

Non-dioxin-like AhR Ligands in a Mouse Peanut Allergy Model

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Recently, we have shown that AhR activation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) suppresses sensitization to peanut at least in part by inducing a functional shift toward CD4⁺CD25⁺Foxp3⁺ T cells. Next to TCDD, numerous other AhR ligands have been described. In this study, we investigated the effect of three structurally different non-dioxin-like AhR ligands, e.g., 6-formylindolo[3,2-*b*]carbazole (FICZ), β -naphthoflavone (β -NF), and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF), on peanut sensitization. Female C57BL/6 mice were sensitized by administering peanut extract (PE) by gavage in the presence of cholera toxin. Before and during peanut sensitization, mice were treated with FICZ, β -NF, or 6-MCDF. AhR gene transcription in duodenum and liver was investigated on day 5, even as the effect of these AhR ligands on CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in spleen and mesenteric lymph nodes (MLNs). Mice treated with TCDD were included as a positive control. Furthermore, the murine reporter cell line H1G1.1c3 (CAFLUX) was used to investigate the possible role of metabolism of TCDD, FICZ, β -NF, and 6-MCDF on AhR activation *in vitro*. TCDD, but not FICZ, β -NF, and 6-MCDF, suppressed sensitization to peanut (measured by PE-specific IgE, IgG1, IgG2a and PE-induced interleukin (IL)-5, IL-10, IL-13, IL-17a, IL-22, and interferon- γ). In addition, FICZ, β -NF, and 6-MCDF treatments less effectively induced AhR gene transcription (measured by gene expression of AhR, AhRR, CYP1A1, CYP1A2, CYP1B1) compared with TCDD-treated mice. Furthermore, FICZ, β -NF and 6-MCDF did not increase the percentage of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in spleen and mesenteric lymph nodes compared with PE-sensitized mice, in contrast to TCDD. Inhibition of metabolism *in vitro* increased AhR activation. Together, these data shows that TCDD, but not FICZ, β -NF, and 6-MCDF suppresses sensitization to peanut. Differences in metabolism, AhR binding and subsequent gene transcription might explain these findings and warrant further studies to investigate the role of the AhR in food allergic responses.

Key Words: aryl hydrocarbon receptor; peanut sensitization; food allergy; FICZ; β -NF; 6-MCDF; TCDD; CD4⁺CD25⁺Foxp3⁺ T_{reg} cells.

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor and is best known for its role in mediating the toxicity of xenobiotics such as halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs). Exposure to the HAH 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent AhR ligand known, results in the induction of a range of species- and tissue-specific toxic and biological effects, the majority of which are AhR-dependent (Denison and Nagy, 2003). One of these effects is the suppression of immune responses, including T-cell dependent immune responses (e.g., food allergy) (Kerkvliet, 1995).

Food allergy affects about 5% of young children and 3–4% of adults, and the prevalence is increasing. Most of these people have only mild reactions after exposure to a food allergen, but in severe cases, anaphylaxis can be developed (Koplin *et al.*, 2011; Lee and Burks, 2009; Shaker and Woodmansee, 2009; Sicherer and Sampson, 2010). Peanut allergy is responsible for the majority of fatal food-induced allergic reactions (Sicherer and Sampson, 2010). Recently, we have shown that activation of the AhR by TCDD suppresses sensitization to peanut and that this is partly mediated by the induction of a functional shift within the CD4⁺ population toward CD4⁺CD25⁺Foxp3⁺ T_{reg} cells (Schulz *et al.*, 2011). Similar effects of this persistent AhR ligand on T_{reg} cells have been described previously in a mouse model for experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveoretinitis and graft versus host disease (GvHD) (Funatake *et al.*, 2005; Marshall *et al.*, 2008; Quintana *et al.*, 2008; Zhang *et al.*, 2010). Like TCDD, an increase of the percentage of T_{reg} cells has been observed with the AhR ligands VAG539, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) and indirubin in a GvHD model, EAE model and in healthy mice, respectively (Hauben *et al.*, 2008; Quintana *et al.*, 2010; Zhang *et al.*, 2007). In addition, it has been shown that the AhR ligands 6-formylindolo[3,2-*b*]carbazole (FICZ) (in the presence of transforming growth factor- β) and kynurenine induce Foxp3⁺

T_{reg} cells *in vitro* (Kimura *et al.*, 2008; Mezrich *et al.*, 2010). Furthermore, FICZ (administrated ip) has been shown to ameliorate trinitrobenzene sulfonic acid (TNBS)-induced colitis and dextran sulfate sodium (DSS)-induced colitis by suppressing inflammatory cytokines and increasing interleukin (IL)-22 (Monteleone *et al.*, 2011). The AhR ligand β -naphthoflavone (β -NF) has also been demonstrated to attenuate DSS-induced colitis (Furumatsu *et al.*, 2011). In contrast, activation of the AhR by FICZ has also been described to induce Th17 cells *in vitro* and to enhance disease in an EAE model by inducing Th17 cells (Mezrich *et al.*, 2010; Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). Thus, depending on the AhR ligand and the disease, activation of the AhR appears to result in attenuation or initiation of T-cell dependent immune responses. Therefore, we investigated whether the non-dioxin-like AhR ligands FICZ, β -NF, and 6-methyl-1,3,8-trichlorodibenzofuran (6-MCDF) suppress peanut sensitization similar to the classical AhR ligand TCDD.

MATERIALS AND METHODS

Mice and reagents. Female C57BL/6 mice (4–5 week old) purchased from Charles River (France) were maintained under controlled conditions (relative humidity of 50–55%, 12 h light/dark cycle, temperature of 23°C $\pm$ 2°C) in filter-topped macrolon cages with wood chip bedding. Food pellets and drinking water were available *ad libitum*. Prior to the start of the experiments, mice were acclimatized. All experiments were approved by the animal experiments committee of the Faculty of Veterinary Medicine, Utrecht University.

2,3,7,8-TCDD (Cambridge Isotope Lab) was dissolved in anisole (Sigma-Aldrich, The Netherlands) at 20.5 μ g/ml and diluted in corn oil (Sigma-Aldrich) to the final exposure concentration (0.07% vol/vol anisole). Anisole diluted in corn oil was used as vehicle control (0.07% vol/vol). FICZ (Biomol International) was dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml and diluted in corn oil to the final exposure concentration (1% vol/vol DMSO). For oral treatment, FICZ was dissolved in DMSO at 2 mg/ml and diluted in corn oil to the final exposure concentration (2.5% vol/vol DMSO). β -NF (Sigma-Aldrich) was dissolved in DMSO at 20, 60, or 200 mg/ml and diluted with corn oil to the final exposure concentration (2.5% vol/vol DMSO). 6-MCDF was dissolved directly in corn oil to the final exposure concentration. Final exposure concentrations of FICZ, β -NF, and 6-MCDF were based on previously published *in vivo* studies (Bannister *et al.*, 1989; McDougal *et al.*, 2001; Quintana *et al.*, 2008; Sugihara *et al.*, 2008).

Peanut extract (PE) (30 mg/ml) was prepared from peanuts from the Golden Peanut Plant (provided by Intersnack Nederland BV, The Netherlands) as described previously (Van Wijk *et al.*, 2005). Peanut extracts were prepared according to standard procedures and checked for protein content by BCA analysis (Pierce, Rockford, IL). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc. (Campbell, CA).

Experimental design. According to our standard PE sensitization protocol, mice were sensitized to PE by intragastric exposure to PE (6 mg PE, 200 μ l/mouse) with CT (15 μ g/mouse) on three consecutive days (day 0, 1, and 2) followed by weekly dosing (days 7, 14, 21, and 28). One day before termination, mice were challenged intragastrically with PE (12 mg/mouse). Mice were killed on day 31 or day 36 by cervical dislocation and blood and spleens were isolated. From experience, we know that the day of termination (31 or 36) has no influence on the results obtained with this peanut allergy model. Before and during sensitization to peanut, mice ($n = 6-8$ per group) were treated with TCDD (15 μ g/kg body weight [BW]) by gavage, on days -1 and 11, FICZ (50 μ g/kg BW, ip, on days -1 and 11 or on days -1, 2, 6, 9, 13, 16, 20, 23, 27, 30, and 33) (Fig. 1, setup 1), β -NF (5, 15, or 50 mg/kg BW, by

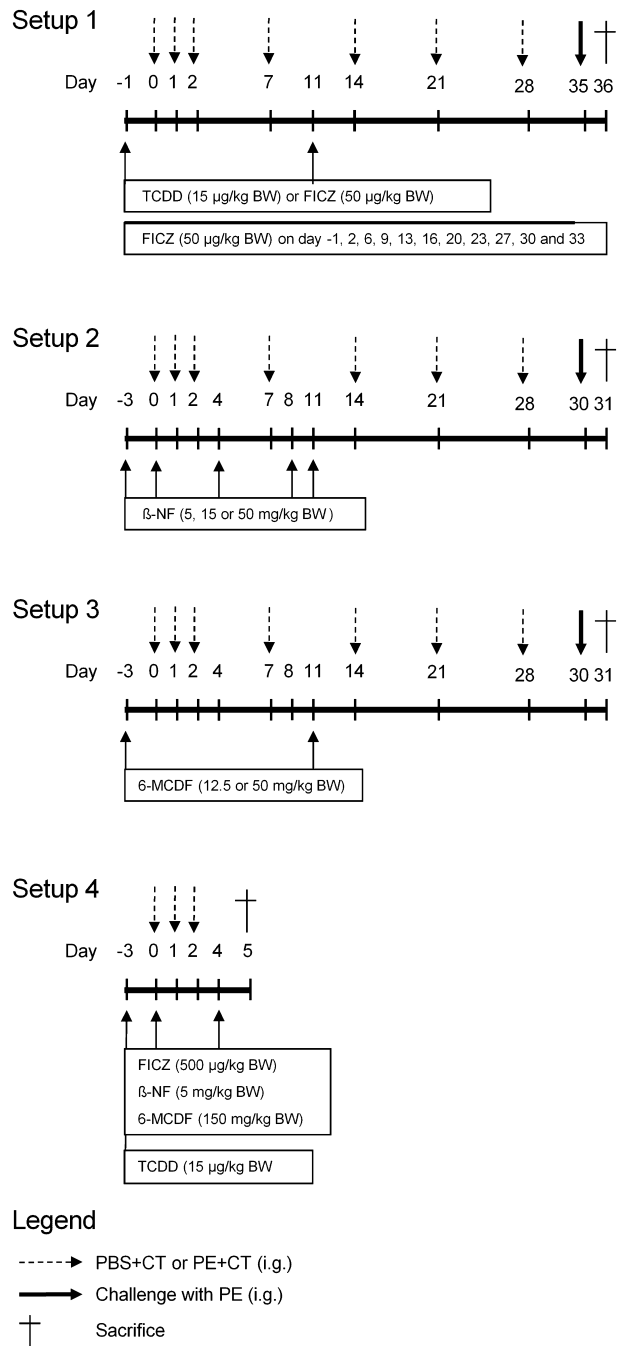


FIG. 1. Treatment protocols as described in the Materials and Methods section.

gavage, on days -3, 0, 4, 8, and 11) (Fig. 1, setup 2), or 6-MCDF (12.5 or 50 mg/kg BW, by gavage, on days -3 and 11) (Fig. 1, setup 3). To detect the effect of FICZ, β -NF, and 6-MCDF on CD4⁺CD25⁺Foxp3⁺ T_{reg} cells, early cytokine production and AhR-related gene expression, mice were exposed before and during initiation of sensitization to peanut to FICZ (500 μ g/kg BW), β -NF (5 mg/kg BW), or 6-MCDF (150 mg/kg BW) by gavage on days -3, 0, and 4. TCDD (15 μ g/kg BW) was given by gavage only on day -3. On day 5, mice were sacrificed by cervical dislocation and blood, spleen, mesenteric lymph nodes (MLN), duodenum, and liver were isolated (Fig. 1, setup 4).

Preparation of liver microsomes. Excised livers were homogenized in Tris/HCl (50mM, 1.15% KCl, pH = 7.4). The microsomal fraction was obtained from the homogenate by successive centrifugation for 25 min at $9000 \times g$ and 85 min at $100,000 \times g$ with a Beckman Coulter Optima L-90 K centrifuge. The microsomal fraction was resuspended in a sucrose solution (0.25M). Protein concentration of the microsomes was determined by the method of Lowry using bovine serum albumin (BSA) as protein standard (Lowry *et al.*, 1951).

Ethoxyresorufin-O-deethylase activity in liver microsomes. Transcriptional activation of the AhR *in vivo* was determined by measuring ethoxyresorufin-O-deethylase (EROD) activity in liver microsomes in 10 μ l sample containing 10–40 μ g protein with 90 μ l 50mM Tris buffer (pH 7.4) containing 5mM $MgCl_2$, 20 μ M dicumarol, 2 μ M 7-ethoxyresorufin, and 1.5mM NADPH. A standard curve using resorufin was generated to quantify the EROD activity. Fluorescence was measured at 37°C at an excitation wavelength of 530 nm and an emission wavelength of 590 nm, every 80 s for 16 min in a FLUOstar plate reader (BMG Labtechnologies GmbH, Germany). EROD activity was calculated as picomoles resorufin per minute per milligram protein.

Measurement of PE-specific IgE, IgG1, and IgG2a antibody levels in serum. PE-specific IgE, IgG1, and IgG2a antibodies in serum were detected as previously described (Smit *et al.*, 2011).

Splenic cell culture and analysis of cytokine production. After red blood cell lysis, single-cell spleen suspensions (2.5×10^6 cells/ml) were cultured in 200 μ l complete RPMI 1640 (10% fetal calf serum) in the presence of medium or PE (100 μ g/ml) for 96 h at 37°C and 5% CO_2 . Levels of IL-5, IL-10, IL-13, IL-17a, IL-22, and interferon (IFN)- γ in collected supernatant were determined by commercially available sandwich ELISA (eBioscience, Austria) according to the manufacturer's instructions. Levels of IL-4 were below detection limit.

Flow cytometry analysis. Single-cell suspensions of spleen (after red blood cell lysis) and MLNs (1×10^7 cells/ml) were stained with anti-CD4-FITC (clone L3T4; eBioscience) and anti-CD25-PE (clone PC61; eBioscience) in fluorescence activated cell sorting (FACS) buffer (PBS containing 0.25% BSA, 0.05% NaN_3 , 0.5mM EDTA) for 30 min at 4°C. Subsequently, cells were washed with fluorescence activated cell sorting (FACS) buffer and stained intracellularly for Foxp3 (Foxp3-APC, clone FJK-16s; eBioscience) according to the manufacturer's instructions. Analysis was performed on a BD FACSCanto II using BD FACS Diva software (BD Biosciences).

RNA isolation. Isolated duodenum and liver were homogenized two times for 1 min at 20 Hz in 500 μ l RNA Insta-Pure (Eurogentec) using a Mixermill (Retsch, Germany). The homogenized tissue was centrifuged for 10 min at $12,000 \times g$. The supernatant, containing RNA in RNA Insta-Pure was transferred to a new vial and RNA was isolated using phenol-chloroform extraction. The amount of RNA was determined using the NanoDrop 2000 Spectrophotometer (ThermoScientific).

Real-time PCR. Complementary DNA (cDNA) was synthesized from 1 μ g RNA using the iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, CA). Amplification reactions were set up with 9 μ l mastermix (7.5 μ l iQ SYBR Green Supermix from Bio-Rad, 0.3 μ l dH_2O , 0.6 μ l (10 μ M) forward primer [FW], 0.6 μ l (10 μ M) reverse primer [RV]) and 6 μ l first strand cDNA ($\times 10$ diluted). Primer sequences: AhR, FW-5'-CGGCTTCTTGCAAAA-CACAGT-3' and RV-5'-GTAATGCTCTCGTCTTCTTCATC-3'; AhRR, FW-5'-GTTGGATCCTGTAGGAGCA-3' and RV-5'-AGTCCAGAGGCT-CACGCTTA-3'; CYP1A1, FW-5'-GGTAAACATGACCGGGAAC-3' and RV-5'-TGCCCCAACCAAGAGAGTGA-3'; CYP1A2, FW-5'-ACATTCC-CAAGGAGCGTGTATCT-3' and RV-5'-GTCGATGGCCGAGTTGTTAT-TGGT-3'; CYP1B1, FW-5'-GTGGCTGCTCATCCTTTTACC-3' and RV-5'-CCCACAACCTGGTCCAAC-3'; and β -actin, FW-5'-ATGCTCCCCGGG-CTGTAT-3' and RV-5'-CATAGGAGTCTTCTGACCCATTC-3'. Reactions were performed in hard-shell PCR 96-well plates (Bio-Rad) capped with optical cap and amplified for 40 cycles with the standard PCR parameters (thermal profile: 95°C for 3 min [$1 \times$ per sample], 95°C for 10 s [$40 \times$ per sample], 60°C

for 45 s [$40 \times$ per sample]). Following amplification, the melting curves of PCR products were determined from 60°C to 95°C to detect possible contaminations in the samples and to determine the specificity of the amplification. The data generated from reactions was analyzed by plotting ΔRn (normalized) fluorescence signal versus cycle number. An arbitrary threshold was set at the midpoint of the log ΔRn versus cycle number plot. The C_t values (thresholds) calculated from this plot were used to determine relative quantitation of gene expression by applying comparative C_t method ($\Delta\Delta C_t$). ΔC_t was calculated by subtracting the C_t of the reference gene from the C_t of the target gene. As a reference gene, β -actin was used. Next, the average ΔC_t of the control group was subtracted from treated-groups ($\Delta\Delta C_t$) and fold changes in gene expression relative to the control group were calculated according to the $2^{-\Delta\Delta C_t}$ method.

CAFLUX cell line. The murine reporter cell line H1G1.1c3 was created by stable transfection of mouse hepatoma cells (Hepa1c1c7) with the AhR-eGFP (enhanced green fluorescent protein) reporter plasmid pGreen and were kindly provided by Dr M.S. Denison (UC Davis, CA). Cells were cultured in 75 cm^2 Tissue Culture Flasks (Greiner Bio-One, The Netherlands) in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, The Netherlands) supplemented with 10% fetal calf serum, 1% Pen/Strep (Pen 10,000 U/ml, Strep 10,000 μ g/ml, Invitrogen) in an incubator (37°C, 5% CO_2).

Exposure of the CAFLUX cell line to AhR ligands and CYP1A2 inhibitor. For exposure, 1×10^5 cells/ml were seeded in flat-bottom 96-well plates and the next day exposed to concentrations series of various AhR ligands for 24 h. TCDD (Cambridge Isotope Lab), FICZ (Biomol International), and 6-MCDF (kindly provided by S. Safe) were dissolved in DMSO. β -NF (Sigma-Aldrich) was dissolved in acetone. The final exposure concentration of each compound was 0.5% vol/vol. Every sample concentration was assayed in triplicate. After 24 h, fluorescence was measured in PBS with a spectrophotometer (POLARstar Galaxy; BMG Labtechnologies, Germany) using excitation at 485 nm and emission at 510 nm. Optical density values were obtained using corresponding software (FLUOstar Galaxy, version 4.30–0) and corrected for background levels. The role of metabolism of TCDD, FICZ, β -NF, and 6-MCDF on AhR activation was investigated by exposing cells to the half maximal effective concentration (EC_{50}) value of these compounds in the presence of the CYP1A2 inhibitor furafylline (3-(2-furanylmethyl)-3,7-dihydro-1,8-dimethyl-1H-purine-2,6-dione) (Calbiochem, Germany) (dissolved in DMSO, 0.5% vol/vol). EROD activity was measured by adding EROD medium (DMEM, containing 0.1% 10mM dicumarol [dissolved in DMSO] and 0.5% 1M $MgCl_2$, all supplied by Sigma-Aldrich) to the cells. Resorufin concentrations were measured eight times during 609 s, with excitation at 530 nm and emission at 590 nm at 37°C. Optical density-values were corrected for background levels. A standard curve using resorufin was generated to quantify the EROD activity. EROD activity was calculated as picomoles resorufin per minute per milligram protein.

Statistical analysis. Results are presented as the mean $\pm$ standard error of the mean of six to eight mice per group. All data were logarithmically transformed to achieve normal distribution. Data were analyzed by one-way ANOVA followed by a Bonferroni post hoc test. A value of $p < 0.05$ was considered as statistically significant. All statistical analyses were performed using GraphPad Prism software.

RESULTS

Immunomodulation of Peanut Sensitization by β -NF and 6-MCDF, but Not FICZ

We first investigated the effect of the AhR ligands FICZ, β -NF, and 6-MCDF on sensitization to peanut in an established mouse model of food allergy. As a positive control, the potent AhR ligand TCDD was included. PE sensitization of the vehicle-control group resulted in increased serum levels of PE-specific IgE, IgG1, and IgG2a (Fig. 2) and increased cytokine

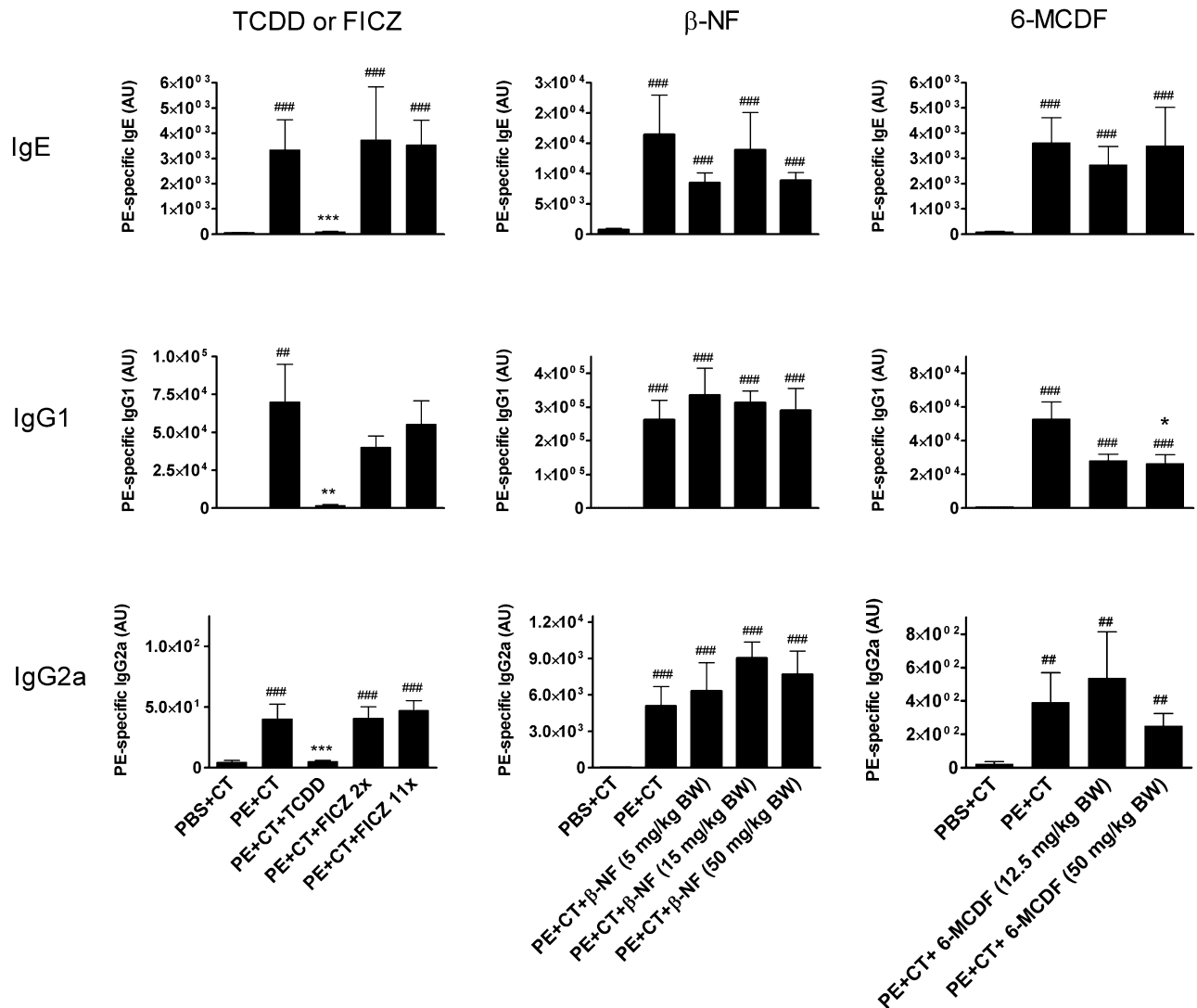


FIG. 2. Effect of FICZ, β -NF, and 6-MCDF treatments before and during peanut sensitization on peanut-specific IgE, IgG1, and IgG2a levels. Before and during sensitization to peanut (PE + CT) mice were treated with TCDD (15 μ g/kg BW, setup 1), FICZ (50 μ g/kg BW, setup 1), β -NF (5, 15, or 50 mg/kg BW, setup 2), or 6-MCDF (12.5 or 50 mg/kg BW, setup 3). Peanut specific IgE, IgG1, and IgG2a were determined in the serum collected on the day of sacrificing by ELISA. Values are presented as mean $\pm$ standard error of the mean ($n = 6-8$ per group). ## $p < 0.01$ compared with PBS + CT, ### $p < 0.001$ compared with PBS + CT, * $p < 0.05$ compared with PE + CT, ** $p < 0.01$ compared with PE + CT, and *** $p < 0.001$ compared with PE + CT.

(IL-5, IL-10, IL-13, IL-17a, IL-22, and IFN- γ) production by spleen cultures incubated with PE (Fig. 3) compared with the nonsensitized vehicle control group. Treatment with TCDD during sensitization to PE suppressed all these parameters, except IL-22 and IFN- γ (Figs. 2 and 3). PE-specific IgE, IgG1, and IgG2a levels were not affected in mice treated with FICZ and β -NF during sensitization to peanut, whereas treatment with 6-MCDF decreased PE-specific IgG1 levels (Fig. 2). Interestingly, treatment of mice with β -NF increased PE-induced IL-5, IL-13 (at 1000 μ g β -NF), and IL-17a production (at 300 μ g and 1000 μ g β -NF) (Fig. 3). FICZ and 6-MCDF treatments of mice during PE-sensitization showed no effect on PE-induced cytokine production (Fig. 3). In addition, repeated oral treatment of mice with FICZ (500 μ g/kg BW, on days -3,

0, 4, 8, and 11), before and during PE sensitization, did also not affect the peanut allergic response (data not shown). Together, these results show that β -NF and 6-MCDF, but not FICZ, affect parameters of peanut sensitization to limited extent at the dose and frequency of administration used in these experiments. However, these ligands are not as effective as TCDD in lowering peanut sensitization.

FICZ, β -NF, and 6-MCDF Do Not Increase the Percentage of CD4⁺CD25⁺Foxp3⁺ T_{reg} Cells in Spleen and MLN in PE-Sensitized Mice

Next, we investigated the effects of FICZ, β -NF, and 6-MCDF treatments on the induction of CD4⁺CD25⁺Foxp3⁺

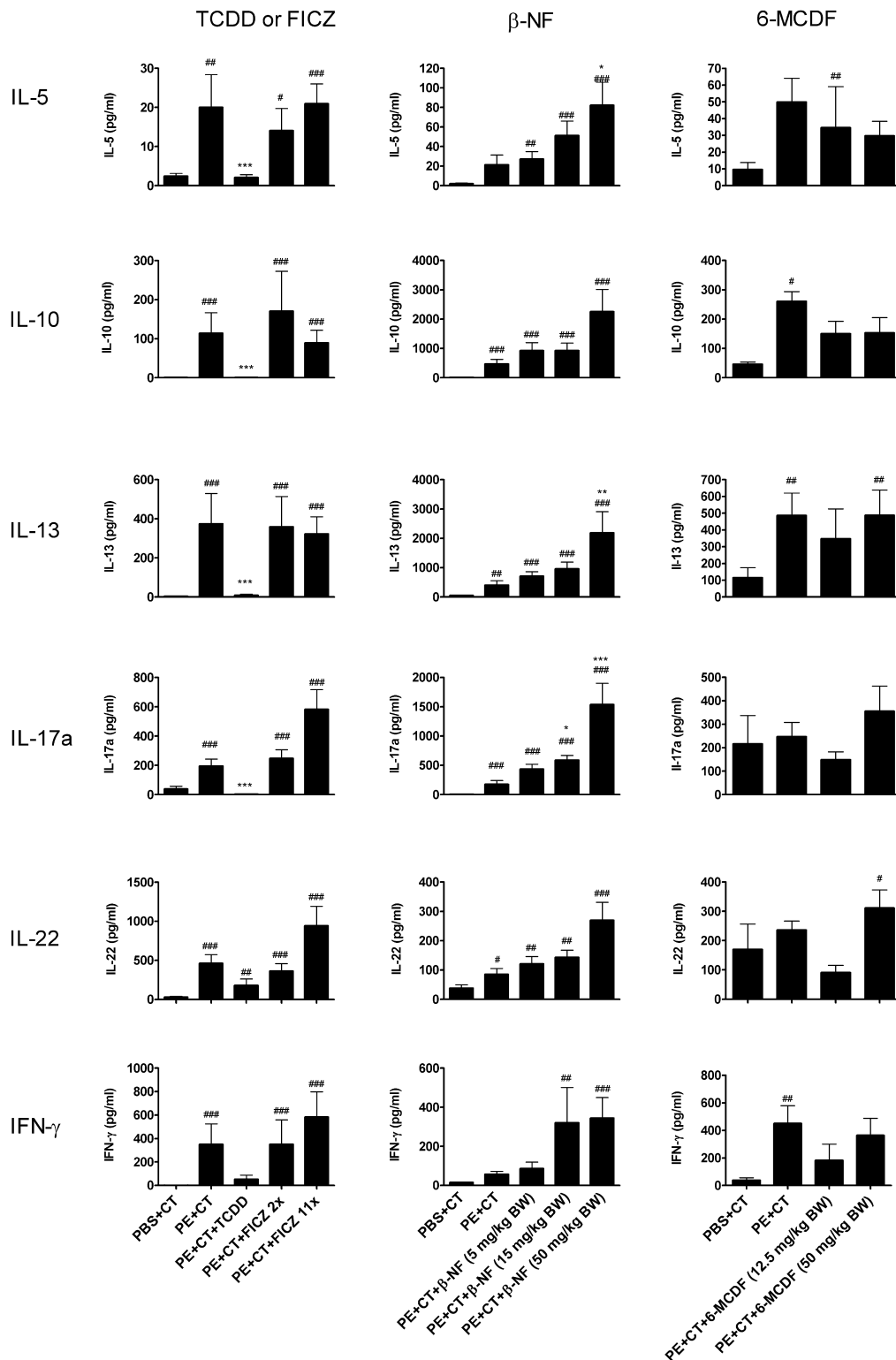


FIG. 3. Effect of FICZ, β -NF, and 6-MCDF treatments before and during peanut sensitization on PE-induced IL-5, IL-10, IL-13, IL-17a, IL-22, and IFN- γ cytokine responses. Mice were sensitized for peanut (PE + CT) and treated with TCDD (15 μ g/kg BW, setup 1), FICZ (50 μ g/kg BW, setup 1), β -NF (5, 15, or 50 mg/kg BW, setup 2), or 6-MCDF (12.5 or 50 mg/kg BW, setup 3). Single-cell suspensions of spleen cells isolated on the day of sacrificing were cultured for 96 h in the presence of PE. The supernatant was analyzed for IL-5, IL-10, IL-13, IL-17a, IL-22, and IFN- γ . No cytokine production could be detected when splenocytes were cultured in the absence of PE. Values are presented as mean $\pm$ standard error of the mean ($n = 6-8$ per group). # $p < 0.05$ compared with PBS + CT, ## $p < 0.01$ compared with PBS + CT, ### $p < 0.001$ compared with PBS + CT, * $p < 0.05$ compared with PE + CT, and *** $p < 0.001$ compared with PE + CT.

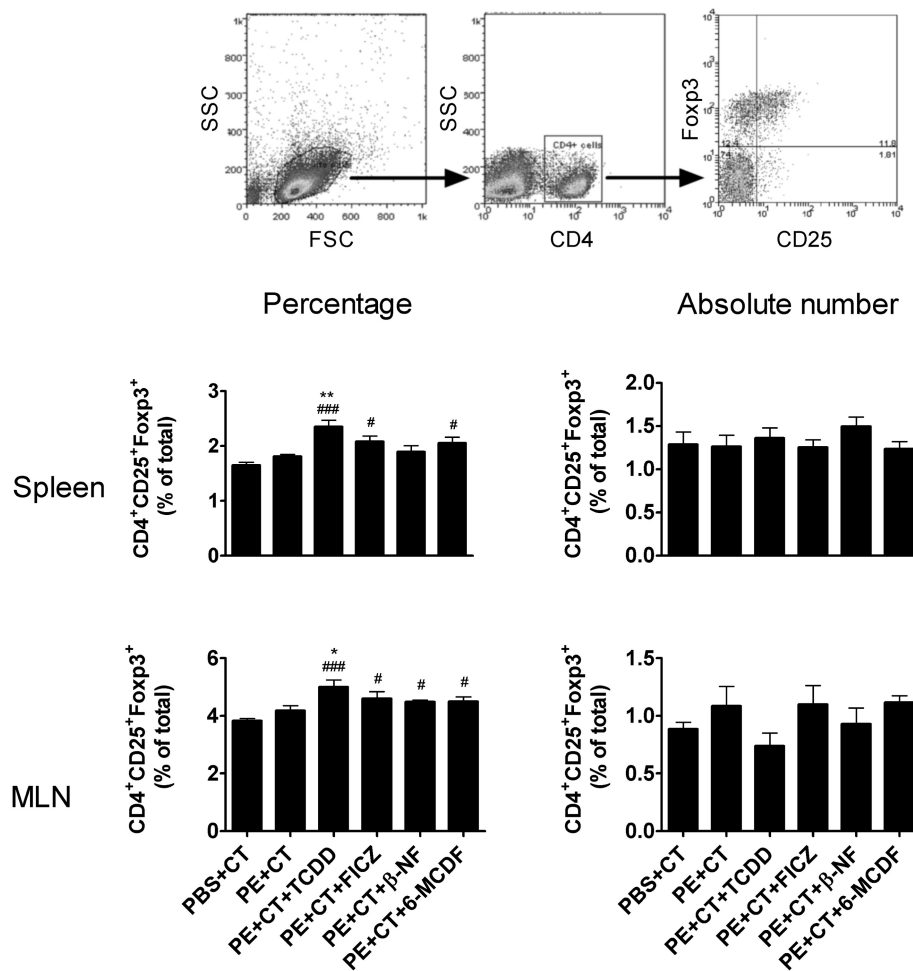


FIG. 4. Effect of FICZ, β -NF, and 6-MCDF treatments before and during peanut sensitization on CD4⁺CD25⁺Foxp3⁺ T_{reg} cells. Before and during sensitization to peanut (PE + CT) mice were treated with TCDD (15 μ g/kg BW), FICZ (500 μ g/kg BW), β -NF (5 mg/kg BW), or 6-MCDF (150 mg/kg BW) (setup 4). On day 5, mice were sacrificed and the percentage and absolute number of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in the spleen and MLN were examined by FACS analysis. Values are presented as mean $\pm$ standard error of the mean ($n = 4$ per group). # $p < 0.05$ compared with PBS + CT, ### $p < 0.001$ compared with PBS + CT, * $p < 0.05$ compared with PE + CT, and ** $p < 0.01$ compared with PE + CT.

T_{reg} cells during peanut sensitization in the spleen and MLN on day 5. In addition, the effect of FICZ, β -NF, and 6-MCDF treatments on PE-induced cytokine production of splenic cells on day 5 was also investigated. Again, TCDD was included as a positive control. PE-sensitization did not increase the percentage of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in the spleen and MLN on day 5 (Fig. 4). As expected, TCDD treatment increased the percentage of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in the spleen and MLN compared with PE-sensitized mice (Fig. 4). FICZ, β -NF, and 6-MCDF did not increase the percentage of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in the spleen and MLN compared with PE-sensitized mice. However, FICZ and 6-MCDF treatments before and during PE-sensitization increased the percentage of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in spleen and MLN compared with nonsensitized vehicle control mice, whereas β -NF treatment only increased the percentage of

CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in MLN (Fig. 4). In addition, we investigated the early effects of FICZ, β -NF, and 6-MCDF treatments on initiation of peanut sensitization by measuring PE-induced cytokine production on day 5. PE sensitization of the vehicle-control group resulted in increased cytokine (IL-5, IL-10, IL-13, IL-17a, and IFN- γ) production by spleen cultures incubated with PE (Supplementary data 1) compared with the nonsensitized vehicle control group. Again, treatment with TCDD prior to PE-sensitization suppressed this increase (Supplementary data 1). FICZ, β -NF, and 6-MCDF, however, did not affect PE-induced IL-5, IL-10, IL-13, IL-17a, and IFN- γ on day 5 (Supplementary data 1). Together, these data show that in contrast to TCDD, treatment of mice with FICZ, β -NF, and 6-MCDF does not increase the percentage of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in PE-sensitized mice and does not affect PE-induced cytokine production during the initiation of sensitization.

In Vivo Treatment of Mice With FICZ, β -NF, and 6-MCDF Less Effectively Induce AhRR and Cytochrome P450 Gene Expression Compared With Mice Treated With TCDD

Because FICZ, β -NF, and 6-MCDF were not as effective as TCDD in lowering peanut sensitization, we investigated whether or not these AhR ligands were able to activate the AhR in the duodenum and liver on day 5, i.e., 24 h after the last administration of a non-dioxin-like AhR ligand. Mice treated with TCDD were included as a positive control. TCDD treatment strongly increased AhRR, CYP1A1, CYP1A2, and CYP1B1 gene expression in both the duodenum and the liver compared with vehicle-treated mice (Fig. 5). Also, EROD activity measured in liver microsomes was strongly increased after TCDD treatment (Fig. 5). Treatment of mice with FICZ, unlike treatment with β -NF, increased CYP1A1 expression in the liver and both FICZ and β -NF treatment increased CYP1A2 expression in the duodenum and liver (Fig. 5). In contrast, 6-MCDF treatment decreased CYP1A1 expression in the duodenum, whereas 6-MCDF did not affect CYP1A2 expression in either the duodenum or the liver. FICZ, β -NF, and 6-MCDF treatment enhanced EROD activity measured in liver microsomes. Together, these results show that treatment of mice with FICZ, β -NF, and 6-MCDF results in limited AhR activation compared with mice treated with TCDD.

Inhibition of Metabolism In Vitro Increases AhR Activation

Because sensitization to peanut could not be suppressed by repeated administration of FICZ, β -NF, and 6-MCDF, we investigated the possible role of metabolism of FICZ, β -NF, and 6-MCDF on AhR activation *in vitro*, as many non-halogenated ligands for the AhR facilitate their own metabolism by inducing CYP450 enzymes. Treatment of the mouse H1G1.1c3 cells with TCDD, FICZ, β -NF, or 6-MCDF resulted in concentration-dependent activation of the AhR after 24 h exposure, as indicated by the production of eGFP controlled by AhR transactivation (Fig. 6a). The estimated EC_{50} value to activate the AhR was lowest for TCDD (19.9×10^{-12} M), followed by FICZ (9.65×10^{-10} M), β -NF (4.69×10^{-10} M), and 6-MCDF (4.66×10^{-7} M). The concentration-response curve for TCDD had a maximal height compared with the other AhR ligands, indicating that the efficacy of TCDD to activate the AhR was highest. The role of metabolism on AhR activation by TCDD, FICZ, β -NF, and 6-MCDF was investigated by using the CYP1A2 inhibitor furafylline. Cells were incubated for 24 h with the EC_{50} values of TCDD, FICZ, β -NF, and 6-MCDF and with an increasing concentration of furafylline. As expected, no effect on AhR activation (measured by eGFP) by TCDD was observed when CYP1A2 was inhibited because it is generally known that TCDD is very slowly metabolized (Figs. 6b and 6c). Inhibition of CYP1A2 activity increased AhR activation by FICZ, β -NF, and 6-MCDF (Figs. 6b and 6c). In all test conditions, cell viability was not affected (data not shown). Together, these data show

that inhibition of CYP1A2 metabolism results in increased *in vitro* AhR activation by FICZ, β -NF, and 6-MCDF, but not by TCDD.

DISCUSSION

Previously, we have shown that activation of the AhR by TCDD suppresses sensitization to peanut and that this is partly mediated by a TCDD-induced increase of the percentage of $CD4^+CD25^+Foxp3^+$ cells (Schulz *et al.*, 2011). Besides TCDD, numerous other exogenous and endogenous AhR ligands are described. Here, we examined the effect of a number of non-dioxin-like AhR ligands that are structurally distinctly different from TCDD, i.e., FICZ, β -NF, and 6-MCDF, on peanut sensitization. The present study shows that in contrast to TCDD, these AhR ligands do not suppress sensitization to peanut, do not increase the percentage of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in PE-sensitized mice and less effectively induce AhR-related gene expression *in vivo* and *in vitro*.

Compared with TCDD, the AhR ligands FICZ and β -NF only marginally increased AhR-related gene expression and EROD activity *in vivo*. The selective AhR modulator (SAHRM) 6-MCDF, which retains the antiestrogenic effects but lacks the transcriptional effects of TCDD associated with toxicity, had no effect on AhR-related expression in the liver and down-regulated CYP1A1 expression in the duodenum, although EROD activity in the liver was increased. The finding that inhibition of CYP1A2 metabolism *in vitro* increased AhR activation by FICZ, β -NF, and 6-MCDF, but not by TCDD, indicates a clear link between metabolism and AhR activation by FICZ, β -NF, and 6-MCDF. This might suggest that these AhR ligands are metabolized too fast *in vivo* to have a comparable effect as TCDD on peanut sensitization and $CD4^+CD25^+Foxp3^+$ T_{reg} cells. In relation to this, it could be argued that the frequency of administration and the doses of FICZ, β -NF, and 6-MCDF used were not high enough to exert similar effects as TCDD on peanut sensitization and $CD4^+CD25^+Foxp3^+$ T_{reg} cells. However, in other disease models, effects of FICZ, β -NF, and 6-MCDF on the immune system have been described at comparable doses and frequency of administration. For example, in our model, FICZ was administered multiple times ip (50 μ g/kg BW), whereas Monteleone *et al.* have shown that a single administration of approximately 50 μ g/kg BW FICZ ip already ameliorates TNBS-induced colitis and relapses DSS-induced (Monteleone *et al.*, 2011). In addition, it has been shown that the presence of 600 ng FICZ in the antigen emulsion accelerates and increases pathology of EAE (Veldhoen *et al.*, 2008). Furthermore, in the peanut allergy model, administration of 50 mg/kg BW β -NF every 2–3 days affected some allergic parameters, as β -NF treatment increased PE-induced IL-5, IL-13, and IL-17a production of splenocytes. However, no effect was observed

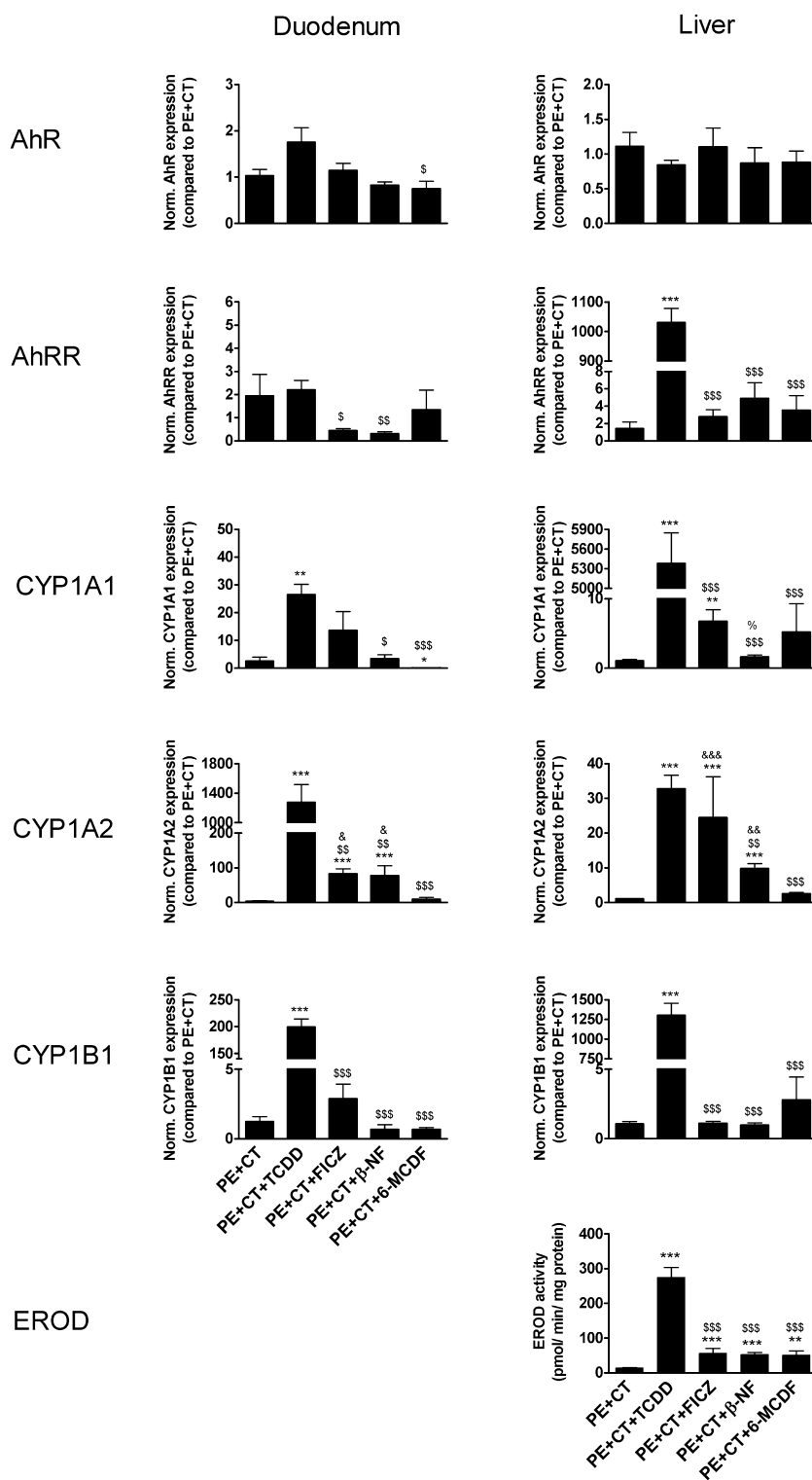


FIG. 5. Effect of FICZ, β -NF, and 6-MCDF treatments on AhR-related gene expression in liver and duodenum. Before and during sensitization to peanut (PE + CT) mice were treated with TCDD (15 μ g/kg BW), FICZ (500 μ g/kg BW), β -NF (5 mg/kg BW), or 6-MCDF (150 mg/kg BW) (setup 4). On day 5, mice were sacrificed and duodenum and liver were isolated. Total RNA was extracted and the expression of AhR, AhRR, CYP1A1, CYP1A2, and CYP1B1 compared with vehicle-treated mice (PE + CT) was examined by real-time PCR. EROD activity was also measured in liver microsomes. Values are presented as mean $\pm$ standard error of the mean ($n = 6$ per group). * $p < 0.05$ compared with PE + CT, ** $p < 0.01$ compared with PE + CT, *** $p < 0.001$ compared with PE + CT, \$ $p < 0.05$ compared with PE + CT + TCDD, \$\$ $p < 0.01$ compared with PE + CT + TCDD, \$\$\$ $p < 0.001$ compared with PE + CT + TCDD, & $p < 0.05$ compared with PE + CT + 6-MCDF, && $p < 0.01$ compared with PE + CT + 6-MCDF, &&& $p < 0.001$ compared with PE + CT + 6-MCDF, and % $p < 0.05$ compared with PE + CT + FICZ.

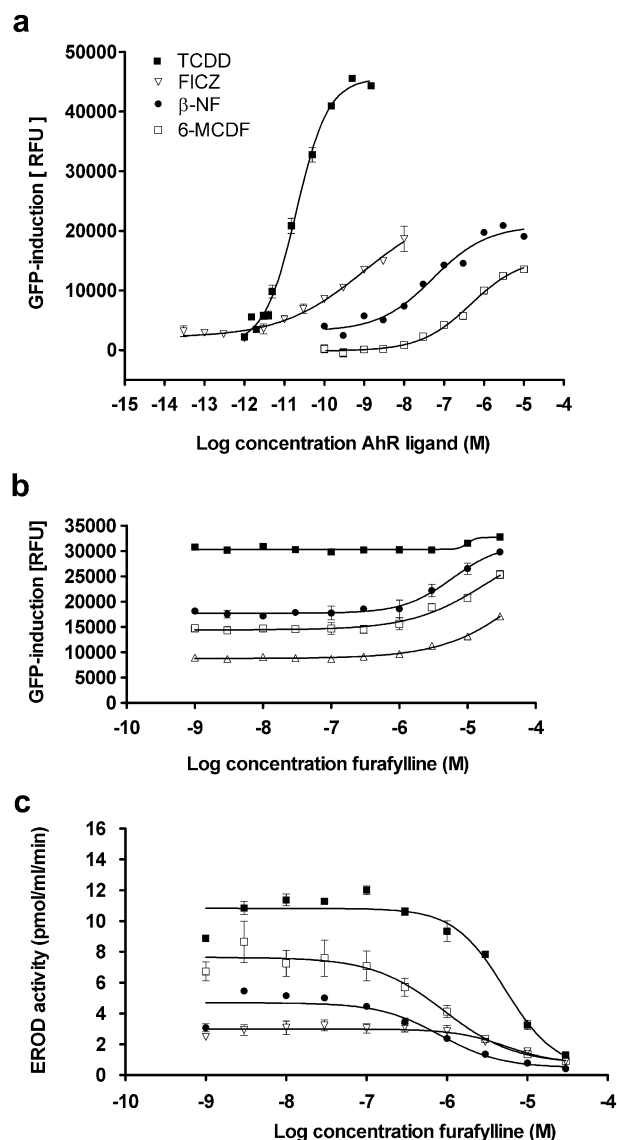


FIG. 6. Effect of FICZ, β -NF, and 6-MCDF on AhR activation and the role of CYP1A2 metabolism on AhR activation. H1G1.1c3 cells containing the AhR-eGFP reporter plasmid pGreen were seeded in a 96-well plate. The next day, cells were exposed to concentration series of TCDD, FICZ, β -NF, and 6-MCDF (0.5% vol/vol) and after 24-h exposure AhR activation was examined by measuring eGFP (a). The role of CYP1A2 metabolism on AhR activation was investigated by exposing cells for 24 h to the EC₅₀ values of TCDD (15pM), FICZ (1nM), β -NF (200nM), and 6-MCDF (2 μ M) in the presence of an increasing dose of furafylline. After 24 h of exposure, AhR activation was examined by measuring eGFP (b) and CYP1A2 metabolism was examined by measuring EROD activity (c). Values are presented as mean $\pm$ standard error ($n = 3$ per concentration).

on antibody levels, suggesting that the effect of β -NF on cytokine level is not sufficient to affect the food allergic response. Interestingly, in a DSS-induced colitis model, a similar dose of β -NF (oral administration, every day) has been shown to suppress IL-6, TNF- α , and IL-1 β expression in colon tissue resulting in attenuated disease (Furumatsu *et al.*,

2011). Furthermore, while 6-MCDF is mainly known for its anti-(breast) cancer effects (McDougal *et al.*, 2001; Zhang *et al.*, 2012), it has also been reported that a single administration of approximately 10 mg/kg BW 6-MCDF caused 26% inhibition of the splenic plaque-forming cell response to sheep erythrocytes (Bannister *et al.*, 1989). This suggests that 6-MCDF might induce modest immunosuppressive effects. However, in our studies, 6-MCDF suppressed PE-specific IgG1 at a dose of 50 mg/kg BW, but did not affect PE-specific IgE, PE-specific IgG2a and *ex vivo* PE-cytokine production, indicating that 6-MCDF does not suppress allergic sensitization to peanut. Together, these findings indicate that next to metabolism, the type of the disease might play a role in determining the effect of FICZ, β -NF, and 6-MCDF on the immune response.

Another possibility for the finding that TCDD, but not FICZ, β -NF, and 6-MCDF, suppresses sensitization to peanut is that these three ligands interact differently with the AhR compared with TCDD because it has been reported that high- and low-affinity ligands interact with different residues of the AhR ligand-binding pocket (Backlund and Ingelman-Sundberg, 2004; Salzano *et al.*, 2011). As a result, it is possible that TCDD induces different genes or distinct pathways than FICZ, β -NF, and 6-MCDF, which has been reported for TCDD compared with FICZ, 3,3'-diindolylmethane and PCB126 (Kopeck *et al.*, 2008; Laub *et al.*, 2010; Okino *et al.*, 2009). Furthermore, proinflammatory cytokines have been shown to affect the expression of most AhR-dependent xenobiotic metabolizing enzymes, thereby affecting metabolism of xenobiotics and thus binding of these xenobiotics to the AhR and the subsequent effect on the immune system (Vondráček *et al.*, 2011). In addition, cytokines can influence the level of AhR expression itself, which could also impact effects of AhR ligands on the immune system (Döhr *et al.*, 1997; Kimura *et al.*, 2008; Kobayashi *et al.*, 2008; Quintana *et al.*, 2008). For the rest, it should be taken into account that metabolism of AhR ligands such as FICZ and β -NF results in the generation of metabolites, which could subsequently interact with the AhR or other receptors and pathways, thereby possibly exerting beneficial or harmful effects. This, together with the different expression of AhR in species, tissues, and various cell types and the importance of transcriptional cross talk in shaping cell-specific AhR responses, makes the effect of AhR ligands on the immune system still very unpredictable (Frericks *et al.*, 2006, 2007, 2008; Head and Lawrence, 2009; Van der Heiden *et al.*, 2009). All together, the diversity of the AhR-linked pathways may result in different effects on immune responses, depending on the AhR ligand, the cytokine milieu, the type of disease, the route of administration, and probably the timing of administration.

In summary, the present study shows that TCDD, but not FICZ, β -NF, and 6-MCDF, suppresses sensitization to peanut. Differences in metabolism, AhR binding and subsequent gene transcription might explain the difference between TCDD and the AhR ligands FICZ, β -NF, and 6-MCDF on the peanut

allergic response. This warrants further studies to investigate the role of the AhR in food allergic responses.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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Supplementary data 1

