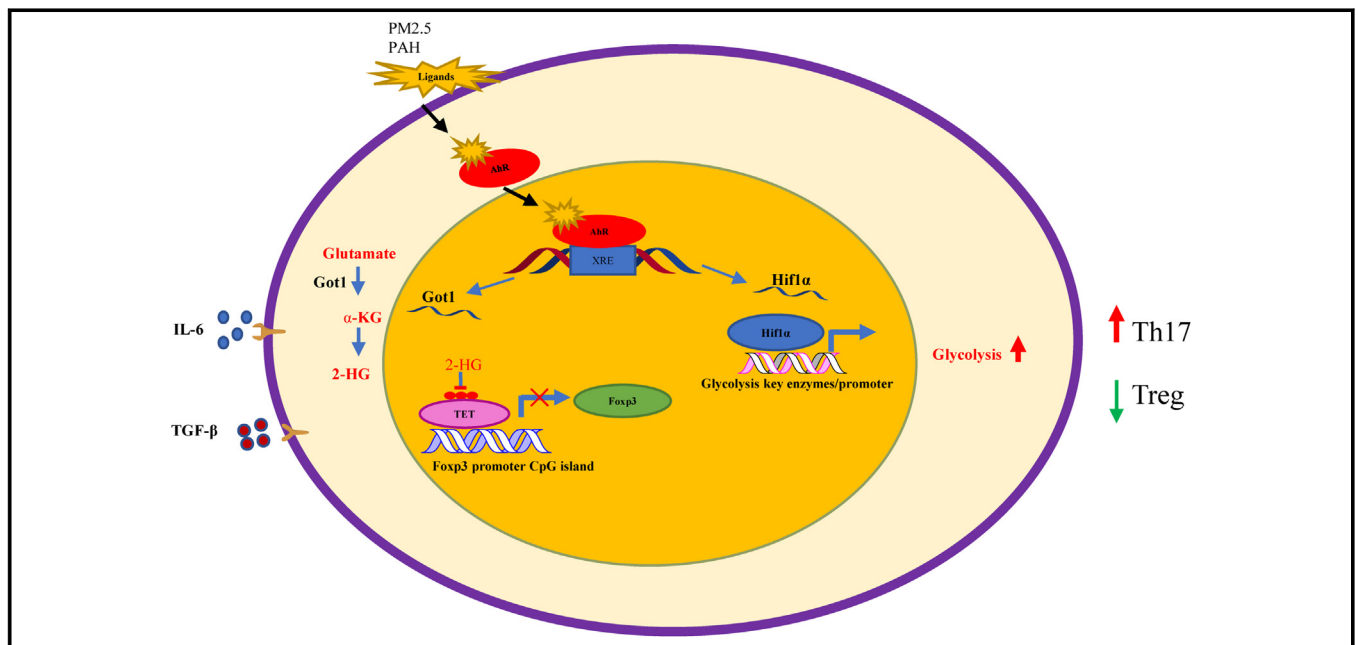


Particulate matter of 2.5 μm or less in diameter disturbs the balance of $\text{T}_\text{H}17$ /regulatory T cells by targeting glutamate oxaloacetate transaminase 1 and hypoxia-inducible factor 1 α in an asthma model



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GRAPHICAL ABSTRACT



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Background: Epidemiologic evidence suggests that exposure to particulate matter of 2.5 μm or less in diameter (PM_{2.5}) aggravates asthma.

Objective: We sought to investigate the underlying mechanisms between PM_{2.5} exposure and asthma severity.

Methods: The relationship between PM_{2.5} exposure and asthma severity was investigated in an asthma model with CD4⁺ T cell-specific aryl hydrocarbon receptor (AhR)-null mice. Effects of PM_{2.5} and polycyclic aromatic hydrocarbons (PAHs) on differentiation of T_H17/regulatory T (Treg) cells were investigated by using flow cytometry and quantitative RT-PCR. Mechanisms were investigated by using mRNA sequencing, chromatin immunoprecipitation, bisulfite sequencing, and glycolysis rates.

Results: PM_{2.5} impaired differentiation of Treg cells, promoted differentiation of T_H17 cells, and aggravated asthma in an AhR-dependent manner. PM_{2.5} and one of its prominent PAHs, indeno[1,2,3-cd]pyrene (IP), promoted differentiation of T_H17 cells by upregulating hypoxia-inducible factor 1 α expression and enhancing glycolysis through AhRs. Exposure to PM_{2.5} and IP enhanced glutamate oxaloacetate transaminase 1 (Got1) expression through AhRs and accumulation of 2-hydroxyglutarate, which inhibited ten-eleven translocation methylcytosine dioxygenase 2 activity, resulting in hypermethylation in the forkhead box P3 locus and impaired differentiation of Treg cells. A GOT1 inhibitor, (aminoxy)acetic acid, ameliorated asthma by shifting differentiation of T_H17 cells to Treg cells. Similar regulatory effects of exposure to PM_{2.5} or IP on T_H17/Treg cell imbalance were noted in human T cells, and in a case-control design PAH exposure appeared to be a potential risk factor for asthma.

Conclusions: The AhR-hypoxia-inducible factor 1 α and AhR-GOT1 molecular pathways mediate pulmonary responses on exposure to PM_{2.5} through their ability to disturb the balance of T_H17/Treg cells. (J Allergy Clin Immunol 2020;145:402-14.)

Key words: Asthma, particulate matter of 2.5 μm or less in diameter, aryl hydrocarbon receptors, T-cell differentiation, glycolysis, DNA methylation

Asthma is a reversible chronic inflammatory disease characterized by airway obstruction and hyperresponsiveness.^{1,2} A wealth of evidence links exposure to outdoor and indoor air pollution, especially airborne particulate matter (PM), to the development and exacerbation of asthma. However, the causal relationship and mechanisms have not been fully established.^{3,4}

Recent discoveries regarding PM-associated polycyclic aromatic hydrocarbons (PAHs) and interactions with the aryl hydrocarbon receptor (AhR) and its influence on immune responses highlight the potential importance of the AhR-PAH axis in the development of allergic and inflammatory diseases.⁵⁻⁸ AhR is a unique cellular “chemical sensor” and a ligand-activated transcription factor. It is expressed universally in multiple cell types: mast cells, macrophages, dendritic cells, T cells, and epithelial cells.⁵⁻¹³ Particulate matter of 2.5 μm or less in diameter (PM_{2.5}) is very small and can enter the airways and reach the alveoli. These small PM sizes are partly eliminated by alveolar macrophages, but some of them (especially those of <1 μm in diameter [like ultrafine particles]) pass into the blood circulation.

Abbreviations used

2-HG:	2-hydroxyglutarate
AhR:	Aryl hydrocarbon receptor
AOA:	(Aminoxy)acetic acid
BALF:	Bronchoalveolar lavage fluid
BaP:	Benzo- α -pyrene
CRE:	Cockroach extract
Cyp1a1:	Cytochrome P450 1A1
FoxP3:	Forkhead box P3
Glut1:	Glucose transporter 1
GOT1:	Glutamate oxaloacetate transaminase 1
HIF-1 α :	Hypoxia-inducible factor 1 α
IDH:	Isocitrate dehydrogenase
IP:	Indeno[1,2,3-cd]pyrene
KO:	Knockout
PAH:	Polycyclic aromatic hydrocarbon
PM:	Particulate matter
PM _{2.5} :	Particulate matter of 2.5 μm or less in diameter
ROR γ t:	Retinoic acid receptor-related orphan nuclear receptor γ t
TET:	Ten-eleven translocation methylcytosine dioxygenase
Treg:	Regulatory T
XRE:	Xenobiotic response element

Furthermore, some components of PM_{2.5} (including PAHs) can be released from the particle and interact with immune cells and affect cellular function directly.¹⁴

Skewing of the balance of T_H17/regulatory T (Treg) cells is correlated with asthma severity. Bronchoalveolar lavage fluid (BALF) from patients with severe asthma contains more IL-17⁺ cells compared with BALF from those with mild asthma or healthy control subjects.^{15,16} In patients with severe asthma, low frequency or impaired function of forkhead box P3 (FoxP3)⁺ Treg cells is often observed. Studies have shown that intense inflammation in an experimental model of asthma can reduce the activity of Treg cells or convert Treg cells to proinflammatory phenotypes.¹⁷⁻²⁰ Moreover, it has been suggested that AhR can regulate the differentiation of T_H17/Treg cells in a ligand-specific manner.^{5,21} Also, PM-associated PAHs have been suggested to aggravate asthma in mice.¹² Nevertheless, how the intrinsic AhR in T cells responds to PM or PAHs and regulates allergic inflammation is not known.

Here we report that PM_{2.5} and its associated PAHs can promote differentiation of proinflammatory T_H17 cells and impair differentiation of anti-inflammatory Treg cells through activation of the AhR and, in part, through metabolic disturbance *in vitro* and *in vivo*. PM_{2.5} and indeno[1,2,3-cd]pyrene (IP) enhanced cell glycolysis and promoted differentiation of T_H17 cells through an AhR-hypoxia-inducible factor 1 α (HIF-1 α) axis and enhanced hypermethylation in the *Foxp3* locus to impair differentiation of Treg cells through a novel regulatory axis: AhR-glutamate oxaloacetate transaminase 1 (GOT1)-2-hydroxyglutarate (2-HG).

METHODS

Ethical approval of the study protocol

Experiments complied with the relevant laws and institutional guidelines, as overseen by the Animal Studies Committee of the Children's Hospital of Fudan University (Shanghai, China).

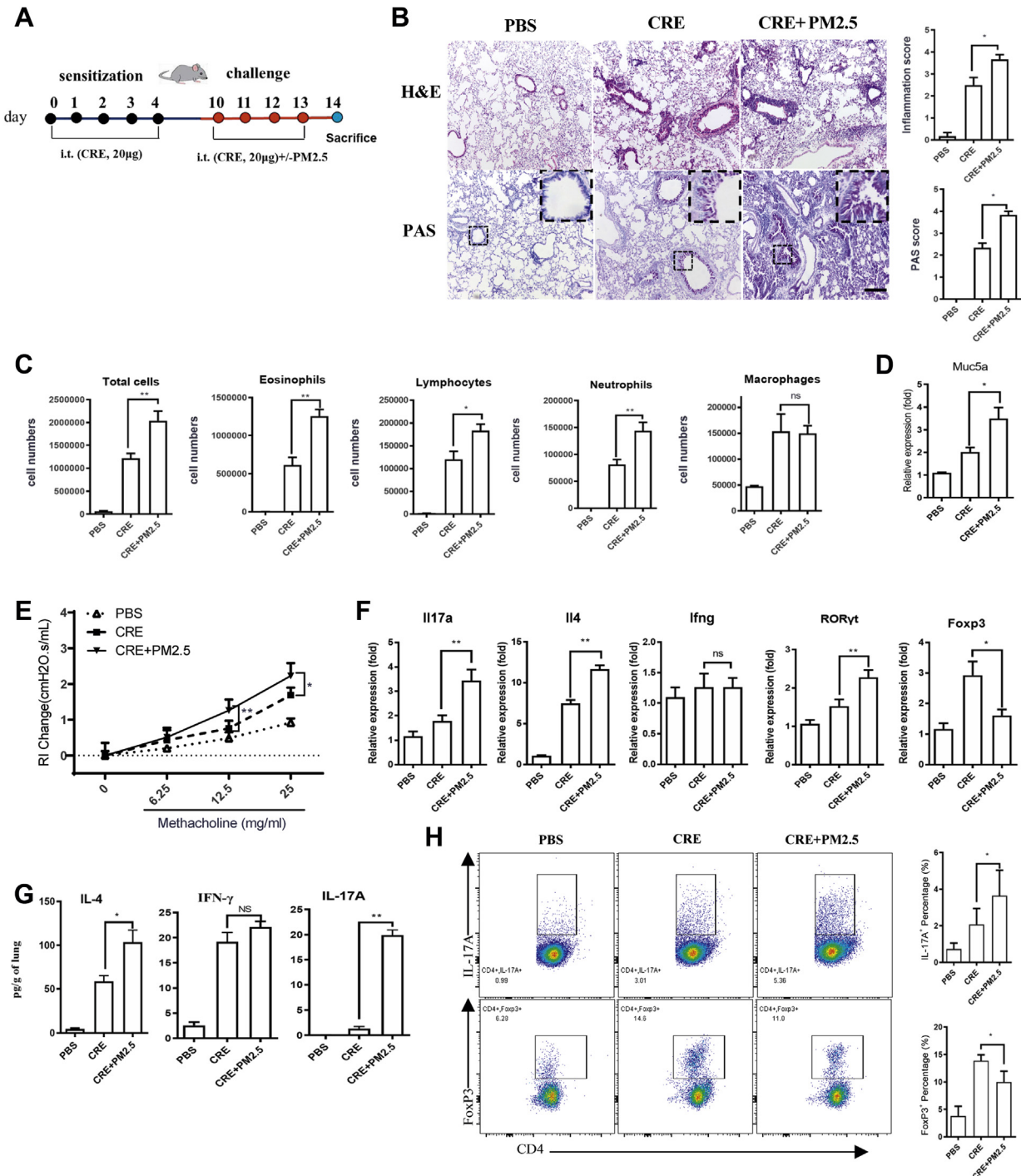


FIG 1. PM2.5 exposure aggravates asthma symptoms in a murine model. **A**, Asthma models used in our study (schematic). C57BL/6 mice were sensitized and challenged as shown with or without PM2.5 treatment, and 24 hours after the last allergen challenge, mice were killed and evaluated at different end points. *i.t.*, Intratracheal. **B**, In lung sections (stained with hematoxylin and eosin [H&E] and periodic acid-Schiff [PAS]) lung tissue inflammation and PAS staining were scored. Scale bar = 250 µm. Small boxes show locations of big boxes in these panels. **C**, Cell counts in BALF. **D**, Quantitative RT-PCR for mRNA expression of MUC5A in lung tissues. **E**, Assessment of methacholine-induced airway hyperresponsiveness in mice. The value of lung airway resistance (RI) is shown. Data were normalized and represented as deviation from 0 mg/mL. **F**, Quantitative RT-PCR for mRNA expression of *Il17a*, *Il4*, *Ifng*, *RORγt*, and *Foxp3* in lung tissues. **G**, Cytokine expression in lung homogenates was measured by using a cytometric bead array. **H**, Percentages of CD4⁺IL-17A⁺ or CD4⁺CD25⁺FoxP3⁺ cells in CD4⁺ cells in the hilar lymph nodes of lungs were measured by using flow cytometry. PM2.5, 250 µg/d per mouse. Data are shown as means ± SEMs from 3 independent experiments. *ns*, Not significant. **P* ≤ .05 and ***P* ≤ .01, unpaired Student *t* test (Fig 1, C, D, and F-H) or 1-way ANOVA with the Tukey *post hoc* test (Fig 1, E). *n* = 12 to 15 mice per group.

Model creation

A cockroach extract (CRE)-induced asthma model in C57BL/6 mice was established, as described previously.^{22,23} Briefly, female C57BL/6 mice were sensitized by means of intratracheal inhalation of CRE (20 µg per mouse) on days 0 to 4 and challenged daily on days 10 to 13 with CRE. Control mice received PBS instead of CRE. In some experiments mice were treated with PM2.5 or IP and assessed by use of established protocols (see the Methods section in this article's Online Repository at www.jacionline.org) during CRE challenge by means of intratracheal inhalation (Fig 1, A). Mice were killed on day 14.

Methods used

Measurement of airway hyperresponsiveness, BALF analyses, lung pathology, chromatin immunoprecipitation assays, T-cell differentiation, methylation analyses, quantitative RT-PCR, glycolysis analyses, 2-HG quantification, mRNA sequencing, measurement of PAH metabolites, cytokine analyses, IgE measurement, flow cytometry, and statistical analyses are provided in the Methods section in this article's Online Repository.

RESULTS

PM2.5 exposure aggravates pulmonary allergic inflammation in a murine model of asthma

Female C57BL/6 mice were challenged intratracheally with PBS, CRE (as a model allergen), PM2.5, or CRE plus PM2.5 daily from day 10 to day 13 to determine the effect of PM2.5 exposure on asthma (Fig 1, A). Compared with the PBS group, mice in the CRE group showed significant recruitment of inflammatory cells in the lungs, with dense peribronchial infiltrates and goblet cell hyperplasia (Fig 1, B). In the preliminary experiments for the CRE plus PM2.5 group, we tested 2 doses of PM2.5 (50 and 250 µg/d per mouse) and found that 50 µg/d per mouse of PM2.5 could aggravate asthma slightly (see Fig E1, A, in this article's Online Repository at www.jacionline.org), but at 250 µg/d per mouse, PM2.5 aggravated asthma significantly, as evidenced by significantly enhanced pulmonary inflammation, mucus production, airway hyperresponsiveness, and total IgE and CRE-specific IgE levels (Fig 1, B-E, and see Fig E1, B). In subsequent *in vivo* experiments the dose of 250 µg/d per mouse was used. Exposure to PM2.5 only did not induce appreciable inflammation or enhance airway resistance, although the number of neutrophils in BALF increased slightly (see Fig E1, C and D). When house dust mite allergens were used instead of CRE, similar results were obtained (see Fig E2 in this article's Online Repository at www.jacionline.org).

The aggravated pulmonary allergic responses seen in PM2.5-exposed mice were found to be associated with significantly increased expression of *Il17a*, retinoic acid receptor-related orphan nuclear receptor γ t (*ROR γ t*), and *Il4*, whereas expression of *Foxp3* was decreased significantly, and *Ifng* expression was not altered significantly in lung tissues in the CRE plus PM2.5 group compared with those in the CRE group (Fig 1, F and G). Furthermore, PM2.5 exposure enhanced the percentage of IL-17A⁺ cells but decreased the percentage of FoxP3⁺ cells in the CD4⁺ population in the hilar lymph nodes of the lungs (Fig 1, H).

Chesne et al¹⁵ reported that skewing of the balance of T_H17/Treg cells is correlated with asthma severity. We hypothesized that increased expression of *Il17a* might be responsible for the asthma exacerbation induced by PM2.5. To test this hypothesis, we used an inhibitor of ROR γ t, SR2211. The latter reduced the number of inflammatory cells in BALF and decreased the airway hyperresponsiveness in PM2.5-exposed mice significantly (see Fig E1, C and D). These data suggested that PM2.5-aggregated

asthma might be mediated (at least in part) through an increased number of T_H17 cells.

PM2.5 affects the balance of T_H17/Treg cells and aggravates asthma through the AhR in CD4⁺ T cells

Considering that T_H17 and Treg cells share a similar differentiation pathway,²⁴ we hypothesized that PM2.5 might affect the balance of T_H17/Treg cells, thereby aggravating asthma. To test this hypothesis, we undertook polarization assays for T_H17 and Treg cells *in vitro* in the presence of PM2.5. The latter could promote differentiation of T_H17 cells significantly but reduced differentiation of Treg cells in a dose-dependent way (Fig 2, A). Consistent with flow cytometric results, PM2.5 also increased expression of *Il17a* and ROR γ t but reduced *Foxp3* expression (Fig 2, B).

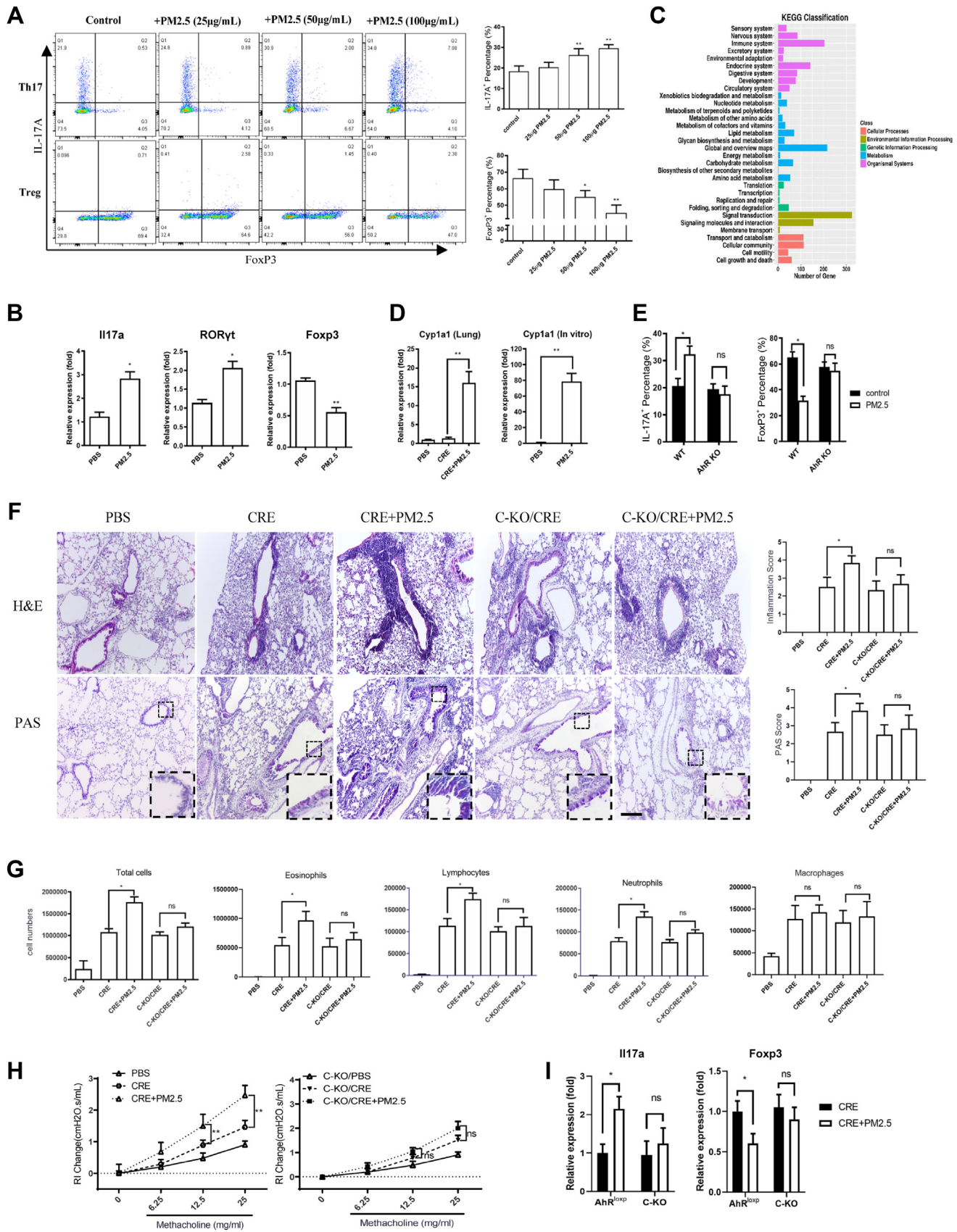
To delineate the mechanism further, we undertook mRNA sequencing of differentiating T_H17 cells in the presence of PM2.5. Gene-enrichment analyses using the Kyoto Encyclopedia of Genes and Genomes database showed that the most differentially expressed genes were those involved in "environmental information processing" and "metabolism" classifications (Fig 2, C), and a "xenobiotic biodegrading metabolism" classification was also noted. Ferecatu et al²⁵ reported that PAHs in PM2.5 can activate xenobiotic biodegrading metabolism through AhRs.

To ascertain whether PM2.5 could activate AhRs to affect the balance of T_H17/Treg cells, expression of cytochrome P450 1A1 (*Cyp1a1*), a known AhR-targeted gene, was measured. PM2.5 could upregulate expression of *Cyp1a1* significantly in lung tissues from C57BL/6 mice *in vivo* (Fig 2, D, left) and in CD4⁺ T cells isolated from the spleens of C57BL/6 mice *in vitro* (Fig 2, D, right). Importantly, the effect of PM2.5 on the differentiation of T_H17 and Treg cells disappeared in CD4⁺ T cells of AhR-null mice (AhR knockout [KO]; Fig 2, E), thereby suggesting that the effect of PM2.5 on the differentiation of T_H17 and Treg cells was AhR dependent *in vitro*.

Next, to test whether the AhR in CD4⁺ T cells was important for the effect of PM2.5 on pulmonary allergic responses *in vivo*, we generated mice with a CD4-specific deletion of AhR (CD4 AhR-KO) by crossing AhR^{loxP} mice with CD4-Cre mice. In the CRE group there was no significant difference in the level of lung inflammation and airway resistance between AhR^{loxP} mice (as a wild-type control) and CD4 AhR-KO mice (Fig 2, F-H). However, in the PM2.5 plus CRE coexposure group, CD4 AhR-KO mice revealed markedly reduced lung inflammation and airway resistance compared with that seen in control AhR^{loxP} mice (Fig 2, F-H). In lung tissues there was lower expression of *Il17a* but higher expression of *Foxp3* in CD4 AhR-KO mice compared with those in AhR^{loxP} mice exposed to PM2.5 (Fig 2, D). These results suggested that PM2.5 affected the balance of T_H17/Treg cells and aggravated asthma further through the AhR in CD4⁺ T cells.

PM2.5 enhances cell glycolysis and promotes differentiation of T_H17 cells through the AhR-HIF-1 α axis

In Kyoto Encyclopedia of Genes and Genomes analyses we noted an additional enriched pathway involving carbohydrate metabolism (Fig 2, C). It has been reported that glycolysis regulates T_H17 cell differentiation.^{24,26} Next, we measured expression of several key enzymes in the glycolytic pathway: glucose transporter



1 (Glut1), hexokinase 2, lactate dehydrogenase A, and enolase 1. Expression of all of these enzymes was upregulated in the PM2.5-exposed group (Fig 3, A). We also undertook an assay to measure the glycolysis rate using a Seahorse XF system (Agilent Technologies, Santa Clara, Calif) to confirm the results. Basal glycolysis and compensatory glycolysis were enhanced by PM2.5 (Fig 3, B) under T_H17 cell-polarizing conditions.

Shi et al²⁷ reported that *Hif1a* regulates cell glycolysis and differentiation of T_H17 cells.²⁷ We hypothesized that PM2.5 might activate the AhR–HIF-1 α pathway to enhance cell glycolysis and further affect differentiation of T_H17/Treg cells. Indeed, *Hif1a* expression was upregulated considerably in differentiation conditions for T_H17 and Treg cells on exposure to PM2.5 (Fig 3, C and D).

Next, we investigated whether knockdown of *Hif1a* expression by short hairpin RNA could restore the imbalance of T_H17/Treg cells exposed to PM2.5. We found that PM2.5 could no longer enhance differentiation of T_H17 cells in the *Hif1a* knockdown group (Fig 3, E), suggesting that PM2.5 could regulate differentiation of T_H17 cells through the AhR–HIF-1 α axis. In contrast, PM2.5-mediated reduction in the number of FoxP3⁺ Treg cells could not be reversed in the *Hif1a* knockdown group. We also found that PM2.5 enhanced *Hif1a* expression in Treg cells, but it was not as dramatic as that noted in T_H17 cells (Fig 3, D). Conversely, PM2.5 did not enhance glycolysis in Treg cells significantly (see Fig E3 in this article's Online Repository at www.jacionline.org). These results suggested that an alternative signaling pathway could be important in mediating the effect of PM2.5 on differentiation of Treg cells.

PM2.5 enhances hypermethylation of DNA CpG in the *Foxp3* locus to impair differentiation of Treg cells through the AhR-GOT1 axis

Xu et al²⁶ reported that hypermethylation of DNA CpG in the *Foxp3* locus can impair *Foxp3* expression and further impair Treg cell differentiation. We measured methylation of the *Foxp3* promoter and its intronic CpG islands using the bisulfite assay in Treg cells.²⁸ Consistent with previous reports,^{28,29} the *Foxp3* promoter in Treg cells was methylated partially, and its intronic conserved noncoding sequence 2 regions were highly methylated. Notably, PM2.5 increased methylation of these regions in Treg cells markedly (Fig 4, A).

Ten-eleven translocation methylcytosine dioxygenases (TETs) can convert 5-methylcytosine to 5-hydroxymethylcytosine and regulate *Foxp3* expression by demethylating the *Foxp3* promoter and its intronic CpG island.^{26,29} We investigated whether this regulatory pathway was involved in the effects of PM2.5 on Treg cell differentiation. Expression of *Tet1* and *Tet2* was not altered in the

PM2.5-exposed group (Fig 4, B), whereas a significant increment in accumulation of 2-HG (the direct product of α -ketoglutaric acid by isocitrate dehydrogenase [IDH] 1/2 and known to inhibit TET activity²⁵) was noted in Treg cells exposed to PM2.5 (Fig 4, C and D). Also, expression of *Got1* (the major transaminase catalyzing the conversion of glutamate to α -ketoglutaric acid in T cells), but not expression of *Idh1* or *Idh2*, was induced significantly by PM2.5 *in vitro* and *in vivo* (Fig 4, D–F). Furthermore, knockdown of *Got1* expression could promote differentiation of Treg cells and rescue the inhibitory effect of PM2.5 on their differentiation (Fig 4, G and H). Therefore these results suggested that an enhanced glutamate metabolic pathway involving GOT1 and an increased level of 2-HG were important in mediating the effect of PM2.5 on Treg cell differentiation.

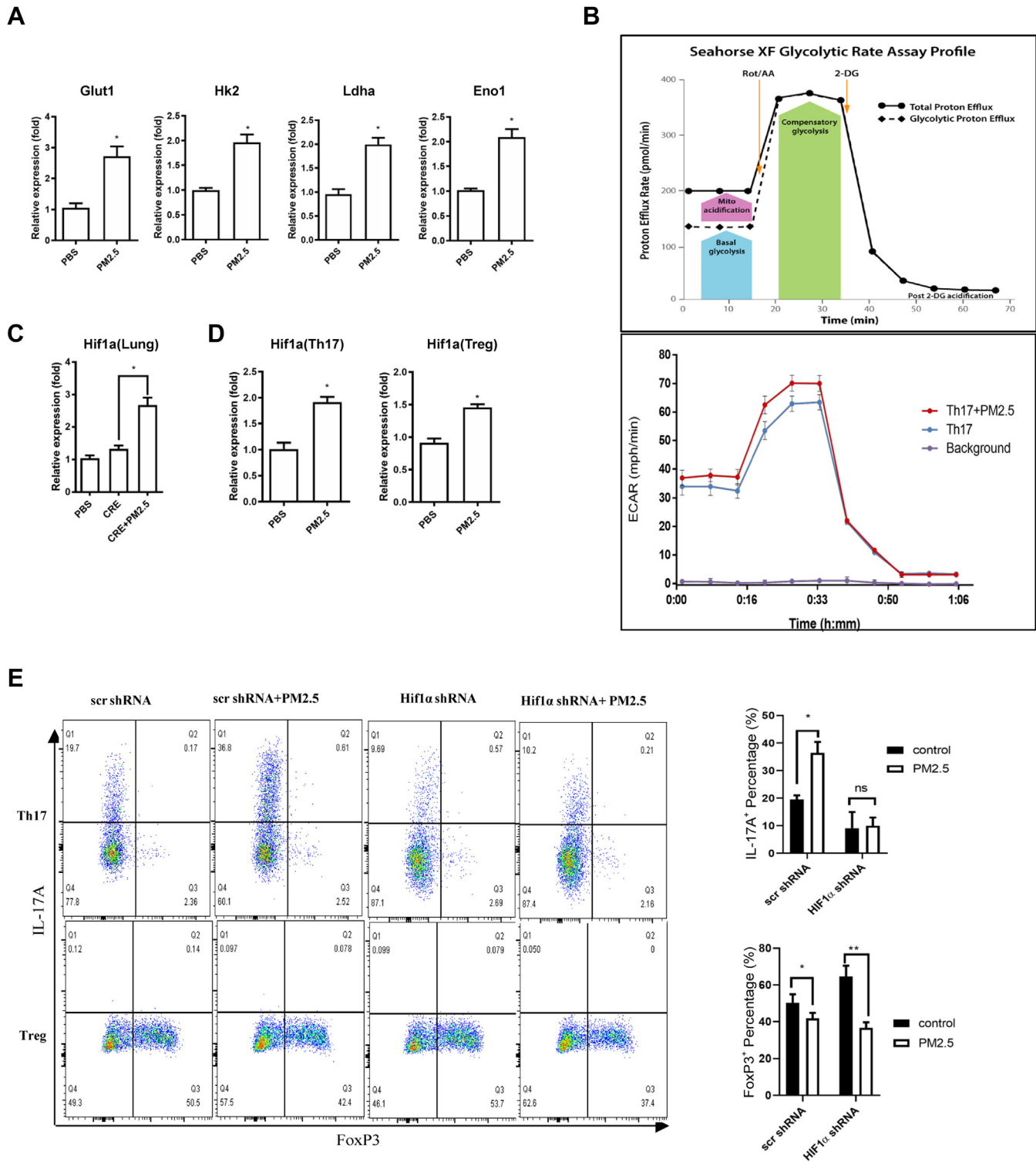
Next, through bioinformatics analyses, we found that there were 4 xenobiotic response elements (XREs) in the *Got1* promoter (Fig 4, I, and see Table E1 in this article's Online Repository at www.jacionline.org). Chromatin immunoprecipitation assays were carried out to assess binding of the AhR to XRE sequences of the *Got1* promoter in Treg cells. We found that the AhR could bind to XRE-2 and XRE-3 of the *Got1* promoter. This binding was absent in AhR-KO cells but enhanced in the PM2.5-exposed wild-type group (Fig 4, I). These results suggested the existence of a likely sequential event by which PM2.5 exposure upregulated *Got1* expression through AhR, which resulted in accumulation of the metabolite 2-HG and subsequent inhibition of TET2 activity and hypermethylation in the *Foxp3* locus, thereby impairing Treg cell differentiation.

A GOT1 inhibitor can restore the differentiation of Treg cells impaired by PM2.5 and ameliorate asthma in a murine model

(Aminoxy)acetic acid (AOA), an inhibitor of GOT1, was used together with PM2.5 to examine whether the AhR-GOT1 axis and impaired differentiation of Treg cells were important in conferring the PM2.5-aggravating effect on airway allergic inflammation. Addition of AOA could reverse the inhibitory effect of PM2.5 on Treg cell differentiation *in vitro*. The population ratio of Treg cells in the PM2.5 plus AOA group was even greater than that in the PBS control group (Fig 5, A).

Next, we investigated the effect of AOA on a murine model of asthma. AOA or control vehicle was injected intraperitoneally daily to mice in the challenge period (Fig 5, B). Notably, AOA administration could ameliorate asthma in the CRE and CRE plus PM2.5 groups, as indicated by reduced pulmonary allergic inflammation, including fewer peribronchial infiltrates and less goblet cell hyperplasia, as well as fewer eosinophils,

FIG 2. PM2.5 can affect the balance of T_H17 and Treg cells through the AhR in T cells. **A**, Differentiation of T_H17 and Treg cells in the presence of different doses of PM2.5 suspensions *in vitro*. Each column is compared with that of the control group. **B**, Cells were cultured for 5 days. Expression of T_H17 and Treg cell-related genes was measured by using quantitative RT-PCR. **C**, Classification of the Kyoto Encyclopedia of Genes and Genomes pathway: PM2.5 versus PBS. **D**, mRNA expression of *Cyp1a1* in lung tissues and cell cultures, respectively. **E**, T_H17 and Treg cell *in vitro* polarization assays were done by using CD4⁺ naive T cells from wild-type (WT) or AhR-null (*Ahr* KO) mice, respectively. **F**, In lung section (stained with hematoxylin and eosin [H&E] and periodic acid–Schiff [PAS]) lung tissue inflammation and PAS were scored. Scale bar = 250 μ m. **C-KO**, CD4-AhR KO. **G**, Cell counts in BALF. **H**, Methacholine-induced airway hyperresponsiveness (CRE vs CRE plus PM2.5). Data were normalized and represented as deviation from 0 mg/mL. **I**, Expression of *Il17a* and *Foxp3* in lung tissues was measured by using quantitative RT-PCR. Data are means $\pm$ SEMs from 3 independent experiments. ns, Not significant. * P < .05 and ** P < .01, unpaired Student *t* test or 1-way ANOVA with the Tukey *post hoc* test (Fig 2, H). n = 12 mice per group (control) and n = 10 mice per group (C-KO; Fig 2, G, H, and I). For the *in vitro* assay, there were 3 samples per group.



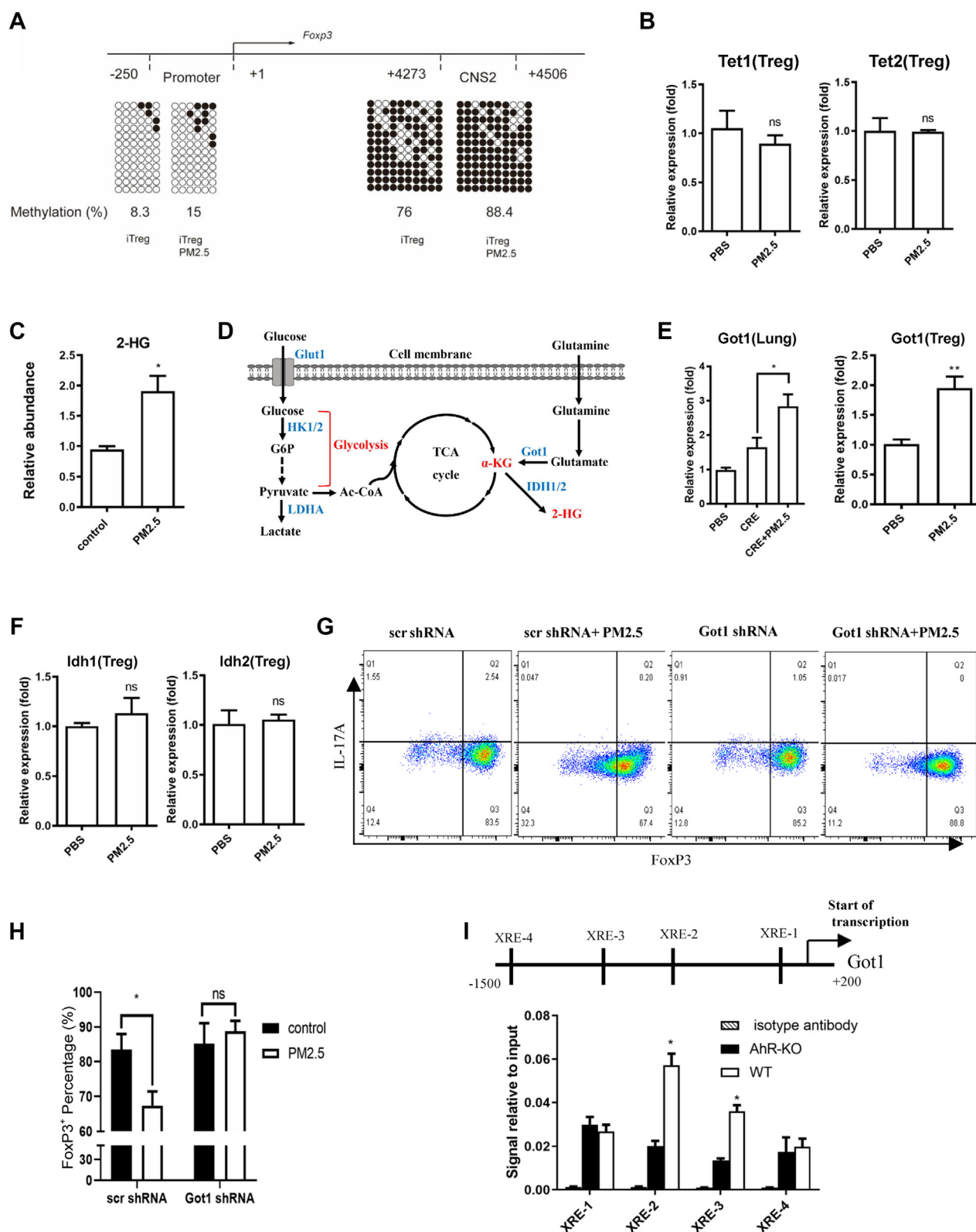


FIG 4. PM2.5 impairs differentiation of Treg cells by inducing hypermethylation of DNA CpG at the *Foxp3* locus. **A**, CpG methylation at the *Foxp3* locus was assessed by using bisulfite sequencing. *iTreg*, Induced regulatory T cells. **B**, mRNA expression of *Tet1* and *Tet2* in Treg cells. Cells were cultured for 48 hours before collection. **C**, Relative abundance of 2-HG in Treg cells. **D**, Cell glycolysis and 2-HG biosynthesis (schematic). **E**, mRNA expression of *Got1* in lungs *in vivo* and Treg cells *in vitro*. **F**, mRNA expression of *Idh1* and *Idh2* in Treg cells *in vitro*. **G**, Treg cell *in vitro* polarization assay with knockdown of *Got1* expression. *scr shRNA*, Scrambled short hairpin RNA. **H**, Quantitation of flow cytometry. **I**, Chromatin immunoprecipitation (ChIP) analyses of the interaction of the AhR with *Got1* promoters after 48 hours under Treg cell polarizing conditions in the presence of PM2.5. Data are shown as means $\pm$ SEMs from 2 to 3 independent experiments. *ns*, Not significant. **P* < .05 and ***P* < .01, unpaired Student *t* test.

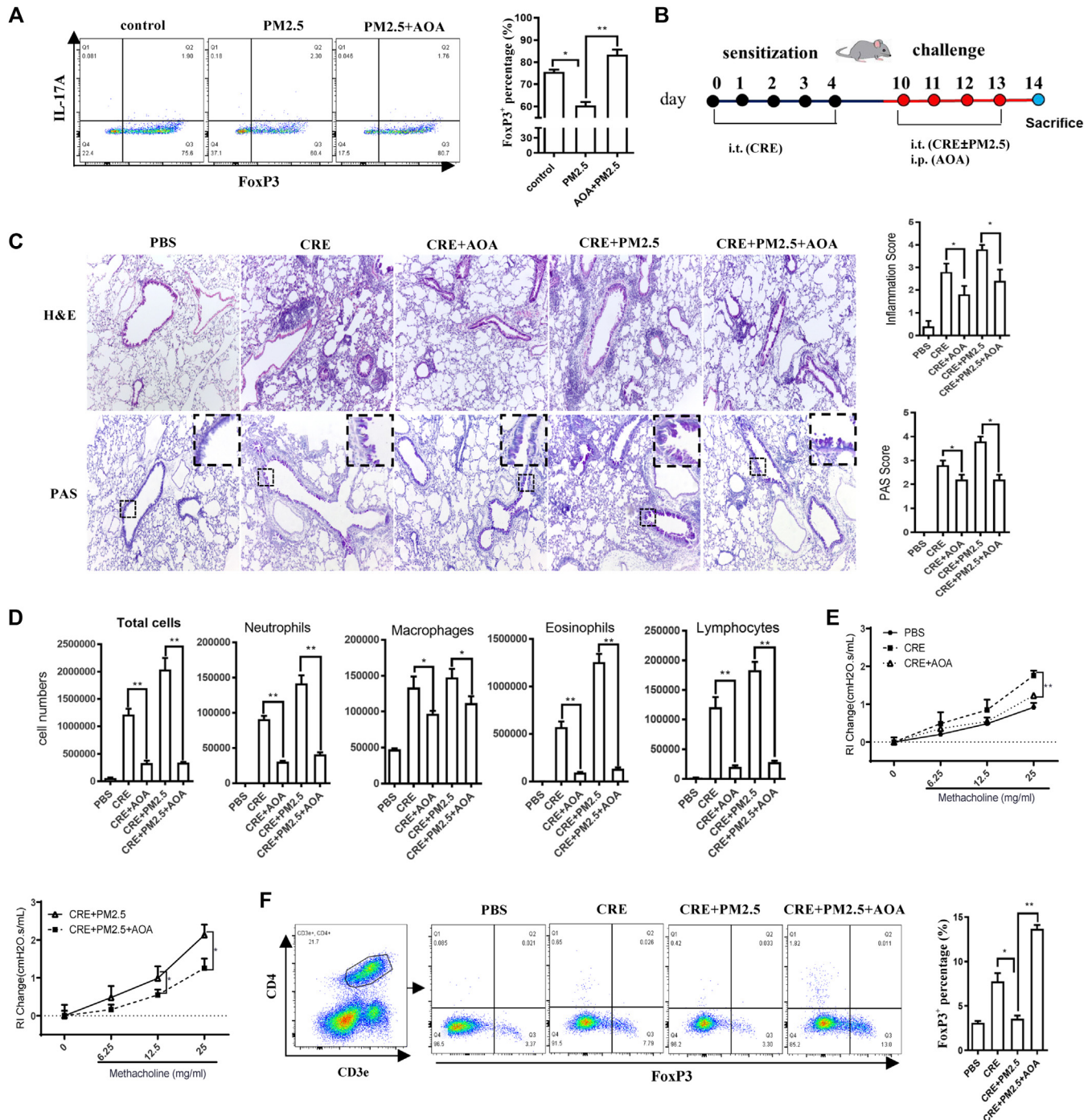


FIG 5. AOA can restore PM2.5-mediated reduction of differentiation of Treg cells *in vitro* and *in vivo*. **A**, Differentiation of Treg cells *in vitro* in the presence of PM2.5 or AOA. PM2.5, 100 μ g/mL; AOA, 0.75 mmol/L. **B**, Experimental approach (schematic): CRE (20 μ g administered intratracheally [*i.t.*]) and AOA (750 μ g administered intraperitoneally [*i.p.*]). **C**, In lung sections (stained with hematoxylin and eosin [*H&E*] and periodic acid-Schiff [*PAS*]) lung-tissue inflammation and PAS were scored. Scale bar = 250 μ mol/L. **D**, Cell counts in BALF. **E**, Methacholine-induced airway hyperresponsiveness. Data were normalized and represented as deviation from 0 mg/mL. **F**, FoxP3⁺ populations in the hilar lymph nodes of lungs were assessed by using flow cytometry. Data are means $\pm$ SEMs from 3 independent experiments. * $P \leq .05$ and ** $P \leq .01$, unpaired Student *t* test or 1-way ANOVA with the Tukey *post hoc* test (Fig 4, E). $n = 12$ to 13 mice per group.

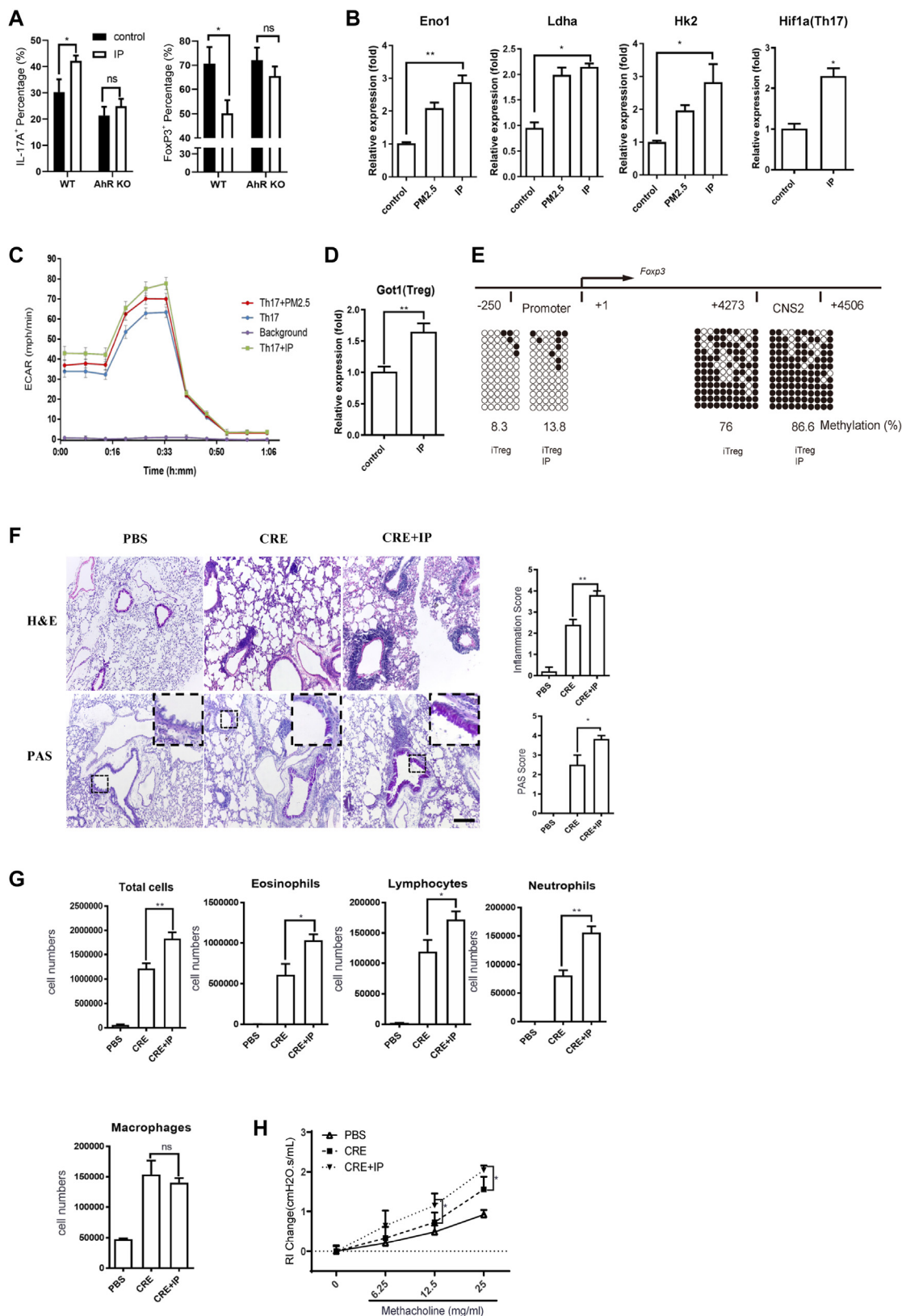


FIG 6. IP has a similar function as PM2.5, can affect the TH17/Treg cell balance, and aggravates asthma. **A**, Differentiation of TH17 and Treg cells *in vitro* in the presence of IP (1 μ mol/L) using CD4⁺ naive T cells from wild-type or AhR-KO mice, respectively. **B**, mRNA expression of various genes. *Eno1*, Enolase 1; *Hk2*, hexokinase 2; *Ldha*, lactate dehydrogenase A. **C**, The glycolysis rate in cells was assessed by using the Seahorse system. ECAR, Extra-cellular acidification. **D**, *Got1* expression induced by IP. **E**, CpG methylation at the *Foxp3* locus assessed by using bisulfite sequencing. **F**, In lung sections (stained with hematoxylin and eosin [H&E] and periodic acid–Schiff [PAS]) lung tissue inflammation and PAS were scored. Scale bar = 250 μ m. **G**, Cell counts in BALF. **H**, Methacholine-induced airway hyperresponsiveness. Data were normalized and represented as deviation from 0 mg/mL. All data are shown as means $\pm$ SEMs from 3 independent experiments. ns, Not significant. * $P \leq .05$ and ** $P \leq .01$, the unpaired Student *t* test or 1-way ANOVA with the Tukey *post hoc* test (Fig 6, H). $n = 10$ to 12 mice per group.

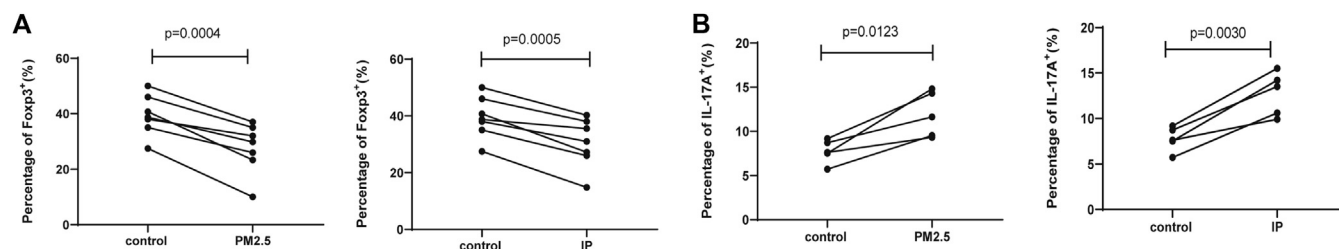


FIG 7. Exposure to PM_{2.5} or IP regulates the balance of T_H17 and Treg cells in human subjects. Differentiation of T_H17 cells (A) and Treg cells (B) *in vitro* in the presence of IP (500 nmol/L) or PM_{2.5} (50 μg/mL) by using CD4⁺ naive T cells from human PBMCs, respectively. The *P* value was calculated by using the paired Student *t* test (*n* = 5-7).

lymphocytes, and neutrophils in BALF (Fig 5, C and D). In addition, airway resistance was reduced markedly in AOA-treated mice compared with that in mice from other groups (Fig 5, E), which was concomitant with a significantly increased percentage of Foxp3⁺ Treg cells in the hilar lymph nodes of the lungs (Fig 5, F).

IP, a prominent PAH associated with PM_{2.5}, impairs the balance of T_H17/Treg cells and aggravates asthma

Several PAHs with 5- or 6-membered rings were tested in T-cell polarization assays to determine which individual PAHs could affect differentiation of T_H17/Treg cells. Considering its effect, solubility, and cytotoxicity, IP was the best candidate to further investigate this mechanism (see Fig E4 in this article's Online Repository at www.jacionline.org). Moreover, analyses of the composition of PAHs associated with ambient PM_{2.5} showed that, among the 16 PAHs (US Environmental Protection Agency priority) assessed, IP was a prominent PAH associated with ambient PM_{2.5} in all seasons (see Table E2 in this article's Online Repository at www.jacionline.org).

Next, *in vitro* and *in vivo* assays were carried out to test the effect of IP on T-cell differentiation. As with PM_{2.5}, IP could promote differentiation of T_H17 cells and inhibit differentiation of Treg cells in an AhR-dependent pathway *in vitro* (Fig 6, A, and see Fig E5, A, in this article's Online Repository at www.jacionline.org). IP could enhance glycolysis by activating the AhR-HIF-1α axis to promote T_H17 cell differentiation (Fig 6, B and C, and see Fig E5, B). Conversely, IP could enhance *Got1* expression and impair Treg cell differentiation by enhancing hypermethylation of DNA CpG at the *Foxp3* locus (Fig 6, D and E, and see Fig E5, C). For the *in vivo* assay, IP could aggravate asthma in a murine model of asthma similarly to PM_{2.5} (Fig 6, F-H, and see Fig E5, D).

PM_{2.5} exposure is associated with asthma severity in patients

To ascertain whether PM_{2.5} or PAHs could disturb the balance of T_H17/Treg cells in human subjects, we undertook an assay to measure T-cell polarization *in vitro*. CD4⁺ naive T cells derived from the PBMCs of healthy human volunteers were polarized under T_H17 or Treg cell conditions in the presence of PM_{2.5} or IP. As expected, the effects of PM_{2.5} or IP on human T cells were similar to those noted in mouse T cells. They could reduce the

population of Foxp3⁺ Treg cells and promote differentiation of T_H17 cells (Fig 7, A and B). Moreover, in a case-control study analyses of urinary levels of 1-hydroxypyrene (a widely used marker of exposure to PAHs) showed significantly higher levels in patients with asthma currently compared with those in healthy control subjects (see Fig E6, A, in this article's Online Repository at www.jacionline.org). This result suggested that PAH exposure could pose a significant risk for asthma (see Fig E6, B, and Table E3 in this article's Online Repository at www.jacionline.org), particularly for severe and very severe asthma (adjusted odds ratio, 2.63; 95% CI, 1.50-4.63).

DISCUSSION

Here we provided evidence in support of a potential causal relationship between PM_{2.5}/PAH exposure and asthma severity in which the AhR-mediated imbalance of T_H17/Treg cells through targeting HIF-1α and GOT1, respectively, was a prominent feature.

HIF-1α is a highly conserved transcription factor that can promote glucose uptake and reinforce aerobic glycolysis by upregulating expression of Glut1 and pyruvate dehydrogenase kinase 1, respectively. Conversely, expression of several glycolysis-related genes encoding rate-limiting enzymes (*Glut1*, *Hk2*, *Ldha*, and pyruvate kinase) was downregulated in *Hif1a*-deficient mice.^{27,30,31} Our data showing that PM_{2.5} mediated increases in glycolysis and differentiation of T_H17 cells in murine and human T cells are consistent with the contention that aerobic glycolysis is crucial for polarization of T_H cells (especially T_H17).²⁴ In our study panel of asthmatic patients and healthy control subjects, 16 of 68 cases had detectable levels of IL-17A in plasma versus only 5 of 68 healthy control subjects. By calculation with undetectable samples set as the half amount of the detection limit for IL-17A, we found a significant difference between the case and control groups (*P* = .029, Mann-Whitney *U* test). Additional studies with larger study populations and other types of specimens are needed to confirm this result.

Epidemiologic studies have shown that PM_{2.5} is associated with hypermethylation of the *Foxp3* locus, impaired function of Treg cells, and increased asthma morbidity,^{18,32} but the underlying mechanisms are incompletely understood. We reported a novel pathway by which PM_{2.5} or IP impaired differentiation of Treg cells through the AhR-GOT1 axis through an epigenetic mechanism. We found that PM_{2.5} could enhance *Got1* expression and accumulation of its downstream metabolite, 2-HG. Xu et al²⁶

stated that in addition to mutated IDHs (IDH1 and IDH2), transamination through GOT1 can lead to much greater accumulation of 2-HG. The latter is an antagonist of TET1-TET3 that does not change mRNA expression^{26,33} and regulates *Foxp3* expression by demethylating the *Foxp3* promoter and its intronic CpG island.²⁶

Furthermore, it has been reported that Treg cells can become exTreg cells expressing FoxP3⁺ and IL-17 in patients with several chronic autoimmune diseases,^{34,35} particularly in the context of TET deficiency.³⁶ PM2.5 exposure inhibited TET activation and impaired development of Treg cells, but we did not observe exTreg cells in our model. The reason might be that exTreg cells are often found in patients with chronic diseases, and thus the development of exTreg cells might require longer periods of time.³⁴

Xu et al²⁶ showed that AOA could rescue experimental autoimmune encephalomyelitis in mice efficiently. In our model AOA reversed the inhibitory effect of PM2.5 on differentiation of Treg cells completely, thereby suggesting its potential clinical utility against asthma.

Traffic exhaust fumes are the main source of IP in outdoor air.³⁷ Cooking-related activities can also cause atmospheric exposure to IP. Although IP is distributed widely in various environments, studies of its toxicity are rare. The toxic equivalent factor with respect to benzo- α -pyrene (BaP) for IP was assessed to be 0.1 by Tsai et al,³⁸ but the functional effect of IP on T cells appears to be more potent than that of BaP (see Fig E4).

Taken together, these results suggest that the PAH (IP)-AhR axis integrates the glycolysis and glutamate metabolic pathways in T cells and plays an important role in airway inflammatory responses. In addition to T cells, mast cells, dendritic cells, macrophages, and epithelial cells are AhR-expressing cells. These cell types might also participate in the development of PM2.5/PAH-enhanced allergic inflammation in the airways.^{9,39-43} Indeed, the effects of PM2.5 in promoting an imbalanced response of T_H1/T_H2 cells,^{44,45} induction of the inflammasome,⁴⁴ and impaired function of Treg cells^{18,46} have been noted. Also, previously, we reported that exposure to AhR ligands resulted in enhanced activation of mast cells and the anaphylactic response *in vivo*.⁹⁻¹¹ Xu et al^{7,8} reported that ambient ultrafine particles could promote differentiation of T_H17 and T_H2 cells in a Jagged 1/Notch 4-dependent manner. Also, Wang et al¹³ reported that the PM-associated PAH BaP facilitates Der f 1-induced epithelial thymic stromal lymphopoietin and IL-33 release through the AhR-reactive oxygen species axis and enhances lung inflammation. Quintana et al⁵ reported that the AhR in T cells can regulate the balance of T_H17/Treg cells in a ligand-dependent manner.⁵ Thus these results clearly show a complex network of interactions between PM2.5/PAH and tissue-resident and immune cells that might, collectively, contribute to the adverse effect of PM2.5/PAH exposure. Therefore the “ultimate” adverse effect of exposure to PM2.5 or PAH could be more important when considering the likely combinatorial effect from multiple cell sources. Additional studies are needed to ascertain whether similar epigenetic and metabolic regulation noted in T_H17/Treg cells operates in different cell types.

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Key messages

- PM2.5 or PAHs aggravate asthma by disturbing the balance of T_H17/Treg cells.
- PM2.5 or PAHs impair differentiation of T_H17/Treg cells through AhR-HIF-1 α and AhR-GOT1 molecular pathways in a mouse model of asthma.
- Imbalance of differentiation of T_H17/Treg cells occurred in human T cells exposed to PM2.5/PAH, and PAH exposure is a potential risk factor for asthma.

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Online Repository

METHODS

Ethical approval of the study protocol. Experiments complied with the relevant laws and institutional guidelines as overseen by the Animal Studies Committee of the Children's Hospital of Fudan University (Shanghai, China).

Mice. All mice had a C57BL/6 background. AhR-null mice (AhR KO, B6.129-*Ahr*^{tm1Bra}/J) and AhR^{loxP} (B6.129(FVB)-*Ahr*^{tm3.1Bra}/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CD4-Cre mice [Tg(Cd4-cre)1Cwi/BfluJ] were obtained from Shanghai Model Organisms (Shanghai, China). AhR^{loxP} mice and CD4-Cre mice were maintained on a C57BL/6 background and were crossed for CD4-specific deletion of AhR (CD4-AhR-KO). All mice were housed, bred, and maintained under specific pathogen-free conditions.

Murine model of asthma. A CRE-induced asthma mouse (C57BL/6) model was established, as described previously^{1,2}. Briefly, light anesthesia was induced in 6–8-week-old female C57BL/6 mice (Shanghai SLAC Experimental Animals, Shanghai, China) with isoflurane. Then, they were suspended by the front incisors from an inclined board. Using forceps, the tongue was extended gently and held against the lower mandible. Allergen solution was delivered to the hypopharynx in 50-μL aliquots. Inhalation of the solution was confirmed by inspiratory sounds. Mice were sensitized by intratracheal inhalation of 20 μg of CRE (Greer Laboratories, Lenoir, NC, USA) per mouse on days 0, 1, 2, 3, 4 and challenged on days 10, 11, 12 and 13 with the same amount of CRE. Control mice received PBS during sensitization and challenge phases instead of CRE. In some experiments, mice were treated with PM2.5 (winter, 250 μg/mouse) or IP (5 μM/mouse, 50

μL) during CRE challenge in a final volume of 50 μL by intratracheal inhalation (Fig 1A). Unless stated otherwise, 250 μg of PM2.5 was mixed with CRE in 50 μL. On day-14, mice were killed and lung tissues dissected and analyzed to observe pulmonary inflammation. BALF was harvested by two consecutive flushes of the lung with 0.8 mL of ice-cold PBS.

Airway hyperresponsiveness. Invasive measurements of airway responsiveness were made on a Resistance & Compliance system (FinePointe®; Buxco Research Systems, Wilmington, NC, USA), as described previously³. Briefly, Mice were anesthetized with phenobarbital sodium (0.5 μg/g body weight). After the induction of anesthesia, mouse tracheas were cannulated with a steel 18-gauge cannula. Mice were ventilated mechanically at 160 breaths/min and tidal volume of 0.15 mL in a closed chamber. After stabilization for 3 min, PBS or increasing concentrations of aerosolized methacholine were nebulized into mouse airway. Finally, airway resistance (RI) was analyzed using FinePointe software (Buxco Research Systems). Briefly, the normalization function of the software was used and data was represented as deviation from 0mg/mL.

Collection and analyses of BALF. Collected BALF was centrifuged, and the total cell number counted with Countess II (Thermo Fisher, Boston, MA, USA). BALF cells were stained with each of the following antibodies and their respective isotype controls, all of which were from eBioscience (San Diego, CA, USA): PE anti-siglec-F (catalog number: 12-1702-80), fluorescein isothiocyanate anti-alpha-mac3 (85-11-0112-82), eFluor 780 anti-Gr-1 (85-94-5931-71), PE-Cy5 anti-CD3e (85-15-0031-63) and anti-CD19 (85-15-0191-81). The composition of BALF cells was determined by flow cytometry⁴. Eosinophils were defined as side scatter (SSC)^{high}

SiglecF⁺Mac-3⁻ cells, alveolar macrophages cells were identified as SSC^{high} SiglecF⁺ Mac-3⁺ cells, granulocytes were recognized as SSC^{high} Gr-1⁺ cells, and lymphocytes were identified as forward scatter (FSC)^{low}/SSC^{low} CD3⁺ or CD19⁺.

Lung pathology. Mouse lungs were perfused with 20 mL of ice-cold PBS injected into the right ventricle followed by excision and fixation in 10% neutral buffered formalin. Sections (5 μm) were prepared from paraffin-embedded lungs and stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS), respectively, according to manufacturer protocols. The severity of peribronchial inflammation was scored using the following criteria ⁵ for H&E staining: 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell-layer deep; 3, a ring of inflammatory cells 2–4-cells deep; 4, a ring of inflammatory cells of four-cells deep. The severity of peribronchial inflammation was scored using the following criteria for PAS staining: 0, < 5% PAS-positive cells; 1, 5–25% PAS-positive cells; 2, 25–50% PAS-positive cells; 3, 50–75% PAS-positive cells; 4, 75% PAS-positive cells.

ChIP assays were undertaken with a CST SimpleChIP[®] Plus Sonication Chromatin IP kit (56383; TaiGen Biotechnology, Taiwan, China) according to manufacturer instructions. Briefly, CD4⁺-naïve T cells were cultured in a T_{reg} cell-polarizing condition. In addition, PM2.5 or IP at the specified concentration was added to the respective experimental groups. After 2 days of culture, cells were fixed with 1% formaldehyde. Chromatin was sheared by sonication and supernatants were collected after centrifugation and diluted. Each sample was incubated with 5 μg of anti-AhR antibodies (BML-SA210; Enzo Life Sciences, New York, NY, USA) for 12 h,

and then the chromatin–antibody complex was incubated with Protein G Beads for an additional 5 h. Finally, after reversing crosslinking, DNA fragments were purified and analyzed with RT-qPCR.

T-cell differentiation in mice. Splenic CD4-naïve T cells were isolated from 6-week-old female C57BL/6 mice using a CD4⁺ T Cell Isolation kit (130-104-453; Miltenyi Biotec, Bergisch Gladbach, Germany). About 4×10^5 cells were seeded into plates pre-coated with anti-CD28 (553295; Becton Dickinson, Franklin Lakes, NJ, USA) and anti-CD3 ϵ (553058; Becton Dickinson). The differentiation conditions were very specific. For differentiation of T_H17 cells, they were: 5 μ g/mL of anti-interferon (IFN) γ (557530; Becton Dickinson), 5 μ g/mL of anti-IL4 (554432; Becton Dickinson), 20 ng/mL of IL-6 (7666-MB-005; R&D Systems, Minneapolis, MN, USA), 10 ng/mL of IL-23 (1686-MR-050; R&D Systems), 2 ng/mL of transforming growth factor (TGF) β ; 406-ML-025l R&D Systems) and 5 ng/mL of IL-2 (402-ML-020; R&D Systems). For differentiation of T_{reg} cells, the conditions were 2 ng/mL of TGF β and 5 ng/mL of IL-2. In some cases, PM2.5, PAHs, or metabolite at the specified concentration was added to the medium 6 h after initial cell plating. After 5 days of culture, cells were re-stimulated for 5 h with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (1 μ g/mL) in the presence of brefeldin A for intracellular staining of cytokines.

Differentiation of human T cells. Peripheral-blood mononuclear cells were isolated from the whole blood of healthy controls by Ficoll (GE Healthcare, Piscataway, NJ, USA). CD4⁺-naïve T cells were isolated using Human CD4⁺ Naïve T cell Isolation Kit II (130-091-155; Miltenyi

Biotech). About 10^5 cells were seeded into 96-well plates. Naïve T cells were activated by CD3/CD28 dynabeads (11131D; Thermo Scientific, Waltham, MA, USA) according to manufacturer instructions. The differentiation conditions were very specific. For differentiation of T_H17 cells, the conditions were: 5 $\mu\text{g/mL}$ of anti-IL4 (554481; Becton Dickinson), 20 ng/mL of IL-6 (206-IL-010; R&D Systems), 15 ng/mL of IL-23 (1290-IL-010; R&D Systems), 5 ng/mL of TGF β (240-B-002; R&D Systems) and 15 ng/mL of IL-1 β (201-LB-005; R&D Systems). For differentiation of induced regulatory T (iT $_{reg}$) cells, 10 ng/mL of TGF β was employed. PM2.5 (50 $\mu\text{g/mL}$) or PAHs (500 nM) was added to the medium 6 h after initial cell plating. After 5 days, the cell culture medium was changed to fresh medium with cytokines and PM2.5 (or IP). After culture for 8 days, cells were re-stimulated for 5 h with PMA (50 ng/mL) and ionomycin (1 $\mu\text{g/mL}$) in the presence of brefeldin A for intracellular staining of cytokines.

Methylation analyses by bisulfite sequencing. An established method was followed, as described by Xu et al. ⁶ Briefly, bisulfite conversion of genomic DNAs was done using an EpiTect™ Bisulfite kit according to manufacturer instructions (Qiagen, Stanford, VA, USA). The primers used to amplify the *Foxp3* promoter and its intronic CpG island are listed in Table E4. Finally, the PCR products were purified and sequenced.

T-cell transfection. Negative control [scramble (scr) shRNA] and shRNA knockdown virus carried with green fluorescent protein (GFP) tags were purchased from Genepharma (Shanghai, China). The shRNA Oligos sequences are listed in Table E4. The lentivirus was added into pre-activated CD4 T cells (20 h after initial plating). Cells were spin-infected at $1000 \times g$ for 2 h

and cultured in an incubator for an additional 2 h. Cells were washed and cultured under T_H17 cell- or iT_{reg} cell-polarizing conditions for an additional 4 days. On day-5, cells were re-stimulated for intracellular staining or mRNA analyses. For flow cytometry, GFP-positive cells were gated for further analyses.

RT-qPCR. For the *in vitro* differentiation assay, cells were re-stimulated with anti-CD3 and anti-CD28 for 6 h, and then collected. RT-qPCR was used to measure mRNA expression of *Foxp3*, *Il17a* and *RORyt*. To measure expression of *Cyp1a1*, *Hif1a* and *Got1*, cells were collected on day-1 or at the indicated time periods as described above. For the *in vivo* assay, lung tissues were collected and mRNAs isolated with TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). Expression was normalized to that of β -actin. The primer pairs are listed in Table E4. Fold-change in expression was calculated using the $2^{-\Delta\Delta C_t}$ method⁷.

PM2.5 and PAHs. A PM2.5 sample was collected twice a month for 1 year from March 2017 to February 2018 using an air sampler (ZR-3930; Qingdao Junray Intelligent Instruments, Shandong, China) on the open-air rooftop of the fourth floor of a research building of the Children's Hospital of Fudan University. Quartz fiber filters (1851-090; Whatman, Little Chalfont, UK) were used to collect PM2.5. The filters were cut into small pieces, immersed in distilled water, and sonicated for 30 min on three occasions. Supernatants of all filter pieces after sonication were filtered through sterile gauze, pooled, lyophilized and stored at -20°C. Levels of PAHs in PM2.5 were measured according to the method of Liu and colleagues⁸. Briefly, PM2.5 and its associated PAHs from quartz fiber filters were extracted with dichloromethane using an

accelerated solvent extractor (ASE 300; Thermo Scientific). Extracts were concentrated to 0.5 mL for gas chromatography–mass spectrometry (GC-MS) (Agilent Technologies, Santa Clara, CA, USA). Sixteen PAHs (US EPA priority) were determined. PAHs were purchased from Sigma–Aldrich (Saint Louis, MO, USA) and included IP (ERI-001), benzo[a]pyrene (B1760), dimethylbenz[a]anthracene (D3254) and phenanthrene (695114).

Glycolysis analyses of CD4⁺ T cells using a Seahorse extracellular flux analyzer. Mouse naïve T cells were cultured in the T_H17 cell- or iT_{reg} cell-polarizing conditions, as described above. For the corresponding wells, IP (1 µM) or PM2.5 (100 µg/mL) were added. Cells were cultured for 2 days, and subjected to glycolytic-rate analyses by a Seahorse XF Glycolytic Rate Assay kit (103344-100; Agilent Technologies) according to manufacturer instructions. Briefly, on the day of the assay, cells were re-stimulated by anti-CD3 and anti-CD28 for 4 h. After stimulation, cells were washed once in XF assay medium and then plated onto Seahorse XF96 cell culture microplates at 1.5×10^5 cells/well. The culture microplate was pre-treated with cell-TAK™ (354240; Corning, Corning, NY, USA). After centrifugation at $400 \times g$ for 5 min, additional XF assay medium was added to a final volume of 175 µL per well. After incubation for 45 min at 37°C, cells were evaluated by a Seahorse Analyzer (XFe96).

Quantification of intracellular 2-HG. CD4⁺ naïve T cells were cultured in differentiation conditions for iT_{reg} cells with or without PM2.5 (100 µg/mL). Cells were collected 2 days after stimulation. A solution of methanol and water (4/1, v/v) was added to each sample to extract intracellular 2-HG. Extracted samples were analyzed by GC-MS. Derivatized samples were

analyzed on a gas chromatography system (7890A; Agilent Technologies) coupled to a 5975C MSD system (Agilent Technologies). A DB-5MS fused-silica capillary column (30 m × 250 µm I.D.; J&W Scientific, Folsom, CA, USA) was utilized to separate derivatives. Helium (>99.999%) was used as the carrier gas at a constant flow rate of 6.0 mL/min through the column. The injector temperature was maintained at 280°C.

mRNA sequencing. CD4⁺-naïve T cells were cultured in differentiation conditions for T_H17 cells with or without PM2.5 (100 µg/mL) for 4 days and then the cells were collected. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Construction and sequencing of a library was undertaken at Shanghai Biotechnology Corporation (Shanghai, China). The library was constructed using a mRNA-seq Library Prep kit (NR602-02; Vazyme Biotech, Nanjing, China) and sequencing was done using a HiSeq 2500 system (Illumina, San Diego, CA, USA). Sequenced reads were trimmed for adaptor sequence, and masked for low complexity or low-quality sequences, then mapped to mm10 whole genome using tophat 2.0.6. Fragments per kilobase of exon model per million mapped reads were calculated using cufflink 2.0.2. Fold-changes of ≥2 and P < 0.05 as cutoff were employed to evaluate the significance of differential gene expression. Also, P = 0.05 was used to denote the significance of enrichment in the list of differentially expressed genes from the Gene Ontology database. Pathway analyses for differentially expressed genes were based on the KEGG database (p = 0.05). mRNA-seq data have been deposited in the GEO under accession number GSE124660.

Patients. The study protocol was approved by the Ethics Review Board of Chang-Hua Christian

Hospital (Chang-Hua, Taiwan). The study was carried out in accordance with the ethical standards laid down in the Declaration of Helsinki. All eligible participants provided written informed consent. The study population comprised adult patients with current asthma (n=68) at the outpatient clinic of Chang-Hua Hospital. We also enrolled healthy controls (n=68) who volunteered and those without respiratory diseases who requested annual physical examinations. All participants were non-smokers. Patients aged ≥ 18 years were enrolled if they had physician-diagnosed asthma. The latter was made according to 2012 Global Initiative for Asthma (GINA) guidelines⁹. Asthma severity was classified on the basis of the intensity of treatment required to achieve good asthma control. As indicated by GINA guidelines, “severe asthma” is asthma that requires high-intensity treatment (e.g., GINA step 4 (severe) or 5 (very severe)) to maintain good control, or if good control is not achieved despite high-intensity treatment. We stratified asthma further into four groups, i.e., asthma that required: GINA step 1 or 2 to maintain good control was termed “mild” asthma; GINA step 3 was called “moderate” asthma; GINA step 4 was termed “severe” asthma; GINA step 5 was called “very severe” asthma. Patients with severe asthma (GINA step 4) were receiving more than two combination controller therapy (Inhaled corticosteroid (ICS)/long-acting β 2-agonist (LABA), leukotriene modifier and sustained-release theophylline). Patients with very severe asthma (GINA step 5) were treated with corticosteroids (p.o.) or anti-IgE antibodies, in addition to GINA step-4 therapies. Urine samples were collected and stored frozen in glass containers until analyses. In this dataset, demographic comparisons revealed no significant differences between healthy controls and patients (Table E5).

Measurement of urinary PAH metabolites. The urinary level of 1-hydroxypyrene (OH-Py) was measured based on a previously published assay and used as a surrogate marker for PAH exposure because the standard IP metabolite was not available commercially at the time of analyses¹⁰. First, the defrosted urine (10 mL) in acetate buffer (0.1 M acetic acid and 0.1 M sodium acetate) was digested with α -glucuronidase/aryl-sulfatase. Then, OH-Py in urine samples was extracted, enriched and purified by cartridges packed with C-18 reversed-phase liquid chromatographic material (SEP-PAK VAC C-18; Waters, Milford, MA, USA) at a loading rate of <3 mL/min. The retained solutes in cartridges were eluted with 6 mL of isopropyl alcohol and dried. The processed sample was dissolved in 2 mL of isopropyl alcohol for analyses using a high-performance liquid chromatography on a 2695 system (Waters) with a fluorescence detector (474; Waters) and a LiChrosorb RP-18 (5 mm) column (150 mm $\times$ 4 mm; Sigma–Aldrich). The mobile phase was methanol:water (65:35). The excitation wavelength and emission wavelength of fluorescence were 281 nm and 388 nm, respectively. This method had a limit of detection of 0.028 ng/mL, with an average recovery rate of 96.18%, and a coefficient of variance <10% for repeat measurements. Urinary levels of OH-Py were normalized by levels of urinary creatinine and expressed as μ g/g urinary creatinine.

Cytokine measurement. Levels of cytokines were measured by a BD Cytometric Bead Array Mouse Cytokine kit (BD 560485) according to manufacturer (Becton Dickinson) instructions. Briefly, lungs were homogenized in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% Tween 20, and Complete Mini, EDTA-Free Protease Inhibitor (Roche Applied Sciences, Basel, Switzerland), and debris-free supernatant was used for cytokine measurement.

Measurement of serum levels of total IgE and CRE-specific IgE. Serum levels of total IgE were measured by a Mouse Total IgE Enzyme-linked Immunosorbent Assay (ELISA) kit (70-EK2752) according to manufacturer (MultiSciences, Beijing, China) instructions. For measurement of CRE-specific IgE levels, serum IgG was first depleted with protein G agarose (GE Healthcare) and levels of CRE-specific IgE were analyzed by ELISA according to our previous work ⁴. Briefly, plates were pre-coated with CRE (10 µg/mL) at 4°C overnight and then, after blockade, samples were incubated at 4°C overnight. Biotinylated anti-mouse IgE (BioLegend, San Diego, CA, USA) was added, followed by streptavidin-horseradish peroxidase and tetramethylbenzidine substrate.

Flow cytometry

For analyses of T_H17 and T_{reg} cells in lung lymph nodes, the latter were collected and teased apart into a single-cell suspension by pressing with the plunger of a 3-mL syringe. For detection of Treg cells, cells were first stained with antibodies against CD4-fluorescein isothiocyanate (FITC) (RM4-5; eBioscience) and CD25-PE (PC61.5; eBioscience), followed by intracellular staining with Foxp3-allophycocyanin (FJK-16s; eBioscience) or allophycocyanin-conjugated rat IgG2a isotype control (eBioscience) using a Foxp3 staining kit (eBioscience). For detection of T_H17 cells, 10⁶ cells/mL in media were stimulated with PMA (50 ng/mL)/ionomycin (1 µg/mL) in the presence of BD GolgiStop™ protein-transport inhibitor for 5 h. Then, cells were incubated with anti-CD4-FITC (RM4-5; eBioscience) antibody at 4°C in the dark for 10 min, washed with PBS, and mixed with 500 µL of fixation/permeabilization solution at 4°C in the dark for 45 min.

After washing with 1×BD Perm/Wash™ buffer, cells were incubated with APC-anti-IL-17A (eBio17B7; eBioscience). Then, samples were analyzed on a BD FACSCanto II flow cytometer (Becton Dickinson).

Statistical analyses. Data are the mean ± SEM from three independent experiments. Statistical analyses were done using the Student's *t*-test or one-way ANOVA for group comparisons with Prism 6.0 (GraphPad, San Diego, CA, USA). For comparison of airway resistance between groups, ANOVA was undertaken for the highest or second highest concentration of methacholine used. Differences were considered significant if $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

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Supplementary figure legends

Fig E1. SR2211 can alleviate the symptoms aggravated by PM2.5 in a murine asthma model. (A) Staining (H&E and PAS) from mouse lung tissues treated with CRE+50 µg/mouse/day of PM2.5. (B) Serum levels of total IgE and CRE-specific IgE. (C) Cell differential counts in BALF. For the corresponding group, 20 mg/kg/day of SR2211 was injected (i.p.) in the challenge phase together with PM2.5. (D) Methacholine-induced airway hyperresponsiveness (deviation from 0mg/mL). Data are the mean ± SEM from two independent experiments. *P ≤ 0.05; **P ≤ 0.01; ns, not significant by the unpaired Student's *t*-test (B, C) or one-way ANOVA with Tukey's *post hoc* test (D). n = 10 mice per group.

Fig E2 PM2.5 has a similar function in the HDM model.

(A) Asthma models used in our study (schematic). Mice were sensitized and challenged as shown with or without PM2.5 treatment and, 24 h after the last allergen challenge, mice were killed and evaluated at different end points. (B) Cell counts in BALF. (C) Assessment of methacholine-induced airway hyperresponsiveness in mice (deviation from 0mg/mL). Value of lung airway resistance are shown. (D) Expression of *Il17a*, *Il4*, *Ifng*, *RORyt* and *Foxp3*. (E) Hilar lymph nodes from lungs were isolated from different groups, and the percentage of

CD4⁺IL-17A⁺ or CD4⁺CD25⁺FoxP3⁺ cells in CD4⁺ cells was calculated *via* flow cytometry. n = 12 mice per group. Data are the mean ± SEM from two independent experiments. *P ≤ 0.05; **P ≤ 0.01; ns, not significant by the unpaired Student's *t*-test (B, D, E) or one-way ANOVA with Tukey's *post hoc* test (C).

Fig E3. The glycolysis rate in cells assessed by the Seahorse system.

Fig E4. Effect of PAHs (US EPA priority) with five or six-membered rings on differentiation of T_H17 cells.

(A) Flow cytometry. (B) Statistical test. Data are the mean ± SD. *P ≤ 0.05; **P ≤ 0.01 by the unpaired Student's *t*-test

Fig E5. IP has a similar function upon T-cell differentiation as PM2.5.

(A) mRNA expression of *Cyp1a1*. (B) mRNA expression of T_H17 cell- and T_{reg}-related genes. (C) Chromatin immunoprecipitation (ChIP) assay. (D) *Hif1a* and *Got1* expression in lung tissues. Data are the mean ± SEM from three independent experiments. *P ≤ 0.05; **P ≤ 0.01 by the unpaired Student's *t*-test

Fig E6. PAH exposure was associated asthma severity.

(A) Analyses of urinary levels of the PAH metabolite, 1-hydroxypyrene (OH-Py), in people currently suffering from asthma and in healthy controls (n = 68 per group). Urinary levels of 1-OH-Py were normalized to urinary levels of creatinine (CRN) and expressed as µg/g CRN. All study participants were non-smokers. *P = 0.0075 by the Mann–Whitney *U*-test. (B) Multiple logistic regression analysis of urinary levels of 1-OH-pyrene stratified by asthma severity. Mi

355 mild asthma; Mo moderate asthma; S, severe asthma; VS, very severe asthma; aOR was adjusted
356 for differences in sex and age. * $P < 0.01$, ** $P < 0.001$; ns, not significant.

357

Table E1.

Four xenobiotic response elements (XRE) in the Got1 promoter

Primers for ChIP analysis of Got1 promoter

Name	Primer
got1_ahr_XRE1F	CCATCCTCATCACCCCAAGG
got1_ahr_XRE1R	TGGGCAGAGGCTAGGATGAT
got1_ahr_XRE2F	TAGACTCTCAGGGGAAGGAGC
got1_ahr_XRE2R	CGGGAAAGCCTAGCCCTAAAG
got1_ahr_XRE3F	TCCTACTCATCAGGAGGCTTTC
got1_ahr_XRE3R	CCTTTCTCCACCTGTTGACC
got1_ahr_XRE4F	AAACAAGGCGATGTTTCTGGA
got1_ahr_XRE4R	CTGGAAGGCGCCAATTCCTT

Table E2

PAHs in PM2.5

PAHs (pg/μg)	Spring	Summer	Autumn	Winter
Acephenanthrylene	12.62	0	18.9	23.38
Acenaphthene	15.29	10.54	19.9	28.88
Anthracene	35.94	30.45	109.25	81.682
Benzo(GHI)fluoranthene	45.883	30.23	108.95	104.28
Naphthalene	50.34	32.41	75.20	50.32
Benzo(j)fluoranthene	30.851	20.56	95.82	57.132
Dibenz(ah)anthracene	65.846	37.07	118.25	127.91
Benz(a)anthracene	115.13	64.82	155.93	223.64
Benzo(a)pyrene	148.09	62.54	152.83	287.67
Indeno(1,2,3-cd)pyrene	154.94	57.17	186.27	300.97
Benzo(k)fluoranthene	158.76	58.78	188.47	308.4
Chrysene	174.85	30.78	109.7	339.64
Phenanthrene	175.67	55.92	198.16	341.25
Pyrene	228.07	78.05	228.21	443.02
Benzo(b)fluoranthene	290.41	104.37	263.96	564.12
Fluoranthene	292.5	202.09	396.66	568.18

Table E3

Multiple logistic regression analysis of urinary 1-OH-pyrene levels stratified by asthma severity.

	aOR (95% CI) [#]	p-value
Asthma (N=68) vs Control (N=68)	1.82 (1.24-2.68)	0.0025
(Mi/Mo*, N=32) vs Control	1.46 (0.95-2.25)	0.0873
(S/VS**, N=33) vs Control	2.63 (1.50-4.63)	0.0008

*Mi/Mo, mild and moderate asthma case; **S/VS, severe and very severe asthma case; #Odds ratio (aOR) adjusted for differences in gender and age.

Table E4

Primer for qPCR

Gene name	qPCR primer sequence
CYP1a1_F	CATCACAGACAGCCTCATTGAGC
CYP1a1_R	CTCCACGAGATAGCAGTTGTGAC
HIF1alph_F	CCTGCACTGAATCAAGAGGTTGC
HIF1alph_R	CCATCAGAAGGACTTGCTGGCT
Got1_F	TGCTACTGGGATGCGGAGAAGA
Got1_R	TGCATGACAGCAGCGATCTGCT
IL-17a_F	CAGACTACCTCAACCGTTCCAC
IL-17a_R	TCCAGCTTTCCCTCCGCATTGA
IDH1_F	CAGGCTCATAGATGACATGGTGG
IDH1_R	CACTGGTCATCATGCCAAGGGA
IDH2_F	GGCTGTCAAGTGTGCCACAATC
IDH2_R	TTGGCTCTCTGAAGACGGTTCC
TET2_F	ACCTGGCTACTGTCATTGCTCC
TET2_R	TGCAGTGACTCCTGAGAATGGC
IFN-g_F	CAGCAACAGCAAGGCGAAAAAGG
IFN-g_R	TTTCCGCTTCCTGAGGCTGGAT
RORytF	GTGGAGTTTGCCAAGCGGCTTT
RORytR	CCTGCACATTCTGACTAGGACG
Foxp3_F	GCGAAAGTGGCAGAGAGGTA
Foxp3_R	GAGGAGCTGCTGAGATGTGA
Glut1_F	GCTTCTCCAACTGGACCTCAAAC
Glut1_R	ACGAGGAGCACCGTGAAGATGA
HK2_F	CCCTGTGAAGATGTTGCCCACT
HK2_R	CCTTCGCTTGCCATTACGCACG
Ldha_F	ACGCAGACAAGGAGCAGTGGAA
Ldha_R	ATGCTCTCAGCCAAGTCTGCCA
Eno1_F	TACCGCCACATTGCTGACTTGG
Eno1_R	GCTTGTTGCCAGCATGAGAACC

Primers for methylation analysis of FOXP3 promoter and CpG island

Name	Primer
Foxp3_cnsF	TTTTGGGTTTTTTTGGTATTTAAGA
Foxp3_cnsR	TTAACCAAATTTTTCTACCATTAAAC
Foxp3_promoterF	TATATTTT TAGATGATTTGTAAAGGGTAAA
Foxp3_promoterR	ATCAACCTAACTTATAAAAAACTACCACAT

shRNA Oligos sequence cloned into lenti-virus vector

Name	Sequence
shGot1	GCCCTATTGCTACTGGGATGC
shHif1a	CTCATCCGTCAAATATTGAAA

Table E5

Demographics and clinical characteristics of the study subjects.

	Control, N=68	Asthma, N=68	p-value
Gender, N(%)			1
Male, N(%)	20(29.41)	20(29.41)	
Female, N(%)	48(70.59)	48(70.59)	
Age, yrs($\pm$ SD)	56.34($\pm$ 12.50)	54.32($\pm$ 16.63)	0.426
BMI, kg/m ² ($\pm$ SD)	24.28($\pm$ 3.37)	24.42($\pm$ 3.42)	0.8093
Onset Age, yrs($\pm$ SD)	NA*	40.48($\pm$ 21.60)	
Smoking Status, N(%)			
Never smoker	68(100)	68(100)	
Ex-smoker	0(0)	0(0)	
Current smoker	0(0)	0(0)	
Atopy, N(%)	14(20.59)	46(67.65)	<.0001
Severity, N(%)			
Mild/Moderate	NA	35(51.47)	
Severe/very severe	NA	33(48.53)	
ACT Score ($\pm$ SD)	NA	20.96($\pm$ 3.88)	
IgE, IU/ml, median(25th-75th)	NA	565.63(66.2-50.)	
Eosinophil count, median(25th-75th)	NA	277.80(100-307)	
FVC% ($\pm$ SD)	84.78($\pm$ 15.47)	82.10($\pm$ 22.89)	0.5181
FEV1 % ($\pm$ SD)	88.19($\pm$ 15.39)	67.09($\pm$ 24.07)	<.0001
FEV1/FVC % ($\pm$ SD)	86.74($\pm$ 8.98)	66.48($\pm$ 10.38)	<.0001

*NA, not applicable