



Cytochrome P4501-inhibiting chemicals amplify aryl hydrocarbon receptor activation and IL-22 production in T helper 17 cells

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ABSTRACT

The aryl hydrocarbon receptor (AHR) controls interleukin 22 production by T helper 17 cells (Th17). IL-22 contributes to intestinal homeostasis but has also been implicated in chronic inflammatory disorders and colorectal cancer, highlighting the need for appropriate regulation of IL-22 production. Upon activation, the AHR induces expression of cytochrome P4501 (CYP1) enzymes which in turn play an important feedback role that curtails the duration of AHR signaling by metabolizing AHR ligands. Recently we described how agents that inhibit CYP1 function potentiate AHR signaling by disrupting metabolic clearance of the endogenous ligand 6-formylindolo[3,2-*b*]carbazole (FICZ). In the present study, we investigated the immune-modulating effects of environmental pollutants such as polycyclic aromatic hydrocarbons on Th17 differentiation and IL-22 production. Using Th17 cells deficient in CYP1 enzymes (*Cyp1a1/1a2/1b1*^{−/−}) we show that these chemicals potentiate AHR activation through inhibition of CYP1 enzymes which leads to increases in intracellular AHR agonists. Our findings demonstrate that IL-22 production by Th17 cells is profoundly enhanced by impaired CYP1-function and strongly suggest that chemicals able to modify CYP1 function or expression may disrupt AHR-mediated immune regulation by altering the levels of endogenous AHR agonist(s).

1. Introduction

The aryl hydrocarbon receptor (AHR) has emerged as a critical physiological regulator of immunity and has been implicated in the development of chronic inflammatory diseases such as inflammatory bowel disease and colorectal cancer [1,2]. The AHR is a ligand-dependent transcription factor renowned for mediating the toxic effects of halogenated aromatic hydrocarbons such as dioxins. Upon activation AHR regulates the expression of numerous target genes including those encoding the cytochrome P4501 (CYP1) biotransforming enzymes [3], that are also involved in control of AHR activity [4]. This negative feedback regulation is of functional significance as sustained AHR activation, exemplified by the non-metabolized AHR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), has detrimental effects resulting in immunotoxicity, teratogenicity and endocrine disruption [5].

The endogenous AHR ligand 6-formylindolo[3,2-*b*]carbazole (FICZ) has the highest AHR activating potency among identified endogenous agonists [6,7]. FICZ is also efficiently metabolized by the AHR-regulated CYP1 enzymes, resulting in transient AHR pathway activation [8].

In a number of reports we have described how CYP1 knockdown or chemicals inhibiting the CYP1 function such as α -naphthoflavone (α NF) and 3-methoxy-4-nitroflavone (MNF) disrupt this negative feedback loop and prolong AHR activity *in vitro* and *in vivo* [8–12]. Furthermore, adverse effects such as immunosuppression [13] and synergistic developmental toxicity have been observed after exposure to an AHR inducer such as β -naphthoflavone, benzo(a)pyrene or FICZ in combination with deletion of the *Cyp1a1* gene or with chemical CYP1 inhibition [14–17]. Altogether, these reports strengthen an important role of CYP1 enzymes in regulation of AHR physiological functions in embryo development and immunity.

The AHR is highly expressed in T helper 17 cells (Th17) and its role in Th17 cell function has been studied extensively [18]. Th17 cells are particularly enriched in the gastrointestinal tract and AHR pathway activation is important for the production of the cytokine IL-22 by Th17 cells. IL-22 promotes epithelial barrier integrity by inducing production of anti-microbial proteins and mucins from epithelial cells, which is crucial for protective immunity to extracellular pathogens such as *Citrobacter rodentium* but also limits the dissemination of commensal

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bacteria to peripheral organs [19,20]. In contrast, IL-22 has been shown to contribute to immune-mediated pathology by promoting the secretion of pro-inflammatory chemokines and cytokines [20]. In addition, IL-22 signaling is involved in the development of colorectal cancer by increasing the proliferation and survival of intestinal stem cells [19]. These complex roles of IL-22 in the context of intestinal homeostasis, immunity and tumorigenesis emphasize the importance of appropriate regulation of IL-22 production.

Based on our previous work demonstrating a crucial role of CYP1 enzymes in AHR signaling [10–12] and downstream regulation of immunity [9], we hypothesized that chemicals interfering with the feedback regulation of AHR activity modulate IL-22 by causing prolonged and potentiated AHR signaling. To this aim, we compared the impact of different CYP1 inhibiting chemicals on AHR signaling and IL-22 production in *in vitro* differentiated Th17 cells generated from wildtype (WT), *Cyp1a1/1a2/1b1* triple knockout (CYP1-KO) or *Ahr* knockout (AHR-KO) mice. Th17 cells generated from these genotypes were exposed to the AHR ligand FICZ with or without co-exposure to CYP1 inhibitors. Subsequently, effects on AHR activation, Th17 cell differentiation, IL-22 expression and CYP1 enzymatic function were determined by flow cytometry, analyses of gene and protein expression and FICZ metabolism studies.

2. Materials and methods

2.1. Mice

Cyp1a1/1a2/1b1 triple knockout (CYP1-KO) and *Ahr* knockout (AHR-KO) mice used in this study were backcrossed to C57BL/6 mice for at least 10 generations. Wild type C57BL/6J mice were used as control. All mice were bred in the Francis Crick Institute animal facility under specified pathogen-free conditions. All animal procedures were conducted under a Project Licence granted by the UK Home Office. Mice were age- and sex-matched and between 6 and 10 weeks old when first used. Both female and male mice were used in experiments. Exclusion criteria such as inadequate staining or low cell yield owing to technical problems were pre-determined.

2.2. Th17 cell differentiation and chemical exposure

CD4 + T cells were isolated from peripheral lymph nodes of C57BL/6 mice using EasySep mouse CD4 + T cell isolation kit (Stemcell Technologies, Cambridge, UK) with the addition of biotinylated anti-CD25 antibody (BioLegend, London, UK). The cells were suspended in IMDM medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.05 mM β-mercaptoethanol and 5% fetal calf serum (Biosera Europe, Nuaille, France) and dispensed on 48-well plates (0.2 × 10⁶ cells/500 µl medium per well) pre-coated with 1 µg/ml anti-CD3 (clone 145-2C11, eBioscience, San Diego, USA) and 10 µg/ml soluble anti-CD28 (clone 37.51, BioLegend, London, UK). To stimulate polarization towards Th17 lineage, the cells were cultured in the presence of 2 ng/ml TGF-β1, 20 ng/ml IL-6, 10 ng/ml IL-1β (all R&D Systems) and 10 µg anti-IFN-γ (BioXCell, West Lebanon, USA). All cytokine stimulation and chemical exposures were started within 30 min after plating the cells by adding an additional 500 µl of IMDM medium containing 2 × cytokine concentrations and 2 × concentrations of FICZ, indolo[3,2-b]carbazole (ICZ) (both from Syntactic, Stockholm, Sweden) or TCDD (LGC standards, Boras, Sweden) and/or the CYP1 inhibitors MNF (kind gift from Michael S. Denison, University of California, Davis, CA), α-NF, fluoranthene (FL), phenanthrene (Phe) or pyrene (PY) (all from Sigma-Aldrich, Stockholm, Sweden), thereby achieving 1 × concentration of respective chemical (0.01 nM–5 µM) and cytokine. All chemicals were dissolved and diluted in DMSO which therefore was included as vehicle control in all exposures (0.1%). At different time points cells were harvested for FACS analysis, gene expression analysis or HPLC analysis. In parallel, IL-22 cytokine levels in

Table 1
TaqMan® Gene Expression Assay IDs.

Gene	Assay ID
<i>hypoxanthine guanine phosphoribosyl transferase (Hprt)</i>	Mm03024075_m1
<i>aryl hydrocarbon receptor (Ahr)</i>	Mm00478932_m1
<i>cytochrome p4501a1 (Cyp1a1)</i>	Mm00487218_m1
<i>interleukin 22 (Il22)</i>	Mm01226722_g1

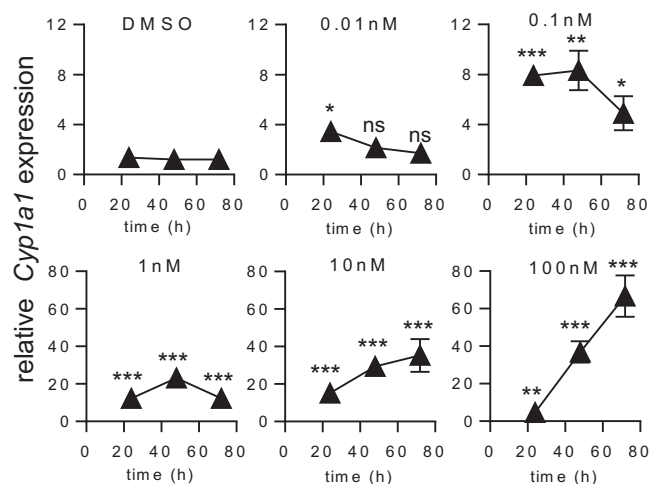


Fig. 1. Time- and dose dependent AHR activation by FICZ. Dose dependent effects of FICZ on *Cyp1a1* expression in CD4 + T cells undergoing Th17 differentiation was determined up to 72 h exposure. Results are shown as mean ± SD from 2 independent experiments each performed in duplicate (n = 4). Relative levels of *Cyp1a1* transcription was calculated using *Hprt* as a reference gene and untreated cells at each time-point as controls. Differences in transcription between DMSO control and respective FICZ-dose were determined using One-way ANOVA followed by Sidak *post hoc* test to correlate for multiple comparisons. Statistically significant differences are shown by asterisks (*p < 0.05, **p < 0.01, and ***p < 0.001. n.s. = not significant).

culture supernatants were determined by ELISA (eBioscience, San Diego, USA).

2.3. Intracellular cytokine staining and flow cytometry

At 4 days post exposure the cells were re-stimulated in the culture medium for 4 h in the presence of 0.1 µg/ml phorbol-12-myristate-13-acetate (PMA), 1 µg/ml ionomycin, 1 µg/ml monensin and 1 µg/ml brefeldin A (all from Sigma-Aldrich, Gillingham, UK). Thereafter, the cells were washed and cell suspensions were incubated with anti-CD16/CD32 (eBioscience, San Diego, USA) and fixable live/dead cell dye (ThermoFisher, Waltham, USA) followed by staining with antibody against the surface marker CD4 (BD Biosciences, Oxford, UK). For intracellular staining, the cells were then fixed in 3.7% formaldehyde in DPBS on ice for 45 min, followed by permeabilization in permeabilization buffer (eBioscience, San Diego, USA) for 45 min on ice. The cells were pelleted and resuspended in fresh permeabilization buffer containing antibodies against IL-17A, IL-22, IFNγ and FoxP3 (all eBioscience, San Diego, USA). Cells were acquired with a BD Fortessa X20 and analysis was performed on viable cells with FlowJo v10 software (Tree Star, Ashland, USA).

2.4. RNA purification and gene expression analysis

At different time points, cells were harvested and total RNA was extracted using the RNeasy plus extraction kit (Qiagen, Sollentuna, Sweden) and cDNA was synthesized using the iScript cDNA Synthesis kit (Biorad, Solna, Sweden), both according to the manufacturer's instructions. Expression of *Hprt*, *Ahr*, *Cyp1a1* and *Il22* was determined by means of quantitative real-time PCR (qRT-PCR) using TaqMan® Gene

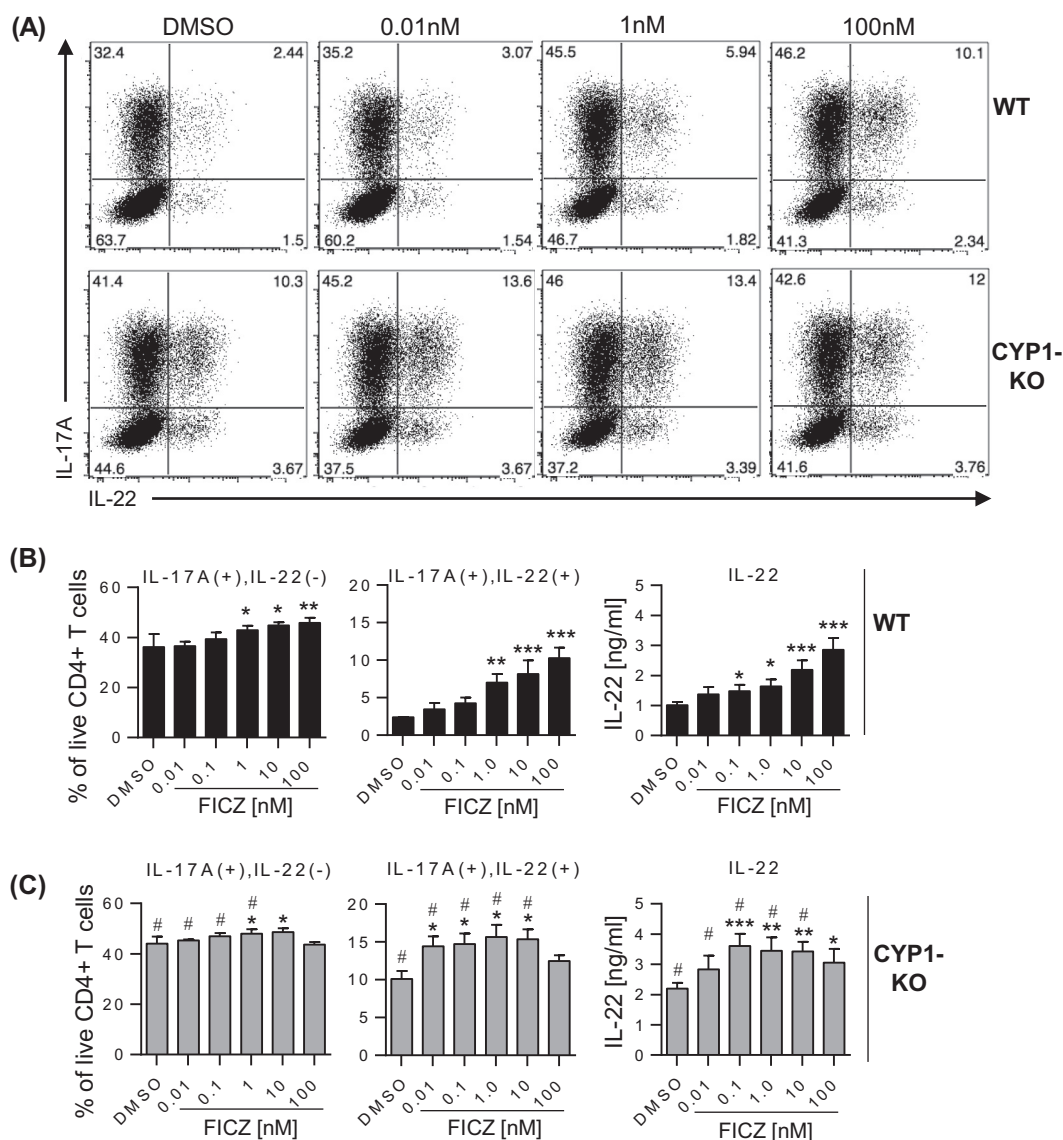


Fig. 2. Role of CYP1 function in Th17 differentiation and IL-22 expression. CD4⁺ T cells isolated from WT mice (A-top panel, B) or CYP1-KO mice (A-bottom panel, C) undergoing Th17 differentiation were exposed to different doses of FICZ. At 96 h exposure frequency of CD4⁺ T cells expressing IL17A and IL-22 was determined by means of FACS and at 72 h exposure cell medium was collected and analysed for content of IL-22 by ELISA. All results are shown as mean $\pm$ SD from 2 independent experiments each performed in duplicate ($n = 4$). All statistical differences were determined using One-way ANOVA followed by Sidak *post hoc* test to correlate for multiple comparisons. Statistically significant differences between DMSO control and respective FICZ-dose in WT cells (B) or CYP1-KO cells (C) are shown by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistically significant differences between WT cells (B) and CYP1-KO cells (C) for respective exposure are shown by hashtags (# $p < 0.05$).

Expression Assay probes (Applied biosystems, Foster City, USA), with detection on an Applied Biosystems 7500 Real-Time PCR system. Gene expression quantification was based on the comparative threshold cycle method ($2^{-\Delta\Delta C_t}$) where level of expression for respective gene was normalized to *Hprt* and given relative to untreated WT cells or WT cells exposed to negative control (DMSO) at each time point. qRT-PCR analysis was performed with the following protocol: 95 °C for 10 min, followed by 35–45 cycles of 95 °C for 15 s and 60 °C for 60 s. Gene expression assay IDs are given in Table 1.

2.5. HPLC analysis

CD4⁺ T cells were cultured under Th17-cell polarizing conditions and exposed as described above. At various time-points cells were collected, washed, extracted and analyzed as previously described [9,10]. In brief, cells were washed in PBS, re-suspended in distilled water, homogenized by sonication on ice, extracted in acetonitrile and analyzed by means of HPLC using an in-line solid-phase extraction

column coupled to a reverse-phase C18 analytical column. FICZ quantity was determined according to a standard curve of FICZ prepared in same matrix as the analyzed samples and normalized to total protein contents determined by PierceTM Coomassie (Bradford) protein assay kit (ThermoFisher, Gothenburg, Sweden) according to the manufacturer's instructions.

2.6. Statistical analysis

All statistical analysis was calculated in Prism 6 (GraphPad Software Inc., La Jolla, USA). The statistical methods used to determine effects on mRNA expression and IL-22 protein levels were one-way ANOVA followed by Sidak *post hoc* tests or by two-way ANOVA followed by Holm-Sidak *post hoc* test. Statistical significance of more than additive effects was determined using Student's *t* test followed by Holm-Sidak *post hoc* test. For this analysis, calculated levels of gene- or protein expression, i.e. the sum of the single exposures (FICZ, TCDD or ICZ alone + CYP1 inhibitor alone), was compared to the observed levels of expression

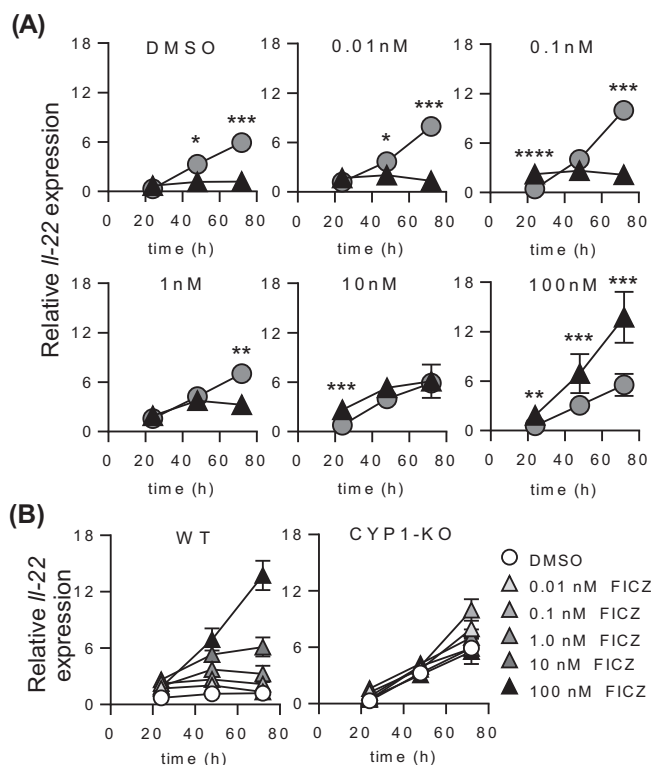


Fig. 3. Impact of CYP1 knockout on FICZ-mediated IL22 expression over time. WT or CYP1-KO CD4⁺ T cells undergoing Th17 differentiation were sampled at 24, 48 and 72 h exposure and expression of IL22 was determined by means of qRT-PCR. (A) Comparison of time-trends in IL22 expression with WT (black triangle) and CYP1-KO (grey circle) cells at exposure to DMSO control or different doses of FICZ. (B) Comparison of dose-responses in WT cells and CYP1-KO cells separately. Level of expression is normalized to *Hprt* and given relative to untreated WT cells at each time point. All results are shown as mean $\pm$ SD from 2 independent experiments each performed in duplicate ($n = 4$). Differences in IL22 expression between WT and CYP1-KO cells for respective exposure were determined using One-way ANOVA followed by Sidak *post hoc* test to correlate for multiple comparisons. Statistically significant differences are shown by asterisks (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

after co-exposure to FICZ, TCDD or ICZ + CYP1 inhibitor. The statistical tests used are given in the figure legends together with the number of experiments and experimental repeats included in the statistical analysis.

3. Results

3.1. Dose- and time-dependent kinetics of FICZ mediated AHR activation, Th17 cell differentiation and IL-22 expression

To investigate the kinetics of AHR activation in Th17 cells, *Cyp1a1* expression was determined over time in CD4⁺ T cells undergoing Th17 cell differentiation while at the same time being exposed to a wide range of FICZ doses (0.01–100 nM).

3.1.1. FICZ-mediated AHR activation during Th17 cell differentiation

A significant induction of AHR signaling, measured as *Cyp1a1* expression, was observed at 0.01 nM FICZ, with maximum induction at 24 h (Fig. 1). A clear dose-dependent difference was observed wherein exposure to 0.01–1.0 nM FICZ displayed a transient induction of *Cyp1a1* expression peaking at 24 to 48 h exposure, while in cells exposed to 10–100 nM FICZ expression levels of *Cyp1a1* were still increasing at 72 h. At 24 h of exposure, a non-linear dose-response was observed where exposure to 100 nM FICZ resulted in lower *Cyp1a1* expression levels as compared to 0.1 nM.

3.1.2. Role of CYP1 function in FICZ-mediated Th17 cell differentiation and IL-22 production

The role of CYP1 enzymes in modulating intracellular levels of FICZ [9,10,21], together with the observed dose-response relationship in FICZ-induced *Cyp1a1* expression (Fig. 1), prompted us to investigate the impact of impaired CYP1 expression on Th17 differentiation. To this end we compared Th17 cell differentiation in WT and CYP1-KO CD4⁺ T cells exposed to 0.01–100 nM FICZ from the start of culture. As expected [9], addition of FICZ to WT CD4⁺ T cells enhanced Th17 cell differentiation and IL-22 expression (Fig. 2A and B). Comparison of IL17A⁺IL-22[−] and IL-17A⁺IL-22⁺ cells revealed that IL-22 expression was more strongly induced by FICZ and at lower concentrations compared to IL-17A expression. In accordance with our previous results [9] CYP1-KO CD4⁺ T cells displayed significantly enhanced Th17 cell differentiation and IL-22 expression compared to WT cells even in the absence of exogenous FICZ (i.e. DMSO control, Fig. 2A–C). Further exposure of CYP1-KO CD4⁺ T cells to FICZ generated an inverted u-shaped dose-response curve characterized by maximal induction of Th17 cell differentiation and IL-22 production observed at 0.1 nM FICZ exposure followed by a decline at higher concentrations (Fig. 2C). At 100 nM FICZ, WT cells and CYP1-KO cells show the same response for all endpoints. The difference in dose-response pattern observed with CYP1-KO cells compared to WT cells suggests that the Th17/IL-22 response in CYP1-KO CD4⁺ T cells is saturated or even reduced at FICZ levels above 10 nM, while WT CD4⁺ T cells are not. In addition, the CYP1-KO cells showed a significantly stronger response to FICZ compared to WT cells, both in Th17 differentiation (0.01–1.0 nM) and IL-22 expression (0.01–10 nM FICZ).

Similar results were observed for IL22 mRNA expression (Fig. 3). The IL-22 expression data reveal differences in dose- and time-response trends with WT cells compared to CYP1-KO. WT cells showed transient induction up to 1 nM FICZ and continuous increase in expression at 10–100 nM while CYP1-KO cells showed a continuous increase over time, at all doses. CYP1-KO cells lacked dose dependent IL-22 induction by FICZ. Only the lower doses of FICZ produced significantly higher levels of IL-22 than the DMSO control (Fig. 3B). Also, CYP1-KO cells showed same response as WT cells at 10 nM and even lower response at 100 nM FICZ, further strengthening a saturated response in absence of a functional CYP1.

3.2. Impact of chemical CYP1-inhibitors on AHR activation and IL-22 expression during Th17 cell differentiation

Exposure to different types of CYP1 inhibiting chemicals and agents has been shown to enhance and/or prolong FICZ-mediated AHR signaling in various human cells and in zebrafish embryos [8,10–12,22,23]. Similar AHR potentiation has been observed after co-exposure to polycyclic aromatic hydrocarbon (PAH)-type AHR activators such as benzo(a)pyrene and β -naphthoflavone and CYP1 inhibitors [14,15,24,25]. Thus, we next set out to determine whether chemical CYP1 inhibitors potentiate AHR signaling during Th17 cell differentiation.

3.2.1. Effects of model CYP1 inhibitors on FICZ-mediated AHR signaling and IL22 gene expression

At first, effects of the previously described potent CYP1 inhibiting chemicals 3-methoxy-4-nitroflavone (MNF) and α -naphthoflavone (α NF) were determined. Expression levels of *Cyp1a1* and IL22 were determined in WT cells exposed to FICZ (0.1 nM) alone or in combination with MNF (0.05–0.5 μ M) or α NF (0.01–0.5 μ M). Both these compounds have previously been shown to activate AHR signaling by reducing the CYP1-dependent cellular clearance of FICZ and to strongly potentiate FICZ-dependent AHR activation [8,10,11]. The FICZ-dose was selected based on the low and transient effect on AHR signaling observed with this dose in WT cells (Fig. 1), allowing for both a potentiated and prolonged AHR response. The cutoff at 0.5 μ M as highest

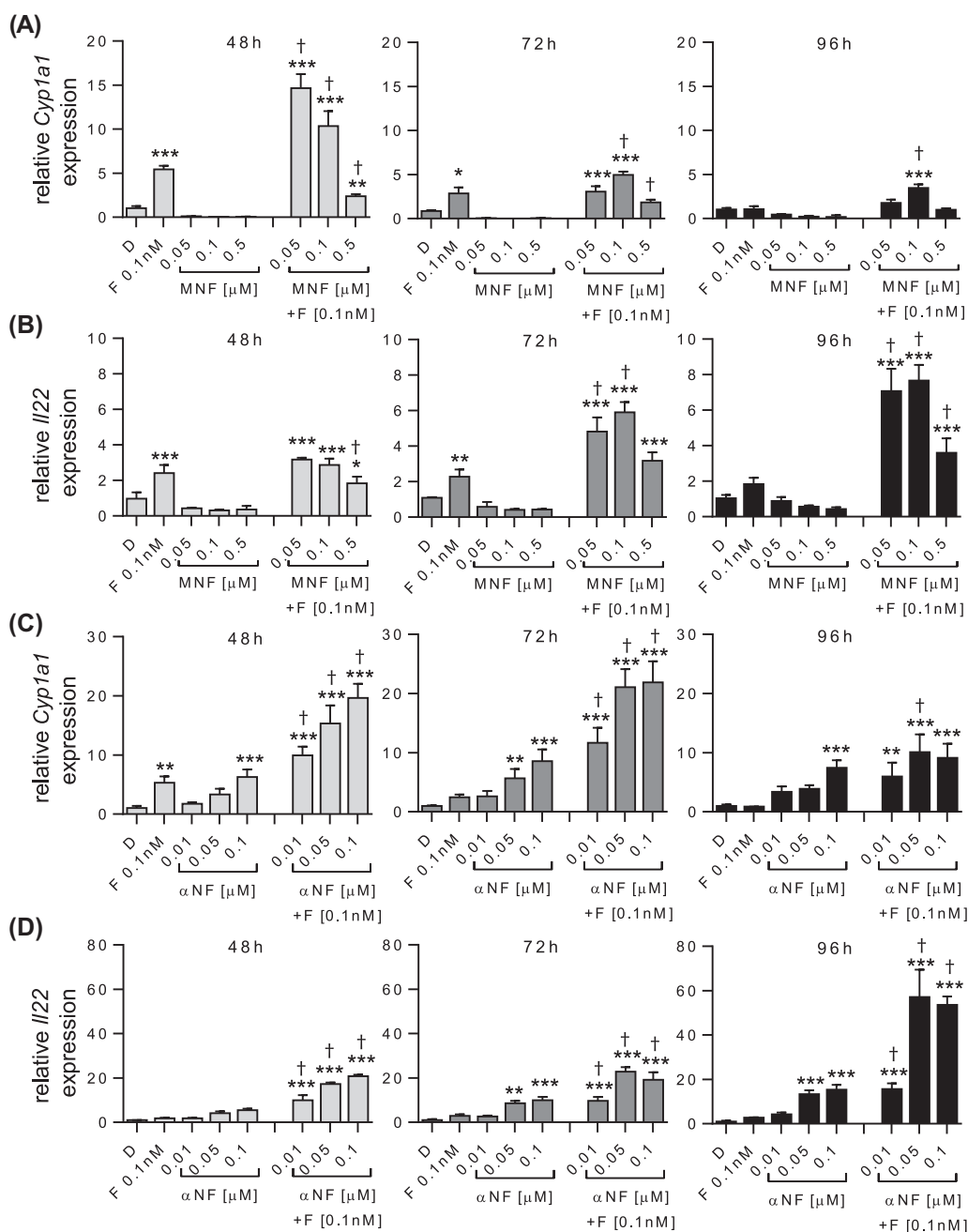


Fig. 4. Effects of model CYP1 inhibitors on FICZ-mediated AHR activation and *Il22* expression. CD4 + T cells (WT) undergoing Th17 differentiation were exposed to different doses of the model CYP1 inhibitors MNF (A–B) or αNF (C–D), with or without co-exposure to 0.1 nM FICZ (abbreviated; F). At 48, 72 and 96 h exposure cells were harvested and expression of *Cyp1a1* and *Il22* was determined. All results are shown as mean ± SD from 2 independent experiments each performed in duplicate (n = 4). Level of expression is normalized to *Hprt* and given relative to DMSO control (abbreviated; D) at each time point. Analysis of statistical differences between each exposure compared to the DMSO control at respective time-point was performed using two-way ANOVA followed by Sidak's *post hoc* test to correlate for multiple comparisons. Student's *t* test followed by Holm-Sidak *post hoc* test was used for statistical analysis of more or less than additive effects. Asterisks indicate statistically significant differences between DMSO control and respective exposure (*p < 0.05, **p < 0.01, and ***p < 0.001). Daggers designate significantly more or less than additive effects in co-exposures compared to sum of single exposures (†p < 0.05).

dose of MNF and αNF was based on reduced viability observed at higher doses (data not shown).

FICZ alone caused induced expression of *Cyp1a1* and *Il22*, with a more transient appearance for *Cyp1a1* compared to *Il22* (Fig. 4A–D). MNF alone did not induce expression of either gene, but rather showed a tendency towards reduced expression compared to DMSO control and an antagonistic effect at co-exposure to FICZ at the highest MNF dose (Fig. 4A and B). At the lower doses of MNF however, co-exposure with FICZ resulted in more than additive induction of gene expression (Fig. 4A and B). These synergistic effects were dependent on both dose and time and differed in kinetics for *Cyp1a1* and *Il22*. In general, effects on *Cyp1a1* expression appeared earlier and were of more transient nature compared to *Il22*. At 48 h exposure, synergistic *Cyp1a1* expression was observed in a reversed dose-response manner, while at 72 h and 96 h co-exposure only 0.1 μM MNF caused synergistic induction. For *Il22* expression, synergistic inductions were observed at 72 h and 96 h co-exposure, with a time-dependent increase in expression levels. In contrast to MNF, αNF alone generated an increase in expression of

both *Cyp1a1* and *Il22* (Fig. 4C and D) and synergistic inductions were observed at 48 h and 72 h co-exposure to FICZ with all doses of αNF. At 96 h co-exposure, *Cyp1a1* expression levels were reduced and synergistic induction was observed only with 0.05 μM αNF, while *Il22* expression levels were almost three times higher compared to 72 h co-exposure.

3.2.2. Functional effects of MNF and αNF on Th17 cell differentiation and *Il-22*

To determine functional effects of chemically induced AHR potentiation, the level of Th17 cell differentiation and IL-22 expression was determined in WT and AHR-KO cells after 96 h exposure to FICZ +/- MNF or αNF. Similar to effects observed at the gene expression level, exposure to MNF alone showed a suppressive tendency compared to DMSO control at the level of Th17 cell differentiation and IL-22 expression (Fig. 5A). However, levels of IL-22 in cell medium did not differ from DMSO control (Fig. 5B). In co-exposure with FICZ, a reversed dose-response was observed for fraction of IL17A⁺IL-22⁺ cells

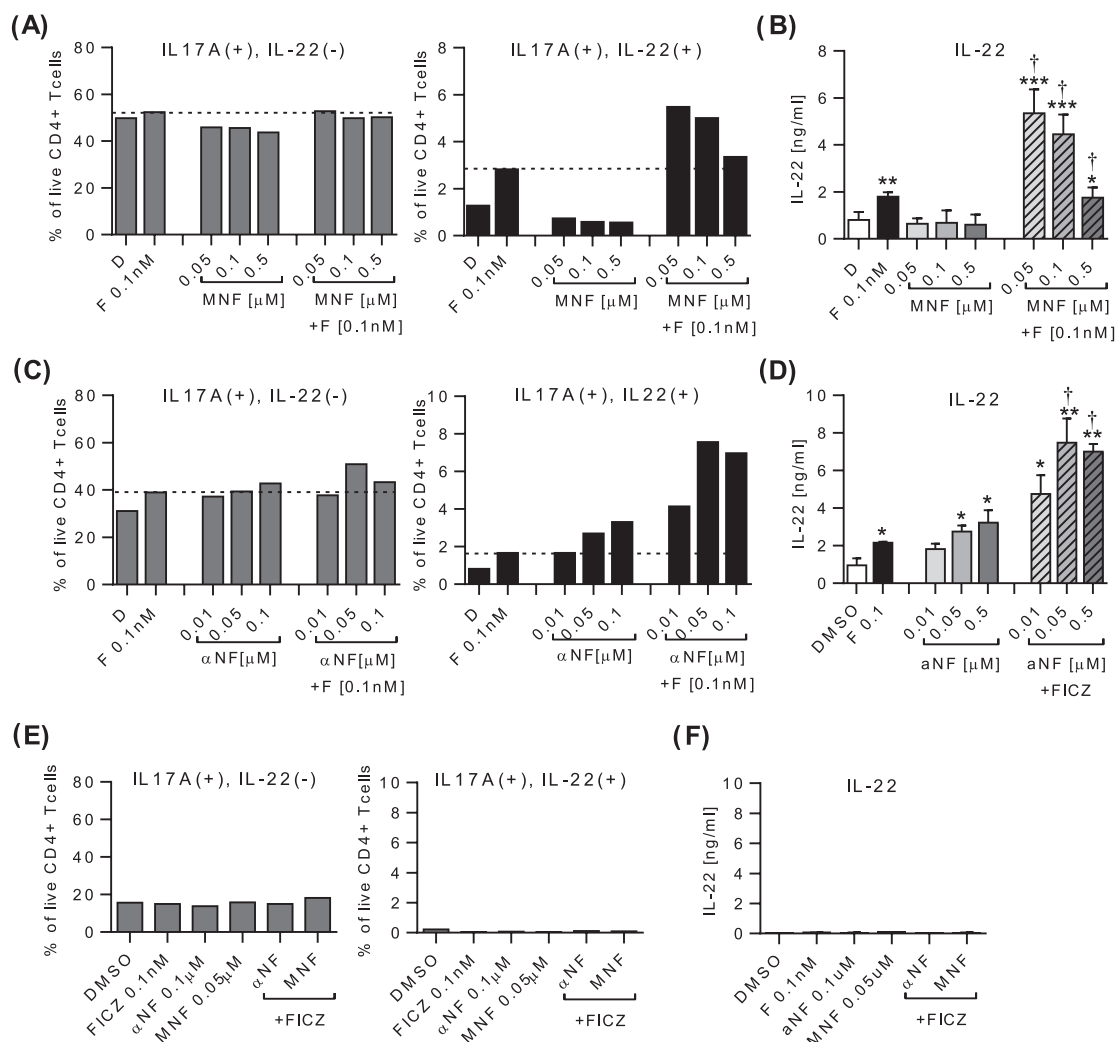


Fig. 5. Impact of MNF and αNF on FICZ-mediated Th17 cell differentiation and IL-22 expression. CD4 + T cells (WT) undergoing Th17 differentiation were harvested at 96 h exposure to different doses of MNF (A-B) or αNF (C-D) with or without co-exposure to 0.1 nM FICZ (abbreviated; F). The frequency of CD4 + T cells expressing IL17A alone (IL17A(+), IL-22(-)) or together with IL-22 (IL17A(+), IL-22(+)) was determined by means of FACS. In parallel, cell medium was harvested and analysed for levels of IL-22 by means of ELISA (B, D). Similar exposures and analysis were performed using CD4 + T cells isolated from *Ahr*^{-/-} mice (E-F). All FACS analysis were performed on pooled duplicates from one experiment while ELISA results are shown as mean ± SD from 2 independent experiments each performed in duplicate or triplicate (n = 4–6). Analysis of statistical differences in IL-22 expression between each exposure compared to the DMSO control was performed using one-way ANOVA followed by Sidak's *post hoc* test to correlate for multiple comparisons. Student's *t* test followed by Holm-Sidak *post hoc* test was used for statistical analysis of more or less than additive effects. Asterisks indicate statistically significant differences between DMSO control and respective exposure (*p < 0.05, **p < 0.01, and ***p < 0.001). Daggers designate significantly more or less than additive effects in co-exposures compared to sum of single exposures (†p < 0.05).

and IL-22 expression (Fig. 5A-B) where the two lower doses of MNF caused a synergistic induction of IL-22 levels in cell medium and the highest dose generated an antagonistic effect (Fig. 5B). No increase in fraction of IL17A⁺IL-22⁻ cells was however observed.

Effects of αNF alone or in co-exposure with FICZ (Fig. 5C-D) also very much resembled the effects at the gene expression level, with dose-dependent induction of IL-22 expression by αNF alone and synergistic induction at co-exposure to FICZ. A tendency toward increased Th17 cell differentiation was also observed.

To confirm the role of AHR in these effects, CD4 + T cells isolated from AHR-KO mice were exposed to FICZ +/- MNF or αNF and analyzed for level of Th17 differentiation and IL-22 expression. As seen in Fig. 5E-F, deletion of *Ahr* strongly reduced the fraction of IL17A⁺IL-22⁻ cells and completely abolished the expression of IL-22, independent of chemical exposure.

3.2.3. Impact of environmentally relevant CYP1 inhibitors on AHR signaling, Th17 cell differentiation and IL-22

The vast number of pharmaceutical drugs, dietary compounds,

metals and environmental chemicals known to impair the function of CYP1 enzymes [10,26–29] suggest this may indeed be an overlooked mechanism of dysregulated IL-22 signaling. To further test this hypothesis we repeated the experimental setup using the environmentally relevant PAHs fluoranthene (FL), pyrene (PY) and phenanthrene (Phe), all known to inhibit CYP1 function [30]. As in the previous experiments, CD4 + T cells were exposed to FICZ +/- CYP1 inhibitor from start of Th17 polarization and samples for analysis of gene expression, cell differentiation and IL-22 expression were taken at 72 h and 96 h exposure, respectively. To cover a wide dose range of the PAHs, three doses differing with a factor of 10 were selected (0.05, 0.5 and 5.0 μM) with the two lowest doses in range of concentrations measured in human blood [31–34].

Regarding AHR activation, none of the three PAHs alone evoked significant induction of *Cyp1a1* expression at 72 h exposure, while they all generated synergistic induction in combination with 0.1 nM FICZ (Fig. 6A), PY showing the strongest and Phe the weakest potentiation. From a dose-response perspective, Phe differed compared to FL and PY by showing a trend for a dose-dependent increase in potentiation while

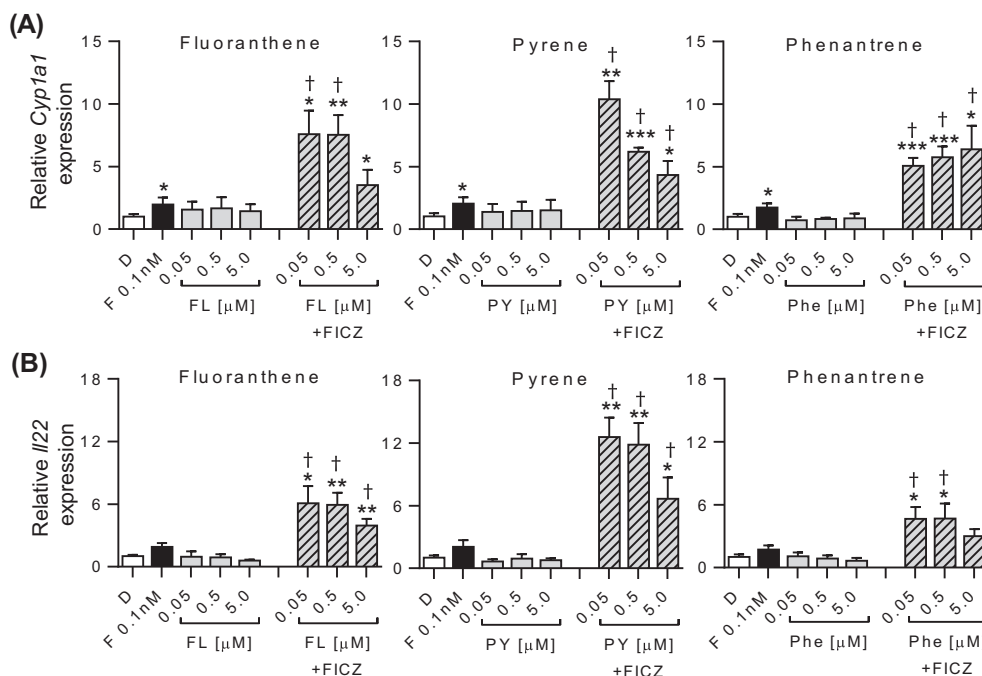


Fig. 6. Impact of PAH-type CYP1 inhibitors on FICZ-mediated AHR activation and *IL22* expression. CD4 + T cells (WT) undergoing Th17 differentiation were exposed to different doses of the PAHs fluoranthene (FL), pyrene (PY) or phenanthrene (Phe) with or without co-exposure to 0.1 nM FICZ (abbreviated; F). At 72 h exposure, cells were harvested and the expression of *Cyp1a1* (A) and *IL22* (B) was determined. All results are shown as mean $\pm$ SD from 2 independent experiments performed in duplicate and triplicate, respectively ($n = 5$). Level of expression is normalized to *Hprt* and given relative to DMSO control (abbreviated; D). Significant differences between DMSO control and respective exposure were determined using One-way ANOVA followed by Sidak *post hoc* test to correlate for multiple comparisons. Student's *t* test followed by Holm-Sidak *post hoc* test was used for statistical analysis of more or less than additive effects. Asterisks indicate statistically significant differences between DMSO control and respective exposure (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Daggers designate significantly more or less than additive effects in co-exposures compared to sum of single exposures ($\dagger p < 0.05$).

the latter two showed a clear reversed dose-response. Similar results were observed with *IL22* expression (Fig. 6B). Here, no induction was observed after exposure to the PAHs alone or to FICZ alone, while different levels of synergy was observed at co-exposure to each separate PAH + FICZ. Unlike the dose-response of *Cyp1a1* expression all three PAHs generated a reverse dose-response trend for the potentiation of *IL22*. Again, PY showed strongest potentiation of expression, followed by FL and Phe.

Regarding functional effects, some of the PAHs showed a tendency to induce Th17 cell differentiation and IL-22 expression at the lower concentrations and to reduce Th17 differentiation at the highest concentration (Fig. 7). At co-exposure to FICZ, all three PAHs generated synergistic induction of IL-22 expression in a reversed dose-response manner (Fig. 7B, D, F). AHR-KO cells were used to confirm the role of AHR in effects of FL, PY and Phe on Th17 differentiation and IL-22 expression (Fig. 7G). As with MNF and α NF, fraction of Th17 cells were strongly reduced, independent of exposure, and IL-22 expression was close to abolished.

3.2.4. Impact of CYP1 inhibitors on AHR signaling and *IL22* gene expression induced by TCDD or ICZ

To further investigate the impact of CYP1 inhibition on AHR signaling and *IL22* gene expression we next compared the effect of MNF and PY on AHR activation mediated by TCDD and indolo[3,2-*b*]carbazole (ICZ). TCDD is a potent AHR ligand known to be poorly metabolized by CYP1 enzymes while ICZ, a derivative of the dietary component indole-3-carbinol, represents an AHR ligand readily metabolized by CYP1 enzymes [21,35,36]. CD4 + T cells were exposed to TCDD or ICZ +/- MNF or PY from the start of Th17 polarization and samples for analysis of gene expression were taken at 48 h, 72 h and 96 h exposure (Fig. 8). Due to differences in AHR potency, TCDD was used at 0.1 nM while ICZ was used at 1 nM. The dose for MNF and PY (0.05 μ M) were based on previous experiments. Exposure to TCDD generated a sustained induction of both *Cyp1a1* (Fig. 8A) and *IL22* (Fig. 8B), with expression levels still increasing at 96 h exposure. In contrast, ICZ showed maximum induction at 72 h exposure, followed by a reduced induction at 96 h. Similar to our observations with FICZ, co-exposure of Th17 cells to ICZ and MNF or PY led to synergistic induction of *Cyp1a1* and *IL22* expression. By contrast, co-exposure of TCDD and MNF or PY inhibited AHR-mediated gene expression in Th17 cells. Although we

currently do not understand the mechanisms by which MNF or PY abrogate TCDD potency our data strongly suggest that CYP1 inhibition by PY and MNF potentiates signaling only in response to metabolizable AHR ligands.

3.3. Chemical CYP1 inhibition as a mechanism of induced IL-22 expression and reduced FICZ metabolism in Th17 cells

Although the selected chemicals are known inhibitors of CYP1 function, we next wanted to confirm the impact of their innate inhibitory function on IL-22 production. In theory, if CYP1 inhibition is the mechanism underlying their capacity to potentiate FICZ-mediated induction of IL-22 expression, two qualities need to be fulfilled; i) they should not be able to induce IL-22 expression in a *Cyp1*-depleted system, and ii) they should reduce cellular clearance of FICZ.

3.3.1. Impact of chemical CYP1 inhibitors on IL-22 production in CYP1-knockout cells

As shown in the previous experiments, exposure to several of the CYP1-inhibitors alone generated induced expression of *IL22* and/or levels of IL-22 excreted in cell medium. To investigate whether these inductions are due to impaired CYP1 function, we next compared effects of MNF, α NF, FL, PY or Phe alone on IL-22 in WT cells with effects in CYP1-KO cells. Here, exposure to FICZ or α NF induced *IL22* expression (Fig. 9A), while all exposures except MNF and PY caused induced levels of IL-22 excretion in WT cells (Fig. 9B). Exposure to MNF showed antagonistic effects at both gene- and protein expression levels. Notably, responses in CYP1-KO cells differed completely (Fig. 9C and D). In these cells, only FICZ was able to induce gene- and protein expression of IL-22 (compared to DMSO) and the potent induction by α NF alone observed in WT cells was reversed into a significant antagonistic response. MNF showed antagonistic effects similar to WT cells while all three PAHs showed no effects compared to DMSO control.

3.3.2. Impact of chemical CYP1 inhibitors on FICZ metabolism in Th17 cells

Our previous studies have shown a clear link between MNF- and α NF-mediated CYP1 inhibition and reduced cellular clearance of FICZ, with potentiation of AHR signaling as consequence [8,10,11]. To investigate whether the CYP1 inhibitors used in this study act in the same

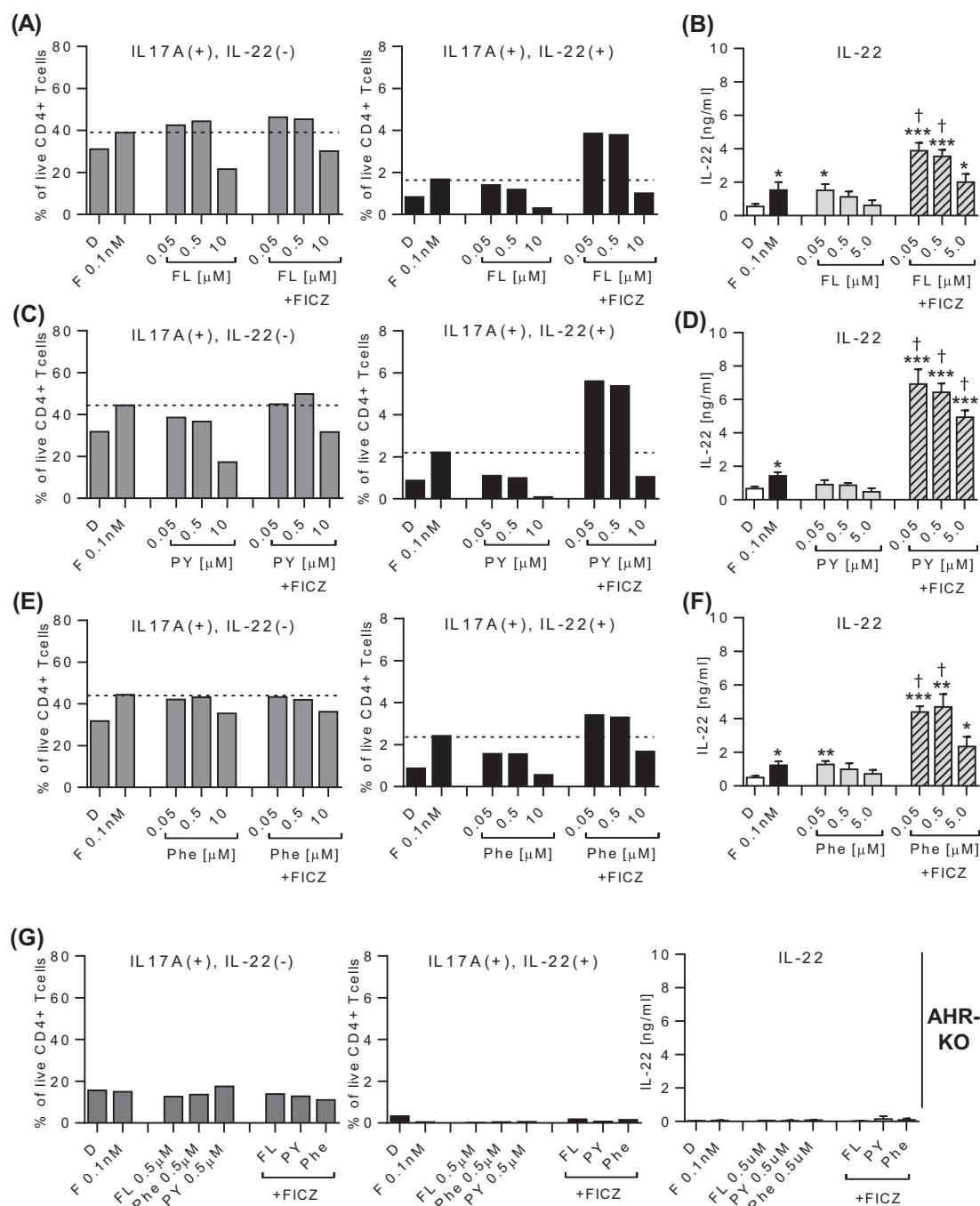


Fig. 7. Effects of PAH-type CYP1 inhibitors on FICZ-mediated Th17 cell differentiation and IL-22 expression. CD4 + T cells isolated from isolated from WT or *Ahr*^{-/-} mice were exposed to different doses of the PAHs fluoranthene (FL; A-B, G), pyrene (PY; C-D, G) or phenanthrene (Phe; E-F, G), with or without co-exposure to 0.1 nM FICZ (abbreviated; F). At 96 h exposure cells were harvested and the fraction of CD4 + T cells with intracellular expression of IL17A alone (IL17A(+), IL-22(-)) or with simultaneous expression of IL-22 (IL17A(+), IL-22(+)) were determined using FACS. In parallel, levels of IL-22 excreted to the cell medium were determined using ELISA. All FACS analysis were performed on pooled duplicates from one experiment while ELISA results are shown as mean $\pm$ SD from 2 independent experiments performed in duplicate or triplicate ($n = 4-5$). Analysis of statistical differences in IL-22 expression between each exposure compared to the DMSO control was performed using one-way ANOVA followed by Sidak's *post hoc* test to correlate for multiple comparisons. Student's *t* test followed by Holm-Sidak *post hoc* test was used for statistical analysis of more or less than additive effects. Asterisks indicate statistically significant differences between DMSO control and respective exposure (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Daggers designate significantly more or less than additive effects in co-exposures compared to sum of single exposures ($^{\dagger}p < 0.05$).

manner during Th17 cell differentiation, cellular levels of FICZ were determined after co-exposure to different doses of CYP1 inhibitors (Fig. 10). Cellular uptake of FICZ was observed already at the first sampling point (1h exposure) and reached maximum levels at 5 h, after which levels had slightly declined at 8 h followed by strongly reduced levels at 24 h and only low levels remaining at 48 h. Co-exposure to α NF or MNF showed comparable effects on FICZ clearance. Both

compounds caused significantly higher cellular levels of FICZ at 8–48 h in a dose-dependent manner (Fig. 10A and B), the higher dose showing stronger and more sustained effects for both compounds.

Similar trends were observed at co-exposure to FL and PY. Here, co-exposure to FL caused increased levels of FICZ at 24 h, while PY caused increased levels at both 8 h and 24 h (Fig. 10C and D). As with MNF and α NF, the higher doses were more efficient in reducing cellular clearance

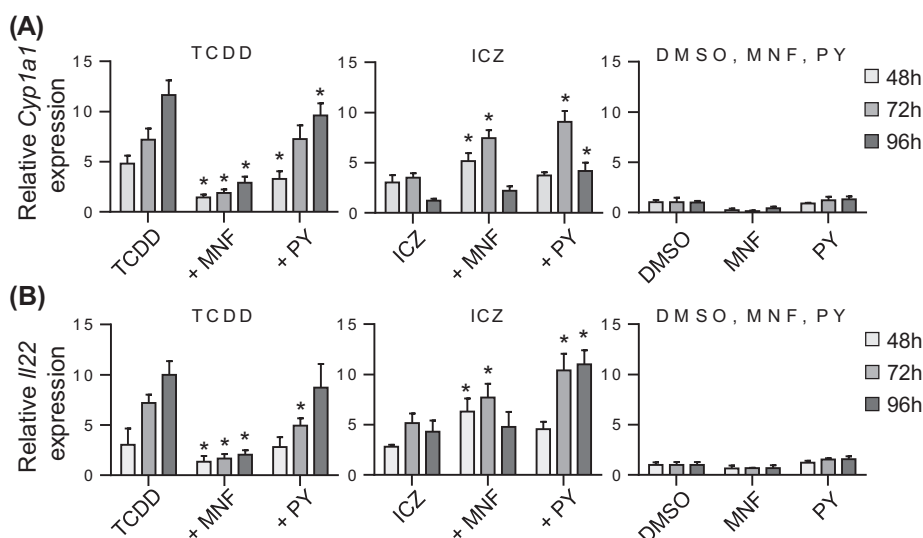


Fig. 8. Impact of CYP1 inhibitors on AHR activation and IL-22 expression induced by TCDD and ICZ. CD4⁺ T cells (WT) undergoing Th17 differentiation were exposed to 0.05 μ M of MNF or PY, with or without co-exposure to 0.1 nM or 1 nM ICZ. At 48, 72 and 96 h exposure cells were harvested and expression of *Cyp1a1* (A) and *IL-22* (B) was determined. All results are shown as mean $\pm$ SD from 2 independent experiments each performed in duplicate (n = 4). Level of expression is normalized to *Hprt* and given relative to DMSO control at each time point. Student's *t* test followed by Holm-Sidak *post hoc* test was used for statistical analysis of more or less than additive effects. Asterisks designate significantly more or less than additive effects in co-exposures compared to sum of single exposures (**p* < 0.05).

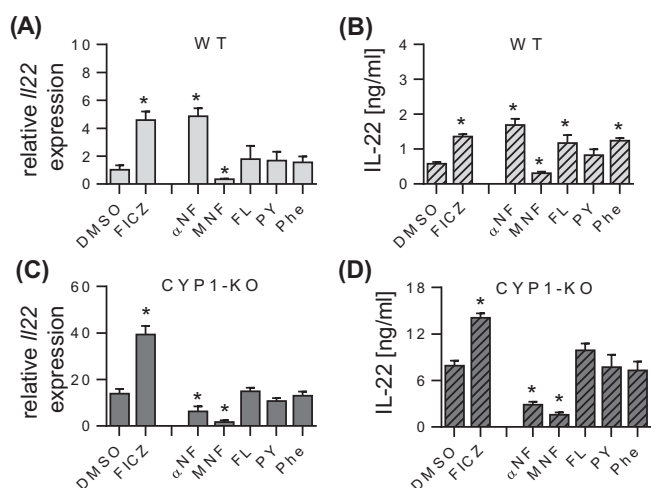


Fig. 9. IL-22 production in response to CYP1-inhibitors in a *Cyp1*-depleted system. CD4⁺ T cells isolated from WT- or CYP1-KO mice were exposed to FICZ (0.1 nM) or 0.05 μ M CYP1 inhibitor (MNF, α NF, FL, PY or Phe) while undergoing Th17 differentiation. At 72 h exposure cells were harvested and expression of *IL22* was determined by means of qRT-PCR (A-B). Level of expression is normalized to *Hprt* and given relative to DMSO control. At 96 h exposure, cell medium was collected and analysed for content of IL-22 by ELISA (C-D). Gene expression- and ELISA analyses were performed with samples from separate experiments performed in triplicate and are shown as mean $\pm$ SD (n = 3). Significant differences between DMSO control and respective exposure were determined using One-way ANOVA followed by Sidak *post hoc* test to correlate for multiple comparisons and are shown by asterisks (**p* < 0.05).

of FICZ. Phe showed increased levels of FICZ at 24 h, although only at the highest dose (Fig. 10D).

4. Discussion

In this study we showed that FICZ-dependent AHR activation and downstream IL-22 production is strongly enhanced when CYP1 enzyme function is impaired. Together with our previous report of a critical role for CYP1 enzymes in feedback-regulation of AHR signaling in intestinal immunity [9], these data suggest that CYP1 enzymes may be essential for other AHR-regulated functions in the immune system. In this context, reduced catalytic activity of CYP1 enzymes may result in elevated systemic levels of endogenous AHR ligands such as FICZ, which could contribute to immune-related adverse effects.

In the present study, the distinct dose- and time dependent effects of FICZ on AHR signaling in Th17 cells demonstrates two critical aspects

of AHR signaling in Th17 cells. Firstly, Th17 cells are very sensitive to FICZ-induced AHR activation, supporting previous findings of high AHR expression in this cell type [18]. Secondly, *Cyp1a1* is inducible very early in the Th17 differentiation process which is in line with previous reports describing *Ahr* expression as early as 4 hrs following initiation of Th17 differentiation [37]. Furthermore, the transient nature of AHR activation by lower concentrations vs. sustained activation with higher concentrations of FICZ indicates a functional but limited CYP1 metabolic capacity throughout the differentiation process, resulting in antagonistic effects at a high dose. In comparison, dose- and time-response studies using human keratinocytes (HaCaT) [10] or hepatoma cells (HepG2) [8] showed steeper transience in response to FICZ with maximum induction around 2 h and complete termination of activation at 24 h. Although an antagonistic response by FICZ was observed also in HaCaT cells, this occurred at much higher doses [10], further pointing to a more limited metabolic capacity in Th17 cells.

A lower capacity to metabolize FICZ would cause not only a prolonged AHR activation but also an enhanced Th17 cell differentiation and IL-22 production, as was evident when using CYP1-KO CD4⁺ T cells. Notably, the difference in dose-response pattern between CYP1-KO and WT Th17 differentiation suggests that CYP1-deficient cells become saturated or even inhibited in their Th17/IL-22 response at FICZ levels above 10 nM, while WT cells do not. A possible explanation for these results is AHR antagonism due to cellular accumulation of FICZ resulting in an “antagonistic internal dose”. However, the mechanism behind this suppression is not clear and it remains a possibility that higher doses of FICZ induce an alternative transcriptional program in CD4⁺ T cells at the expense of Th17 differentiation. No changes in Foxp3 induction were however observed, ruling out competing differentiation into regulatory T cells (Treg) (data not shown).

Altogether, the data generated using the CYP1-KO cells clearly demonstrate an essential role of CYP1 metabolic activity in regulation of IL-22 production and support the notion that altered CYP1 function may play a role in IL-22 regulated physiological and pathological processes. The relatively smaller impact of CYP1-KO on Th17 reiterates our previous finding that Th17 cell differentiation can be enhanced by FICZ but is not strictly dependent on AHR activation [18].

We and others have previously demonstrated synergistic AHR activation and AHR-mediated toxicity at combined exposure to a CYP1-inhibitor with a metabolizable AHR agonist, and reduced receptor activation and toxicity in combination with a poorly metabolized agonist [8,10,11,14,15,17,24,38–40]. However, the impact of chemical CYP1 inhibition on the Th17/IL-22 response remained unknown. In this project we clearly demonstrate that chemical CYP1 inhibitors reduce the cellular clearance of FICZ and strongly potentiate IL-22 production

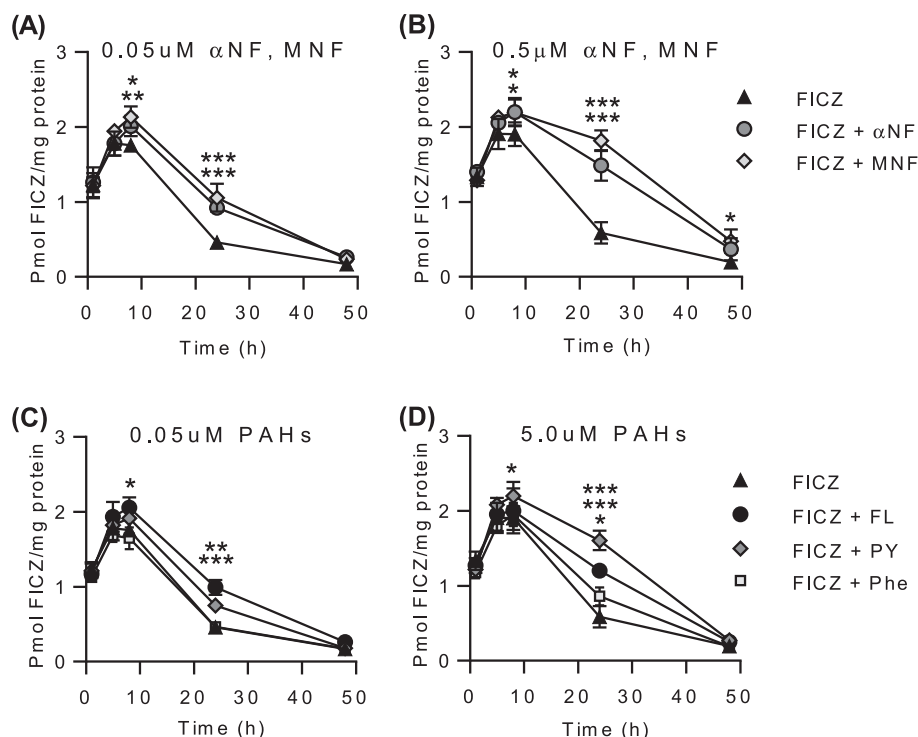


Fig. 10. Impact of CYP1 inhibitors on cellular clearance of FICZ. Effects of low and high concentrations of α NF or MNF (A-B) and the PAHs fluoranthene (FL), pyrene (PY) or phenanthrene (Phe) (C-D) on FICZ metabolism was determined by measuring cellular levels of FICZ (pmol/mg protein) upon co-exposures to respective CYP1 inhibitor. Results are shown as mean $\pm$ SD from one experiment performed in triplicate ($n = 3$). Differences in levels of FICZ in co-exposures compared to exposure to FICZ alone were determined using Two-way ANOVA followed by Dunnett's *post hoc* test to correlate for multiple comparisons. Statistically significant differences are shown by asterisks (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

in an AHR dependent manner in Th17 cells. The results obtained when exposing CYP1-deficient cells to the separate inhibitors, together with their impact on cellular clearance of FICZ, support our suggestion of CYP1-inhibition *per se* being the mechanism underlying potentiation of IL-22 expression by these compounds. This was further corroborated by the lack of potentiation observed after co-exposure to the poorly metabolized ligand TCDD.

The CYP1 inhibitors were selected based on previously demonstrated effects on CYP1/AHR-feedback regulation (MNF and α NF) or environmental relevance (FL, Phe and PY). Importantly, our data demonstrate that environmentally relevant PAHs, at doses detected in human blood [31–34], potentiate AHR activation and subsequent IL-22 production by an endogenous receptor ligand. While some PAHs such as benzo(a)pyrene and 3-methylcholanthrene are known to activate AHR signaling [41], AHR-potentiation through CYP1 inhibition demonstrates an additional mechanism through which PAHs and other environmental pollutants may impair immune homeostasis. Our findings postulate a novel mechanism of IL-22 regulation by environmental factors that could potentially promote the tissue protective functions of IL-22 in intestinal homeostasis or exacerbate IL-22-driven adverse effects. Moreover, measurements of maternal blood and cord blood revealed levels of FL, PY and Phe in the same range as included in this study, suggesting a possible effect of these PAHs on immune system maturation during early development [34]. It is also important to point out that in this study cells were exposed to one CYP1 inhibitor at a time, while humans and wildlife are exposed to complex mixtures that together may reach higher CYP1-inhibiting potencies.

While all CYP1 inhibitors generated synergistic effects on AHR signaling and IL-22 expression at co-exposure to FICZ, they differed in effects when used as single exposures. The antagonistic effect observed with MNF is in line with previous reports on MNF acting as an AHR antagonist [10,42] in addition to being a CYP1 inhibitor. In contrast, α NF has been suggested to act as a weak agonist and partial antagonist, which could explain the observed induction [43–45] by α NF alone. However, we recently revealed that AHR activation by α NF is dependent on receptor agonists in the cell culture medium [10] and that *in vitro* differentiation of Th17 cells are highly influenced by same medium ligands [46], confirming that the observed effects by α NF most

likely is not due to AHR agonism *per se*. Thus, differences in dose- and time-response observed with MNF and α NF alone and in co-exposure to FICZ are likely due to their innate differences in potency to inhibit the function of CYP1 enzymes [10,26,47] and to antagonize AHR signaling [48,49]. The antagonistic switch observed with α NF in CYP1-KO cells may partly be explained by altered metabolism of α NF itself since CYP1 enzymes has shown to metabolize α NF [50–52] into even stronger CYP1 inhibitors [53]. The discrepancy between effects of single exposures of PAHs on IL22 gene expression and IL-22 excretion is not fully clear but is likely due to the difference in length of exposure (72 h for gene expression vs. 96 h for FACS and ELISA analysis) and the cumulative levels of IL-22 measured in the cell medium compared to the time-point specific measurement of gene expression.

Collectively, our data demonstrate that chemical CYP1-inhibition potentiates IL-22 production and this finding could have important implication for intestinal homeostasis. The intestinal immune system is exposed to a plethora of CYP1 inhibitory agents derived from environmental pollution [10] that may induce prolonged AHR pathway activation and IL-22 production. The receptor for IL-22 is restricted to non-hematopoietic cells such as intestinal epithelial cells (IEC) and dysregulated IL-22 signaling is associated with the development of colorectal cancer (CRC) through increased proliferation and survival of intestinal stem cells [19]. The use of CYP1 enzymes as therapeutic target in cancer prevention has been discussed extensively based on the overexpression of *Cyp1* in different tumors and the role of CYP1 in metabolic activation of pro-carcinogens [54–57]. However, CYP1-KO mice are reported to have increased DNA adduct levels following exposure to the PAH benzo[a]pyrene (BaP) [58] and CYP1A1 induction in the GI tract is absolutely required for detoxification of oral BaP [59], suggesting that functional CYP1 enzyme can also act as a tumor suppressor. Thus, CYP1 inhibition and subsequent potentiation of IL-22 production may represent an additional mechanism by which by environmental pollutants promote cancerogenesis.

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