# IL-17F, rather than IL-17A, underlies airway inflammation in a steroid-insensitive toluene diisocyanate-induced asthma model

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**In a TDI-induced steroid-insensitive murine asthma model, IL-17A restricts allergic responses through suppressing Th2 inflammation and eosinophil recruitment, while IL-17F modulates airway inflammation by driving Th17 response and neutrophil infiltrates** http://ow.ly/vP2z30nk7Z3

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ABSTRACT Steroid insensitivity constitutes a major problem for asthma management. Toluene diisocyanate (TDI) is one of the leading allergens of asthma that induces both T-helper Th2 and Th17 responses, and is often associated with poor responsiveness to steroid treatment in the clinic.

We sought to evaluate the effects of inhaled and systemic steroids on a TDI-induced asthma model and to find how interleukin (IL)-17A and IL-17F function in this model. BALB/c mice were exposed to TDI for generating an asthma model and were treated with inhaled fluticasone propionate, systemic prednisone, anti-IL-17A, anti-IL-17F, recombinant IL-17A or IL-17F.

Both fluticasone propionate and prednisone showed no effects on TDI-induced airway hyperresponsiveness (AHR), bronchial neutrophilia and eosinophilia, and epithelial goblet cell metaplasia. TDI-induced Th2 and Th17 signatures were not suppressed by fluticasone propionate or prednisone. Treatment with anti-IL-17A after TDI exposure led to increased AHR, aggravated mucus production and airway eosinophil recruitment, accompanied by amplified Th2 responses, whereas anti-IL-17F ameliorated TDI-induced AHR and airway neutrophilia, with decreased Th17 responses. Recombinant IL-17A and IL-17F showed opposite effects to the monoclonal antibodies.

IL-17A and IL-17F exert distinct biological effects during airway inflammation of a TDI-induced asthma model, which is unresponsive to both inhaled and systemic steroids.

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## Introduction

About 10–25% of adult-onset asthma is occupational associated, with diisocyanates (including toluene diisocyanate (TDI) and methylene diphenyl diisocyanate) the most commonly reported causes [1], which often responds poorly to steroid treatment, leading to poor prognosis even after cessation of exposure [2]. A deeper understanding of the disease is urgently needed to find more therapeutic targets. TDI-induced asthma is often characterised by accumulation of a large number of neutrophils and a smaller number of eosinophils in the airways [3]. Multiple mechanisms are thought to be involved in the induction of TDI-induced asthma, including immunological, genetic, neurogenic, *etc.*, leading to the definition of two subtypes of TDI-induced asthma: immunological and nonimmunological [4]. Evidence from human and animal models suggests that different subgroups of $CD4^+$ T-cells (mainly T-helper Th1, Th2 and Th17 cells), together with their secreted cytokines, critically contribute to TDI-induced asthma [4], and therefore further studies are needed to uncover their complex functions.

During the past decade, the role of interleukin (IL)-17 in immune and allergic disorders has been attracting increasing attention. IL-17 is a family of cytokines mainly produced by Th17 cells and consists of six members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F [5]. Among them, IL-17A and IL-17F share the closest amino acid sequence identity and function through IL-17 receptors. Recent clinical and animal studies support the idea that IL-17 is critically involved in asthma pathogenesis. Elevated IL-17 concentrations were found in the airways of allergic asthma patients [6], and correlate positively with asthma severity and steroid insensitivity [7]. Inhibition of IL-17 using gene knockout mice or blocking antibody reduces ovalbumin (OVA)-induced airway hyperresponsiveness (AHR), bronchial inflammatory cell infiltration as well as airway vascular remodelling [8–10]. However, Schnyder-Candrian *et al.* [11] discovered that exogenous IL-17 inhibited OVA-induced pulmonary eosinophil recruitment, Th2 inflammation and bronchial hyperreactivity. These opposite findings revealed that IL-17 conveys dual effects, which may be attributed to the conflicting biological functions of individual IL-17 family members, especially for IL-17A and IL-17F [12]. Researchers have already demonstrated an increased level of IL-17 in TDI-induced asthma. Anti-IL-17 neutralising antibody could decrease TDI-induced airway inflammation and AHR [13, 14], suggesting important roles of IL-17 in TDI-induced asthma. However, the separate functions of different subtypes of IL-17 have not been assessed.

We have previously established a TDI-induced asthma model with pronounced airway neutrophilia and eosinophilia. The aims of this study were 1) to evaluate the effects of inhaled and systemic steroids on TDI-induced asthmatic responses, and 2) to examine the roles of IL-17A and IL-17F in TDI-induced asthma.

## Methods

For detailed methods including animal protocols and experimental procedures, see the supplementary material.

### *Ethics statement*

All animal experiments described here complied with the guidelines of the Committee on the Use and Care of Animals of Guangzhou Medical University (Guangzhou, China), and were approved by the Animal Subjects Committee of Guangzhou Medical University.

### *Statistics*

Data are expressed as mean with standard deviation. Results were interpreted using one-way ANOVA and Bonferroni's difference *post hoc* test with SPSS version 22.0 (SPSS, Chicago, IL, USA). Differences were considered statistically significant when $p<0.05$.

## Results

### *TDI-induced AHR and pathological changes were not prevented by inhaled fluticasone propionate or systemic prednisone*

Allergic airway inflammation was induced by exposing BALB/c mice to TDI. As expected, this led to significant neutrophil and eosinophil accumulation with structural and functional abnormalities of the airways. Despite their known effects in mild and moderate asthma, corticosteroids do not seem to be of any help in our TDI-induced asthma model. Intraperitoneal injection of 5 $mg{\cdot}kg^{-1}$ prednisone or intranasal instillation of 300 $\mu g{\cdot}kg^{-1}$ fluticasone propionate once daily beginning from the first inhalation for a period of 1 week did inhibit TDI-induced airway mucus production as assessed by Periodic acid–Schiff staining and *Muc5ac* mRNA expression (figure 1a, d and e), yet TDI-induced airway inflammation, epithelial hyperplasia, AHR and smooth muscle thickening did not change after prednisone or fluticasone propionate treatment (figure 1a–c and f). Similarly, neither inhaled fluticasone propionate nor systemic prednisone affected the increased numbers of neutrophils and eosinophils in bronchoalveolar lavage fluid (BALF) of TDI-sensitised

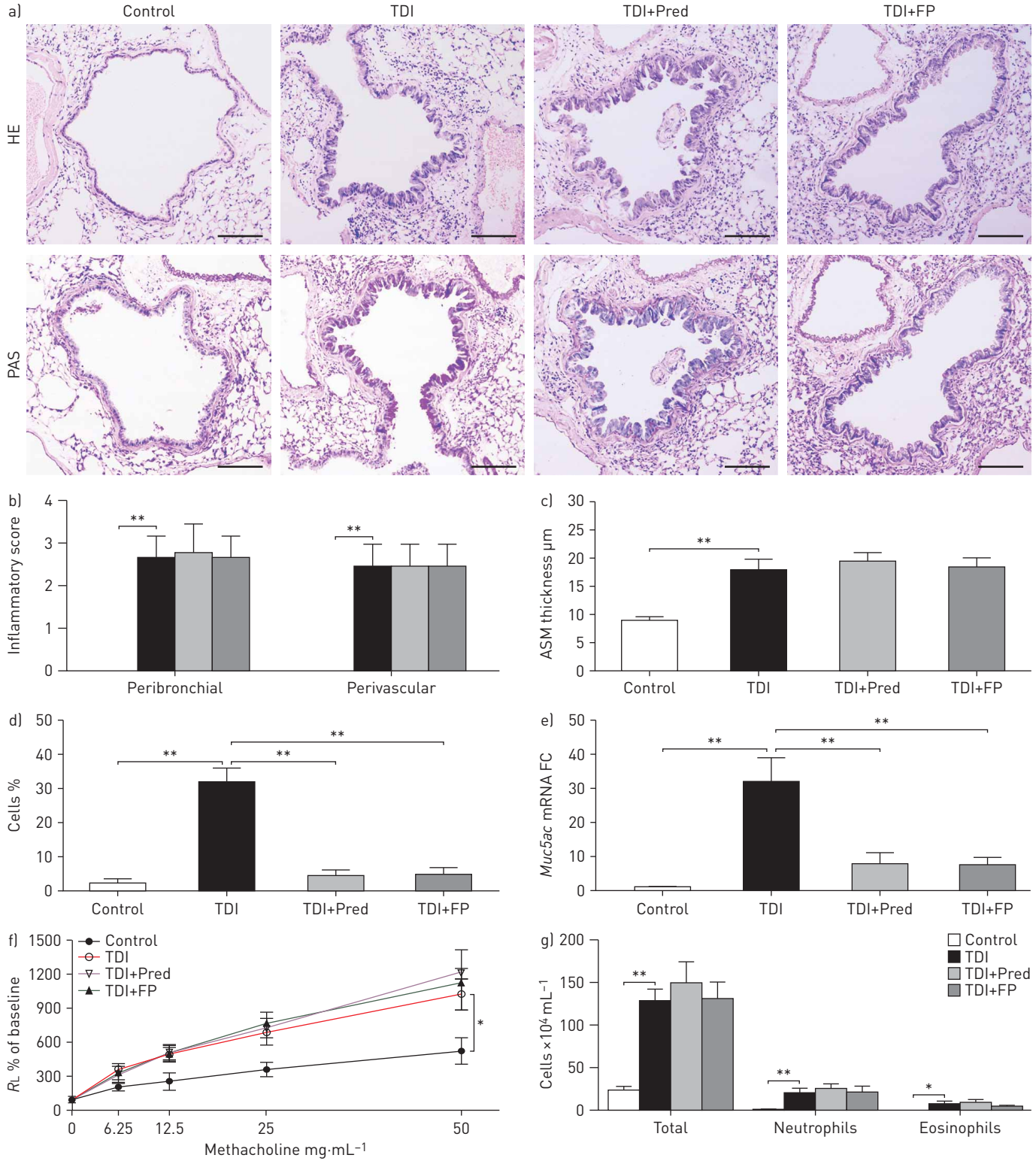


FIGURE 1 Inhaled fluticasone propionate (FP) or systemic prednisone (Pred) had no effects on toluene diisocyanate (TDI)-induced airway inflammation and bronchial hyperreactivity. HE: haematoxylin and eosin; PAS: Periodic acid–Schiff; ASM: airway smooth muscle; FC: fold change; $R_L$: lung resistance. a) Representative HE- and PAS-stained lung sections of different groups. Scale bar: 120 µm. b) Semiquantitative analysis of airway inflammation and c) analysis of ASM thickness. n=8–10 mice per group. d) Quantification of PAS-positive staining was determined by counting the number of PAS-positive epithelial cells. n=8–10 mice per group. e) Expression of *Muc5ac* (quantitative PCR) in the lung. n=4 mice per group. f) Airway hyperresponsiveness was measured by $R_L$. Results are shown as percentage of baseline value. n=5 mice per group. g) Numbers of total inflammatory cells, neutrophils and eosinophils. n=8–10 mice per group. *: p<0.05; **: p<0.01.

and challenged mice (figure 1g). In spite of this, greater numbers of neutrophils were seen in steroid-treated mice ($25.55\pm1.97\times10^4$ mL$^{-1}$ (TDI+prednisone group) *versus* $21.76\pm2.33\times10^4$ mL$^{-1}$ (TDI+fluticasone propionate group) *versus* $20.20\pm2.13\times10^4$ mL$^{-1}$ (TDI group); nonsignificant). Total serum IgE titres did not differ among all TDI-exposed groups (table 2).

### *Both Th2 and Th17 responses are involved in TDI-induced asthma that cannot be suppressed by inhaled fluticasone propionate or systemic prednisone*

TDI-induced asthma is thought to be mediated by a mixed Th1, Th2 and Th17 response [13, 15]. Here, we set out to determine the percentages of those subtypes of T-helper cells in this asthma model and to evaluate the effect of steroid treatment. BALF and lung single-cell suspensions were harvested and stained for flow cytometry analysis. Results revealed that the percentages of CD4 interferon (IFN)-$\gamma^+$ (Th1), CD4 IL-4$^+$ (Th2) and CD4 IL-17A$^+$ (Th17) cells in BALF CD4$^+$ cells of TDI-exposed mice were 4.60±0.65%, 2.61±0.67% and 7.88±1.08% and the percentages in lung CD4$^+$ cells were 0.97±0.21% (CD4 IFN-$\gamma^+$), 0.61±0.10% (CD4 IL-4$^+$) and 1.99±0.39% (CD4 IL-17A$^+$) compared with 2.02±0.46% (CD4 IFN-$\gamma^+$), 0.09±0.08% (CD4 IL-4$^+$) and 2.43±0.25% (CD4 IL-17A$^+$) in BALF of control mice and 0.20±0.07% (CD4 IFN-$\gamma^+$), 0.11±0.02% (CD4 IL-4$^+$) and 0.73±0.10% (CD4 IL-17A$^+$) in lung CD4$^+$ cells of control mice, whereas treatment with either inhaled fluticasone propionate or systemic prednisone had no effects on those TDI-induced T-helper cell subsets (table 1).

Th1-, Th2- and Th17-related cytokines were also quantified. Levels of Th2-related IL-4, IL-5 and IL-13 and Th17-related IL-17A and IL-17F in both BALF and lung homogenates were higher in TDI-sensitised and challenged mice compared with vehicle-exposed mice (table 2). The same was found for IL-6 and IL-1β, which are critical for the differentiation and maturation of Th17 cells, although IL-1β was not detected in BALF of this model. Other cytokines, including IL-18, as well as the eosinophil chemoattractants CCL11 and CCL24, also significantly increased in TDI-induced asthma. Fluticasone propionate or prednisone blunted the release of IL-5 but promoted IL-6 production in BALF, yet had no significant effects on the other cytokines (table 2). IFN-γ, IL-22 and IL-23 were not detected.

Th2 and Th17 signatures would result in distinct gene expression patterns in lung epithelia [16]. Thus, we assessed the relative expression of those genes in the whole lung. As shown in table 3, the mRNA levels of Th2 markers *Ccl11* and *Clca3* and Th17 markers *Cxcl1*, *Cxcl3* and *Csf3* were extensively upregulated by TDI. However, inhaled fluticasone propionate or systemic prednisone did not alter those gene expression patterns. The same was found for *Il17a* and *Il17f* mRNA expression.

### *Blockade of IL-17A exacerbates TDI-induced airway inflammation*

As IL-17A and IL-17F were both increased in TDI-induced asthma, we wondered whether blocking each of them would help to ameliorate the disease. Monoclonal antibodies (100 μg per mouse) against IL-17A and IL-17F were administered to the mice after each airway challenge. Surprisingly, we observed completely different outcomes after neutralising IL-17A and IL-17F in TDI-exposed mice. Despite decreasing the level of secreted IL-17A in BALF, IL-17A monoclonal antibody exacerbated TDI-induced AHR and inflammation, led to more severe epithelial cell hyperplasia and remodelling, and drove greater numbers of eosinophils into the airway lumen (figure 2), coupled with markedly enhanced expression of eosinophil chemokine CCL24 in the lung (figure 3c). Administration of IL-17A monoclonal antibody did

TABLE 1 T-helper cell subsets in CD4$^+$ cells in steroid-treated mice

| | Control group | TDI group | TDI+prednisone group | TDI+fluticasone propionate group |
|---|---|---|---|---|
| **BALF** | | | | |
| CD4 IFN-$\gamma^+$ % | 2.02±0.46 | 4.60±0.65** | 4.32±0.65 | 4.34±1.19 |
| CD4 IL-4$^+$ % | 0.09±0.08 | 2.61±0.67** | 2.80±0.68 | 2.73±0.52 |
| CD4 IL-17A$^+$ % | 2.43±0.25 | 7.88±1.08** | 8.24±0.92 | 7.93±1.46 |
| **Lung** | | | | |
| CD4 IFN-$\gamma^+$ % | 0.20±0.07 | 0.97±0.21** | 1.05±0.24 | 0.91±0.38 |
| CD4 IL-4$^+$ % | 0.11±0.02 | 0.61±0.10** | 0.69±0.21 | 0.91±0.16 |
| CD4 IL-17A$^+$ % | 0.73±0.10 | 1.99±0.39** | 1.79±0.25 | 1.84±0.43 |

Data are presented as mean±SD. TDI: toluene diisocyanate; BALF: bronchoalveolar lavage fluid; IFN: interferon; IL: interleukin. **: $p<0.01$, compared with the control group. No significant differences were observed between the TDI group and the TDI+prednisone group or between the TDI group and the TDI+fluticasone propionate group.

TABLE 2 Cytokine levels in steroid-treated mice

| | Control group | TDI group | TDI+prednisone group | TDI+fluticasone propionate group |
|---|---|---|---|---|
| **BALF IL-4 pg·mL$^{-1}$** | 0.38±0.42 | 19.43±4.15** | 16.77±4.20 | 17.10±4.54 |
| **Lung IL-4 pg·mL$^{-1}$** | 5.15±1.10 | 27.88±7.66* | 21.64±3.05 | 23.01±2.62 |
| **BALF IL-5 pg·mL$^{-1}$** | 4.92±1.36 | 64.37±17.06** | 15.40±2.92** | 14.54±5.78** |
| **Lung IL-5 pg·mL$^{-1}$** | 1.29±3.37 | 43.14±7.80** | 39.55±8.82 | 43.88±6.46 |
| **BALF IL-13 pg·mL$^{-1}$** | 2.39±0.48 | 18.68±5.46** | 18.26±4.66 | 19.25±4.17 |
| **Lung IL-13 pg·mL$^{-1}$** | 1.89±4.05 | 74.68±15.80** | 58.91±12.05 | 63.19±16.39 |
| **BALF IL-17A pg·mL$^{-1}$** | 2.72±1.02 | 14.68±3.72** | 20.99±6.05 | 17.39±4.35 |
| **Lung IL-17A pg·mL$^{-1}$** | 10.63±2.35 | 21.23±1.79** | 26.32±3.88 | 24.07±4.20 |
| **BALF IL-17F pg·mL$^{-1}$** | 0.00±0.00 | 34.90±4.09** | 38.72±11.94 | 41.78±22.52 |
| **Lung IL-17F pg·mL$^{-1}$** | 6.70±0.31 | 37.32±10.50* | 31.50±2.66 | 30.48±2.83 |
| **BALF IL-6 pg·mL$^{-1}$** | 8.76±2.20 | 98.57±29.93** | 325.79±109.18## | 347.92±112.60## |
| **BALF IL-18 pg·mL$^{-1}$** | 8.46±1.94 | 310.75±98.40** | 325.75±40.97 | 276.21±65.13 |
| **BALF CCL11 pg·mL$^{-1}$** | 8.31±2.02 | 140.16±21.06** | 110.76±23.16 | 106.23±37.58 |
| **BALF CCL24 pg·mL$^{-1}$** | 8.88±5.05 | 223.81±47.17* | 183.97±24.44 | 201.24±14.32 |
| **Lung IL-1β pg·mL$^{-1}$** | 31.14±6.91 | 70.00±7.92* | 73.28±10.97 | 70.68±30.47 |
| **Serum IgE ng·mL$^{-1}$** | 2.31±1.17 | 54.78±11.65** | 41.57±6.93 | 44.29±10.00 |

Data are presented as mean±SD. TDI: toluene diisocyanate; BALF: bronchoalveolar lavage fluid; IL: interleukin. *: p<0.05; **: p<0.01, compared with the control group; ##: p<0.01, compared with the TDI group.

not inhibit the percentages of CD4 IL-17A$^+$ cells in BALF and lung, but gave rise to a number of CD4 IL-4$^+$ cells (figure 3a and b). At the same time, we detected larger amounts of Th2 cytokines IL-4 and IL-5 in BALF after the mice were treated with IL-17A monoclonal antibody, whereas levels of IL-6, IL-18 and IL-1β in TDI asthmatic mice were not affected by IL-17A monoclonal antibody (figures 3c and 4a). Accordingly, TDI-induced increased mRNA expression of Th2 markers *Ccl11* and *Clca3* was enhanced, in contrast with suppressed expression of Th17 markers *Cxcl1*, *Cxcl3* and *Csf3* (figure 4b). We found the opposite results after treating the TDI-sensitised and challenged mice with recombinant IL-17A (supplementary figures S2 and S3), except for the unaffected number of CD4 IL-4$^+$ cells in BALF and lung despite their suppressed functions of secreting Th2 cytokines (supplementary figure S3a–c). These results suggest that IL-17A is restraining TDI-induced AHR and allergic responses.

### *Anti-IL-17F attenuates AHR and airway neutrophil inflammation in TDI-induced asthma*

Blocking IL-17F produced a list of promising protective functions. Intraperitoneal injection of IL-17F monoclonal antibody at a dose of 100 µg per mouse per time after each TDI challenge for a total of 3 times resulted in dramatically decreased airway inflammation and AHR, extensively compromised epithelial hyperplasia, goblet cell metaplasia and mucus production, as well as a significantly small number of neutrophils in BALF, while airway eosinophil recruitment was not inhibited (figure 2). In addition, the percentages of CD4 IFN-γ$^+$, CD4 IL-4$^+$ and CD4 IL-17A$^+$ cells in BALF and lung were also lowered after IL-17F monoclonal antibody treatment (figure 3a and b). Interestingly, IL-4, IL-5, IL-13, CCL11 and CCL24 in BALF or lung homogenates did not show obvious differences between mice treated with isotype

TABLE 3 Fold change of mRNA expression in lungs of steroid-treated mice

| | Control group | TDI group | TDI+prednisone group | TDI+fluticasone propionate group |
|---|---|---|---|---|
| ***Ccl11*** | 1.00±0.26 | 7.85±2.22** | 7.21±2.27 | 6.28±2.52 |
| ***Clca3*** | 1.00±0.12 | 41.45±8.52** | 47.30±11.95 | 35.32±19.47 |
| ***Cxcl1*** | 1.00±0.14 | 8.37±2.99** | 7.57±2.54 | 8.97±3.36 |
| ***Cxcl3*** | 1.00±0.25 | 4.30±1.17** | 7.06±3.64 | 7.50±3.65 |
| ***Csf3*** | 1.00±0.35 | 18.15±4.22** | 32.99±12.71 | 32.91±11.33 |
| ***Il17a*** | 1.00±0.42 | 28.71±4.68** | 33.59±5.78 | 35.18±5.65 |
| ***Il17f*** | 1.00±0.09 | 2.61±0.72* | 2.68±0.76 | 2.12±0.46 |

mRNA expression was normalised to control values; data are presented as mean±SD fold change. *: p<0.05; **: p<0.01, compared with the control group. No significant differences were observed between the toluene diisocyanate (TDI) group and the TDI+prednisone group or between the TDI group and the TDI +fluticasone propionate group.

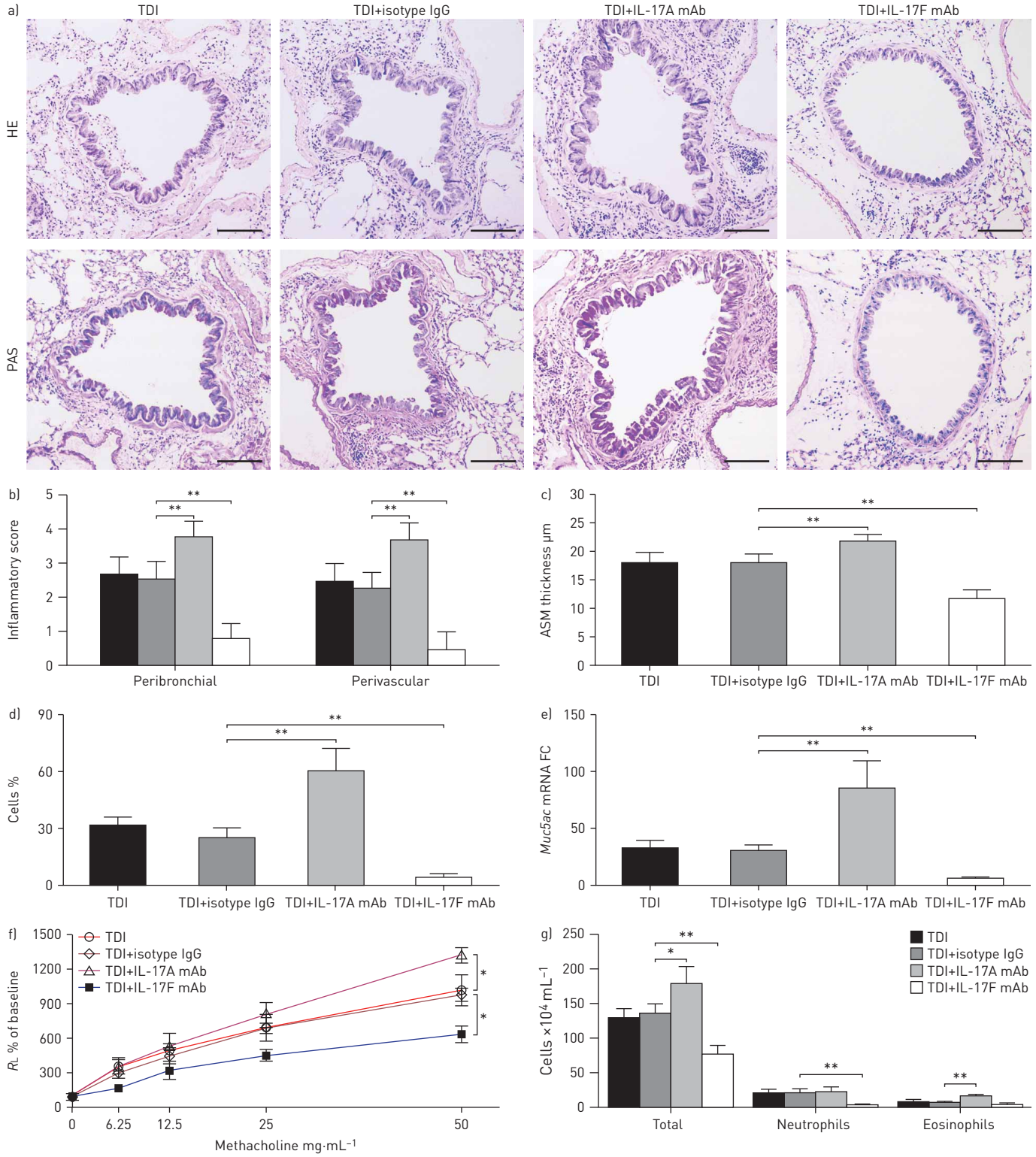


FIGURE 2 Interleukin (IL)-17A monoclonal antibody (mAb) and IL-17F mAb had opposite effects on toluene diisocyanate (TDI)-induced airway inflammation and bronchial hyperreactivity. HE: haematoxylin and eosin; PAS: Periodic acid–Schiff; ASM: airway smooth muscle; FC: fold change; $R_L$: lung resistance. a) Representative HE- and PAS-stained lung sections of different groups. Scale bar: 120 µm. b) Semiquantitative analysis of airway inflammation and c) analysis of ASM thickness. n=8–10 mice per group. d) Quantification of PAS-positive staining was determined by counting the number of PAS-positive epithelial cells. n=8–10 mice per group. e) Expression of *Muc5ac* (quantitative PCR) in the lung. n=4 mice per group. f) Airway hyperresponsiveness was measured by $R_L$. Results are shown as percentage of baseline value. n=5 mice per group. g) Numbers of total inflammatory cells, neutrophils and eosinophils. n=8–10 mice per group. *: p<0.05; **: p<0.01. No significant differences were observed between the TDI group and the TDI+isotype IgG group.

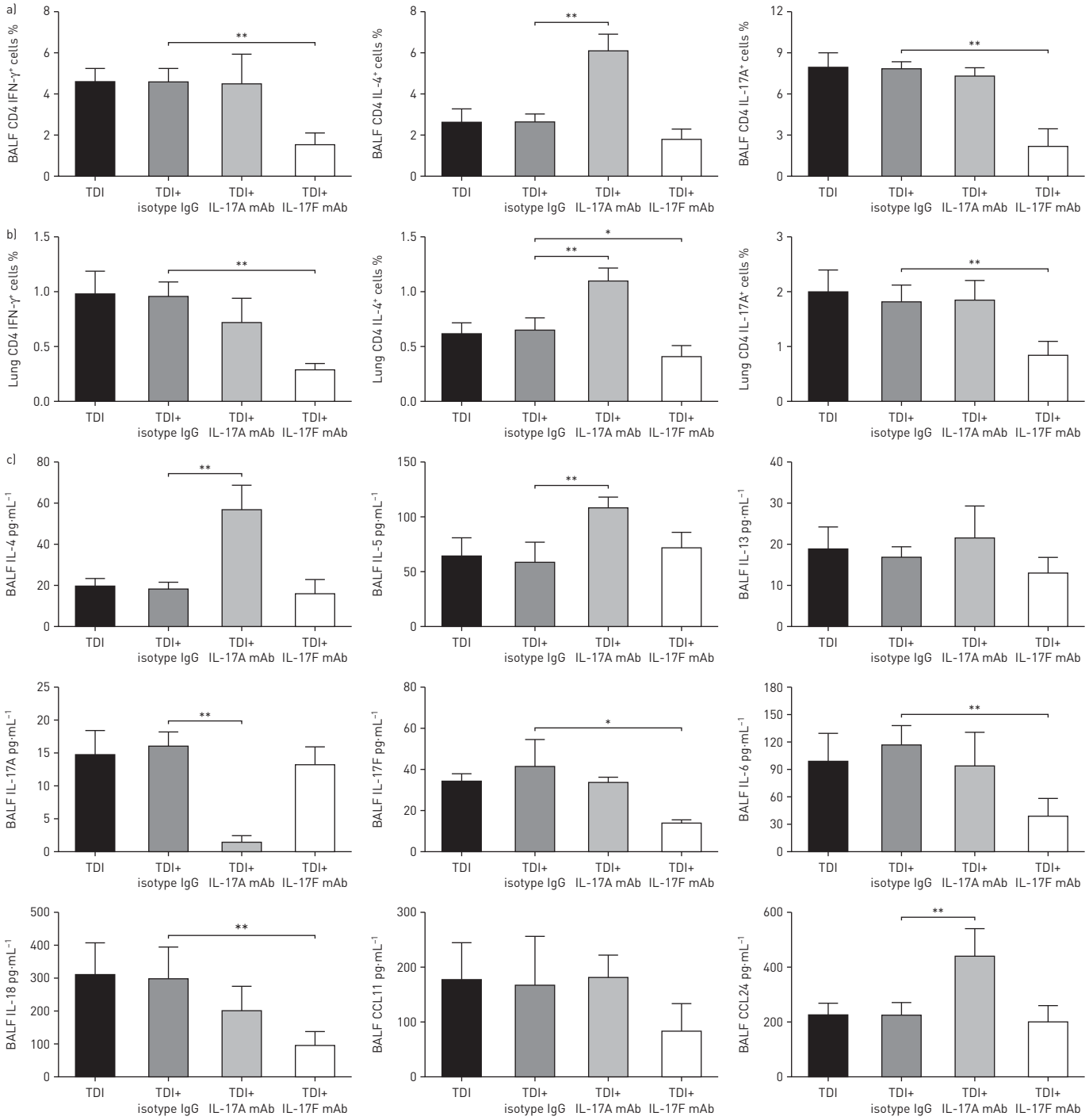


FIGURE 3 Blocking interleukin (IL)-17A and IL-17F displayed different capacity for orchestrating T-helper Th2 and Th17 responses. BALF: bronchoalveolar lavage fluid; IFN: interferon; mAb: monoclonal antibody. a) CD4 IFN-$\gamma^+$ (Th1), CD4 IL-$4^+$ (Th2) and CD4 IL-$17A^+$ (Th17) cells in $CD4^+$ cells of BALF. n=5 mice per group. b) CD4 IFN-$\gamma^+$, CD4 IL-$4^+$ and CD4 IL-$17A^+$ cells in $CD4^+$ cells of lung single-cell suspensions. n=5 mice per group. c) IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-6, IL-18, CCL11 and CCL24 levels in BALF were quantified by multiplex immunoassays or ELISA. n=8–10 mice per group. *: p<0.05; **: p<0.01. No significant differences were observed between the TDI group and the TDI+isotype IgG group.

IgG and mice treated with IL-17F monoclonal antibody (figures 3c and 4a), yet gene expression of Th17 markers *Cxcl1*, *Cxcl3* and *Csf3* was downregulated after neutralising IL-17F with monoclonal antibody in TDI-sensitised and challenged mice (figure 4b). At the same time, IL-17F monoclonal antibody treatment also inhibited the release of IL-6 and IL-18 in BALF and IL-1β expression in lung (figures 3c and 4a). Conversely, treatment with recombinant IL-17F aggravated the TDI-induced airway neutrophilic inflammation and Th17-related responses (supplementary figures S2 and S3), although AHR to methacholine was not significantly increased by IL-17F (supplementary figure S2f).

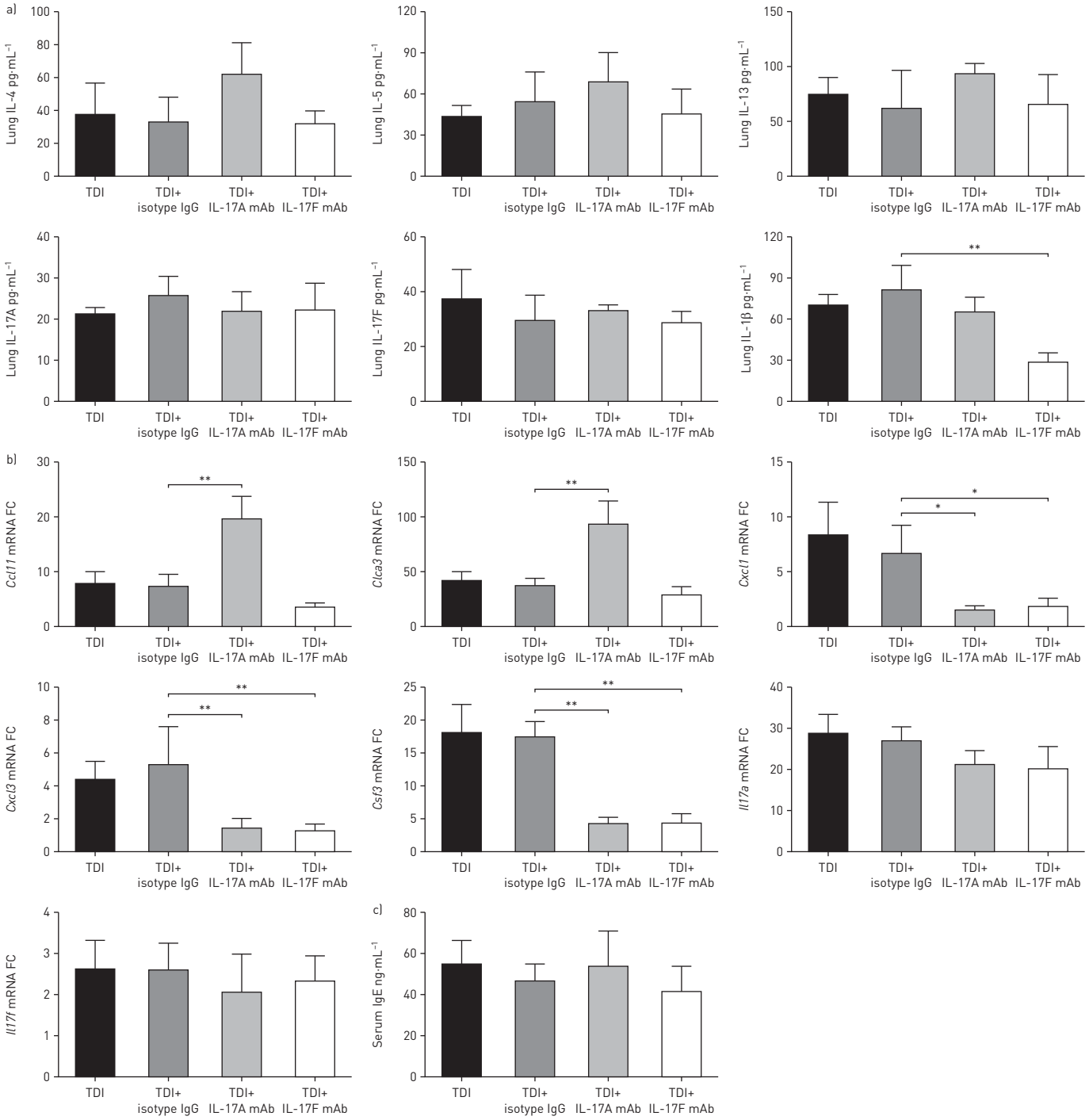


FIGURE 4 Neutralising interleukin (IL)-17A and IL-17F had different effects on toluene diisocyanate (TDI)-induced pulmonary expression of T-helper Th2 and Th17 markers. mAb: monoclonal antibody; FC: fold change. a) IL-4, IL-5, IL-13, IL-17A, IL-17F and IL-1β levels in whole lung homogenates were quantified by multiplex immunoassays or ELISA. n=4 mice per group. b) Whole lung tissue expression of Th2 markers *Clca3* and *Ccl11* and Th17 markers *Cxcl3*, *Cxcl1* and *Csf3*, as well as *Il17a* and *Il17f*, was assessed by quantitative PCR. n=4 mice per group. c) Total serum IgE concentrations were determined. n=8–10 mice per group. *: $p<0.05$; **: $p<0.01$. No significant differences were observed between the TDI group and the TDI+isotype IgG group.

## Discussion

In the present study, we assessed the effects of inhaled and systemic corticosteroids on TDI-induced asthmatic responses. Unexpectedly, both administration routes displayed few effects, indicating that we have identified a new steroid-insensitive asthma model that can be generated by a single allergen. In the meantime, we found for the first time that IL-17A restrains while IL-17F contributes to TDI-induced airway inflammation and AHR, demonstrating distinct roles for IL-17A and IL-17F.

The family of diisocyanates consists of a list of highly reactive chemicals that have commonly been used in industrial production, including TDI, hexamethylene diisocyanate, methylene diphenyl diisocyanate, *etc.* They are all well-established important respiratory allergens and are connected with the majority of occupational asthma cases [17]. Here, we prepared a TDI-induced asthma model through dermal sensitisation followed by inhalation (supplementary material). Without neglecting the importance of respiratory sensitisation, the skin also acts as an important exposure route for initiating immune sensitisation. Skin exposure is well recognised as a mechanism for inducing immune sensitisation in TDI-induced asthma, including production of allergen-specific IgE [4]. Although the reported incidence of dermal exposure to TDI varies substantially [18], it predisposes the body with a very low concentration of TDI and is technically much easier.

As the mainstay therapy in asthma, corticosteroids display a great capacity for inhibiting allergic Th2 responses and inducing eosinophil apoptosis [19]. However, there are ~10% of asthma patients that do not respond to steroid treatment, incurring a disproportionate amount of asthma-associated healthcare costs [20]. Studies have shown that the number of neutrophils correlates with poor responses to corticosteroids and decreasing lung function [21–23]. High numbers of neutrophils may reflect a non-Th2-dominated mechanism and, possibly, a steroid-resistant asthma phenotype, predicting more severe disease. Here, we used the TDI-induced asthma model (supplementary material). Allergic airway inflammation is dominated by accumulation of eosinophils and neutrophils, paralleled by increased numbers of Th2 and Th17 cells in both BALF and lungs of TDI-exposed mice. Compared with eosinophils, more neutrophils were seen in BALF of this model, together with predominant Th17 cells. Yet, to our surprise, treatment with inhaled fluticasone propionate (300 $\mu g \cdot kg^{-1}$) or systemic prednisone (5 $mg \cdot kg^{-1}$) failed to inhibit TDI-induced airway eosinophil infiltration and most of the Th2-related responses, although IL-5 and airway mucus production were actually blunted. At the same time, TDI-induced AHR, epithelial damage and hyperplasia, and Th17 signatures also showed no responsiveness to inhaled or systemic steroids. We even detected greater numbers of neutrophils (although not significant) in steroid-treated mice, accompanied by markedly higher levels of IL-6, an upstream cytokine for Th17 maturation, indicating that steroids not only exhibit no effects on TDI-induced eosinophilic inflammation, but also tend to propagate neutrophil inflammation and Th17 responses. Similar results were obtained when the TDI asthmatic mice were treated with systemic prednisone at a higher dose of 10 $mg \cdot kg^{-1}$ (data not show). The results indicate that our TDI-induced asthma model is actually a steroid-insensitive asthma model, which agrees with findings in clinical patients [2, 24]. However, this sounds contradictory to an earlier published paper showing potent anti-inflammatory effects of dexamethasone for TDI-induced eosinophilia [25]. Although it has been well recognised that eosinophils would undergo apoptosis after glucocorticoid treatment in asthma [19], steroid-resistant eosinophilic airway inflammation has also been described in both patients and animal models [26, 27]. Further studies are needed to determine the reasons why eosinophils may have different responses to steroid treatment.

As one of the most important features of TDI-induced asthma, the large numbers of neutrophils accumulating in the airway make huge contributions to steroid resistance and greatly hamper control of the disease. Depletion of neutrophils could prevent TDI-induced AHR, epithelial damage and significantly reduce airway inflammation [28]. Therefore, targeting neutrophils might have potential therapeutic effects. Members of the IL-17 family, mainly IL-17A and IL-17F, are known to be potent neutrophil-mobilising chemokines [5]. They attract neutrophils on to mucosal surfaces by increasing the release of neutrophil-modulating cytokines and chemoattractants (including IL-1β, IL-6, IL-8, granulocyte colony-stimulating factor, *etc.*) from epithelial and endothelial cells [29]. Genetic deletion or neutralisation of IL-17A diminishes neutrophil invasion and confers protection against organ injury and infection in mice [30, 31]. IL-17A can also enhance neutrophil fungal killing functions and induce the production of reactive oxygen species [32]. Studies have already demonstrated an important role of IL-17 in TDI-induced asthma [13, 14] and we previously detected higher levels of IL-17A in this TDI-induced asthma model [33]. Thus, in the current study, we treated the TDI-sensitised and challenged mice with IL-17A monoclonal antibody, hoping to find some therapeutic effects. Unexpectedly, IL-17A antibody not only showed no effects on TDI-induced airway neutrophilic inflammation, but also augmented eosinophil aggregation, Th2-related responses, AHR, bronchial epithelial hyperplasia and mucus production, indicating that IL-17A functions to counteract eosinophilia and Th2 inflammation, which was verified a second time after we treated the TDI-inhaling mice with exogenous IL-17A. These findings appear to be in striking contrast to the aforementioned studies [13, 14] and differ from observations in OVA- or house dust mite (HDM)-induced asthma models [10, 34], but might help to account for the controversial dual roles of IL-17 (which consists of not only IL-17A) in allergic airway inflammation [8–11]. Consistent with our study, Nakae *et al.* [8] and Hellings *et al.* [9] discovered that IL-17 deficiency or neutralisation significantly enhanced BALF IL-4 and IL-5, and aggravated OVA-induced eosinophilia. A recent study in a mouse model of allergic bronchopulmonary aspergillosis

also supports the notion that antagonising IL-17A can boost allergen-induced airway eosinophilia and Th2 responses [12].

In contrast to the effects of IL-17A monoclonal antibody, IL-17F monoclonal antibody treatment had completely different outcomes. As mentioned earlier, IL-17F, another member of the IL-17 family, is also capable of driving neutrophilic inflammation [29]. With the greatest sequence homology (>50%) shared between IL-17A and IL-17F, few studies have disentangled the specific effects of these two IL-17 family members in pathological inflammation. Compared with IL-17A, IL-17F is much less studied. It was originally discovered in 2001 in bronchoalveolar lavage cells from asthma patients upon ragweed allergen challenge [35]. Later, in 2002, a Japanese team demonstrated that a coding region variant of the IL-17F gene is linked to chronic inflammatory airway diseases [36]. Recent studies proved that airway IL-17F expression positively correlates with the number of neutrophils and asthma severity [37, 38]. In this study, the BALF IL-17F level in TDI-exposed mice was higher than in control mice. In line with the findings in the HDM-induced model [34], neutralising IL-17F with monoclonal antibody resulted in considerably diminished bronchial neutrophil infiltration, ameliorated airway epithelial injury and hyperplasia, decreased AHR, and blunted Th17-related responses, together with lower eosinophils (though not significant) in the airway. At the same time, IL-17F monoclonal antibody treatment also suppressed the excessive secretion of IL-6 and IL-18 and the upregulated IL-1β expression in TDI asthmatic mice, all of which are critical mediators for neutrophil migration and activation [39]. The opposite results were seen when the TDI-sensitised and challenged mice were treated with recombinant IL-17F. These data suggest that IL-17F is the major culprit for TDI-induced neutrophilic inflammation and pathological changes.

In conclusion, we discovered a novel steroid-insensitive asthma model that is induced by TDI, providing an alternative approach for investigating mechanisms involved in severe asthma. In addition, we also found that IL-17A restricts TDI-induced allergic airway inflammation through counteracting Th2 responses and preventing eosinophil influx, while IL-17F contributes to TDI-induced airway neutrophilic inflammation and bronchial hyperresponsiveness, which might be effective therapeutic targets for severe asthma in the future.

Author contributions: L. Yao and H. Tang designed the experiment, analysed the results and wrote the manuscript. L. Yao, S. Chen and H. Tang performed the experiments. R. Chen, Q. Zhang, P. Huang and S. Wei helped with the experiment and data analysis. R. Chen and Q. Zhang helped to revise the manuscript. Z. Liang, X. Chen and A. Tao helped with data analysis and image processing. All the authors agreed upon the final approval of the version to be published, and to ensure questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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**IL-17F, rather than IL-17A, underlies airway inflammation in a steroid insensitive toluene diisocyanate-induced asthma model**

**Online supplementary material**

**Methods**

*Mice, allergen and treatments*

6~8-week-old male BALB/c mice were purchased from Guangdong Medical Laboratory Animal Center. They were housed under specific pathogen-free conditions and maintained on a 12-hour light-dark cycle with free access to food and water. A TDI-induced asthma model was prepared based on our previous work [1]. In short, BALB/c mice were dermally sensitized with 0.3% TDI on the dorsum of both ears (20 μL per ear) on days 1 and 8. After that, on days 15, 18 and 21, the mice were placed in a horizontal rectangle chamber and challenged for 3 h each time via the airways with 3% TDI through compressed air nebulization (NE-C28, Omron). TDI was diluted in a mixture of 3 volumes of olive oil and 2 volumes of acetone for the sensitization and 4 volumes of olive oil and 1 volume of acetone for the challenge. Control mice were sensitized and challenged with the same amount of vehicle.

Fluticasone propionate (FP, SigmaAldrich, St. Louis, Missouri, US.) was dissolved in sterile 0.05% Tween-20 in PBS and administered i.n. at a dose of 300 μg/kg/mouse in a volume of 20 μL in mice lightly anesthetized with isofluorane. Prednisone (Pred, SigmaAldrich, 5 mg/kg), anti-IL-17A monoclonal antibody (IL-17A mab, eBioscience, 100 μg/mouse), anti-IL-17F monoclonal antibody (IL-17A mab, eBioscience, 100 μg/mouse), or the isotype control antibody (100 μg/mouse) was administered separately via the intraperitoneal (i.p.) route. Recombinant mouse IL-17A or IL-17F (PeproTech, Rocky Hill, NJ) was dissolved in sterile PBS and administered intranasally at the dose of 1 μg/mouse per time. FP and Pred were administrated once daily, beginning immediately after the first challenge to the last day of challenge for a consecutive of seven days; while the monoclonal antibodies and recombinant cytokines were given immediately after each inhalation. FP, Pred, monoclonal antibodies and recombinant cytokines were given at different time points and with different routes of administration because of their intrinsic pharmacodynamics and pharmacokinetic characteristics. These treatments are all aimed to be therapeutic and not prophylactic.

*Airway responsiveness measurements*

As previously described [2], twenty-four hours after the third inhalation, airway responsiveness was assessed by lung resistance ($R_L$) measurement (Buxco Electronics, Troy, NY, USA) mice in response to grading doses of aerosolized methacholine (6.25, 12.5, 25 and 50 mg/mL). $R_L$ was recorded every five minutes following each nebulisation step until a plateau phase was reached. Results were expressed as percentage of baseline value (value at 0 mg/mL methacholine) for each increased concentration of methacholine.

*Specimens collection and cell isolation*

For bronchoalveolar lavage fluid (BALF), mice were cannulated through the trachea to the left main bronchus and lavaged *in situ* by instilling and retrieving 0.5 mL of sterile saline into the left lung. BALF recovered exclusively from the left lobe was centrifuged at 500 rpm for 10 min at room temperature, and supernatants were stored at -80°C until further use. Whereas the pellet was immediately resuspended in 0.2 ml of sterile saline. Cell counts were then determined for each BALF sample, and differential cell counts were performed on cytospin preparations stained with haematoxylin and eosin (H&E).

To remove the intravascular pool of cells, lungs were perfused with 5 ml of sterile saline via the pulmonary circulation, through catheterization of the right heart. Then, the right lungs (not undergone BAL) were removed.

To prepare single cell suspensions, right lungs were diced using iridectomy scissors. Lungs were enzymatically digested with collagenase (200U/mL, Sigma) in RMPI 1640 medium for 30 minutes at 37°C. Pulmonary cell suspensions were obtained by grinding the tissue through 100 μm nylon sieves, and red blood cells were lysed using ammonium chloride buffer. Cell counts were then performed before staining for flow cytometry analysis.

*Flow cytometry analysis*

Bronchoalveolar lavage fluid and lung single-cell suspensions were fixed, permeabilized (Cytofix/Cytoperm buffer; BD Biosciences) and stained for 30 min with antibodies for CD4, IFNγ, IL-4, IL-17A (BD Biosciences). All appropriate isotype controls were used. Data were collected on a BD Biosciences Fortessa flow cytometer and analyzed using FlowJo software.

*Histopathological analysis*

The left lungs were harvested, fixed overnight in 4% neutral formalin and embedded in paraffin. Sections (4 μm) were stained with H&E to show morphological changes and inflammation. As

previously described [1], two criteria were scored to quantify airway inflammation: peribronchial inflammation and perivascular inflammation. A value of 0 was allocated when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by one layer (1 to 10 cells) of inflammatory cells, a value of 3 for most bronchi or vessels were surrounded by 2 layers (10 to 20 cells) of inflammatory cells and a value of 4 for most bronchi or vessels were surrounded by more than two layers (more than 20 cells) of inflammatory cells. Thickness of airway smooth muscle was measured by a modification of Cho JY's method [5]. Briefly, the thickness of the peribronchial smooth muscle layer (the transverse diameter) in large airways was measured from the innermost aspect to the outermost aspect of the circumferential smooth muscle layer. 20~24 image fields of eight sections from 8~10 mice per group were analyzed.

To visualize airway mucus production, sections were stained with periodic acid-Schiff base (PAS) and semi-quantified. PAS-positive epithelial cells of the total epithelial cells were counted to obtain a percentage and compare groups. 16~20 image fields of eight sections from 8~10 mice per group were analyzed.

*Cytokine and chemokine analysis*

Levels of cytokines and chemokines in BALF and lung homogenates, including Th2-related IL-4, IL-5, IL-13, Th17-related IL-17A, IL-17F, Th17 cell maturation associated IL1β, IL-6 [3, 4], neutrophil chemokine IL-18 [6], as well as eosinophil attractants CCL11 and CCL24, were measured using multiplex immunoassay or ELISA kits (eBioscience) according to Manufacturer's specifications.

*Gene expression analysis*

Lung tissue was homogenized in TRIzol Reagent (Takara, Guangzhou, China). Total RNA were extracted with an RNAiso Plus kit (Takara) and reverse-transcribed to complementary DNA using PrimeScript$^{TM}$ RT reagent kit (Takara). Gene expression was quantified using SYBR Green Premix Ex Taq (Takara) by LightCycler 480 Fast Real-Time PCR System. The primers used were listed in Table S1.

**TABLE S1. Murine qPCR primer sequences**

| Gene | Forward sequence (5'→3') | Reverse sequence (5'→3') |
|---|---|---|
| *Gapdh* | AAGAGGGATGCTGCCCTTAC | CCAATACGGCCAAATCCGTTC |
| *Muc5ac* | CAGGACTCTCTGAAATCGTACCA | AAGGCTCGTACCACAGGGA |
| *Il17a* | GAGAGCTTCATCTGTGTCTCTG | GCGCCAAGGGAGTTAAAGAC |
| *Il17f* | CGTGAAACAGCCATGGTCAAG | GGGACAGAAATGCCCTGGTT |
| *Ccl11* | TGCTCACGGTCACTTCCTTC | CTTGAAGACTATGGCTTTCAGGGTG |
| *Clca3* | AGGAAAACCCCAAGCAGTG | GCACCGACGAACTTGATTTT |
| *Cxc11* | AACCGAAGTCATAGCCACACT | CCGTTACTTGGGGACACCTT |
| *Cxcl3* | CACCCAGACAGAAGTCATAGCC | CCGTTGGGATGGATCGCTTT |
| *Csf3* | GTGCTGCTGGAGCAGTTGT | TCGGGATCCCCAGAGAGT |

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