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

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

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Key message

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.

Abstract

Steroid insensitivity constitutes the major problem for asthma management. Toluene diisocyanate (TDI) is one of the leading allergens of asthma that induces both 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 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 (FP), systemic prednisone (Pred), anti-IL-17A, anti-IL-17F, recombinant IL-17A or IL17F. Both FP and Pred showed no effects on TDI-induced airway hyperreactivity (AHR), bronchial neutrophilia and eosinophilia, epithelial goblet cell metaplasia. TDI-induced Th2 and Th17 signatures were not suppressed by FP or Pred. Treatment with anti-IL-17A after TDI exposure led to increased AHR, aggravated mucus production and airway eosinophil recruitment, accompanied by amplified Th2 responses. While 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. In conclusion, IL-17A and IL-17F exert distinct biological effects during airway inflammation of a TDI-induced asthma model, which is irresponsive to both inhaled and systemic steroids.

**Keywords**

asthma; toluene diisocyanate; steroid insensitive; IL-17A; IL-17F

**Introduction**

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

During the past decade, the role of IL17 in immune and allergic disorders is attracting increasing attention. IL-17 is a family of cytokines mainly produced by Th17 cells and consists of six members, named 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 supported that IL-17 is critically involved in asthma pathogenesis. Elevated IL-17 concentrations were found in the airways of allergic asthma patients [6] and positively correlate to asthma severity and steroid insensitivity [7]. Inhibition of IL-17 using gene knockout mice or blocking antibody reduces OVA-induced airway hyperresponsiveness, bronchial inflammatory cell infiltration as well as airway vascular remodeling [8-10]. However, Schnyder-Candrian S and coauthors discovered that exogenous IL-17 inhibited OVA-induced pulmonary eosinophil recruitment, Th2 inflammation, and bronchial hyperreactivity [11]. Those
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 asthma. Anti-IL17 neutralizing antibody could decrease TDI-induced airway inflammation and hyperresponsiveness [13, 14], suggesting important roles of IL-17 in TDI asthma. Yet, 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 two aims of this study were to evaluate the effects of inhaled and systemic steroids on TDI-induced asthmatic responses, and to examine the roles of IL-17A and IL-17F in TDI asthma.

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

Ethics statement
All animal experiments described here complied with the guidelines of the Committee of Guangzhou Medical University on the use and care of animals and were approved by the Animal Subjects Committee of Guangzhou Medical University.

Statistics
Data are expressed as mean ± SD. Results were interpreted using one-way ANOVA and Bonferroni’s difference post hoc test with SPSS 22.0. Differences were considered statistically significant when \( p < 0.05 \).

Results

TDI-induced airway hyperresponsiveness and pathological changes were not prevented by inhaled fluticasone propionate (FP) or systemic prednisone (Pred)

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 do any help in our TDI-induced asthma model. Intraperitoneal injection of 5 mg/kg prednisone (Pred) or intranasal instillation of 300 \( \mu g/kg \) fluticasone propionate (FP) once daily beginning from the first inhalation for a consecutive of one week did inhibit TDI-induced airway mucus production as
assessed by PAS staining and Muc5ac mRNA expression (Fig 1, a, d and e), yet TDI-induced airway inflammation, epithelial hyperplasia, airway hyperresponsiveness, smooth muscle thickening did not change after Pred or FP treatment (Fig 1, a, b, c and f). Similarly, neither inhaled FP nor systemic Pred affected the increased numbers of neutrophils and eosinophils in bronchoalveolar lavage fluid (BALF) of TDI sensitized and challenged mice (Fig1, g). In spite, greater numbers of neutrophils were seen in steroid-treated mice [25.55 ± 1.97 (TDI+Pred group) vs 21.76 ± 2.33 (TDI+FP group) vs 20.20 ± 2.13 (TDI group) × 10^4/mL, though not significant]. Total serum IgE titers did not differ among all TDI-exposed groups (TABLE 2).

**TABLE 1** Percentages (%) of T helper cell subsets in CD4+ cells in steroid-treated mice

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>TDI</th>
<th>TDI+Pred</th>
<th>TDI+FP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BALF CD4 IFNγ+</strong></td>
<td>2.02 ± 0.46</td>
<td>4.60 ± 0.65**</td>
<td>4.32 ± 0.65</td>
<td>4.34 ± 1.19</td>
</tr>
<tr>
<td><strong>BALF CD4 IL-4+</strong></td>
<td>0.09 ± 0.08</td>
<td>2.61 ± 0.67**</td>
<td>2.80 ± 0.68</td>
<td>2.73 ± 0.52</td>
</tr>
<tr>
<td><strong>BALF CD4 IL17A+</strong></td>
<td>2.43 ± 0.25</td>
<td>7.88 ± 1.08**</td>
<td>8.24 ± 0.92</td>
<td>7.93 ± 1.46</td>
</tr>
<tr>
<td><strong>lung CD4 IFNγ+</strong></td>
<td>0.20 ± 0.07</td>
<td>0.97 ± 0.21**</td>
<td>1.05 ± 0.24</td>
<td>0.91 ± 0.38</td>
</tr>
<tr>
<td><strong>lung CD4 IL-4+</strong></td>
<td>0.11 ± 0.02</td>
<td>0.61 ± 0.10**</td>
<td>0.69 ± 0.21</td>
<td>0.91 ± 0.16</td>
</tr>
<tr>
<td><strong>lung CD4 IL17A+</strong></td>
<td>0.73 ± 0.10</td>
<td>1.99 ± 0.39**</td>
<td>1.79 ± 0.25</td>
<td>1.84 ± 0.43</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. ** in TDI group indicates p<0.01 compared with control group. No significant differences were observed between TDI and TDI+Pred group, nor between TDI and TDI+FP group.

Both Th2 and Th17 responses are involved in TDI-induced asthma that can not be suppressed by inhaled FP or systemic Pred

TDI-induced asthma is thought to be mediated by a mixed Th1, Th2 and Th17 response [13, 15]. Here, we set 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 IFNγ+ (Th1), CD4 IL4+ (Th2), CD4 IL17A+ (Th17) cells in BALF CD4+ cells of TDI-exposed mice were 4.60 ± 0.65, 2.61 ± 0.67, 7.88 ± 1.08, and the percentages (%) in lung CD4+ cells were 0.97 ± 0.21 (CD4 IFNγ+), 0.61 ± 0.10 (CD4 IL4+), 1.99 ± 0.39 (CD4 IL17A+), compared with 2.02 ± 0.46 (CD4 IFNγ+), 0.09 ± 0.08 (CD4 IL4+), 2.43 ± 0.25 (CD4 IL17A+) in BALF of control mice and 0.20 ± 0.07 (CD4 IFNγ+), 0.11 ± 0.02 (CD4 IL4+), 0.73 ± 0.10 (CD4 IL17A+) in lung CD4+ cells of control group. While treatment with either inhaled FP or systemic Pred 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, IL-13 and Th17-related IL-17A, IL-17F in both BALF and lung homogenates were higher in TDI sensitized and challenged mice compared with vehicle-exposed (TABLE 2). It is the same for IL-6 and IL-1β, which are critical for the differentiation and maturation of Th17 cells, though 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 increased significantly in TDI asthma. FP or Pred blunted the release of IL-5 but promoted IL-6 production in BALF, yet had no significant effects on the others (TABLE 2). IFNγ, IL-22, IL-23 were not detected.

Th2 and Th17 signatures would result in distinct gene expression patterns in lung epithelia [16]. So we assessed the relative expression of those genes in the whole lung. As shown in TABLE 3, the mRNA levels of Th2 markers Ccl11, Clca3 and Th17 markers Cxcl1, Cxcl3, Csf3 were extensively up-regulated by TDI. Still, inhaled FP or systemic Pred did not alter those gene expression patterns. It was the same for Il17a and Il17f mRNA expression.

**TABLE 2** Levels of a list of cytokines in BALF in steroid-treated mice

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>TDI</th>
<th>TDI+Pred</th>
<th>TDI+FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF IL-4</td>
<td>0.38±0.42</td>
<td>19.43±4.15**</td>
<td>16.77±4.20</td>
<td>17.10±4.54</td>
</tr>
<tr>
<td>lung IL-4</td>
<td>5.15±1.10</td>
<td>27.88±7.66*</td>
<td>21.64±3.05</td>
<td>23.01±2.62</td>
</tr>
<tr>
<td>BALF IL-5</td>
<td>4.92±1.36</td>
<td>64.37±17.06**</td>
<td>15.40±2.92**</td>
<td>14.54±5.78**</td>
</tr>
<tr>
<td>lung IL-5</td>
<td>1.29±3.37</td>
<td>43.14±7.80**</td>
<td>39.55±8.82</td>
<td>43.88±6.46</td>
</tr>
<tr>
<td>BALF IL-13</td>
<td>2.39±0.48</td>
<td>18.68±5.46**</td>
<td>18.26±4.66</td>
<td>19.25±4.17</td>
</tr>
<tr>
<td>lung IL-13</td>
<td>1.89±4.05</td>
<td>74.68±15.80**</td>
<td>58.91±12.05</td>
<td>63.19±16.39</td>
</tr>
<tr>
<td>BALF IL17A</td>
<td>2.72±1.02</td>
<td>14.68±3.72**</td>
<td>20.99±6.05</td>
<td>17.39±4.35</td>
</tr>
<tr>
<td>lung IL17A</td>
<td>10.63±2.35</td>
<td>21.23±1.79**</td>
<td>26.32±3.88</td>
<td>24.07±4.20</td>
</tr>
<tr>
<td>BALF IL17F</td>
<td>0.00±0.00</td>
<td>34.90±4.09**</td>
<td>38.72±11.94</td>
<td>41.78±22.52</td>
</tr>
<tr>
<td>lung IL17F</td>
<td>6.70±0.31</td>
<td>37.32±10.50*</td>
<td>31.50±2.66</td>
<td>30.48±2.83</td>
</tr>
<tr>
<td>BALF IL-6</td>
<td>8.76±2.20</td>
<td>98.57±29.93**</td>
<td>325.79±109.18**</td>
<td>347.92±112.60**</td>
</tr>
<tr>
<td>BALF IL-18</td>
<td>8.46±1.94</td>
<td>310.75±98.40**</td>
<td>325.75±40.97</td>
<td>276.21±65.13</td>
</tr>
<tr>
<td>BALF CCL11</td>
<td>8.31±2.02</td>
<td>140.16±21.06**</td>
<td>110.76±23.16</td>
<td>106.23±37.58</td>
</tr>
<tr>
<td>BALF CCL24</td>
<td>8.88±5.05</td>
<td>223.81±47.17*</td>
<td>183.97±24.44</td>
<td>201.24±14.32</td>
</tr>
<tr>
<td>lung IL1β</td>
<td>31.14±6.91</td>
<td>70.00±7.92*</td>
<td>73.28±10.97</td>
<td>70.68±30.47</td>
</tr>
<tr>
<td>serum IgE*</td>
<td>2.31±1.17</td>
<td>54.78±11.65**</td>
<td>41.57±6.93</td>
<td>44.29±10.00</td>
</tr>
</tbody>
</table>
Data are presented as mean±SD. * in TDI group indicates p<0.05 compared with control group, and ** indicates p<0.01. ** in TDI+Pred or TDI+FP group indicates p<0.01 compared with TDI group.

The unit for IgE is ng/mL, and pg/mL for all the others.

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

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>TDI</th>
<th>TDI+Pred</th>
<th>TDI+FP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ccl11</strong></td>
<td>1.00±0.26</td>
<td>7.85±2.22**</td>
<td>7.21±2.27</td>
<td>6.28±2.52</td>
</tr>
<tr>
<td><strong>Clca3</strong></td>
<td>1.00±0.12</td>
<td>41.45±8.52**</td>
<td>47.30±11.95</td>
<td>35.32±19.47</td>
</tr>
<tr>
<td><strong>Cxcl1</strong></td>
<td>1.00±0.14</td>
<td>8.37±2.99**</td>
<td>7.57±2.54</td>
<td>8.97±3.36</td>
</tr>
<tr>
<td><strong>Cxcl3</strong></td>
<td>1.00±0.25</td>
<td>4.30±1.17**</td>
<td>7.06±3.64</td>
<td>7.50±3.65</td>
</tr>
<tr>
<td><strong>Csf3</strong></td>
<td>1.00±0.35</td>
<td>18.15±4.22**</td>
<td>32.99±12.71</td>
<td>32.91±11.33</td>
</tr>
<tr>
<td><strong>Il17a</strong></td>
<td>1.00±0.42</td>
<td>28.71±4.68**</td>
<td>33.59±5.78</td>
<td>35.18±5.65</td>
</tr>
<tr>
<td><strong>Il17f</strong></td>
<td>1.00±0.09</td>
<td>2.61±0.72*</td>
<td>2.68±0.76</td>
<td>2.12±0.46</td>
</tr>
</tbody>
</table>

mRNA expression was normalized to control values and expressed as n-fold. Data are presented as mean±SD. * in TDI group indicates p<0.05 compared with control group, and ** indicates p<0.01. No significant differences were observed between TDI and TDI+Pred group, nor between TDI and TDI+FP group.

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

As IL-17A and IL-17F were both increased in TDI asthma, we wondered whether blocking each of them would help to ameliorate the disease. Monoclonal antibodies (100 μg/mouse) against IL-17A and IL-17F were respectively given to the mice after each airway challenge. Surprisingly, we observed completely different outcomes after neutralizing IL-17A and IL-17F in TDI-exposed mice. Despite decreasing the level of secreted IL-17A in BALF, IL-17A mab exacerbated TDI-induced airway hyperresponsiveness and inflammation, led to more severe epithelial cell hyperplasia and remodeling, and drove greater numbers of eosinophils into the airway lumen (Fig 2), coupled with markedly enhanced expression of eosinophil chemokine CCL24 in the lung (Fig 3, c). Administration of IL-17A mab did not inhibit the percentages of CD4 IL17A+ cells in BALF and lung, but gave rise to the number of CD4 IL4+ cells (Fig 3, a 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 mab, whereas levels of IL-6, IL-18 and IL-1β in TDI asthmatic mice were not affected by IL-17A mab (Fig 3, c; Fig 4, a). Accordingly, TDI-induced increased mRNA expression of Th2 markers Ccl11, Clca3 was enhanced, in contrast with suppressed expression of Th17 markers Cxcl1, Cxcl3 and Csf3 (Fig 4, b). We got opposite results after treating the TDI-sensitized and challenged mice...
with recombinant IL-17A (Online supplement, Fig S2 and Fig S3), except for the unaffected number of CD4 IL4+ cells in BALF and lung despite their suppressed functions of secreting Th2 cytokines (Online supplement, Fig S3, a-c). These results suggest that IL-17A is restraining TDI-induced airway hyperreactivity and allergic responses.

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

On the other hand, blocking IL-17F exerted a list of promising protective functions. Intraperitoneal injection of IL-17F mab at the 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 hyperresponsiveness, 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 (Fig 2). Besides, percentages of CD4 IFNγ+, CD4 IL4+ and CD4 IL17A+ cells in BALF and lung were also lowered after IL-17F mab treatment (Fig 3, a and b). Interestingly, IL-4, IL-5, IL-13, CCL11, CCL24 in BALF or lung homogenates did not show obvious differences between mice treated with isotype IgG and mice treated with IL-17F mab (Fig 3, c; Fig 4, a), yet gene expression of Th17 markers Cxcl1, Cxcl3 and Csf3 was downregulated after neutralizing IL-17F with monoclonal antibody in TDI sensitized and challenged mice (Fig 4, b). At the same time, IL-17F mab treatment also inhibited the release of IL-6, IL-18 in BALF and IL-1β expression in lung (Fig 3, c; Fig 4, a). Conversely, treatment with recombinant IL-17F aggravated the TDI-induced airway neutrophilic inflammation and Th17 related responses (Online supplement, Fig S2 and Fig S3), though airway hyperreactivity to methacholine was not significantly increased by IL-17F (Online supplement, Fig S2, f).

**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’ve identified a new steroid insensitive asthma model that can be generated by a single allergen. In the mean time, we found for the first time that IL-17A is restraining while IL-17F is contributing to TDI-induced airway hyperreactivity and inflammation, demonstrating distinct roles of IL-17A and IL-17F.

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

As the mainstay therapy in asthma, corticosteroids display great capacity for inhibiting allergic Th2 response and inducing eosinophil apoptosis [19]. Yet there are approximately 10% of asthma patients that do not respond to steroid treatment, consuming 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 number of neutrophils may reflect a non-Th2-dominated mechanism and, possibly, a steroid resistant asthma phenotype, predicting more severe disease. Here, we prepared the TDI-induced asthma model as previously described. 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 predominated Th17 cells. Yet to our surprise, treatment with inhaled fluticasone propionate (300 μg/kg) or systemic prednisone (5 mg/kg) failed to inhibit TDI-induced airway eosinophil infiltration and most of the Th2-related responses, though 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 (though 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 10mg/kg (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]. But this sounds
contradictory to an earlier published paper, showing potent anti-inflammatory effects of dexamethasone for TDI-induced eosinophilia [25]. Though it has been well recognized 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 find out 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 have made 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 prove potential therapeutical effects. Members of the IL-17 family, mainly IL-17A and IL-17F, are known to be potent neutrophil mobilizing chemokines [5]. They attract neutrophils into mucosal surfaces by increasing the release of neutrophil-modulating cytokines and chemoattractants (including IL1β, IL-6, IL-8, granulocyte colony-stimulating factor, etc.) from epithelial and endothelial cells [29]. Genetic deletion or neutralization 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 its production of reactive oxygen species (ROS) [32]. Studies have already demonstrated an important role of IL-17 in TDI-induced asthma [13, 14], and previously we detected higher levels of IL-17A in this TDI-induced asthma model [33]. So in the current study, we treated the TDI-sensitized and challenged mice with IL-17A monoclonal antibody, hoping to find some therapeutic effects. Unexpectedly, IL-17A antibody not only showed no effects for TDI-induced airway neutrophil inflammation, but also augmented eosinophil aggregation, Th2-related responses, AHR, bronchial epithelial hyperplia and mucus production, indicating that IL-17A functions to counteract eosinophilia and Th2 inflammation, which is verified a second time after we treated the TDI-inhaling mice with exogenous IL-17A. These appear to be in striking contrasts to the aforementioned studies [13, 14] and different from observations in OVA- or house dust mite (HDM)-induced asthma models [34, 35], 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 S et al. and Hellings PW et al. discovered that IL-17 deficiency or neutralization significantly enhanced BALF IL-4, IL-5 and aggravated OVA-induced eosinophilia [8, 9]. One latest study in a
mouse model of allergic broncho-pulmonary aspergillosis (ABPA) also supports the notion that antagonizing IL-17A can boost allergen-induced airway eosinophilia and Th2 responses. In contrast to the effects of IL-17A mab, IL-17F mab treatment had completely different outcomes. As mentioned before, IL-17F, another member of the IL-17 family, is also capable of driving neutrophilic inflammation. 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 pathologic inflammation. Compared with IL-17A, IL-17F is much less studied. It was originally discovered in bronchoalveolar lavage cells from asthma patients upon ragweed allergen challenge. Later, in 2002, a Japanese team demonstrated that a coding region variant of IL-17F gene is linked to chronic inflammatory airway diseases. Recent studies proved that airway IL-17F expression positively correlates the number of neutrophils and asthma severity. In this study, BALF IL-17F level in TDI-exposed mice was higher than in control ones. In line with the findings in HDM-induced model, neutralizing IL-17F with monoclonal antibody resulted in considerably subsided bronchial neutrophil infiltration, ameliorated airway epithelial injury and hyperplasia, decreased AHR and blunted Th17 related responses, together with less eosinophils (though not significant) in the airway. At the same time, IL-17F mab treatment also suppressed the excessive secretion of IL-6, IL-18 and the upregulated IL1β expression in TDI asthmatic mice, all of which are critical mediators for neutrophil migration and activation. Opposite results were seen when the TDI sensitized 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, whilst IL-17F contributes to TDI-induced airway neutrophilic inflammation and bronchial hyperresponsiveness, which might be a effective therapeutical targets for severe asthma in the future.

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Author contributions
Y.L. and T.H. designed the experiment, analyzed the results and wrote the manuscript. Y.L., C.S. and T.H. performed the experiments. C.R., Z.Q., H.P. and W.S. helped with the experiment and data analysis. C.R. and Z.Q. helped to revise the manuscript. All the authors agreed that the final approval of the version to be published and ensured questions relating to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Conflicts of interest
The authors declare that they have no conflicts of interest.

References


FIGURE LEGENDS

FIG 1. Inhaled fluticasone propionate (FP) or systemic prednisone (Pred) had no effects on TDI-induced airway inflammation and bronchial hyperreactivity. **a**, Representative H&E stained and PAS stained lung sections of different groups. Original magnification was 200×. **b-c**, Semiquantification of airway inflammation and analysis of airway smooth muscle thickness were performed. n=8~10. **d**, Quantification of PAS-positive staining was determined by counting the number of PAS-positive epithelial cells. n=8~10. **e**, Expression of Muc5ac gene (qPCR) in the lung. n=4. **f**, Airway hyperresponsiveness was measured by lung resistance (R_L). Results were shown as percentage of baseline value. n=5. **g**, Numbers of total inflammatory cells and neutrophils, eosinophils. n=8~10. *p<0.05; **p<0.01.

FIG 2. IL-17A monoclonal antibody (IL-17A mab) and IL-17F monoclonal antibody (IL-17F mab) had opposite effects on TDI-induced airway hyperreactivity and bronchial inflammation. **a**, Representative H&E- and PAS- stained lung sections of different groups. Original magnification was 200×. **b-c**, Semiquantification of airway inflammation as well as analysis of airway smooth muscle thickness was performed. n=8~10. **d**, Quantification of PAS-positive staining was determined by counting the number of PAS-positive epithelial cells. n=8~10. **e**, Expression of Muc5ac gene (qPCR) in the lung. n=4. **f**, Airway hyperresponsiveness was measured by lung resistance (R_L). Results were shown as percentage of baseline value. n=5. **g**, Numbers of total inflammatory cells, as well as neutrophils, eosinophils in BALF. n=8~10. *p<0.05; **p<0.01. No significant differences were observed between TDI group and TDI+isotype IgG group.

FIG 3. Blocking IL-17A and IL-17F displayed different capacity for orchestrating Th2/Th17 responses. **a**, Percentages of CD4 IFNγ+ (Th1), CD4 IL4+ (Th2), CD4 IL17A+ (Th17) cells in BALF CD4+ cells. n=5. **b**, Percentages of CD4 IFNγ+, CD4 IL4+, CD4 IL17A+ cells in CD4+ cells of lung single cell suspensions. n=5. **c**, Levels of IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-6, IL-18, CCL11 and CCL24 in BALF were quantified by multiplex immunoassays or ELISA. n=8~10. *p<0.05; **p<0.01. No significant differences were observed between TDI group and TDI+isotype IgG group.
FIG 4. Neutralizing IL-17A and IL-17F had different effects TDI-induced pulmonary expression of Th2 and Th17 markers. a, IL-4, IL-5, IL-13, IL-17A, IL-17F and IL1β in whole lung homogenates were quantified by multiplex immunoassays or ELISA. n=4. b, Whole lung tissue expression of Th2 markers Clca3 and Ccl11, and Th17 markers Cxcl3, Cxcl1, and Csf3, as well as Il17a and Il17f were assessed by qPCR. n=4. c, Total serum IgE concentrations were determined. n=8~10. *p<0.05; **p<0.01. No significant differences were observed between TDI group and TDI+isotype IgG group.
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 (RL) measurement (Buxco Electronics, Troy, NY, USA) mice in response to grading doses of aerosolized methacholine (6.25, 12.5, 25 and 50 mg/mL). RL 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™ 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 1.
### TABLE 1. Murine qPCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence (5’→3’)</th>
<th>Reverse sequence (5’→3’)</th>
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<tbody>
<tr>
<td>Gapdh</td>
<td>AAGAGGGATGCTGCCCTTAC</td>
<td>CCAATACGGCCAAATCCGTTC</td>
</tr>
<tr>
<td>Muc5ac</td>
<td>CAGGACTCTCTGAAATCGTACCA</td>
<td>AAGGCTCGTACCACAGGGA</td>
</tr>
<tr>
<td>Il17a</td>
<td>GAGAGCTTCTCTGTGTCTCTCTG</td>
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<tr>
<td>Il17f</td>
<td>CGTGAAACAGCCATGGTCAAG</td>
<td>GGGACAGAAATGCCCTGGTT</td>
</tr>
<tr>
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</tr>
<tr>
<td>Csf3</td>
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<td>TCGGGATCCCCAGAGAGT</td>
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### References


FIGURE LEGENDS

FIG S1. Experimental treatment schedules. a, Mice were dermally sensitized with 0.3% TDI on days 1 and 8. On days 15, 18 and 21, the mice were challenged with 3% TDI through compressed air nebulization. Prednisone (Pred, 5 mg/kg) and Fluticasone propionate (FP, 300 μg/kg) were respectively given to the mice through intraperitoneal injection or intranasal instillation once daily, beginning immediately after the first challenge to the last day of challenge for a consecutive of seven days. b, Mice were dermally sensitized and challenged with TDI. Anti-IL-17A monoclonal antibody, anti-IL-17F monoclonal antibody, or the isotype control antibody was administered separately via the intraperitoneal route at the dose of 100 μg/mouse per time immediately after each airway challenge. c, Mice were dermally sensitized and challenged with TDI. Recombinant IL-17A or IL-17F was administered intranasally at the dose of 1 μg/mouse per time immediately after each airway challenge.

FIG S2. Recombinant IL-17A and IL-17F had different effects on TDI-induced pathological changes as well as airway neutrophilic and eosinophilic inflammation. a, Representative H&E- and PAS-stained lung sections of different groups. Original magnification was 200×. b-c, Semiquantification of airway inflammation and analysis of airway smooth muscle thickness were performed. n=8~10. d, Quantification of PAS-positive staining was determined by counting the number of PAS-positive epithelial cells. n=8~10. e, Expression of Muc5ac gene (qPCR) in the lung. n=4. f, Airway hyperresponsiveness was measured by lung resistance (R_L). Results were shown as percentage of baseline value. n=8~10. *p<0.05; **p<0.01.

FIG S3. Exogenous IL-17A and IL-17F showed distinct roles for TDI-induced Th2 and Th17 responses. a-b, Levels of different cytokines in BALF or lung homogenates were quantified by multiple immunoassays or ELISA. n=6~8. c, Percentages of CD4 IFNγ+ (Th1), CD4 IL4+ (Th2), CD4 IL17A+ (Th17) cells in BALF or lung CD4+ cells. n=5. d, Whole lung tissue expression of Th2 markers Clca3 and Ccl11, Th17 markers Cxcl3, Cxcl1, and Csf3, as well as Il17a, Il17f were assessed by qPCR. n=4. *p<0.05; **p<0.01.
a) 0.3% TDI sensitization
Day 1
8

3% TDI inhalation
15
18
21
22
23
Analysis
Sacrifice
Pred, FP given once daily for 7 days after inhalation

b) 0.3% TDI sensitization
Day 1
8

3% TDI inhalation
15
18
21
22
23
Analysis
Sacrifice
Antibodies given i.p. after each inhalation

c) 0.3% TDI sensitization
Day 1
8

3% TDI inhalation
15
18
21
22
23
Analysis
Sacrifice
Recombinant IL17 given i.n. after each inhalation
**Figure captions**

**Figure a:** H&E and PAS staining of tissue samples from different groups: control, TDI, TDI+IL17A, and TDI+IL17F.

**Figure b:** Bar graphs showing the number of inflammatory cells in peribronchial and perivascular regions.

**Figure c:** Graphs illustrating the thickness of airway smooth muscle.

**Figure d:** Graphs showing the percentage of cells.

**Figure e:** Graphs representing the levels of mRNA expression.

**Figure f:** Graphs depicting the effects of methacholine on baseline resistance and cell counts.