The Ah-receptor: Genetics, structure and function. *Pharmacogenetics* 1993;3:213–30.
- [6] Rushmore TH, Pickett CB. Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene: Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J Biol Chem* 1990;265:14648–53.
- [7] Favreau LV, Pickett CB. Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Identification of regulatory elements controlling basal level expression and inducible expression by planar aromatic compounds and phenolic antioxidants. *J Biol Chem* 1991;266:4556–61.
- [8] Huff J, Lucier G, Tritscher A. Carcinogenicity of TCDD: experimental, mechanistic, and epidemiologic evidence. *Annu Rev Pharmacol Toxicol* 1994;34:343–72.
- [9] Poland A, Knutson JC. 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu Rev Pharmacol Toxicol* 1982;22:517–54.
- [10] Kerkvliet NL, Burleson GR. Immunotoxicity of TCDD and related halogenated aromatic hydrocarbons. In: Dean JH, Luster MI, Munson AE, Kimber I, editors. *Immunotoxicology and Immunopharmacology*. NY: Raven Press; 1994. p. 97–121.
- [11] Miniero R, De Felip E, Ferri F, di Domenico A. An overview of TCDD half-life in mammals and its correlation to body weight. *Chemosphere* 2001;43:839–44.
- [12] Matsumura F, Puga A, Tohyama C. Biological functions of the arylhydrocarbon receptor: beyond induction of cytochrome P450s. Introduction to this special issue. *Biochem Pharmacol* 2009;77:473.
- [13] Singh KP, Casado FL, Opanashuk LA, Gasiewicz TA. The arylhydrocarbon receptor has a normal function of hematopoietic and other stem/progenitor cell populations. *Biochem Pharmacol* 2009;77:577–87.
- [14] Shi LZ, Faith NG, Nakayama Y, Suresh M, Steinberg H, Czuprynski CJ. The arylhydrocarbon receptor is required for optimal resistance to *Listeria monocytogenes* infection in mice. *J Immunol* 2007;179:6952–62.
- [15] Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol* 2003;43:309–34.
- [16] Nguyen LP, Bradfield CA. The search for endogenous activators of the aryl hydrocarbon receptor. *Chem Res Toxicol* 2008;21:102–16.
- [17] Walker C, Kaegi M, Braun P, Blaser K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J Allergy Clin Immunol* 1991;88:935–42.
- [18] Elwood W, Barnes PJ, Chung KF. Airway hyperresponsiveness is associated with inflammatory cell infiltration in allergic brown-Norway rats. *Int Arch Allergy Immunol* 1992;99:91–7.
- [19] Lundgren JD, Shelhamer JH. Pathogenesis of airway mucus hypersecretion. *J Allergy Clin Immunol* 1990;85:399–404.
- [20] Elias JA. Airway remodeling in asthma. Unanswered questions. *Am J Respir Crit Care Med* 2000;161:168–71.
- [21] Luebbe RW, Copeland CB, Daniels M, Lambert AL, Gilmour MI. Suppression of allergic immune responses to house dust mite (HDM) in rats exposed to 2,3,7,8-TCDD. *Toxicol Sci* 2001;62:71–9.
- [22] Fujimaki H, Nohara K, Kobayashi T, Suzuki K, Eguchi-Kasai K, Tsukumo S, et al. Effect of a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin on immune function in male NC/Nga mice. *Toxicol Sci* 2002;66:117–24.
- [23] Moon DO, Kim MO, Lee HJ, Choi YH, Park YM, Heo MS, et al. Curcumin attenuates ovalbumin-induced airway inflammation by regulating nitric oxide. *Biochem Biophys Res Commun* 2008;375:275–9.
- [24] Rannug A, Rannug U, Rosenkranz HS, Wingvist L, Westerholm R, Agurell E, et al. Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. *J Biol Chem* 1987;262:15422–7.
- [25] Oberg M, Bergander L, Håkansson H, Rannug U, Rannug A. Identification of the tryptophan photoproduct 6-formylindolol[3,2-b]carbazole, in cell culture medium, as a factor that controls the background aryl hydrocarbon receptor activity. *Toxicol Sci* 2005;85:935–43.
- [26] Wincent E, Amini N, Luecke S, Glatt H, Bergman J, Crescenzi C, et al. The suggested physiologic aryl hydrocarbon receptor activator and cytochrome P4501 substrate 6-formylindolol[3,2-b]carbazole is present in humans. *J Biol Chem* 2009;284:2690–6.
- [27] Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 2008;453:65–71.
- [28] Veldhoen M, Hirota K, Westendorp AM, Buer J, Dumoutier L, Renauld JC, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 2008;453:106–9.
- [29] Norval M. The challenges of UV-induced immunomodulation for children's health. *Prog Biophys Mol Biol* 2011;107:323–32.
- [30] McGlade JP, Gorman S, Larcombe AN, Sly PD, Finlay-Jones JJ, Turner DJ, et al. Suppression of the asthmatic phenotype by ultraviolet B-induced antigen-specific regulatory cells. *Clin Exp Allergy* 2007;37:1267–76.
- [31] McGlade JP, Strickland DH, Lambert MJM, Gorman S, Thomas JA, Judge MA, et al. UV inhibits allergic airways disease in mice by reducing effector CD4+ T cells. *Clin Exp Allergy* 2010;40:772–85.
- [32] Lee JA, Sung HN, Jeon CH, Gill BC, Oh GS, Youn HJ, et al. A carbohydrate fraction, AIP1 from *Artemisia iwayomogi* suppresses pulmonary eosinophilia and Th2-type cytokine production in an ovalbumin-induced allergic asthma. Down regulation of TNF- α expression in the lung. *Int Immunopharmacol* 2008;8:117–25.
- [33] Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol* 2003;3:721–32.
- [34] Kimura A, Naka T, Nohara K, Fujii-Kuriyama Y, Kishimoto T. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc Natl Acad Sci U S A* 2008;105:9721–6.
- [35] Park HJ, Lee CM, Jung ID, Lee JS, Jeong YI, Chang JH, et al. Quercetin regulates Th1/Th2 balance in a murine model of asthma. *Int Immunopharmacol* 2009;9:261–7.
- [36] Zhang M, Deng CS, Zheng JJ, Xia J. Curcumin regulated shift from Th1 to Th2 in trinitrobenzene sulphonic acid-induced chronic colitis. *Acta Pharmacol Sin* 2006;27:1071–7.
- [37] Hart PH, Gorman S, Finlay-Jones JJ. Modulation of the immune system by UV radiation: more than just the effects of vitamin D. *Nat Rev Immunol* 2011;11:584–96.
- [38] Halliday GM, Lyons JG. Inflammatory doses of UV may not be necessary for skin carcinogenesis. *Photochem Photobiol* 2008;84:272–83.
- [39] Toda M, Wang L, Ogura S, Torii M, Kurachi M, Kakimi M, et al. UV irradiation of immunized mice induces type 1 regulatory T cells that suppress tumor antigen specific cytotoxic T lymphocyte responses. *Int J Cancer* 2011;129:1126–36.
- [40] Fritsche E, Schafer C, Calles C, Bernsmann T, Bernshausen T, Wurm M, et al. Lightening up the UV response by identification of the arylhydrocarbon receptor as a cytoplasmic target for ultraviolet B radiation. *Proc Natl Acad Sci U S A* 2007;104:8851–6.
- [41] Wei Y-D, Helleberg H, Rannug U, Rannug A. Rapid and transient induction of CYP1A1 gene expression in human cells by the tryptophan photoproduct 6-formylindolol[3,2-b]carbazole. *Chem Biol Interact* 1998;110:39–55.
- [42] Nair S, Kekatpure VD, Judson BL, Rifkin AB, Granstein RD, Boyle JO, et al. UVR exposure sensitizes keratinocytes to DNA adduct formation. *Cancer Prev Res* 2009;2:895–902.
- [43] Katiyar SK, Matsui MS, Mukhtar H. Ultraviolet-B exposure of human skin induces cytochromes P450 1A1 and 1B1. *J Invest Dermatol* 2000;114:328–33.
- [44] Diani-Moore S, Ma Y, Labitzke E, Tao H, David Warren J, Andersen J, et al. Discovery and biological characterization of 1-(1H-indol-3-yl)-9H-pyrido[3,4-b]-indole as an aryl hydrocarbon receptor activator generated by photoactivation of tryptophan by sunlight. *Chem Biol Interact* 2011;193:119–28.
- [45] Le Floch N, Otten W, Merlot E. Tryptophan metabolism, from nutrition to potential therapeutic applications. *Amino Acids* 2011;41:1195–205.
- [46] Carlson JR, Yokoyama MT, Dickinson EO. Induction of pulmonary edema and emphysema in cattle and goats with 3-methylindole. *Science* 1972;176:298–9.
- [47] Hankinson O. Role of coactivators in transcriptional activation by the aryl hydrocarbon receptor. *Arch Biochem Biophys* 2005;433:379–86.
- [48] Beischlag TV, Wang S, Rose DW, Torchia J, Reisz-Porszasz S, Muhammad K, et al. Recruitment of the NCoA/SRC-1/p160 family of transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator complex. *Mol Cell Biol* 2002;22:4319–33.
- [49] Wang S, Hankinson O. Functional involvement of the Brahma/SWI2-related gene 1 protein in cytochrome P4501A1 transcription mediated by the aryl hydrocarbon receptor complex. *J Biol Chem* 2002;277:11821–7.
- [50] Wang S, Ge K, Roeder RG, Hankinson O. Role of mediator in transcriptional activation by the aryl hydrocarbon receptor. *J Biol Chem* 2004;279:13593–600.
- [51] Wei YD, Helleberg H, Rannug U, Rannug A. Rapid and transient induction of CYP1A1 expression in human cells by the tryptophan photoproduct 6-formylindolol[3,2-b]carbazole. *Chem Biol Interact* 1998;110:39–55.
- [52] Bergander L, Wahlstrom N, Alsberg T, Bergman J, Rannug A, Rannug U. Characterization of in vitro metabolites of the aryl hydrocarbon receptor ligand 6-formylindolol[3,2-b]carbazole by liquid-mass spectrometry and NMR. *Drug Metab Dispos* 2003;31:233–41.
- [53] Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol* 2003;43:309–34.
- [54] Krstic G. Asthma prevalence associated with geographical latitude and regional insulation in the United States of America and Australia. *PLoS One* 2011;6:e18492(1–9).
- [55] Franco JM, Gurgel R, Sole D, Lucia Franca V, Brabin B, Brazilian ISAAC Group. Socio-environmental conditions and geographical variability of asthma prevalence in Northern Brazil. *Allergol Immunopathol (Madr)* 2009;37:116–21.
- [56] Staples JA, Ponsonby AL, Lim LL, McMichael AJ. Ecologic analysis of some immune-related disorders, including type 1 diabetes, in Australia: latitude, regional ultraviolet radiation, and disease prevalence. *Environ Health Perspect* 2003;111:518–23.
- [57] Zanolin ME, Pattaro C, Corsico A, Bugiani M, Carrozzi L, Casali L, et al. The role of climate on the geographic variability of asthma, allergic rhinitis and respiratory symptoms: results from the Italian study of asthma in young adults. *Allergy* 2004;59:306–14.