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Polycyclic aromatic hydrocarbons reciprocally regulate IL-22 and IL-17 cytokines in peripheral blood mononuclear cells from both healthy and asthmatic subjects

Coline Ple,^{a,b,c,d*} Ying Fan ^{a,b,c,d*}, Saliha Ait Yahia ^{a,b,c,d}, Han Vorng ^{a,b,c,d}, Laetitia Everaere ^{a,b,c,d}, Cécile Chenivesse ^a, Joanne Balsamelli ^a, Imane Azzaoui ^{a,b,c,d}, Patricia de Nadai ^{a,b,c,d}, Benoit Wallaert ^{a,b,c,d,e}, Gwendal Lazennec ^f & Anne Tsicopoulos ^{a,b,c,d,e}

^aInstitut National de la Santé et de la Recherche Médicale, U1019, F-59019, Lille, France; ^bInstitut Pasteur de Lille, Center for Infection and Immunity of Lille, F-59019, Lille, France; ^cCNRS UMR 8204, F-59000, Lille, France; ^dUniv Lille Nord de France, F-59000, Lille, France; ^eClinique des Maladies Respiratoires et Centre Hospitalier Régional et Universitaire de Lille, F-59037, Lille, France; ^fInstitut National de la Santé et de la Recherche Médicale, U844, F-34295, Montpellier, France

Corresponding author:

Anne Tsicopoulos

Center for Infection and Immunity of Lille, U1019 Pulmonary Immunity

Institut Pasteur de Lille

1, rue du Professeur Calmette

59019 Lille Cedex, France

Tel : +33 320 877 221, Fax: +33 320 877 345

Email : anne.tsicopoulos@pasteur-lille.fr

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* These authors contributed equally to the work

ABSTRACT

Background: Pollution, including polyaromatic hydrocarbons (PAH), may contribute to increased prevalence of asthma. PAH bind to the Aryl hydrocarbon receptor (AhR), a transcription factor involved in Th17/Th22 polarization. IL-17 allows neutrophil recruitment and IL-22, tissue repair. Increased IL-17 and IL-22 productions have been associated with asthma.

Objective: To evaluate if PAH might enhance, through their effects on AhR, Th17 and/or Th22 polarization and contribute to asthma.

Methods: Activated peripheral blood mononuclear cells (PBMCs) from nonallergic nonasthmatic (NA) and allergic asthmatic (AA) subjects were incubated with PAH, and Th17/Th22 type cytokines and transcription factors evaluated by ELISA and Q-PCR respectively. Cytokine cell origin was determined by flow cytometry. The involvement of AhR and different kinases was evaluated using specific antagonists.

Results: PBMCs from AA exhibited an increased Th17/Th22 profile compared with NA subjects. Diesel exhaust particle (DEP)-PAH and Benzo[a]Pyrene (B[a]P) stimulation further increased IL-22 but surprisingly decreased IL-17A production in both groups. Accordingly, Th17 transcription factors *RORA* and *RORC* were down regulated, whereas AhR target gene *CYP1A1* was up-regulated in both groups. *NOTCH* was decreased only in AA patients. HAP-induced IL-22 production originated mainly from Th22 cells. The AhR antagonist reversed almost completely the effects of DEP-PAH, but only partially the effects of B[a]P, on IL-22/IL-17 reciprocal regulation. The kinases PI3K, JNK and ERK participated to the enhancing effect of B[a]P on IL-22 production, whereas p38 MAP kinase had an inhibitory effect.

Conclusion: DEP-PAH- and B[a]P- induced excessive production of IL-22 in AA may contribute to the progression of airway remodeling.

Key messages (30 words)

Capsule summary (35 words)

***Key words* (max 10 words)**

Abbreviations used

INTRODUCTION

Allergic asthma has strongly increased in the last decades in western countries and is considered mainly as a Th2 mediated disease. There is increasing evidence that environmental pollution contributes to this increased prevalence, as well as to new-onset asthma (1). In particular, we and others have shown that Polycyclic Aromatic Hydrocarbons (PAH) contained in diesel exhaust particles (DEP) play an adjuvant role in the development and exacerbation of allergic inflammation by skewing the immune response towards a Th2 profile, through increased IgE production, proTh2 cytokine and chemokine levels, and decreased pro Th1 cytokine and chemokine production (2-8). It is now clear that the biological response to many environmental pollutants is a direct consequence of their interactions with the Aryl hydrocarbon Receptor (AhR), a cytosolic ligand-activated transcription factor which binds exogenous ligands, such as PAH, as well as endogenous ligands, such as 6-formylindolo[3,2-b]carbazole (FICZ), a derivative of tryptophane metabolism (9). Recent data suggest that AhR may be a major transcription factor involved in the development of the recently described Th17 and Th22 subsets (10-13). The human Th17 subpopulation is characterized by the production of IL-17A, IL-17F, IL-22 and CCL20 (14), and the expression of ROR α and RORc (15, 16), whereas the Th22 subpopulation produces only IL-22 but not IL-17 (12, 17). IL-17A and IL-17F are involved in neutrophilic influx (18, 19), in airway smooth muscle cell proliferation and migration (20-22) and in mucus induction (23), through their receptors expressed on both hematopoietic cells and structural cells, whereas IL-22 is involved in tissue repair, smooth muscle cell proliferation (20, 21) and remodeling mechanisms through its receptor expressed only on structural cells. In agreement with the presence of these endpoints in asthma, increased levels of IL-17A, IL-17F and IL-22 are found in sera and at the lung level in allergic asthmatic patients as compared to controls (24-27) and tend to increase with the severity of the disease (24, 28, 29). Although AhR has been consistently involved in IL-22 production by T cells (12, 30), controversial results have been obtained for IL-17A production. Indeed, according to the studies, AhR ligands may induce in humans (31) and in mice (10-13) or inhibit IL-17A production in humans (12, 30). Moreover, AhR has been shown to favor regulatory T cells according to other studies (11, 32, 33).

These apparently contradictory results were partly explained by the ligand used, the exogenous ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) inducing functional T regulatory cells in mice (11) and in humans (34), whereas activation of AhR by the endogenous ligand FICZ boosted Th17 differentiation in mice (13), but not in humans (12). Despite these discrepancies, these studies link environmental pollution to the Th17/Th22 program, opening intriguing possibilities regarding the potential of such factors to initiate or augment allergic asthma. Therefore, we hypothesized that DEP-PAH and some purified PAH previously shown to be involved in asthma, might enhance, through their effects on AhR, Th17 and/or Th22 polarization and contribute to allergic asthma. In the present study, we show for the first time to our knowledge, that DEP-PAH and Benzo-a-pyrene exacerbated Th22 polarization in stimulated mononuclear cells, particularly in allergic asthmatic patients, whereas Th17 polarization was inversely down regulated. The mechanisms of reciprocal regulation were partly dependent upon AhR, and also involved the MAP Kinase p38.

METHODS

Human donors

Venous blood was collected from healthy nonallergic non asthmatic (NA) subjects, with negative skin prick tests to environmental allergens, total IgE levels <100KU/l and negative specific IgE towards environmental allergens and from allergic asthmatic (AA) patients. These patients had a clinical history of asthma, elevated IgE levels and exhibited positive skin prick tests and positive specific IgE (more than 3 KU/L) towards *Dermatophagoides pteronyssinus* (Dpt). In order to exclude the potential confounding effects of steroid treatment, only untreated asthmatic subjects taking only β 2 agonists as needed were included. The study was approved by the CHRU of Lille Ethical Review Committee (Number 2008/010) and all donors signed an informed consent form.

Cell purification and culture

Peripheral blood mononuclear cells (PBMCs) were prepared from blood collected on heparin as previously described (35). To activate the potentially circulating Th17/Th22 cells, we used a surrogate of antigen stimulation, i.e. stimulation of TCR through CD3 combined with costimulation by CD28, which has the advantage of activating all T cells. It is of note that activation of PBMC with the purified major allergen of Dpt Der p 1 alone did not induce the production of IL-17 or IL-22 in both NA and AA subjects. Preliminary experiments were performed to determine the minimal concentrations of anti CD3/CD28 able to induce IL-17 and IL-22 production, and PAH were added to these selected doses. PBMCs (2.10^6 /ml) were then stimulated in 48-well flat-bottomed microculture plates coated with anti-CD3 (100ng/ml, OKT3 clone) and soluble anti-CD28 (2 μ g/ml; BD Biosciences) in complete IMDM (Invitrogen Corporation, Carlsbad, CA, USA). This medium has been shown to be the best suited to assess Th17 expansion in both mouse and human cells (36). Preliminary kinetics experiments showed that the highest level of IL-17 was observed 72 hrs after stimulation. A mix of DEP-PAH commercially available SRM 1975 (Interchim, Montluçon, France) as well as different purified PAH previously shown to increase allergen-induced asthmatic responses, Benzo[a]Pyrene (B[a]P) (37), 9-10 Phenanthrene

quinone (PHEQ) (38), and anthracene (ANT) (39) were dissolved in 0.02% DMSO and used (all from Sigma-Aldrich, St Louis, MO, USA) at optimal non-cytotoxic doses enabling the production of Th17/Th22 cytokines (150ng/ml for SRM1975, 250ng/ml for B[a]P, 75nM for PHEQ and 1 μ M for ANT). PAH solvent (DMSO) was used as negative control. After 72hr of culture, the supernatants were collected and cells were harvested in TRIzol[®] Reagent (Invitrogen). In some experiments, AhR antagonist CH-223191 (3 μ M; Calbiochem, Darmstadt, Germany), inhibitors of p38 kinase (SB203580, 1 μ M; Merck KGaA, Darmstadt, Germany), c-jun N-terminal kinase (JNK) (SP600125, 20 μ M; Merck KGaA), MAP kinase kinase (MEK/ERK) (PD98059, 25 μ M; Merck KGaA) or Phosphatidylinositol 3-kinase (PI3K) (LY294002, 10 μ M; Merck KGaA) all used at non-cytotoxic concentrations were added to the cultures.

Cytokine secretion assays

All cytokines were measured by ELISA according to the manufacturer's recommendations, and expressed as nanograms per milliliter. IL-17A, IL-22, CCL20, IL-13, IL-10, TGF- β and CCL18 kits were from R & D systems (Minneapolis, MN, USA), IL-17F from eBiosciences (San Diego, CA, USA), and IFN- γ from BD Biosciences (Franklin Lakes, NJ, USA). The level of sensitivity was 15.6 pg/ml for IL-17A, IL-17F and CCL20, 31.2 pg/ml for IL-22, IFN- γ , and TGF- β , 93.8 pg/ml for IL-13 and 7.8 pg/ml for CCL18.

Quantitative Real Time PCR

Total RNA was isolated from stimulated PBMCs. RNA isolation was performed using TRIzol[®] reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was determined using the NanoVue spectrophotometer (GE Healthcare, Buckinghamshire, United Kingdom) and quality was evaluated by electrophoresis in a 0.8% agarose gel stained with Gelstar. Reverse transcription was performed using random primers and M-MLV enzyme (Invitrogen). Real-time PCR quantification was then performed using a SYBR Green approach (Light Cycler; Roche, Penzberg, Germany), as previously described (40). For each sample, target mRNA levels were normalized with RS9 house keeping gene

mRNA levels. The sequences of the oligonucleotides used are shown in the online repository (Table E1). Results were expressed as mean Relative Expression (RE) of $2^{(-\Delta Ct)} \pm SEM$.

Flow Cytometry

The cell origin of the production of IL-17 and IL-22 was determined by flow cytometry. Stimulated PBMCs ($2 \cdot 10^6$ /ml) were incubated with SRM 1975 (150ng/ml) or DMSO. After 2 days, IL-2 (20U/ml; PeproTech, London, United Kingdom) was added to the culture. At day 7, cells were stimulated by PMA (50ng/ml; Sigma-Aldrich) and Ionomycin ($1\mu\text{g}/\text{ml}$; Calbiochem) in the presence of Brefeldin A ($10\mu\text{g}/\text{ml}$; Sigma-Aldrich) for 4h30. Cell surface and intra cellular staining was performed as previously described (41, 42). Specific cell populations were identified using CD4-FITC⁺, CD8-PE⁺ for T cells; CD3-FITC⁺CD56-PE⁺ for NK cells; iNKT-PE⁺ for iNKT cells; Lin2-FITC⁺CD123-PE⁺/HLA-DR-V450⁺ for dendritic cells (DCs) (BD Biosciences) and intracellular staining using IL-17A-APC and IL-22-PERCP eFluor710 (eBiosciences). Mouse IgG1-APC and mouse IgG1-PERCP eFluor710 isotypes were from eBiosciences. Results were expressed as percentage of stimulated control cells set at 100%.

Statistical Analyses

Comparisons within NA and AA subjects for the different PAH stimulations were evaluated using one way ANOVA followed by post hoc multiple comparison tests, and between samples from NA and AA using unpaired t-test. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). Values of $p < 0.05$ were regarded as statistically significant.

RESULTS

Stimulated PBMCs from AA patients exhibit an exacerbated Th17 type cytokine profile compared to AA subjects

Previous studies have evidenced that allergic asthmatics exhibit higher levels of IL-17 in the lung than healthy subjects (24, 26, 27). To evaluate if this difference was also found in PBMC, we measured the production of IL-17A, IL-17F, CCL20 and IL-22 in anti-CD3/CD28 stimulated PBMCs of AA patients and NA control subjects. Stimulated PBMCs from AA patients exhibited significant increases in IL-17A, IL-17F and IL-22 as compared with PBMCs from NA subjects, whereas no difference was observed for CCL20 secretion (Figure 1A). Besides Th17/Th22- type cytokines, levels of Th2-, Th1- and Tregulatory-type cytokines were also determined. Anti-CD3/CD28- induced production of IL-13, IFN- γ , TGF- β and IL-10 were not significantly different between NA and AA donors (data not shown). Furthermore, the lineage-specifying Th17/Th22 transcription factors *ROR α* , *ROR γ* and AhR target gene *CYP1A* were evaluated by Q-PCR. As shown in figure 1B, *ROR α* and *CYP1A* mRNA were upregulated in AA compared to NA subjects, whereas *ROR γ* was not differentially expressed.

DEP-PAH and B[a]P drive reciprocal regulation of IL-17A and IL-22 production by PBMCs from both AA and NA subjects

To assess the ability of different PAH to modulate the production of Th17/Th22 type cytokines, activated PBMC were coincubated with SRM 1975 or with purified PAH. It is of note that PAH were not able to induce IL-17 or IL-22 production by unstimulated PBMCs (data not shown?). Surprisingly, B[a]P decreased IL-17A secretion of stimulated PBMCs from both AA and NA subjects, with a similar effect observed for SRM 1975 although to a lesser extent and only in AA patients (Figure 2A). No effects were observed on IL-17A production for the two other PAH, ANT and PHEQ (Figure 2A). IL-17F was slightly decreased in the presence of B[a]P and ANT, and only in AA patients (Figure E1A), and CCL20 was not modified (data not shown). The opposite effect was observed on IL-22 production for SRM 1975 and B[a]P with increasing secretion of IL-22 in both groups of subjects, but higher levels in AA than in NA subjects (Figure 2B). As for IL-17A, PHEQ and ANT did not modify IL-22 production. To evaluate if the

inhibition of IL-17A by PAH was related to the induction of suppressive mediators, the presence of two regulatory cytokines, IL-10 and TGF- β , and of CCL18, a chemokine with immunoregulatory properties (35, 42, 43) were assessed. However, there was no upregulation of IL-10 (figure 2C) or TGF- β (Figure E1B), and in contrast a strong inhibition of CCL18 in response to B[a]P was observed in both NA and AA subjects (figure E1C). As for the other cytokines, PHEQ and ANT did not modify the production of IL-10 and CCL18 and therefore were not furthermore evaluated. Collectively, these results show that B[a]P and DEP-PAH promote the secretion of IL-22 (produced by both Th17 and Th22 cells) and concomitantly inhibit IL-17A production (produced only by Th17 cells) independently of IL-10, suggesting that the dissociated effect may be linked to a differential effect on the two subpopulations.

DEP-PAH increase IL-22 production in Th22 cells, CD8⁺ T cells and dendritic cells

To evaluate which sub population was producing IL-22 in response to PAH stimulation, IL-22 cell origin was determined by flow cytometry following SRM 1975 stimulation. BaP stimulation appeared cytotoxic in the 7 day protocol necessary to ascertain IL-22/IL-17 production by intracellular staining, and therefore was discarded. Besides Th22 and Th17 cells, CD8, NK, NKT and DCs have been shown to produce IL-22 (44-47). In antiCD3/CD28- stimulated PBMC without PAH, as expected, IL-22 was mainly secreted by T cells ($76.0 \pm 6.7\%$ CD4⁺ T cells including $49.8 \pm 5.1\%$ Th22 cells and $26.2 \pm 5.3\%$ Th17 cells and $20.7 \pm 5.9\%$ CD8⁺ T cells) as well as from from DCs ($3.2 \pm 0.9\%$), whereas IL-17 was preferentially produced by Th17 cells ($74.9 \pm 5.7\%$) and less by CD8⁺ T cells ($16.4 \pm 5.0\%$) and dendritic cells ($3.0 \pm 0.3\%$). SRM 1975 stimulation induced an increase in IL-22 single positive cells in CD4⁺ T cells (Th22 cells), CD8⁺ T cells and dendritic cells, but no modification in IL-17A single positive cells and IL-17A/IL-22 double positive cells (Th17 cells) as compared with control cells (Figure 3). There was no difference of cytokine secretion between plasmacytoid and myeloid DC subtypes. No expression of IL-22 was observed in NK cells, and NKT cells did not survive in our culture conditions. Thus, the majority of DEP-PAH-induced IL-22 in PBMC came from Th22 cells ($60.6 \pm 4.1\%$), whereas the remaining was produced by CD8⁺T cells ($38.7 \pm 2.8\%$) and by DCs ($0.7 \pm 0.5\%$).

B[a]P and DEP-PAH inhibit *RORC*, *RORA* and *NOTCH* whereas they increase AhR target gene *CYP1A1* expression.

To understand the mechanisms underlying the modified cytokine profile in response to PAH, a number of transcription factors related to the Th17/Th22 profile were evaluated. Besides the key lineage transcription factors *ROR γ* and *RORC* for Th17 cells and *AHR* for Th17Th22 cells, *BATF* and *Notch* have been associated to Th17 differentiation in mice and also humans for the latter (48-50) and *BNC2* and *Notch* to Th22 differentiation in humans and mice respectively (51, 52). In agreement with the IL-17A protein data, SRM stimulation inhibited the Th17 associated transcription factors *RORA* and *RORC* in AA patients, whereas it had no effect in NA subjects. SRM stimulation induced the expression of the AhR target gene in both groups. The differential effect observed for B[a]P on IL-17 and IL-22 production was reflected at the transcriptional level, with inhibition of *RORA* and *RORC* in both groups and also of *NOTCH* in AA patients, whereas AhR target gene *CYP1A1* was increased in both groups (Figure 4). *NOTCH* was the only gene differentially expressed after PAH stimulation between NA and AA subjects. In contrast, the Th17 and Th22 associated transcription factor *BATF* and *BNC2* respectively were not modified by PAH stimulation. Collectively, these results suggest that PAH-induced down regulation of IL-17A may be related to transcriptional inhibition of *RORA* and *RORC*, as well as *NOTCH* in AA patients, whereas upregulation of IL-22 may relate to activation of AhR.

AhR participates to both induction of IL-22 and inhibition of IL-17 by PAH, albeit to a different extent

To more precisely determine the contribution of AhR to Th17 and Th22 cytokine regulation by PAH, we used the specific AhR antagonist CH-223191 (53). Control activated cells treated with CH-223191 exhibited decreased production of IL-22 (Figure 5A), consistent with previous observations showing that endogenous AhR ligands are induced by anti-CD3/CD28 stimulation and present in culture medium (30, 36). Surprisingly, control activated cells treated with CH-223191 only, exhibited highly increased level of IL-17A whatever the donor status (Figure 5B). AhR antagonist did not significantly

modify CCL18, IL-17F (Figure E2) and CCL20 secretion in control activated cells (data not shown). In SRM-stimulated conditions, IL-22 induced production was completely inhibited by the AhR antagonist in both AA and NA subjects, reaching the same level as of antagonist-treated control cells (dotted line) (Figure 5A), showing a total dependency upon AhR. Moreover, the small inhibitory effect of SRM 1975 observed on IL-17A production in AA patients, was also restored but not completely to the level of the antagonist-treated control cells (dotted line) (Figure 5B). In B[a]P-stimulated conditions, the inhibitory effect on IL-17A production and the enhancing effect on IL-22 secretion were only very partially reversed by the AhR antagonist, in both NA donors and AA patients, and failed to reach the level of antagonist-treated control cells (Figure 5A and 5B). The inhibitory effect of B[a]P on CCL18 production by activated PBMC was not dependent on AhR in both groups, as well as for B[a]P-induced IL-17F decrease in AA patients (Fig E2). Altogether, these results suggest that besides DEP-PAH effects that are almost entirely dependent upon AhR, other pathways are involved in B[a]P -induced IL-22/IL-17A imbalance in both groups.

PI3K, JNK and ERK participate to the enhancing effect of B[a]P on IL-22 production, whereas p38 MAP kinase has an inhibitory effect

B[a]P has been shown to have effects independent of the AhR pathway, in particular through the activation of MAP kinases (MAPK) (54). The involvement of different kinases was investigated in PBMC from NA subjects. PI3K and JNK inhibitors totally inhibited IL-22 induction in both control- and B[a]P-stimulated cells. ERK inhibitor also displayed a potent inhibitory effect although to a lesser extent. In contrast p38 inhibitor increased IL-22-production by control cells and further in B[a]P-stimulated cells, showing that p38 act as an inhibitor of IL-22 secretion and that B[a]P stimulation act on IL-22 secretion through p38 inhibition (Figure 6A). For IL-17 production, the inhibitory effect induced by B[a]P was not restored but reduced to the same level as control cells plus inhibitor, with PI3K, JNK and ERK inhibitors showing that these kinases are inducers of IL-17A, and are not involved in the inhibitory effect of B[a]P on IL-17 production. P38 inhibitor was the only one to slightly restore some IL-17 production above the level of inhibitor-treated control cells indicating that B[a]P may partly act through inhibition of p38

MAPK (figure 6B). Overall, these data show that IL-22 induction by B[a]P is dependent upon activation of AhR, PI3K ERK and JNK, and inhibition of p38 MAPK, whereas IL-17 inhibition is mediated partially by AhR and by p38 MAPK inhibition. Among these pathways AhR and p38 MAPK reciprocally regulate IL-17 and IL-22 production in response to PAH.

DISCUSSION

The aim of this study was to evaluate the involvement of PAH in the Th17/Th22 type profile in asthmatic patients, as recent studies point out a participation of these populations in some endpoints of asthma. Anti CD3/CD28 stimulated T cells from AA patients exhibited higher expression of the master Th17 transcription factors *RORC* and of the AhR target gene *CYP1A1* than NA subjects, which translated in increased levels of IL-17A, IL-17F and IL-22 cytokines, suggesting that AA patients display a higher sensitivity to the induction of the Th17/Th22 profile than NA subjects. Although the chosen PAH have been shown to enhance allergen-induced experimental asthma, (37-39), only DEP-PAH and B[a]P modulated IL-17A and IL-22 productions. This may be related to different activity of the PAH, phenanthrene and anthracene being weak inducers of AhR mediated activity (55). In contrast to our hypothesis of an inducing effect of PAH on both cytokines, an inhibitory effect on the production of IL-17A and an enhancing effect on IL-22 secretion were observed, which originated mainly from Th22 cells and not Th17 cells, suggesting that the two sub populations are differentially regulated by PAH. Similar differential regulation of IL-17A and IL-22 has been previously observed in human PBMC stimulated with the high affinity AhR ligand TCDD (30, 56), but not in mouse studies, where AhR ligands induced both cytokines (11-13). This indicates possible species-specific effects of AhR agonists. Although some AhR ligands such as TCDD have been shown to favor regulatory T cells that promote IL-10 in humans (34), PAH stimulation did not modify IL-10 production in PBMCs. Studies on human Th17 differentiation have revealed that absence of TGF- β inhibits IL-17 but promotes IL-22 production (57, 58). However, PAH stimulation did not modify TGF- β secretion, ruling out such a mechanism. Altogether, these data suggested that the mechanism of differential regulation of IL-17 and IL-22 was not related to post translational mechanisms involving inhibitory cytokines but rather to the transcriptional events occurring at earlier steps of PAH activation. In mice, IL-22 is produced mainly by Th17 cells and the transcription factors ROR γ t and ROR α , which controls the generation of Th17 cells, seem to be critical for IL-22 production, as transfer of ROR γ t and ROR α into mouse naïve T cells endows these cells with the ability to produce IL22 (16). However, transduction of RORc into human naïve CD4⁺

T cells does not lead to the production of IL-22 (59), whereas downregulation of RORc and AhR by siRNA in already differentiated human Th17 cells, leads to decrease in IL-22 production (12). In our conditions, including the presence of both naïve and memory differentiated T cells, PAH stimulation led to decreased transcription of both *RORA* and *RORC* genes, in agreement with the inhibitory effect observed on IL-17, and to increased AhR target gene *CYP1A1* expression concomitantly to IL22 enhancement suggesting that PAH differentially regulate these cytokines at the transcriptional level in humans. Among the other genes evaluated, *NOTCH* expression appeared to be associated with the inhibition of IL-17, but only in AA patients, suggesting an activation of this pathway specifically in these patients. In favour of this hypothesis, polymorphisms of *NOTCH* have been associated with asthma (60). To evaluate the role of AhR in IL-22 induction by PAH, a specific antagonist was used. As expected from our previous data, AhR antagonist inhibited PAH- as well as endogenous AhR ligand- induced IL-22 production but it also increased IL-17 production. This result is different from another study that found that the same antagonist was able to inhibit IL-17 production by human CD4⁺ cells cultured in Th17 polarizing conditions (36), suggesting that AhR activation may differ according to the presence of some cytokines or to the cell differentiation status. It also suggests that AhR may be more related to Th22 than to Th17 polarization in humans. Besides AhR, p38 MAPK was also able to antagonistically regulate IL-22 and IL-17 production in response to B[a]P, although inversely. The role of IL-17 and IL-22 in asthma is still controversial. Animal models of asthma have shown that both cytokines can be deleterious or protective. IL-17 has been shown to mediate steroid resistant inflammation and airway hyperresponsiveness (61) and to abrogate regulatory T cells mediated tolerance (62), whereas it can also negatively regulate established asthma (63). IL-22 has been shown to contribute to allergic asthma during the sensitization phase (25) and to promote lung immunopathology during fungal allergy (64), whereas it can also inhibit antigen-induced eosinophil airway inflammation through inhibition of IL-25 (65), of IL-10 (66), or during the challenge phase (25). Interestingly, this ambivalence has also been shown recently in a model of epithelial tumorigenesis, where IL-22 induction at baseline was protective, whereas sustained high levels of IL-22 caused prolonged epithelial proliferation promoting

intestinal tumors (67). Contrasting results have also been described in humans. It has been recently shown that IL-22 inhibits IFN- γ -induced expression of proinflammatory chemokines in human bronchial epithelial cells and that the levels of IL-22 in the broncho-alveolar lavage fluid of asthmatic patients are inversely correlated with the levels of proinflammatory chemokines, suggesting a protective role of IL-22 in asthma (68). On the other hand, increase in the two cytokines has been associated with more severe asthma (24, 26, 28, 29), and IL-22 enhances the proliferation and migration of human airway smooth muscle cells (20, 21), suggesting that IL-22 may be involved in smooth muscle cell hyperplasia. Taken together, these studies suggest that limited production of IL-22 may play an inhibitory role on the bronchial cell infiltration but that prolonged secretion may also promote airway remodeling, a feature thought to explain the lack of corticosteroid sensitivity in severe asthmatics. In our study, PAH stimulation led to increased levels of IL-22 in NA subjects, which were further increased in AA patients. These data suggest that in asthmatic patients, PAH-induced excessive production of IL-22 may contribute to the progression of airway remodeling.

FIGURE LEGENDS

FIG 1. Th17/Th22 type profile of anti-CD3/CD28-stimulated PBMCs. **A,** Cytokine secretion by stimulated PBMCs from NA subjects (n=7-8) and AA patients (n=8), expressed as means \pm SEM. **B,** Gene mRNA level in stimulated PBMCs from NA subjects (n=7) and AA patients (n=7), expressed as mean Relative Expression (RE) \pm SEM. * P <.05.

FIG 2. Cytokine profile of PAH-stimulated PBMCs. **A,** IL-17A **B,** IL-22 **C,** IL-10 (**C**) secretion by activated PBMCs from NA subjects (n=7-8) and AA patients (n=9) incubated or not with PAH. Results are expressed as means \pm SEM. * P <.05 and ** P <.01 versus control.

FIG 3. Intra cellular distribution of IL-17A and IL-22 after DEP-PAH activation of PBMCs. Results are expressed as mean % of control set at 100% (dotted line) \pm SEM. One representative flow cytometry experiment per cell subset out of 3 to 6 is shown.

FIG 4. Transcript levels of genes involved in Th17/Th22 polarization in PBMCs. Activated PBMCs from NA subjects (n=8) and AA patients (n=7) were incubated with or without PAH. Results are expressed as mean RE \pm SEM. * P <.05, ** P <.01, ### p <.01 NA versus AA subjects.

FIG 5. Effects of AhR antagonist on IL-22 (**A**) and IL-17A (**B**) secretion by PBMCs. Activated PBMCs from NA subjects (n=7-8) and AA patients (n=9) were incubated or not with PAH, in the presence or not of AhR antagonist CH-223191. The dotted line is set on the level of the antagonist-treated control cells. Results are expressed as means \pm SEM. * P <.05, ** P <.01.

FIG 6. Effects of kinase inhibitors on IL-22 (**A**) and IL-17A (**B**) secretion by PBMCs. Activated PBMCs from 8 NA subjects were incubated or not with PAH and with or without inhibitors of kinases. The dotted line is set on the level of inhibitor-treated control cells. Results are expressed as mean % of control set at 100% \pm SEM. * P <.05, ** P <.01 *** P <.001

FIG E1. Cytokine profile of PAH-stimulated PBMCs. **A**, IL-17F **B**, TGF- β **C**, CCL18 secretion by activated PBMCs from NA subjects (n=7-8) and AA patients (n=9) incubated or not with PAH. Results are expressed as means \pm SEM. * P <.05 and ** P <.01 versus control.

FIG E2. Effects of AhR antagonist on IL-17F (**A**) and CCL18 (**B and C**) secretion by PBMCs. Activated PBMCs from NA subjects (n=7-8) and AA patients (n=9) were incubated or not with PAH, in the presence or not of AhR antagonist CH-223191. The dotted line is set on the level of the antagonist-treated control cells. Results are expressed as means \pm SEM.

Acknowledgments

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Table E1
Oligonucleotide sequences for qPCR amplification of target mRNA

mRNA	Oligonucleotide sequences
<i>BATF</i>	Forward: 5' - ACGCAGGGGTCAGAGGTGGCTACA -3' Reverse: 5'- GGCTCTTCTGGGCGGCAATACGA -3'
<i>BNC2</i>	Forward: 5' - GACTCGTCGCAGCCCTCCCTTAG -3' Reverse: 5'- ACTGTCACTCGCCCCGTCAATGTC -3'
<i>CYP1A1</i>	Forward: 5'- GGTCAAGGAGCACTACAAAACC -3' Reverse: 5'- TGGACATTGGCGTTCTCAT -3'
<i>NOTCH2</i>	Forward: 5' - CTGGCTTTGCTGGGGAGCGTT -3' Reverse: 5'- TCCCGGGGACAACGGCAA -3'
<i>RORA</i>	Forward: 5' - ATGCCTTGCCGTAGGGATGT -3' Reverse: 5'- CCGTTGGCCGAGATGTTGTA -3'
<i>RORC</i>	Forward: 5' - GCATGTCCCGAGATGCTGTC -3' Reverse: 5'- CTGGGAGCCCCAAGGTGTAG -3'
<i>RS9</i>	Forward: 5' - AAGCCGCCCCGGAACTGCTGAC -3' Reverse: 5'- ACCACCTGCTTGC GGACCCTGATA -3'

Table E1

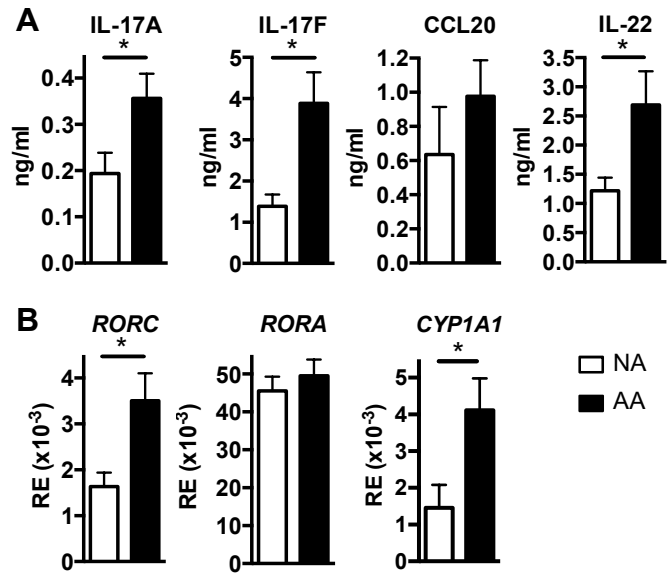


Figure 1

Figure 2

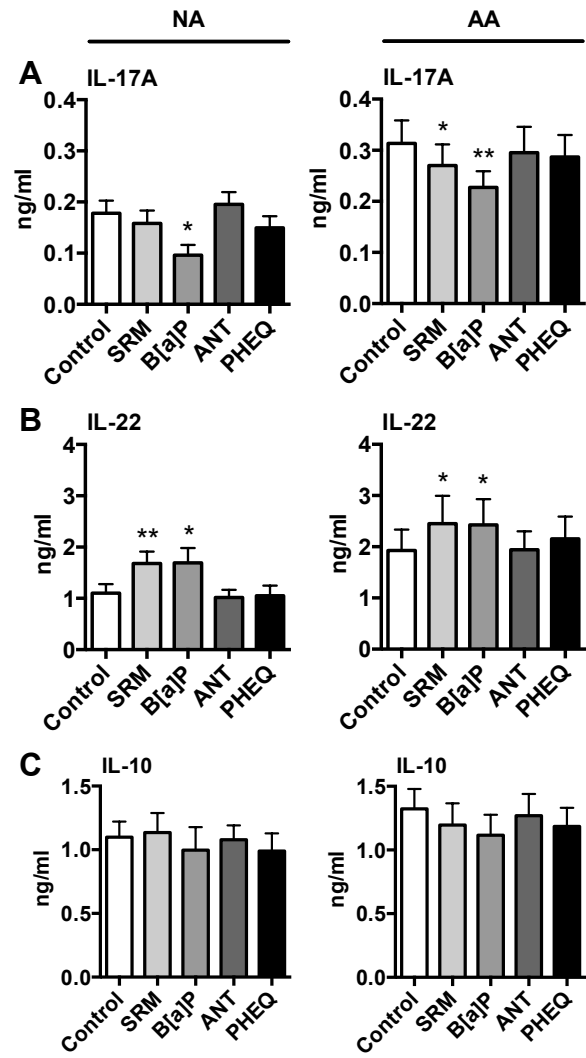


Figure E1

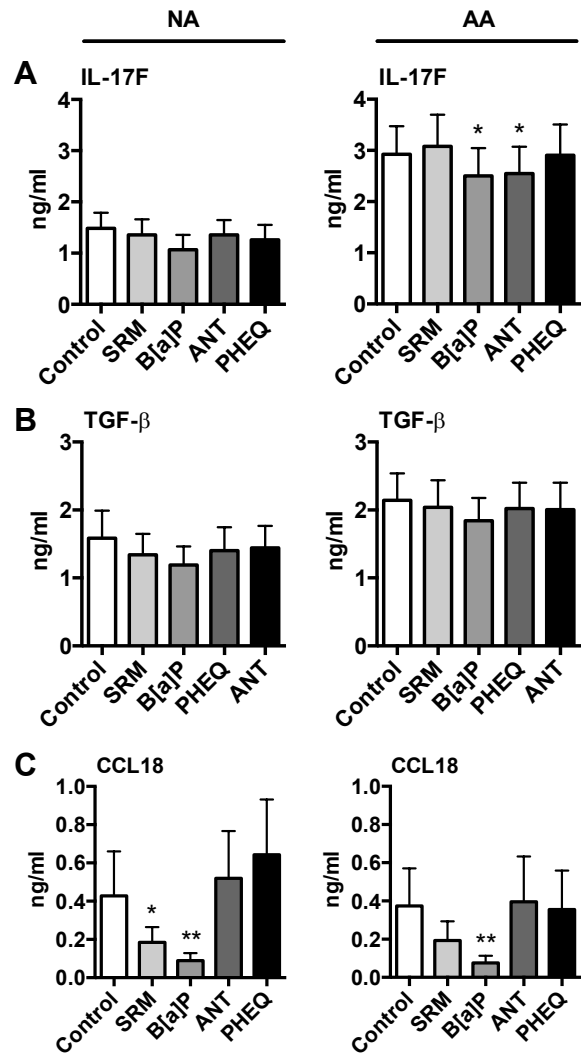


Figure 3

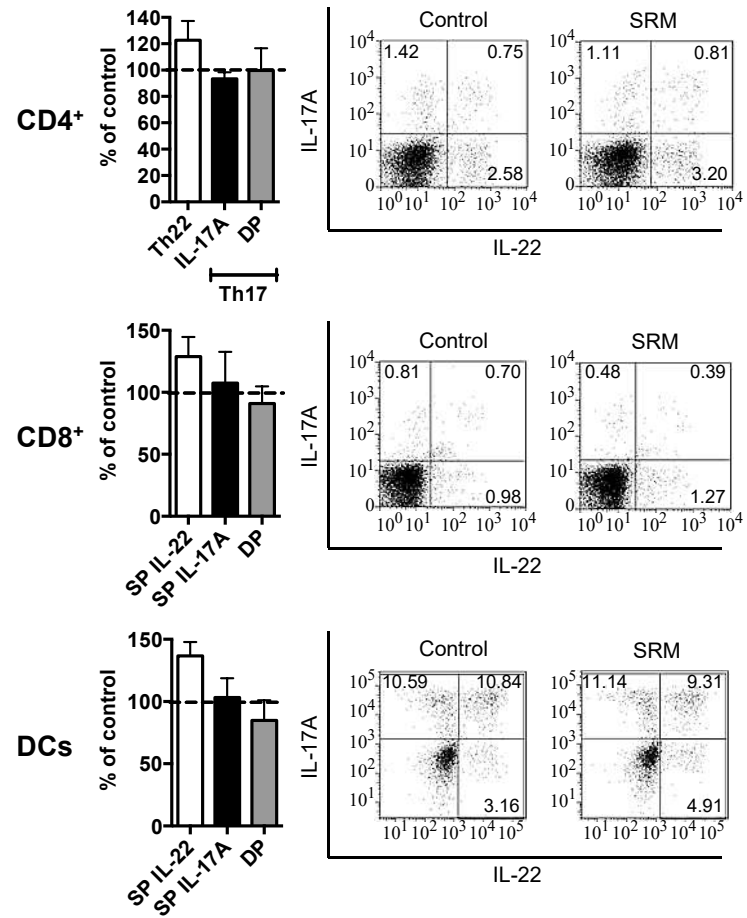
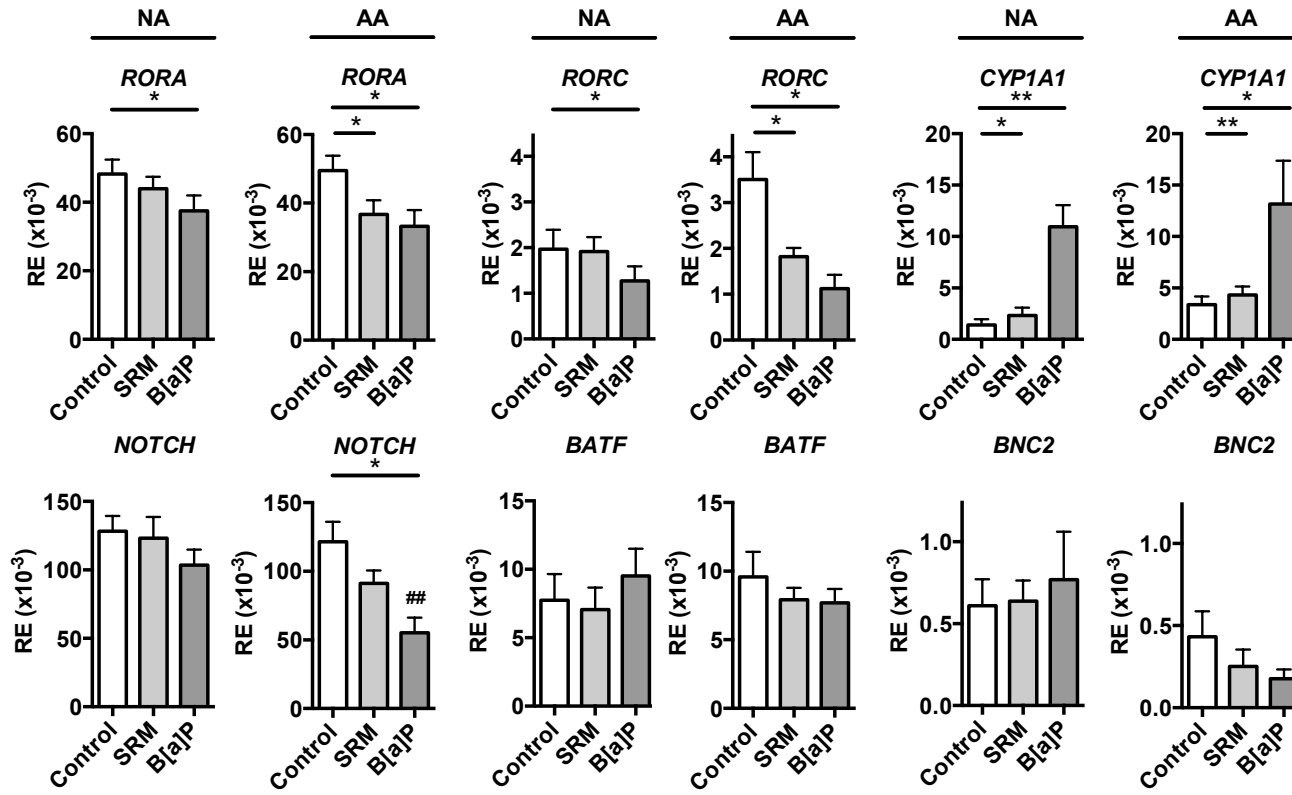


Figure 4



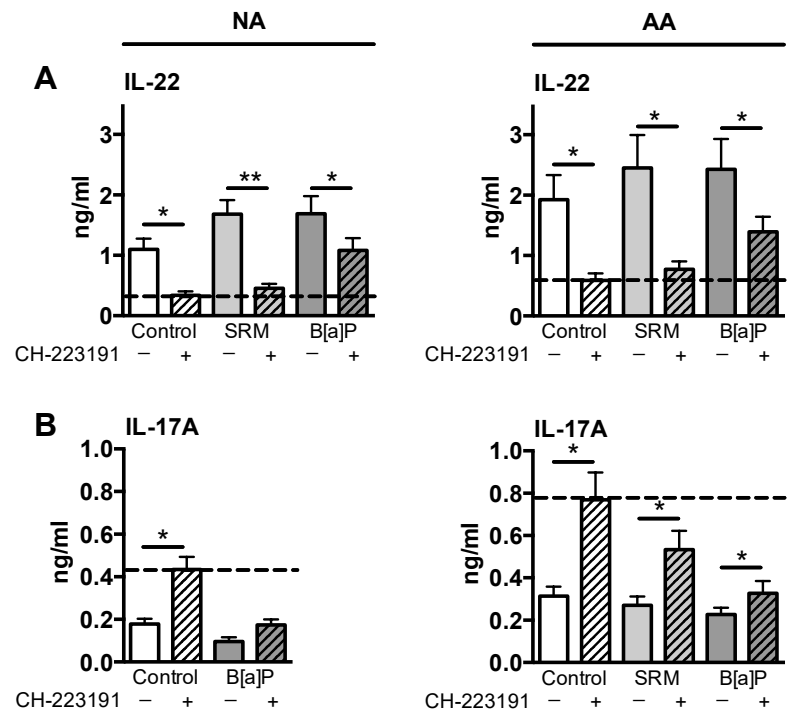


Figure 5

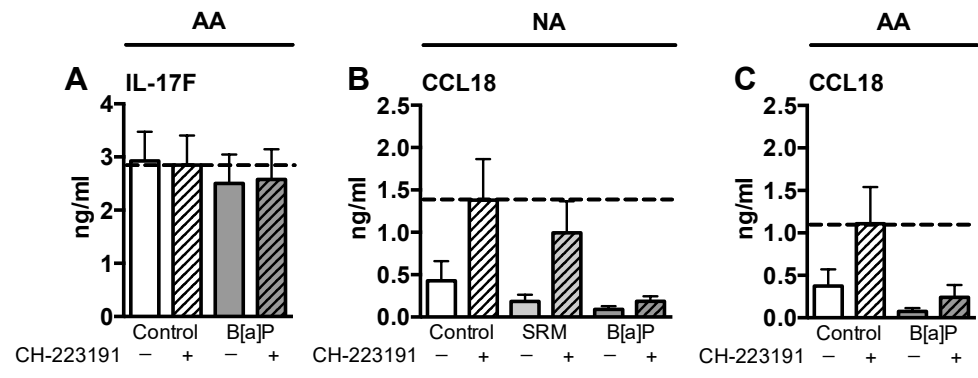


Figure E2

Figure 6

