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™ 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**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence (5'→3')</th>
<th>Reverse sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>AAGAGGGATGCTGCCCTTAC</td>
<td>CCAATACGCGCCAATCCGTTC</td>
</tr>
<tr>
<td>Muc5ac</td>
<td>CAGGACTCTCTGAAATCGTACCA</td>
<td>AAGGCTCGTACCACAGGGGA</td>
</tr>
<tr>
<td>Il17a</td>
<td>GAGAGCTTCATCTGTCTCTTG</td>
<td>GCCGCAAGGGAGTTAAGAC</td>
</tr>
<tr>
<td>Il17f</td>
<td>CGTGAAACAGCCATGGGTAAGAG</td>
<td>GGGACAGAAATGCCCTGTT</td>
</tr>
<tr>
<td>Ccl11</td>
<td>TGCTCACGTCACTTCCCTTC</td>
<td>CTTGAAGACTATGGCTTTCAGGGTG</td>
</tr>
<tr>
<td>Clca3</td>
<td>AGGAAAACCCCAAGCAGTG</td>
<td>GCACCGACGAACCTGTATTTTT</td>
</tr>
<tr>
<td>Cxc11</td>
<td>AACCAGGTAGCCACACT</td>
<td>CCGTTACTTGGGGGACACCTT</td>
</tr>
<tr>
<td>Cxcl3</td>
<td>CACCCAGACAGAAGTACAGCC</td>
<td>CCGTGGGATGGATCGCTTT</td>
</tr>
<tr>
<td>Csf3</td>
<td>GTGCTGCTGGAGCAGTTGT</td>
<td>TCGGGATCCCCAGAGAGT</td>
</tr>
</tbody>
</table>

**References**


