



Perfluorooctanesulfonate and perfluorooctanoate exacerbate airway inflammation in asthmatic mice and in vitro

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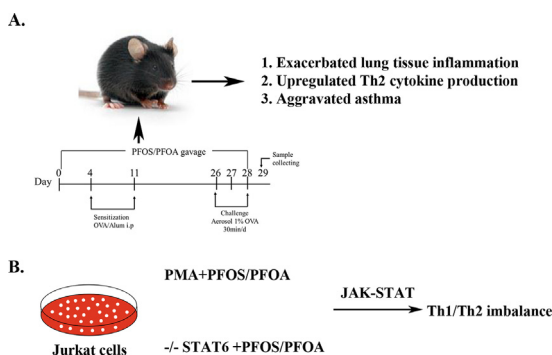
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HIGHLIGHTS

- PFOS and PFOA exacerbated lung tissue inflammation, upregulated Th2 cytokine production, aggravated asthma in asthmatic mice.
- PFOS and PFOA regulated the balance of Th1/Th2 through the JAK-STAT signaling pathway and aggravated asthma.
- PFOS induced Th1/Th2 imbalance through up-regulation of p-STAT6 level, while PFOA through another pathway.

GRAPHICAL ABSTRACT



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ABSTRACT

Emerging evidence suggests associations between Perfluoroalkyl substances (PFASs) exposure and asthma, but the findings are inconsistent. The current study sought to investigate whether perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) could contribute to asthma exacerbation and to clarify the underlying biological mechanisms. The objectives are a) to determine whether PFOS or PFOA could aggravate the mouse asthma and pulmonary inflammation b) to investigate whether PFOS or PFOA regulate the balance of Th1/Th2 through the JAK-STAT signaling pathway and aggravated asthma. Ovalbumin (OVA) induced asthmatic mice were exposed to PFOS or PFOA by gavage. PFOS and PFOA serum level and toxicity in organs were assessed; and the impacts on respiratory symptoms, lung tissue pathology, T helper cell (Th2) response, and STAT6 pathway activity were also evaluated. In vitro Jurkat cells were used to study the mechanisms of PFOS and PFOA mediated Th1 and Th2 responses. Both PFOS and PFOA exacerbated lung tissue inflammation (greater number of eosinophils and mucus hyperproduction), upregulated Th2 cytokine production (IL-4 and IL-13), and promoted Th2 cells and STAT6 activation. Furthermore, PFOS and PFOA enhanced the Th2 response in Jurkat cells via STAT6

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activation; and the effect of PFOS exposure on GATA-3, IL-4 and IFN- γ was blocked after the expression of STAT6 was suppressed in Jurkat cells, however, the effects of PFOA exposure were only partially blocked. PFOS and PFOA aggravated inflammation among OVA-induced asthmatic mice, by promoting the Th2 response in lymphocytes and disturbing the balance of Th1/Th2 through the JAK-STAT signaling pathway.

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1. Introduction

Perfluoroalkyl substances (PFASs), are widely used in industrial manufacturing processes and consumer products due to their joint hydrophobic and oleophobic properties (Lindstrom et al. 2011). Because of their ubiquitous use and long biological half-life, PFASs have been detected in the environment, wildlife, and humans (Boiteux et al. 2017; Cao et al. 2018; Pan et al. 2018). Human populations are exposed to PFASs by ingesting contaminated drinking water and food, inhalation of contaminated indoor air and dust, and contacting contaminated media (Averina et al. 2018; Dassuncao and Hu 2018; Schwanz et al. 2016). Among the various PFASs, perfluorooctanesulfonate (PFOS; $C_8F_{17}SO_3^-$) and perfluorooctanoate (PFOA; $C_8F_{17}COO^-$) have been the most commonly studied types of these compounds in association with adverse health effects (Sunderland et al. 2019). Association have been reported for PFASs exposure and a wide variety of adverse human health outcomes, including cancer, immune dysfunctions, neurodevelopmental delays, and respiratory disease (Chang et al. 2016; Dong et al. 2013; Humblet et al. 2014; Negri et al. 2017). Especially PFOS and PFOA, may be associated with worsened asthma symptoms, but the results are inconsistent (Anderson-Mahoney et al. 2008; Dong et al. 2013; Humblet et al. 2014; Negri et al. 2017).

Asthma is a major global health issue, affecting about 235 million people worldwide, and causing approximately 383,000 annual deaths (Aghaei et al. 2019). Environmental factors, such as exposure to hazardous chemicals, may contribute to the increasing prevalence of asthma and the exacerbation of airway symptoms (Carrillo et al. 2018; Lee et al. 2019; Miller and Marty 2010; Murrison et al. 2019). Epidemiological and experimental studies have investigated the association between PFASs exposure and asthma prevalence, but the findings are inconsistent (Anderson-Mahoney et al. 2008; Dong et al. 2013; Fairley et al. 2007; Humblet et al. 2014; Impinen et al. 2018; Lee et al. 2017; Ryu et al. 2014; Singh et al. 2012). In the previous in vivo experimental studies, only two studies have reported the effects of PFOS and PFOA exposure on the exacerbation of asthma in mice (Fairley et al. 2007; Ryu et al. 2014). However, their findings were inconsistent. Therefore, importance of PFOS and PFOA in aggravating asthma damage and the mechanisms behind these effects are very insufficiently studied. Pathogenesis of asthma is complex and the ratio of Th1/Th2 imbalance is generally accepted pathological basis for asthma (Kubo 2017). Previous research suggests that IFN- γ and IL-4 are representative of Th1 and Th2 cytokines (Akdis et al. 2016). Transcription factors T-bet and GATA3 regulate the Th1 and Th2 differentiation, and secretion of their characteristic cytokine (Zhu et al. 2010). JAK-STAT is an important pathway that mediates cytokine signaling and participates in regulating the differentiation, proliferation and survival of Th1 and Th2 cells. JAK2/TYK2/STAT4 regulates Th1 cell differentiation by up-regulating the expression of T-bet, while JAK1/JAK3/STAT6 regulates Th2 cell differentiation by up-regulating GATA3 expression (Howell et al. 2018). At present, JAK/STAT signaling pathway is one of the important pathways in the pathogenesis of asthma, and the key molecules on the pathway have been used as key targets for the treatment of asthma (Vale 2016). In asthmatic mice, STAT6 is involved in regulating Th2 cell differentiation and cytokine secretion as well as regulating various pathological changes associated with asthma, including increased airway eosinophilia and increased mucus secretion (Goenka and Kaplan 2011; Walford and Doherty 2013). Therefore, our goal was to use an allergic asthmatic mouse model with exposure to PFOS or PFOA, to determine

whether PFOS or PFOA could aggravate asthma and pulmonary inflammation by evaluating symptoms, lung tissue histopathology, cytokine production, helper T cell subsets, and JAK/STAT signaling pathway biomarkers. Moreover, we used Jurkat cells to further investigate whether PFOS and PFOA regulated the ratio of T-bet/GATA-3 through the JAK-STAT signaling pathway, thereby inducing Th1/Th2 balance disorders.

2. Methods and materials

2.1. Animals and exposure

Male C57BL/6 mice (7 weeks old; weighing 18–22 g) were obtained from the Guangdong Medical Laboratory Animal Center (Guangzhou, China), housed in a pathogen-free facility, and allowed them to acclimate to the new treatment room for one week before the study was initiated. Forty-eight mice were randomly divided into eight treatment groups (6 mice per group): 1) 2% Tween 80 (control group); 2) 7 mg/kg total administered dose (TAD) PFOS group; 3) 70 mg/kg TAD PFOS group; 4) OVA group; 5) 7 mg/kg TAD PFOS + OVA group; 6) 70 mg/kg TAD PFOS + OVA group; 7) 7 mg/kg TAD PFOA + OVA group; and 8) 70 mg/kg TAD PFOA + OVA group. We calculated the daily dose of PFOS and PFOA based on the body weight and exposure time of the mice. Mice were exposed to PFOS, PFOA, or 2% Tween 80, via gavage from day 1 to 28. The OVA and OVA combined PFOS or PFOA groups were first sensitized with OVA and Al(OH) $_3$ by subcutaneous injection on days 4 and 11; and then challenged with an aerosol of 1% OVA using an ultrasonic nebulizer on day 26 to 28 (Fig. E1). We exsanguinated the mice on the 29th day (24 h after last treatment). The mouse lung function test and processing of tissue/blood samples are described in the eMethods 2,3 (Supplementary materials). The chemicals and reagents used in the experiments are listed in eMethods 1 and Table E3, 4 (Supplementary materials). All animal experiment protocols were approved by the Institutional Animal Ethics Committee of Sun Yat-sen University.

2.2. Serum PFOS and PFOA analysis

PFOS or PFOA in serum of mice were analyzed by HPLC-MS/MS as reported previously (Bao et al. 2014). The details of sample preparation and analysis are described in the eMethods 4 (Supplementary materials).

2.3. Measurement of peripheral blood cells

The number of red blood cells, platelets, and white blood cells (WBC) as well as the percentages of different types of WBC were analyzed by automated hematology analyzer (HEMAVET 950, Drew Scientific, Waterbury, CT).

2.4. Measurements of IgE and cytokines

Concentrations of OVA-specific IgE and total IgE in serum, and cytokines (IL-4, IL-13, IFN- γ) in mice serum and bronchoalveolar lavage fluids (BALF) were analyzed with corresponding ELISA kits (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturers' instruction. The detection limits for anti-OVA IgE, total IgE, IL-4, IL-13, and IFN- γ were 3.12 ng/mL, 4.0 ng/mL, 2.0 pg/mL, 1.5 and 2.0 pg/mL, respectively.

2.5. T cell subsets analyzed by flow cytometry assay

Lung cell suspensions were stimulated with Cell Stimulation Cocktail (including phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A, and monesin) (eBioscience, San Diego, CA, USA). Different T cell subsets, including Th1 (CD4⁺ IFN- γ ⁺), Th2 (CD4⁺ IL-4⁺), Th17 (CD4⁺ IL-17⁺), Tc1 (CD8⁺ IFN- γ ⁺), Tc2 (CD8⁺ IL-4⁺), Tc17 (CD8⁺ IL-17⁺), and Treg (CD4⁺ CD25⁺ Foxp3⁺) were detected using Flow cytometry (Beckman Coulter, USA) panels of monoclonal antibodies and analyzed by FlowJo software (Treestar, San Carlos, CA).

2.6. Protein and mRNA detection

Tissue expression of T-bet, IL-4R α , p-Jak1, p-Jak3, GATA3, p-STAT6 proteins, and cell expression of T-bet, p-Jak1, p-Jak3, GATA3, STAT6, p-STAT6 proteins were determined by Western blot. Lung tissue p-STAT6 was stained using immunohistochemistry. T-bet, GATA3, ROR γ t, STAT6, IL-4 and IFN- γ mRNA expressions were determined by quantitative PCR. Details of the above experiments are described in the eMethods 5–8 (Supplementary materials).

2.7. Jurkat cells culture and stimulation experiment

Jurkat cells were grown in RPMI-1640 medium (Thermo Fisher Science, Grand Island, NY, USA) containing 10% fetal bovine serum (Biowest, Loire Valley, France) and 1% penicillin-streptomycin (Thermo Fisher, USA). Cells were first primed with cell stimulation cocktail and then stimulated with PFOS or PFOA at concentrations of 0.1, 1.0, 10, and 50 μ M for 48 h, respectively. The medium and cell lysates from stimulated Jurkat cells were collected for measurement of cytokines, qPCR of target genes' mRNA levels and western blot analysis.

2.8. STAT6 siRNA transfection experiment

We diluted 1.25 μ L 20 μ M siRNA stock solution with 30 μ L 1 $\times$ riboFECTTM CP Buffer, then added 3 μ L of riboFECTTM CP Reagent, mixed by gently pipetting, and incubated at room temperature for 15 min to prepare transfection complex. STAT6 siRNA sequence and related reagents were purchased from Guangzhou Ruibo Biotechnology Company. We added the riboFECTTM CP transfection complex to the Jurkat cells without penicillin-streptomycin complete medium, and placed the culture plate in the incubator. Cells were transfected with STAT6 siRNA for 24 h, and then infected with PFOS and PFOA for 48 h. We set up blank control group, siRNA negative control group, siRNA group, siRNA + PFOS exposure group (0.1, 1, 10, 50 μ M), siRNA + PFOS exposure group (0.1, 1, 10, 50 μ M).

2.9. Statistical analysis

All data were analyzed by using SAS software (Version 9.4; SAS Institute Inc., Cary, NC). A one-way analysis of variance (ANOVA), along with the Dunnett's *t*-test, was used to compare means among different groups. The level for statistical significance was set at *p* < 0.05. Results were expressed as the mean $\pm$ standard deviation (SD).

3. Results

3.1. Toxicity of PFOS and PFOA in the mice

Only PFOS or PFOA exposed mice presented a strong serum PFOS or PFOA signal, while limited PFOS and PFOA signals were detected in the control or OVA only groups (Fig. 1). PFOS serum levels of exposed mice in 7 mg/kg PFOS group and 7 mg/kg PFOS + OVA group were 8.43 $\pm$ 1.86 mg/L and 7.68 $\pm$ 1.41 mg/L, in 70 mg/kg PFOS group and 70 mg/kg PFOS + OVA group were 78.52 $\pm$ 11.40 mg/L and 74.41 $\pm$ 19.01 mg/L, respectively (Fig. 1K). PFOA serum levels in 7 mg/kg PFOA

+ OVA group was 6.38 $\pm$ 1.23 mg/L, and in 70 mg/kg PFOA + OVA group was 60.38 $\pm$ 6.32 mg/L, respectively (Fig. 1L). Compared to control and OVA only mice, animals exposed to the highest concentration of PFOS or PFOA had significant liver weight gain; while the body and other organ (spleen, lung, and thymus) weights were similar across groups (Table E1). In addition, peripheral blood examination showed similar proportions of white blood cells, eosinophils, neutrophils, lymphocytes, monocytes, and basophils, among all the groups (Table E2).

3.2. Lung function in OVA-induced asthmatic mice

Lung function tests were conducted in the OVA-sensitized mice treated with PFOS or PFOA (Fig. E2). While the OVA alone challenge induced the lung function decrease; FVC and FEV1 were similar among asthmatic mice exposed to PFOS or PFOA and OVA-sensitized mice. PFOS and PFOA treatment did not alter the severity of OVA-induced lung function decrease in asthmatic mice.

3.3. Lung eosinophils and goblet cells expression in mice

To test the effects of PFOS or PFOA on lung inflammation in asthmatic mice, we checked the leukocyte numbers in BALF and lung tissues. In BALF, the total cell count, eosinophil, macrophage and neutrophil numbers were increased in the asthmatic mice as compared to the control group or the mice treated with PFOS alone (Fig. 2). In addition, the total cell count and the eosinophil numbers were higher in the 7 or 70 mg/kg PFOS + OVA, and 7 or 70 mg/kg PFOA + OVA exposed asthmatic mice than in the mice induced by OVA alone (Fig. 2). In lung tissues, the OVA-induced mice showed significant tissue eosinophilia and increased mucus production compared to the control group or the mice exposed to PFOS alone (Fig. 3). PFOS or PFOA exposure can further promote the eosinophil infiltration (33% and 19% increase, respectively) and mucus production (67% and 60%, respectively) in mice treated with 70 mg/kg PFOS + OVA or 70 mg/kg PFOA + OVA group as compared to OVA alone challenged mice (Fig. 3).

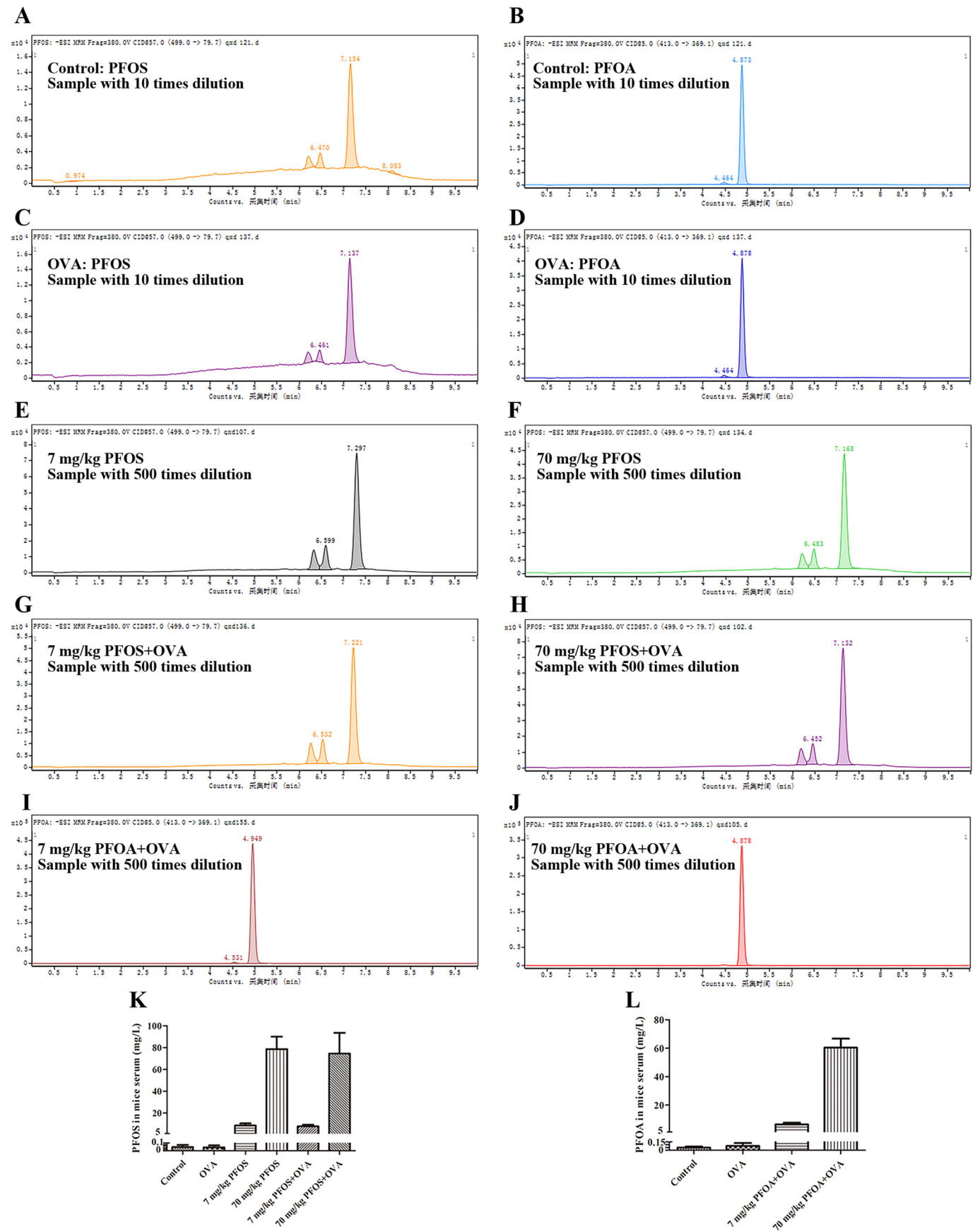
3.4. Serum IgE and Th2 cytokine production in mice

Mice exposed to PFOS or PFOA alone showed a low level of serum IgE, similar to the control group (Fig. 4A). As expected, OVA-induced mice demonstrated greater serum IgE and OVA-specific IgE compared to the control group. Asthmatic mice showed a significant up-regulation of serum IgE and OVA-specific IgE when they were exposed to PFOS (54% and 67% increase in mice treated with 70 mg/kg PFOS, respectively) or PFOA (42% and 73% increase in mice treated with 70 mg/kg PFOA, respectively) (Fig. 4A, B). The production of signature Th1 cytokine IFN- γ was decreased in serum from OVA-induced asthmatic mice or PFOS only treated mice, and PFOS or PFOA did not further affect the IFN- γ levels in serum or BALF from asthmatic mice (Fig. 4C, F). The Th2 cytokine IL-4 and IL-13 were elevated in serum and BALF from asthmatic mice compared to control, and PFOS and PFOA treatments significantly enhanced levels of IL-4 and IL-13 levels in serum and BALF of asthmatic mice (Fig. 4D-E, G-H).

3.5. Th2 cells and Th2-related gene expression in mice

The proportions of T cell subsets (including Th1, Th2, Th17, and Treg) in lung tissues from the different treatment groups were analyzed by flow cytometry. The proportion of Th2 cells (CD4⁺ IL-4⁺) was significantly elevated (51% increase) in the asthmatic mice exposed to PFOS, but not PFOA, compared to the OVA only treated group (Fig. 5A, C). However, the proportions of Th1 (CD4⁺ IFN- γ ⁺), Th17 (CD4⁺ IL-17⁺) and Treg (CD4⁺ CD25⁺ FOXP3⁺) were similar among the control, OVA, PFOS + OVA, and PFOA + OVA groups (Fig. 5A, B-E).

mRNA levels of Th1-, Th2-, Th17-, and Treg-associated transcription factors were also evaluated in lung tissues from the above groups.



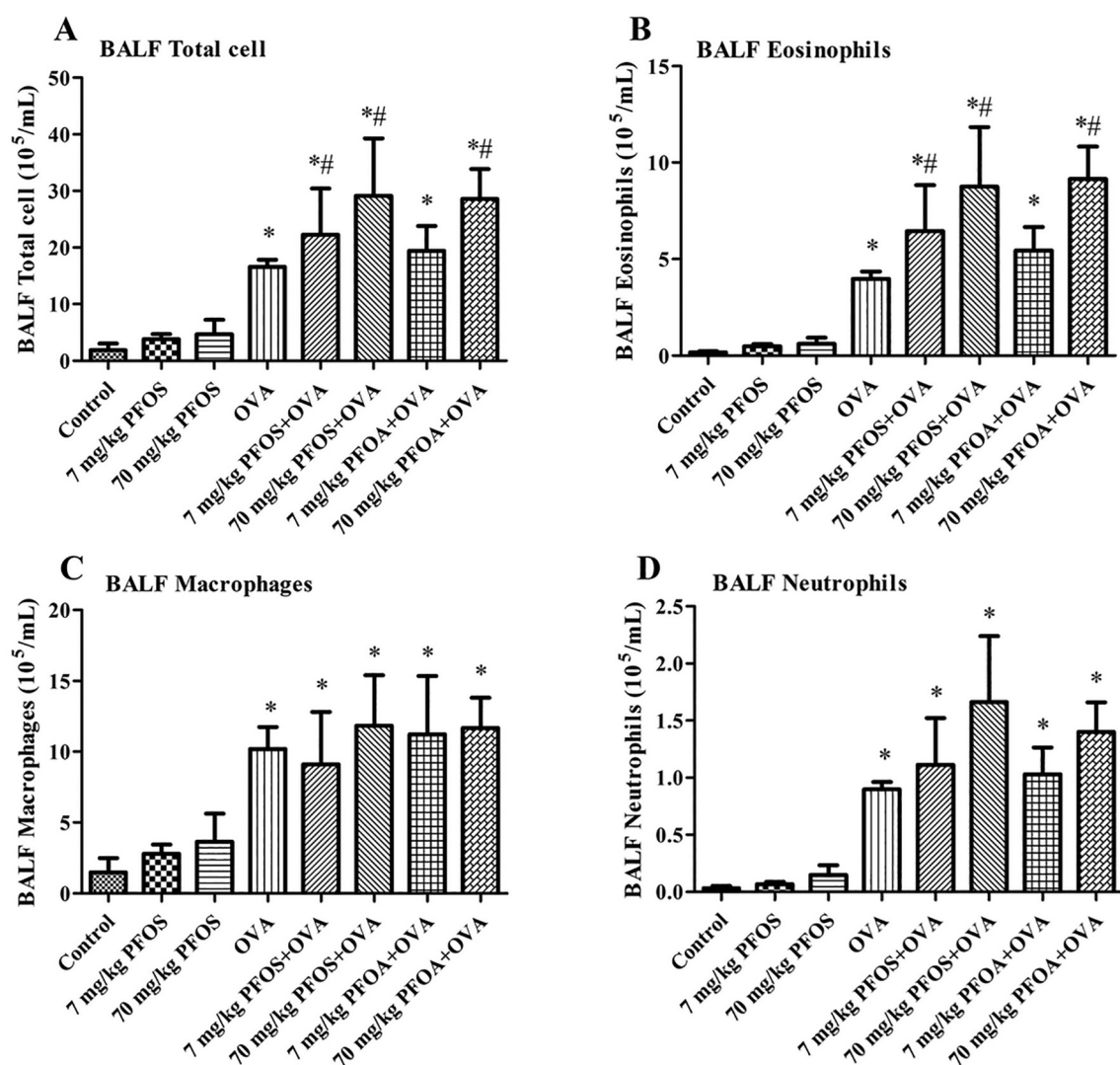


Fig. 2. Cell counts in BALF from different exposure groups. The numbers of total cells (A), eosinophils (B), macrophages (C), and neutrophils (D) were evaluated in control, PFOS or PFOA only group, OVA only group, and PFOS or PFOA plus OVA groups. Data are shown as mean $\pm$ standard deviation ($n = 6$ for each group). * $p < 0.05$, compared with control group; # $p < 0.05$, compared with OVA group.

Significant down-regulation of Th1 cell transcription factor (T-bet) and up-regulation of Th2 cell transcription factor (GATA3) were found in PFOS or PFOA exposed asthmatic mice compared to OVA-induced or control mice (Fig. E3). In contrast, expressions of the Th17 (ROR- γ) and Treg (FOXP3) transcription factors were not different between OVA-induced mice treated or untreated with PFOS or PFOA.

3.6. p-STAT6 expression in lung tissues from mice

We also investigated the alteration of the key proteins in Th2 signaling pathway (IL-4R α -Jak1-Stat6) and Th cell transcription factors in asthmatic mice exposure to PFOS or PFOA. Protein levels of IL-4R α , activation forms of Jak1 (phosphorylated Jak1, p-Jak1) and STAT6 (phosphorylated STAT6, p-STAT6), GATA3, and T-bet were all up-regulated in OVA-induced mice compared to control group (Fig. 6A-F). PFOS and PFOA treatment further enhanced p-STAT6 protein expression in

OVA-induced mice (Fig. 6A, F). Immunostaining for p-STAT6 was also performed in lung tissues to evaluate the distribution of the protein in specific cell types. p-STAT6 was observed in both epithelial cells and leukocytes in the tissue sections. The percentage of p-STAT6+ cells among the leukocytes (usually infiltrated around the bronchi) was significantly elevated in the OVA-induced group or in PFOS only-treated group compared to the control group. Moreover, PFOS treatment could further promote p-STAT6+ leukocytes in OVA-induced mice (Fig. 6G-L). These results indicated that PFOS had positive effects on STAT6 activation (p-STAT6) in leukocytes from asthmatic mice, but the effect may not have been mediated by IL-4R α and Jak1.

3.7. Th2 response in Jurkat cells

PFOS and PFOA exposure significantly enhanced expression of Th2 related genes (IL-4, GATA3, and STAT6) at 1.0 μM , 10 μM , 50 μM

Fig. 1. Detection of mouse serum PFOS and PFOA concentrations by HPLC-MS/MS. Representative chromatogram plots for 1) serum PFOS level were shown in control group (A), OVA group (C), only PFOS exposed group (E & F), PFOS plus OVA group (G & H); and 3) serum PFOA level were shown in control group (B), OVA group (D), PFOA plus OVA group (I & J). Samples in control group and OVA groups were diluted 10 times, while those in PFOS or PFOA exposed groups were diluted 500 times. The X-axis was the peak time and the Y-axis was the response value. Comparisons of serum PFOS and PFOA levels in different groups were shown in K and L, respectively. Data were presented as mean $\pm$ standard deviation ($n = 6$ for each group).

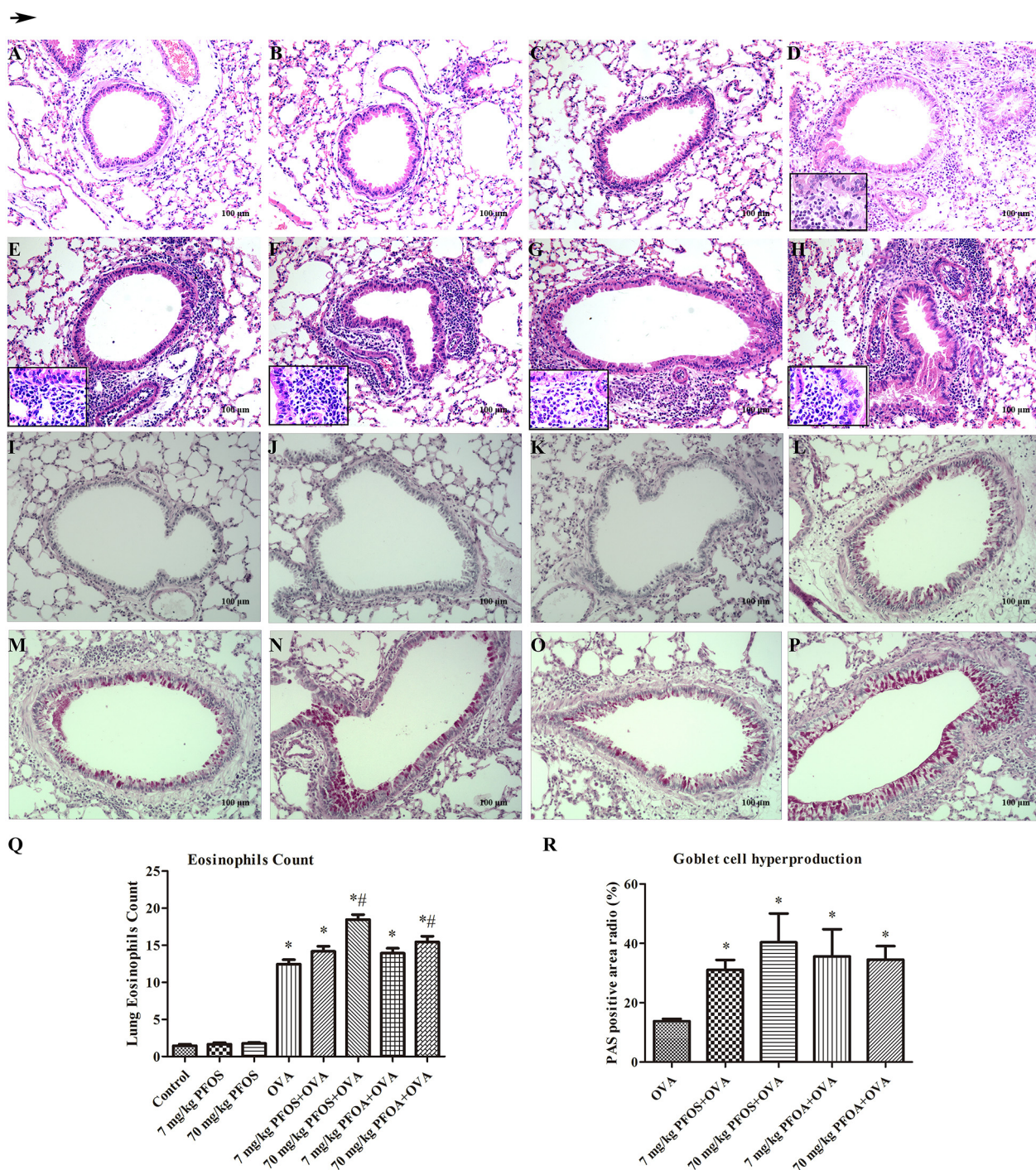


Fig. 3. Histopathological changes in mice treated with OVA and PFOS or PFOA. Representative pictures of H&E stains showing the inflammatory cell infiltration in control (A), PFOS (7 mg/kg or 70 mg/kg) only group (B & C), OVA only group (D), PFOS (7 mg/kg or 70 mg/kg) plus OVA group (E & F), and PFOA (7 mg/kg or 70 mg/kg) plus OVA groups (G & H). Representative pictures of PAS stains showing mucus production in control (I), PFOS (7 mg/kg or 70 mg/kg) only group (J & K), OVA only group (L), PFOS (7 mg/kg or 70 mg/kg) plus OVA group (M & N), and PFOA (7 mg/kg or 70 mg/kg) plus OVA groups (O & P). Original magnification is $\times 400$. The bar charts demonstrate the differences between eosinophil infiltration (Q) and goblet cell mucus production (R) among the above-mentioned groups. Data are shown as mean $\pm$ standard deviation ($n = 5$ for each group). * $p < 0.05$, compared with control; # $p < 0.05$, compared with OVA group.

concentrations, but inhibited Th1 related gene expression (IFN- γ and T-bet) in a dose dependent manner (Fig. E4). To study the effects of PFOS and PFOA on Jurkat cells Th2 response, the cells were first primed by PMA and then exposed to PFOS or PFOA at 1.0 μM , 10 μM , and 50 μM . IFN- γ mRNA and protein expression were decreased, but IL-4 expression was increased, in PFOS and PFOA exposed cells relative to PMA only

primed cells (Fig. 7A-H). p-STAT6 was up-regulated in PMA stimulated cells versus unstimulated cells; and further enhanced in cells exposed to PFOS or PFOA in a dose dependent manner (Fig. 7I-S). Interestingly, up-regulation of GATA3 but down-regulation of T-bet were found in cells exposed to PFOS or PFOA compared to those stimulated by PMA only (Fig. 7I-S). Expressions of p-Jak1 and p-Jak3 were significantly increased

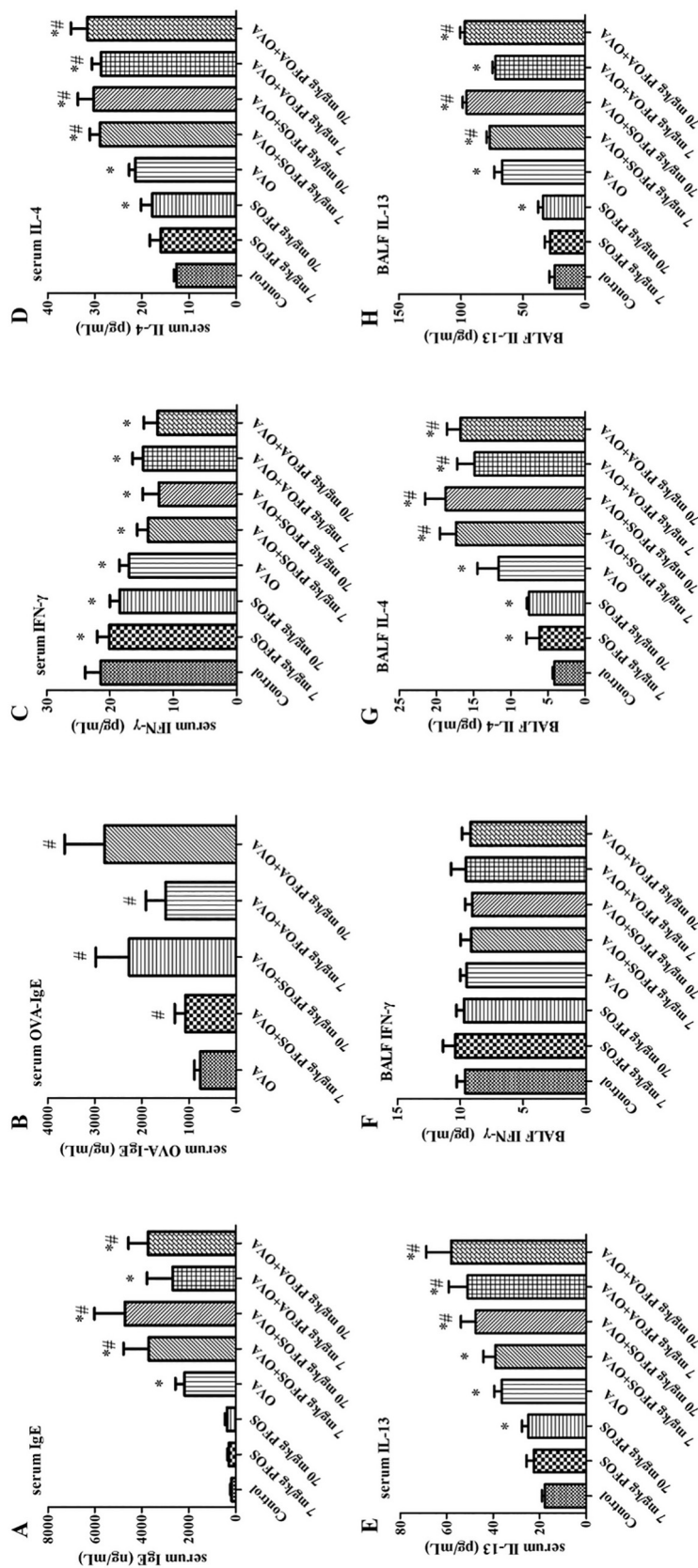


Fig. 4. Effects of PFOA or PFOA on serum IgE and cytokine concentrations in serum or BALF. Levels of serum total IgE (A) and OVA specific IgE (B) were shown in different stimulation groups. Serum and BALF IFN-γ (C & F), IL-4 (D & G), and IL-13 (E & H) were compared in different stimulation groups. Data are shown as mean ± standard deviation (n = 6 for each group). * p < 0.05, compared with control; # p < 0.05, compared with OVA group.

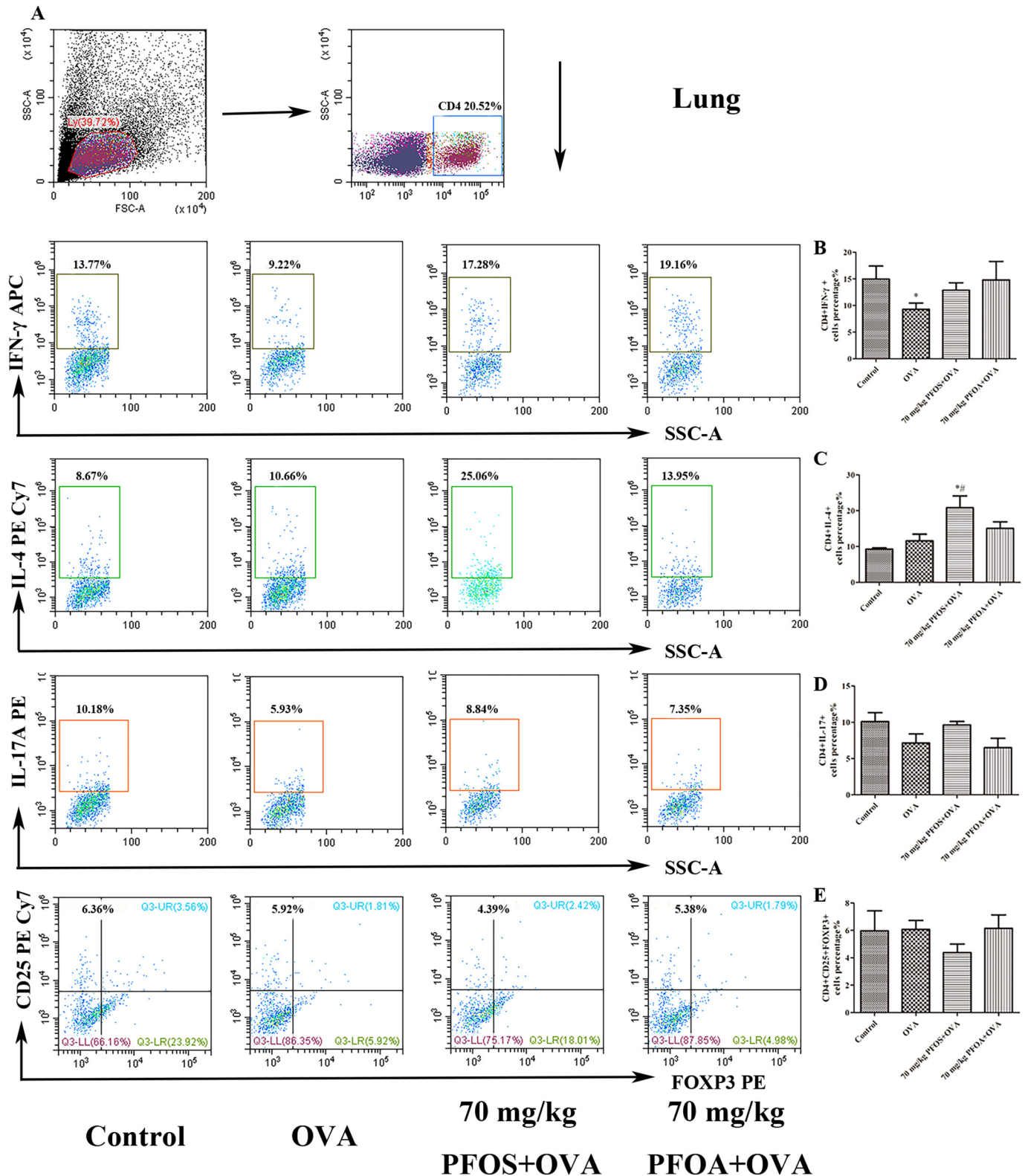
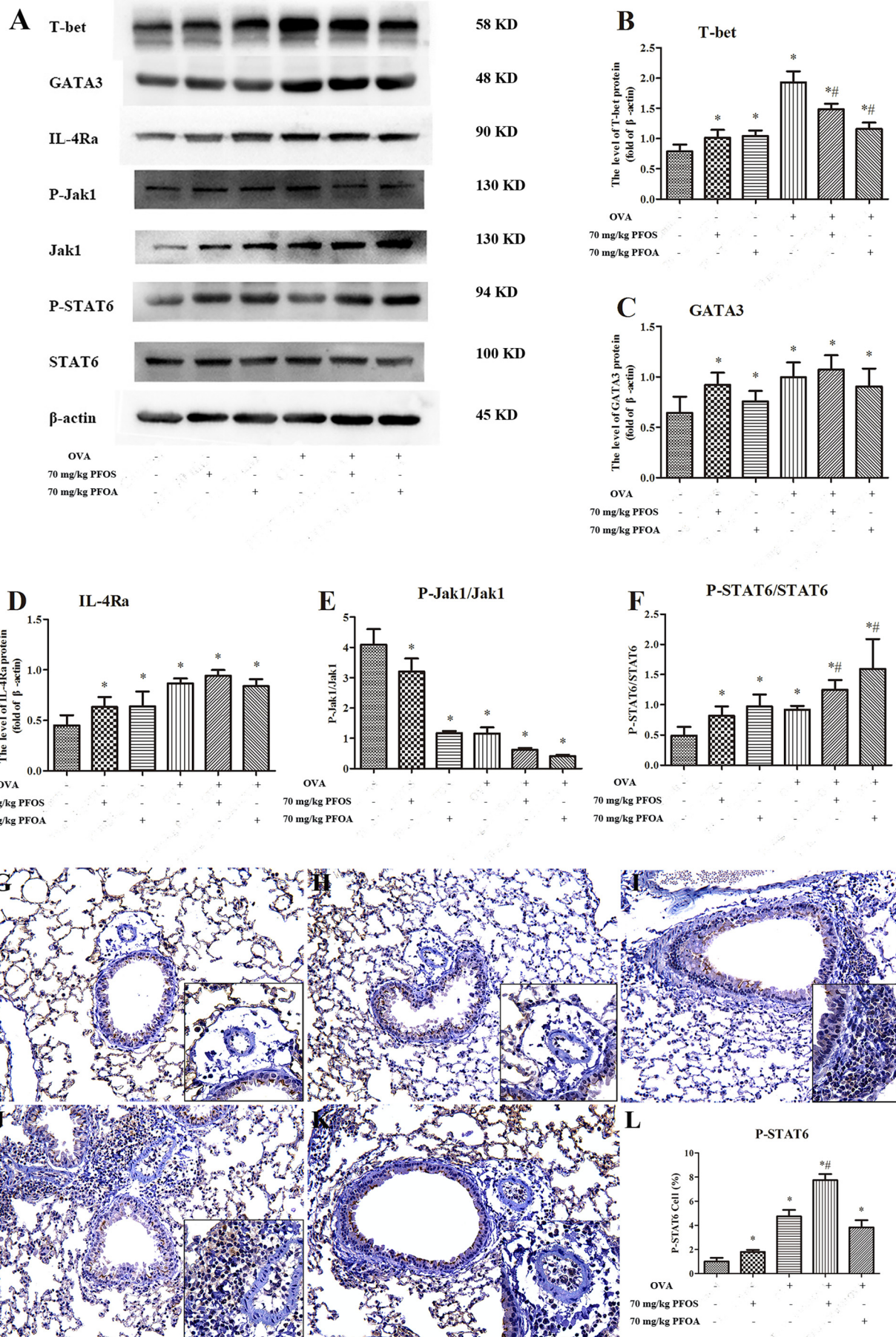


Fig. 5. Effects of PFOS and PFOA on modulating helper T (Th) cell subsets in the lung tissues. Cells isolated from lung tissues were gated by CD4+ cell population (A). Representative pictures show the changes of Th cell subsets in mice under treatment with OVA and OVA plus PFOS or PFOA groups, including Th1 (CD4+ IFN- γ +) (B), Th2 (CD4+ IL-4+) (C), Th17 (CD4+ IL-17+) (D), and Treg (CD4+ CD25+ FOXP3+) (E). Data are shown as means $\pm$ standard deviation ($n = 6$ for each group). * $p < 0.05$, compared with control; # $p < 0.05$, compared with OVA group.

in the PFOS exposed groups (Fig. 7J, K, O, P). T-bet was decreased in the PFOA exposed group (Fig. 7S). These results suggested that PFOS and PFOA enhanced the Th2 response in PMA primed Jurkat cells, indicating an exacerbation of Th2 dominant allergic airway inflammation.

3.8. Th1/Th2 response in Jurkat cells lacking STAT6

PFOS and PFOA exposure significantly enhanced gene expression of IL-4 at 50 μ M concentrations, but inhibited IFN- γ gene expression



(Fig. 8A–D). Th1 and Th2 gene expressions in STAT6 siRNA group were close to the control group. IFN- γ mRNA expression was decreased, but instead IL-4 was increased, in STAT6 siRNA + PFOA (10 and 50 μ M) exposed cells relative to STAT6 siRNA only cells (Fig. 8C, D). Compared to the PFOS 50 μ M group, IFN- γ mRNA expression was increased, but IL-4 was decreased in STAT6 siRNA + PFOS 50 μ M group (Fig. 8A, B). T-bet protein among the STAT6 siRNA + PFOS exposed groups were significantly lower than that of the STAT6 siRNA only (Fig. 8F), although T-bet was decreased in cells treated with STAT6 siRNA + PFOA (0.1 mM, 1.0 mM, 10 mM) compared to those with STAT6 siRNA only (Fig. 8J). GATA3 protein was lower in STAT6 siRNA + PFOS (0.1 mM, 1.0 mM, 10 mM, 50 mM) than in the PFOS 50 mM and STAT6 siRNA groups (Fig. 8G). Very little p-STAT6 protein was expressed by the STAT6 siRNA, STAT6 siRNA + PFOS, and STAT6 siRNA + PFOA groups (Fig. 8H, I). However, STAT6 protein was still expressed in the STAT6 siRNA + PFOS/PFOA groups, although expression was decreased (Fig. 8I, M).

4. Discussion

The current study used both animal and cell models to investigate the impact and mechanism of exposure of PFOS or PFOA in OVA-induced asthmatic mice and Jurkat cells. Our results demonstrated that PFOS or PFOA exposure further enhanced lung inflammation, including eosinophil infiltration, local Th2 cytokine production and disturbed the balance of Th1/Th2 in asthmatic mice. We found that PFOS and PFOA exposure could induce the expression of JAK-STAT pathway related to Th2 inflammation in asthmatic mice and Jurkat cells. Especially after we suppressed the expression of STAT6 in Jurkat cells, we found the effects of PFOS exposure on GATA-3, IL-4 and IFN- γ was blocked. However, the effects of PFOA exposure on GATA-3, IL-4 and IFN- γ were only partially blocked. These results suggested that the mechanism of Th1/Th2 balance disturbance induced by PFOS exposure may be through up-regulation of p-STAT6 level to regulate the expression of GATA-3 and cytokines, and then induced Th1/Th2 imbalance. PFOA can still regulate Th1/Th2 cytokine secretion even with low STAT6 expression, affecting Th1/Th2 balance.

To our knowledge, only two previous articles have studied the effects of PFOS and/or PFOA on asthmatic mice. However, they mainly focused on the effect of PFOS and/or PFOA on lung inflammation in asthmatic mice, and did not deeply study the mechanisms of PFOS causing/ aggravating asthma. Fairley et al. reported that the acute dermal exposure to PFOA augmented airway symptoms in asthmatic mice (Fairley et al. 2007). Our observation supported Fairley's findings, but shed additional light on the mechanisms of PFOS and PFOA mediated lung inflammation in OVA-induced asthmatic mice. In contrast to Fairley et al., Ryu et al. reported that PFOA exposure did not exacerbate the OVA-induced lung inflammation. Instead, PFOA alone could induce airway inflammation and altered lung function (Ryu et al. 2014). Our results seem to contradict these findings and the difference may be attributed to different study questions and different exposure protocol. In a study by Ryu et al., the authors used pretreated model to explore if PFOA was a predisposed factor on asthma exacerbation. The pregnant mice were fed by PFOA contaminated diets, and the pups were exposed until adulthood (12 weeks); and OVA challenge was performed from 8 to 9 weeks. Compared to our observations, protocol of Ryu et al. had a longer exposure duration (105 days versus 28 days) and a higher cumulative dose (105 mg/kg versus 70 mg/kg) of PFOA. However, the internal serum

PFOA level was lower than in the current mouse model (4.8 mg/L versus 60.38 mg/L). In fact, in our study, the highest levels of PFOS and PFOA exposure in the asthmatic mice were designed based on the level of PFOS/PFOA in the serum of the occupational populations and people in high-exposure areas. At present, it is reported that the exposure levels of PFOS and PFOA in occupational populations and for people in high-exposure areas can reach as high as 31,400 ng/mL and 92,303 ng/mL (Olsen and Zobel 2007; Zhou et al. 2014). In our study, the concentrations of PFOS and PFOA in the serum of mice in the highest exposure groups were close to the levels of occupational populations and populations in high-exposure areas. Indeed, "pretreated" mice in the study by Ruy et al. was suitable if PFOA is an etiologic factor for asthma development, and chronic PFOA exposure may alone elicit lung pathology. However, the asthma exacerbation effects of PFOS or PFOA may have been confounded by the pretreatment approach. In addition, low serum PFOA levels in their study, may have been insufficient to elicit the PFOA exacerbation effect in OVA-induced asthmatic mice. Contrary to Ryu et al., our study was designed to investigate the additional effects and mechanism of PFOS or PFOA among mice already experiencing allergic asthma. Nonetheless, the results by us and the other studies all agreed that PFOA exposure should be involved in the lung inflammation.

More importantly, we found that PFOS and PFOA induce airway inflammation of asthma, possibly by JAK-STAT pathway to disturb the balance of Th1/Th2. T-bet as a Th1-specific T-box transcription factor controls the expression of the hallmark Th1 cytokine IFN- γ (Zhu et al. 2010). GATA-3 has been shown to regulate Th2 development via induction of Th2 cytokine production, selective growth of Th2 cells, and inhibition of Th1 cell-specific factors (Muehling et al. 2017). In this context, we found that PFOS and PFOA exposure promoted GATA3 expression, and inhibited T-bet level in asthmatic mice and Jurkat cells. JAK-STAT is an important cytokine signaling pathway that can transmit most cytokine information. JAK-STAT6 signaling pathway as an important member of JAK-STAT, participates in Th2 cell differentiation and regulates the expressions of IL-4, IL-13 and other cytokines, and participates in multiple stages of asthma. The mechanism is that activated STAT6 up-regulates the expression of GATA3, and then activates the expression of Th2-specific genes such as IL-4 and IL-13 to induce the functional differentiation of Th0 cells into Th2, while inhibiting Th1-specific genes such as IFN- γ and T-bet expression eventually leads to the imbalance of Th1/Th2 and aggravates the asthma response (Goenka and Kaplan 2011; Vale-Pereira et al. 2011). STAT-6 is involved in the initiation of Th2 differentiation and can maintain the Th2 phenotype by inducing the expression of chemokines and other Th2-related genes (Maier et al. 2012). Goenka and Kaplan found that after activation of the STAT6 signaling pathway, the level of Th2 type cytokines in BALF of mice was significantly increased, and the degree of Th2 cell activity was positively correlated with the severity of asthma (Goenka and Kaplan 2011). In addition, they found that Th2 cytokine levels in STAT6-deficient mice were suppressed, and airway inflammation was significantly reduced in the experiments with STAT6 knockout mice (Goenka and Kaplan 2011). We found similar results in the Jurkat cells lacking STAT6. After we inhibited STAT6 in Jurkat cells, the gene expressions of GATA-3, IL-4 and IFN- γ caused by PFOS exposure decreased, which partially affected the gene expressions of GATA-3, IL-4 and IFN- γ caused by PFOA.

One intriguing finding of our study was that airway reactivity and immune response induced by PFOS or PFOA -OVA co-exposure may be mediated by an adjuvant effect of PFOS or PFOA. In the absence of

Fig. 6. Effects of PFOS or PFOA on regulating Th1 and Th2 cell transcription factors and IL-4R α -JAK1-STAT6 pathway protein expression in the lung tissues. Representative western blot pictures of T-bet, GATA3, IL-4R α , p-JAK1, JAK1, p-STAT6, and STAT6 (A). The relative proteins expression levels of T-bet (B), GATA3 (C), IL-4R α (D), p-JAK1/JAK1 (E), and p-STAT6/STAT6 (F) were quantified based on the density of the bands. Representative images of immunohistochemical stains for p-STAT6 in the lung tissues from different groups (G–K) and the p-STAT6+ cells infiltrating around bronchi in lung sections. Data are shown as means $\pm$ standard deviation (n = 5). * $p < 0.05$, compared with control; # $p < 0.05$, compared with OVA group.

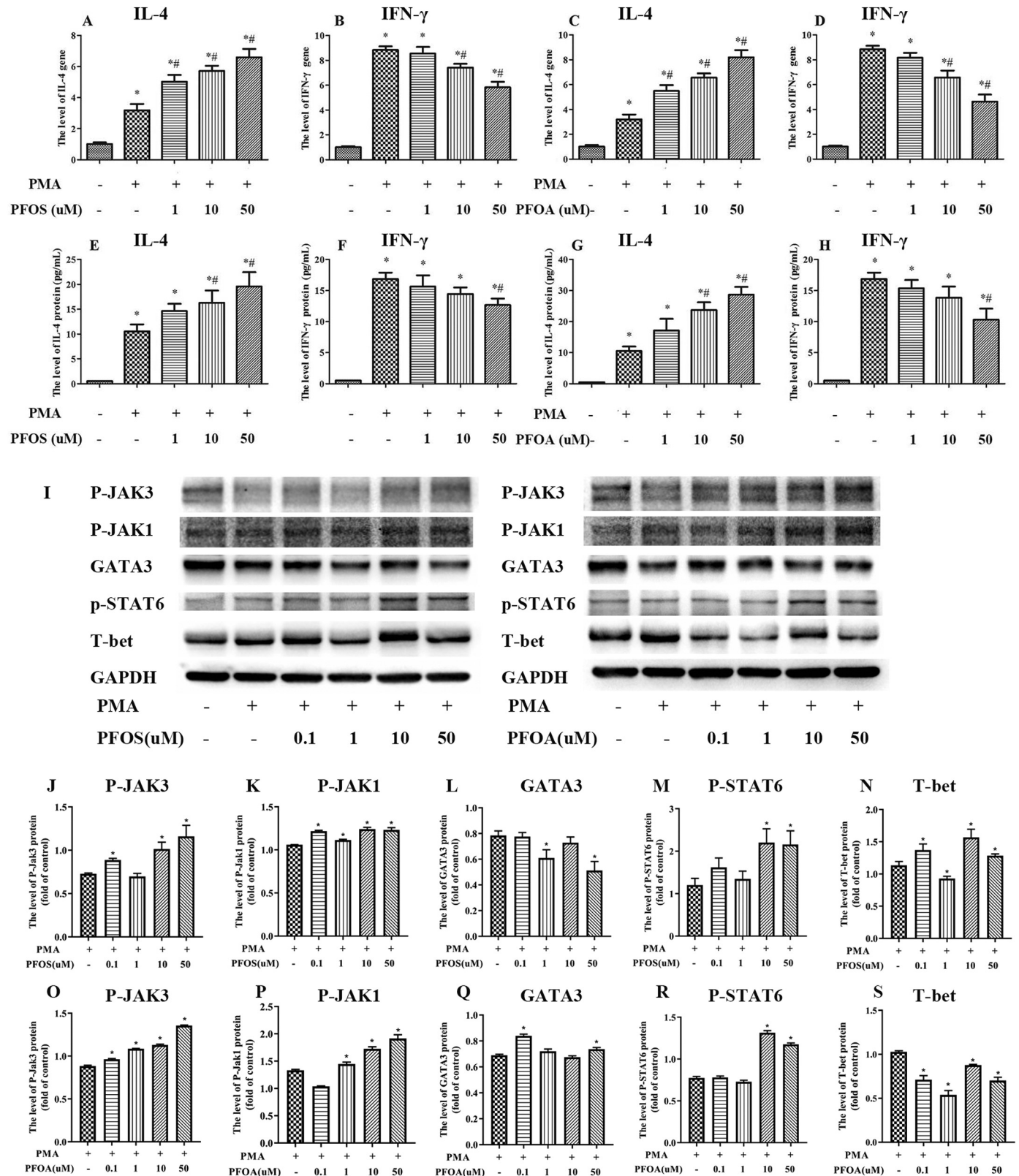


Fig. 7. Effects of PFOS and PFOA on expressions of Th1 and Th2 response genes and transcription factors in PMA primed Jurkat cells. mRNA and protein levels of IL-4 (A, C, E, G) and IFN-γ (B, D, F, H) in PMA and PMA plus different dosages (0.1 μM, 1 μM, 10 μM, 50 μM) of PFOS or PFOA exposure groups. Expressions of mRNAs and proteins were examined by quantitative PCR and ELISA assays. Other Th1 and Th2 genes and transcription factors' protein (p-Jak3, p-Jak1, GATA3, p-STAT6, and T-bet) levels were determined by western blot (I). Relative proteins expression levels of p-Jak3 (J & O), p-Jak1 (K & P), GATA3 (L & Q), p-STAT6 (M & R), and T-bet (N & S) were quantified based on the density of the bands. Data were expressed as means ± standard deviation ($n = 3$). * $p < 0.05$, compared with PMA group.

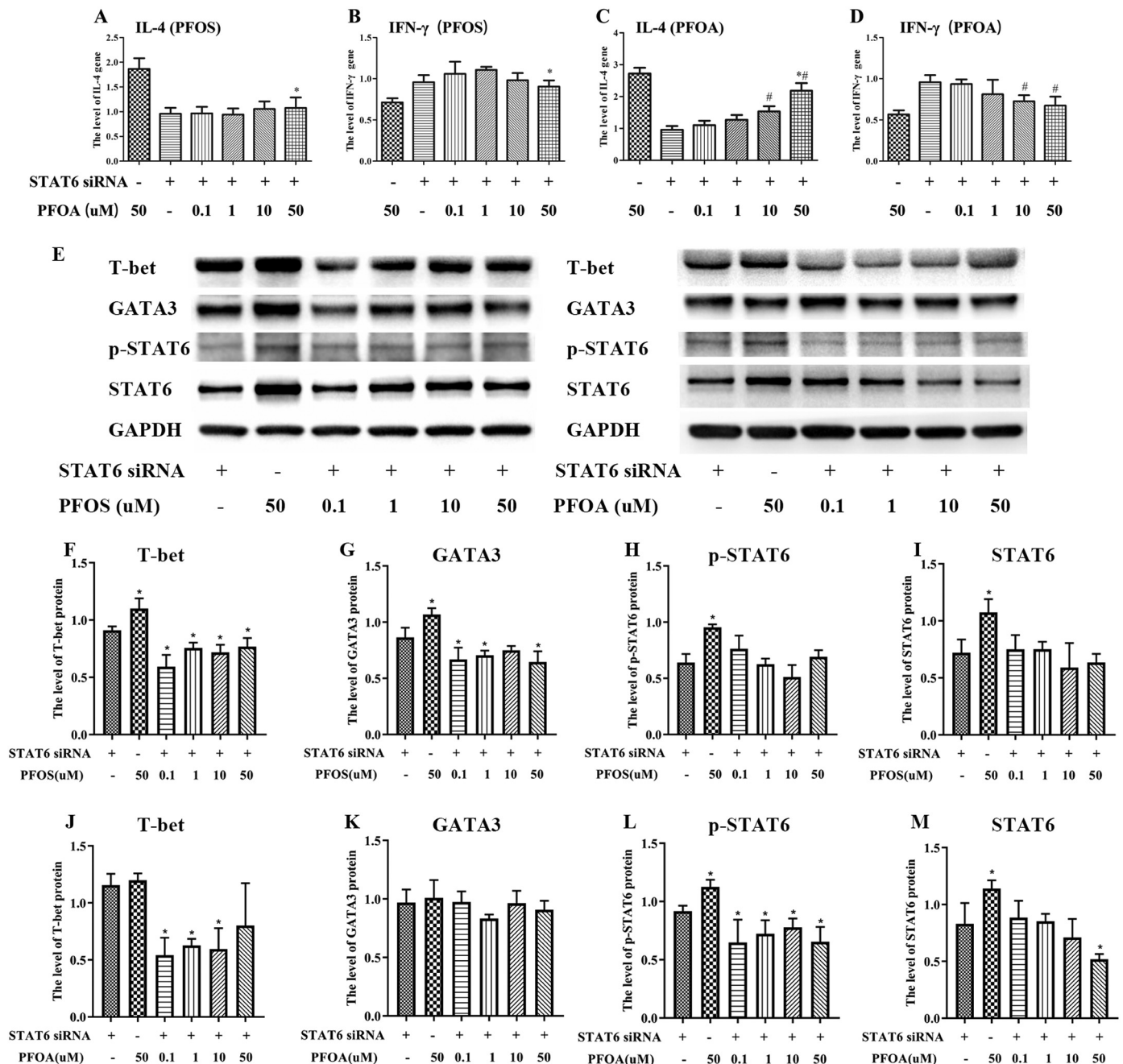


Fig. 8. Effects of PFOS and PFOA on expressions of Th1 and Th2 response genes and transcription factors in Jurkat cells lacking STAT6. mRNA levels of IL-4 (A, C) and IFN- γ (B, D) in 50 μ M, STAT6 siRNA and STAT6 siRNA plus different dosages (0.1 μ M, 1 μ M, 10 μ M, 50 μ M) of PFOS or PFOA exposure groups. Expressions of mRNAs were examined by quantitative PCR. Other Th1 and Th2 transcription protein (T-bet, GATA3, STAT6 and p-STAT6) levels were determined by western blot (E). Relative proteins expression levels of T-bet (F & J), GATA3 (G & K), p-STAT6 (H & L), and STAT6 (I & M) were quantified based on the density of the bands. Data were expressed as means $\pm$ standard deviation ($n = 3$). * $p < 0.05$, compared with STAT6 siRNA group.

OVA, neither PFOS nor PFOA exposure caused significant changes in the mice, but when coupled with OVA, it resulted to elevated inflammatory cell production (especially eosinophils), histological changes including leukocyte infiltration, airway remodeling, goblet cells hyperplasia, and immune response disturbance such as increased T-IgE and OVA-IgE levels. The adjuvant effect of PFOS and PFOA on airway inflammation was dose-dependent.

RoRyt is the key transcription factor of Th17 cells, a distinct lineage of CD4⁺ effector T cells that express IL-17 (Zhu et al. 2010). Many studies have proved that Th17 cells play crucial roles in the pathogenesis of asthma (Ji et al. 2013; Na et al. 2018). In addition, Foxp3 is a T cell development regulator (Treg) and is the most specific Treg marker (Fang and

Zhu 2017). Previous studies have found that induction or restoration of regulatory T-cell function is associated with the tolerization to allergens and autoantigens (Noval Rivas and Chatila 2016). Both Th17 and Treg cells play especially critical roles in the development of asthma, thus, we studied the mRNA expression of RoRyt and Foxp3, and the protein level of IL-17 and Foxp3 in lung tissue of asthmatic mice. But there was just Foxp3 mRNA expression significantly down-regulated in the 70 mg/kg PFOA+OVA group compared with OVA-immunized group. One possible explanation is that Th17 cells play crucial roles in the asthma with the hallmarks of neutrophilic airway inflammation (Papi et al. 2018; Yang and Jiang 2016). However, in our study, the murine asthma model is characterized by eosinophilic airway inflammation,

which may be the reason that we could not observe significantly changes of RoRyt expression.

IL-4 and IL-13 are canonical cytokines of Th2. IL-4 through its role in regulating Th2 cell proliferation and survival, induces IgE production by B cells and has been shown to be essential in the initiation of allergic airway responses (Gould and Sutton 2008; Gour and Wills-Karp 2015). IL-13 recruits mast cells into the airway, induces goblet cell proliferation and airway remodeling, leading to mucus production and airway hyper-responsiveness through the pathogenesis of asthma (Kubo 2017). Conversely, IFN- γ , a predominant cytokine generated by Th1 cells, is vital to the suppression of Th2-mediated responses. IFN- γ has also been shown to be a crucial regulatory cytokine that impedes Th2 cell proliferation and antagonizes Th2-type responses such as IL-4-dependent IgE antibody production (Koch and Finotto 2015). Therefore, the IL-4/IFN- γ and IL-13/IFN- γ ratio can be indicative of the Th1/Th2 balance. In the present study, PFOS or PFOA exposure did not affect IFN- γ levels in BALF, OVA+PFOS or PFOA exposure but did significantly affect IL-4 and IL-13 levels when compared with the OVA-only exposure group. Our results suggested that PFASs precipitated a Th1/Th2 differentiation ratio imbalance, predominantly stimulating Th2 differentiation, which could prove to be the major mechanism responsible for the increased asthma risk following environmental chemical exposure.

Despite the similar structures and functions of PFOS and PFOA, we found evidence of different effects in this study. Firstly, PFOA had more modest effects on Th2 response than PFOS. The difference may be related to differential interactions with peroxisome proliferator activated receptors (PPAR), as PFOS has a higher affinity for binding to PPAR α , but PFOA has a higher affinity for binding to PPAR γ (Li et al. 2017). Secondly, although PFOS and PFOA are both hepatotoxic in mice, exposure to PFOS or PFOA alone did not demonstrate obvious pathological changes in the lung. Thus, PFOS and PFOA exposure are more likely exacerbate existing asthma rather than to cause asthma. Thirdly, PFOS and PFOA had no significant impact on lung function (or symptoms) in OVA-induced mice. However, pathological changes in lung tissue, such as inflammatory cell infiltrations, cytokine production, and tissue remodeling, antecede respiratory symptoms; hence a more extended observation time after OVA challenge may be necessary to more definitively evaluate associations with asthma symptoms.

5. Conclusion

We observed that PFOS or PFOA exposures aggravate inflammation in asthmatic mice: (1) strong positive dose-response associations existed between combined OVA and PFOS or PFOA exposure and airway reactivity and immune response, potentially mediated by an adjuvant effect of PFOS or PFOA; (2) PFOS or PFOA exposure could disturb the balance of Th1/Th2 differentiation, which may be a possible biological mechanism underlying its association with asthma. PFOS and PFOA may cause Th1/Th2 imbalance through the JAK-STAT pathway, which exacerbated asthma. STAT6 played a key role in the expression of Th2-related cytokines caused by PFOS exposure, and PFOA may also affect Th2-related cytokines through other pathways. Our results suggested that although PFOS and PFOA may not be allergenic, they may enhance inflammation among asthmatic patients. Therefore, environmental persistence of PFASs in combination with these findings imply that exposure to PFASs may enhance the adverse responses to other commonly encountered environmental pollutants.

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CRediT authorship contribution statement

Conceptualization: Mo Yang, Xiao-Di Qin, Guang-Hui Dong, Chun-Wei Li; Methodology: Mo Yang, Li-Yue Li, Xiao-Di Qin, Xiao-Yan Ye, Shu Yu, Qing Bao, Lin Sun, Xiao-Wen Zeng, Guang-Hui Dong, Chun-Wei Li; Formal analysis and investigation: Mo Yang, Li-Yue Li, Xiao-Di

Qin, Shu Yu, Chun-Wei Li; Writing-original draft preparation: Mo Yang, Li-Yue Li, Xiao-Di Qin, Zhi-Bin Wang, Michael S. Bloom, Hong-Yao Yu, Guang-Hui Dong, Chun-Wei Li; Writing-review and editing: Mo Yang, Chun-Wei Li; Funding acquisition: Li-Wen Hu, Xiao-Wen Zeng, Bo-Yi Yang, Guang-Hui Dong, Chun-Wei Li; Resources: Mo Yang, Xiao-Di Qin, Guang-Hui Dong, Chun-Wei Li; Supervision: Mo Yang, Guang-Hui Dong, Chun-Wei Li.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Perfluorooctanesulfonate and perfluorooctanoate exacerbate airway inflammation in asthmatic mice and *in vitro*

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Table E1 Organ mass in the mice treated orally with PFOS or PFOA

Table E2 Routine blood test in the mice blood treated orally with PFOS or PFOA

Table E3 Antibody information

Table E4 Primers for quantitative RT-PCR

eMethods 1: Chemicals and reagents

Ovalbumin (Grade V, Grade II), PFOS (purity $\geq 98\%$), PFOA (purity $\geq 96\%$), and $\text{Al}(\text{OH})_3$ were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tetrabutylammonium hydrogensulfate (TBAHS, HPLC grade) and sodium carbonate (Na_2CO_3 , 99.5%) were obtained from Acros Organics (Geel, Belgium). Ammonium acetate (HPLC grade) was obtained from Dikma Technology (Richmond, VA, USA). Methyltert-butyl ether (MTBE, HPLC grade), methanol (HPLC grade), and acetonitrile (HPLC grade) were obtained from Tedia (Fairfield, OH, USA). Information on the primary antibodies used in immunostaining and flow cytometry is listed in **Table E3**; and the primers used in quantitative RT-PCR are described in **Table E4**.

eMethods 2: Lung function test

Mouse lung function tests were performed using an animal pulmonary function analysis system (Beijing Beilanbo Technology Co., Ltd., Beijing, China), evaluating FVC (forced vital capacity), FEV1 (forced expiratory volume in 1 second), FEV1/FVC, FEF25% (forced expiratory flow 25%), FEF50% (forced expiratory flow 50%), FEF75% (forced expiratory flow 75%), FEF25-75% (forced expiratory flow 25%-75%), MMEF (maximum midexpiratory flow), and PEF (peak expiratory flow). The surgical procedures included: 1) mice were deeply anesthetized with 5% nembutal; 2) the head and limbs fixed in place; and 2) the trachea was dissected and connected to the ventilator.

eMethods 3: Preparations of blood sample, bronchoalveolar lavage fluids (BALF), lung tissue extract, and cell suspension from lung

Mice were bled by retro-orbital puncture under light diethyl ether anesthesia and sacrificed by cervical dislocation. Blood samples were collected and centrifuged to isolate the serum. We collected venous blood collection. Half of the mouse blood was collected in EDTA anticoagulation tubes (collecting blood cells), and half was collected in ordinary blood collection tubes (collecting serum). The spleen, thymus, liver, and lungs were dissected and weighed. BALF was collected by gentle aspiration of PBS injected into the airways of mice via a tracheal cannula. After we took the mouse BALF, we put the mouse lungs on the sterile gauze to fully absorb the water before weighing. The lungs were snap-frozen in liquid nitrogen and the tissue extracts were dissolved with RIPA sample buffer (plus PPI cocktail) (Beyotime, Nantong, China). Single cell suspensions were obtained by digesting fresh lung or spleen tissues with collagenase (2 mg/ml) (Sigma) and DNase I (10 µg/ml) (Roche, Mannheim, Germany) for 1h at 37°C.

eMethods 4: Serum PFOS and PFOA preparation and analysis

Firstly, 0.5 mL of serum sample, 1 mL of 0.5M tetrabutylammonium hydrogensulfate (TBAHS), and 2 mL of 0.25M Na₂CO₃ (pH=10) were mixed in a 15 mL polypropylene tube and then the mixture was added to 5 mL of methyltert-butyl ether (MTBE). The sample mixture was centrifuged to separate the organic and aqueous layers and the organic layer was removed. Secondly, the extracts were dried under high purity N₂ gas flow, and then reconstituted in a 0.5 mL mixture of methanol and ammonium acetate. Finally, sample extracts were mixed with ammonium acetate and acetonitrile, and then injected into a C18 column for HPLC-MS/MS analysis using an Agilent 1200 HPLC system (Agilent, Palo Alto, CA, USA).

eMethods 5: Histopathology analysis

Lung tissues sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS)-Masson to examine eosinophil infiltration and mucus production, respectively. Eosinophil count was calculated in the fields around bronchi per lung section, and mucus production level was quantified by evaluating the ratio of PAS-positive areas in the epithelium region. These parameters were evaluated in three randomly selected fields at 1000x magnification and analyzed using Image J software (v1.47, Media Cybernetics, Rockville, MD, USA).

eMethods 6: Western blot analysis

Tissue extract (30 µg protein) from lung tissues were separated by SDS-PAGE gel and the proteins transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked in blocking buffer and then incubated overnight at 4°C with primary antibodies targeting T-bet, IL-4R α , p-Jak3, p-Jak1, GATA3, p-Stat6, and GAPDH. The membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Cell signaling Technology, Danvers, MA, USA) and the reaction detected using enhanced chemiluminescence (Millipore). The blots were exposed to a Tanon 4200 chemiluminescence imaging analysis system (Yuan Ping Hao Biological Co., Ltd., China) and protein band intensities were analyzed by Image J software (v1.47, Media Cybernetics, Rockville, MD, USA).

eMethods 7: Immunostaining and evaluation

Protein expression of pSTAT6 in lung tissues was evaluated by immunohistochemical staining using a modified horseradish peroxidase (HRP) technique with the DakoCytomation EnVision+System-HRP (Dako, Glostrup, Denmark). Antigen retrieval was performed with Target Retrieval Buffer (Dako). Tissue sections were then stained with rabbit anti-mouse and human pSTAT6 antibody (Abcam, City, UK) and incubated overnight at 4°C. The slides were then incubated with DAKO EnVision+System-HRP (Dako) at room temperature for 30 minutes. Diaminobenzidine was used as a substrate for color development. All slides were counterstained with hematoxylin. Proportions of pSTAT6+ cells were determined in three randomly selected fields per lung section for each mouse at 1000× magnification, using Image J software (v1.47, Media Cybernetics, Rockville, MD, USA). The researcher independently assessed all cases in a blind fashion.

eMethods 8: RNA isolation and quantitative PCR (Q-PCR)

Total RNA was isolated from the lungs or Jurkat cells by using the TRIzol Reagent (Thermo Fisher Scientific, City, Country). The concentration and purity were determined by a spectrophotometer (BioPhotometer, Eppendorf, City, Country). The PrimeScript™ RT reagent kit was used for the reverse transcription of RNA (500 ng) to 10 µL cDNA. Quantitative PCR was carried out with the 7500 Real-Time PCR System (Thermo Fisher Scientific) using primer pairs for β -Actin, T-bet, GATA-3, forkhead box P3 (Foxp3), and retinoic acid receptor-related orphan receptor- γ t (ROR γ t) (**Table E4**). Each 20 µL PCR reaction was comprised of 1 µL cDNA, 10 µL SYBR Premix Ex Taq II, 8 µL RNase-Free H₂O, and 0.5 µL per primer, as determined by the manufacturer's protocol. Six samples were included in each treatment, and each PCR reaction was performed in duplicate. A two-step method was adopted for all genes; their reaction conditions were as follows: 95°C for 30 s, 95°C for 5 s, and corresponding annealing temperatures were 60°C for 20 s. Upon the conclusion of the PCR cycle, a dissociation curve was executed in order to verify the specificity of the product. We used β -Actin as the internal standard with relative gene expression calculated using the $\Delta\Delta C_t$ method.

Table E1 Organ mass in the mice treated orally with PFOS or PFOA

Group	Liver ^a	Spleen ^a	Lung ^a	Thymus ^a
Control	4.47±0.54	0.27±0.04	1.23±0.12	0.17±0.05
OVA only	4.60±0.52	0.30±0.07	1.42±0.18	0.14±0.03
PFOS (7 mg/kg)	4.44±0.58	0.24±0.03	1.24±0.11	0.14±0.02
PFOS (70 mg/kg)	7.09±0.62 [#]	0.23±0.05	1.31±0.09	0.13±0.02
PFOS (7 mg/kg) + OVA	4.71±0.74	0.31±0.03	1.39±0.14	0.13±0.03
PFOS (70 mg/kg) + OVA	7.19±0.67 [#]	0.23±0.04	1.36±0.13	0.12±0.02
PFOA (7 mg/kg) + OVA	5.17±0.64	0.26±0.03	1.45±0.16	0.13±0.02
PFOA (70 mg/kg) + OVA	10.52±0.54 ^{*#}	0.18±0.06 [#]	1.38±0.19	0.12±0.02

Data are reported as mean ± SD, *n* indicating the number of animals represented in each group. Body mass and organ mass data did not require transformation for statistical analysis.

^{*}Significantly different from control (*P* < 0.05)

[#]Significantly different from OVA (*P* < 0.05)

^a Calculated as: [organ weight (g)/body weight (g)] × 100

Table E2 Routine blood test in the mice blood treated orally with PFOS or PFOA

Items	Control	PFOS (70 mg/kg)	PFOA (70 mg/kg)	OVA	PFOS (70 mg/kg)+OVA	PFOA (70 mg/kg)+OVA
WBC (white blood cell)	1.80±0.38	3.13±1.57	2.90±1.85	3.15±1.37	2.64±1.32	1.87±0.99 [#]
REC (red blood cell)	7.40±2.49	6.75±3.28	9.97±0.93	8.39±2.61	9.37±2.00	8.26±2.22
Hb (hemoglobin)	120.33±49.70	106.00±57.98	159±14.11	140.63±41.11	154.33±36.75	131.00±38.62
HCT (hematocrit)	40.43±8.69	38.50±11.03	49.67±4.57	42.13±12.81	46.52±10.26	40.38±11.31
MCV (mean corpuscular volume)	56.70±9.28	60.10±12.87	49.80±0.92	50.63±2.29	49.60±0.88	48.68±1.06 [#]
MCH (mean cellular hemoglobin content)	15.90±1.75	15.45±1.06	15.97±0.93	17.19±2.34	16.43±0.88	15.71±1.01
MCHC (mean corpuscular hemoglobin concentration)	287.67±68.41	264.50±74.25	320.67±22.90	338.63±29.31	330.89±16.00	322.80±18.80
RDW (red blood cell distribution width)	25.60±13.95	29.95±17.75	17.20±0.92	17.44±2.17	16.94±0.79	16.66±0.61
PLT (platelet counts)	203.33±79.59	575.50±532.45	568.33±528.22	363.88±102.70 [*]	389.67±138.20	351.80±114.20
MPV (mean platelet volume)	4.73±1.17	4.20±2.97	4.80±1.91	5.25±0.61	4.86±0.75	5.17±0.37
NEUT (neutrophils)	0.32±0.15	0.47±0.36	0.50±0.33	0.76±0.48	0.69±0.38	0.54±0.36
NEUT% (neutrophils%)	17.55±7.96	13.73±4.50	16.88±0.95	23.31±7.85	25.28±2.92	26.46±7.86
LY (lymphocytes)	1.38±0.30	2.53±1.24	2.27±1.42	2.05±0.72	1.69±0.82	1.16±0.56 [#]
LY% (lymphocytes%)	76.77±5.58	80.94±1.07	79.45±2.68	67.61±11.36	65.53±6.08	64.84±9.92
EO (Eosinophils)	0.04±0.04	0.06±0.01	0.05±0.04	0.14±0.16	0.13±0.07	0.08±0.06
EO% (Eosinophils%)	2.22±1.43	2.01±1.14	1.45±0.70	3.67±3.22	4.34±1.89	4.17±1.91
MO (Monocytes)	0.05±0.02	0.07±0.04	0.06±0.05	0.13±0.10	0.10±0.06	0.06±0.04 [#]
MO% (Monocytes%)	2.75±1.54	2.55±2.36	1.65±0.82	3.90±1.97	3.43±1.71	2.89±1.75
Ba (Basophils)	0.01±0.01	0.03±0.02	0.02±0.02	0.06±0.06	0.04±0.03	0.03±0.03
Ba% (Basophils%)	0.71±0.38	0.79±0.06	0.58±0.31	1.52±1.17	1.42±0.83	1.63±0.90

Data are reported as mean ± SD, *n* indicating the number of animals represented in each group. Body mass and organ mass data did not require transformation for statistical analysis.

^{*}Significantly different from control (*P* < 0.05) [#]Significantly different from OVA (*P* < 0.05)

Table E3 Antibody information

Target antigen	Clone	Species	Conjugation	Brand
CD4	GK1.5	Rat	APC-eFluor® 780	eBioscience
IFN- γ	XMG1.2	Rat	APC	eBioscience
IL-4	11B11	Rat	PE-Cyanine7	eBioscience
IL-17	eBio17B7	Rat	PE	eBioscience
CD25	PC61.5	Rat	PE-Cyanine7	eBioscience
p-STAT6	Polyclonal	Rabbit	Not applicable	Abcam
STAT6	YE361	Rabbit	Not applicable	Abcam
T-bet	4B10	Mouse	Not applicable	Abcam
GATA3	D13C9	Rabbit	Not applicable	Cell Signaling technology
IL-4Ra	Polyclonal	Rabbit	Not applicable	absin
P-Jak1	Polyclonal	Rabbit	Not applicable	absin
Jak1	6G4	Rabbit	Not applicable	Cell Signaling technology
β -actin	13E5	Rabbit	Not applicable	Cell Signaling technology
GAPDH	D16H11	Rabbit	Not applicable	Cell Signaling technology

Table E4 Primers for quantitative RT-PCR

Target genes	Forward primer	Reverse primer
CCL22	TGTCCTCGTCCTCCTTGCTGTG	GGCAGGAGTCTGAGGTCCAGTAG
GM-CSF	CTGGTGACAAGCCTTCTG	GGTGCCACTGTTCCGAGATCC
IL-18	TTGACCAAGGAAATCGGCCTC	GCCATACCTCTAGGCTGGCT
IL-6	GGTGTTGCCTGCTGCCTTCC	GTTCTGAAGAGGTGAGTGGCTGTC
IL-1 β	GCGGCATCCAGCTACGAATCTC	AACCAGCATCTTCCTCAGCTTGTC
IL-33	CTGGTACTCGCTGCCTGTCAAC	ACCATCAACACCGTCACCTGATTC
TNF- α	CAATGCCTTACTCTGTGATTGG	CTAATGACTTTGGGGTTACTGC
CCL20	GCTCCTGGCTGCTTTGATGT	GCCGTGTGAAGCCCACAATA
IL-8	GGACCACACTGCGCCAACAC	AACCCTCTGCACCCAGTTTTC
IL-37	ATGAAGCTGGCTGCCCAAAGG	GGGTGAGCCGCCGACTCC
TSLP	CGGCCACATTGCCTTACTGA	TAGCCTGGGCACCAGATAGC
JAK2	GTGGCAGCAACAGAGCCTATCG	GCCGCCACTGAGCAAAGAGG
TYK2	CAGGGACCGTGGGCAGGAG	AGGCTGGCTGTCTCGTAGAAGG
STAT4	GCTCTGCCATTCGCTGACATCC	CGCAAGGCTGAGAGCTGTAGTG
T-bet	TGAACGACGGAGAGCCAGAGG	CTGGTAGGCAGTCACGGCAATG
IFN- γ	AGTGATGGCTGAACTGTGCG	ACTGGGATGCTCTTCGACCT
JAK1	GATGTCCCTCCGGCTCCACTAC	ACGCCACACTGACTGCTCATTG
JAK3	TGCCAGCCGCCTCCTTCTC	TGACGTGTGCCTCGCTCTCC
STAT6	GCCGACATGGTGACAGAGAAGC	CTTGAACAGGGCAGAGCAGCAG
GATA3	CACCACCTACCCGCCCTACG	GCCTGGACTTGCATCCGAAGC
IL-4	ACAGCAGTTCCACAGGCACAAG	CGTACTCTGGTTGGCTTCCTTCAC
ROR- γ	AAGAACGCCATCCGCCACAAC	TCCAGCTCATCCACGGTCCAC
FOXP3	AAGAACGCCATCCGCCACAAC	TCCAGCTCATCCACGGTCCAC