Original Article
Dynamic alteration of CD4+ and CD4- effector/memory T-cell levels in an asthmatic mouse model

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Abstract: Objective: The aim of the current study was to investigate the timing of CD4+ and CD4- effector memory T-cell (TEM) expression levels during an antigen challenge in an asthmatic mouse model. Methods: A total of 51 mice were sensitized with ovalbumin (OVA), then challenged with OVA for 7 consecutive days (asthma group). Another 6 mice were sensitized and challenged with phosphate-buffered saline (PBS) (control group). Spleen and lung cells were harvested, at various time points, up to 30 days after the final challenge. Percentages of CD4+ and CD4- TEM cells (CD3+CD4+/CD4-CD44HighCD62L-), out of the total CD4+ and CD4- T-cell populations, were determined by flow cytometry. Results: Percentages of CD4+ and CD4- TEM cells were higher in OVA-challenged mice than in control mice at 48 hours after the final OVA challenge. In the asthma group, percentages of CD4+ TEM cells in the lungs increased rapidly during the OVA challenge. Levels were continuously elevated after the challenge ended, reaching a peak (55.69 ± 2.18%) at day 6 after the final challenge. Levels then declined, gradually, to 20.04 ± 4.39% at day 30 after the final challenge. Percentages of CD4- TEM cells in the lungs also increased, gradually, during the OVA challenge period. However, levels peaked (61.97 ± 3.22%) earlier at 2 days after the final challenge. They then decreased more rapidly to less than 20% at 2 weeks after the final challenge. Conclusion: CD4+ and CD4- TEM cells exhibited different expression patterns during and after the OVA challenge.

Keywords: Bronchial asthma, immunologic memory, flow cytometry, animal model, T-cell

Introduction

Asthma, one of the most prevalent chronic diseases worldwide, is characterized by chronic airway inflammation. This results in recurrent attacks of coughing and wheezing, as well as chest tightness and variable airflow obstruction. Over time, the airflow obstruction may become irreversible due to airway remodeling [1]. There are many mechanisms involved in the pathogenesis of asthma, including heredity, respiratory infections, neuro-regulation, and immunological mechanisms. For many years, asthma has been considered a T helper (Th) 2 cell-mediated disorder. However, in recent years, other cell types, such as regulatory T (Treg) cells, Th1 cells, and Th17 cells, have been found to be involved in the pathogenesis of asthma [2, 3]. Effector memory T-cells (TEM) play a particularly important role. Airway inflammation in asthma has been associated with activated T-cells, as well as cytokines secreted after bronchial allergen challenge [4]. Some activated T-cells develop into TEM cells, which have been shown to survive for more than a year in a mouse model of asthma [5]. When these TEM cells are re-exposed to the same allergen, they rapidly reactivate and differentiate into Th2 cells, accompanied with secretion of the same cytokines that induced the original symptoms of asthma. A very important cell type in immune response, CD4+ TEM cells play critical roles in many physio-pathological mechanisms, including graft-versus-host disease [6, 7], cancer [8], response to vaccines [9, 10], defense against various pathogens [11-13], and asthma [5, 14]. Although TEM cells can cause eosinophil accumulation, bronchial over-response, and asthma symptoms in asthma [15], few studies have focused on dynamic fluctuations of TEM cells during immune responses in asthma. Therefore, the current study analyzed the timing of TEM cell development, during and after antigen challenge, in a mouse model of asthma. Mouse TEM cells were defined by high expression of the CD44 surface...
Memory T-cells in an asthmatic mouse model

Marker. Mouse TEM cells were further classified as CD62L+ central memory T (TCM) cells and CD62L- TEM cells [16, 17]. Dynamic alteration levels of CD4+ and CD4- TEM cells (defined as CD4+/CD3+CD44HighCD62L- T cells) were investigated in detail. Understanding the development of TEM cells in the pathogenesis of asthma may help to elucidate the mechanisms of asthma, providing theoretical evidence and guidance for new therapeutic strategies.

Methods

Asthmatic mouse model

Female BALB/c mice, aged 6-8 weeks and weighing 18-22 g, were obtained from the Center of Laboratory Animals, School of Basic Medical Sciences, Jilin University. They were maintained in a pathogen-free facility with constant humidity and temperature levels. They were exposed to a 12-hour light/dark cycle and given free access to food and water. Inducing bronchial asthma, 57 mice were randomized into either the asthma (n = 51) group or normal control (n = 6) group. Mice in the asthma group were sensitized by intraperitoneal injections with 10 µg of ovalbumin (OVA, grade V, Sigma-Aldrich, St. Louis, MO, USA) and 2 mg of aluminum hydroxide (Pierce Biotechnology, Rockford, IL, USA) in 100 µL of phosphate-buffered saline (PBS) on days 0, 7, and 14. Daily challenges with aerosolized 2% OVA for 1 hour began one week after the last sensitization. They continued for a total of 7 consecutive days. PBS was used to replace OVA in the normal control group. Levels of IgE, Interleukin-4, and Interferon-γ were detected by ELISA using specific kits, according to manufacturer instructions. Briefly, 50 ul assay diluent was added into each well of a 96-well dish containing testing cells. Next, 50 ul standard, reference, or sample was added to the well and mixed gently for 1 minute. It was then covered with an adhesive strip. The dish was incubated at room temperature for 2 hours after covering with an adhesive strip. It was washed 5 times, added with 100 ul conjugate, sealed with a new adhesive strip, and incubated at room temperature for 2 hours. After washing another 5 times, each well was added with 100 ul substrate solution, incubated at room temperature for 30 minutes avoiding light, and added with 100 ul Termination fluid. Optical density values were detected at 450 nm with a spectrometric reader within 30 minutes.

Fluorescence-activated cell sorting (FACS) analysis

Preparing the spleen cells, mouse spleens were cut into small pieces. They were mashed by two ground glass plates, filtered twice through a 200-mesh strainer, and washed twice with PBS solution. Obtained cells were resuspended in RPMI 1640 culture medium (Hyclone, Omaha, NE, USA), counted, and adjusted to a concentration of 5 × 10^6/mL. Preparing the lung cells, the mice were anesthetized. The right atrium of each heart was punctured with a needle. Hemoperfusion was performed with PBS until the lungs turned white. The right lung was then harvested and single cells were prepared according to the same process used for the isolation of spleen cells.

Regarding flow cytometric analysis, 100 µL of the cell suspension (5 × 10^6 cells) was mixed with fluorophore-conjugated antibodies, as shown in Table 1. For the other tests, cells were labeled with FITC-conjugated anti-mouse CD4, PE-Cy7-conjugated anti-mouse CD3e.
Memory T-cells in an asthmatic mouse model

Table 2. Levels of serum interleukin (IL)-4, interferon (IFN)-γ, and IgE in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IL-4 (pg/mL)</th>
<th>IFN-γ (pg/mL)</th>
<th>IgE (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>6</td>
<td>47.95 ± 5.03*</td>
<td>2.15 ± 1.02*</td>
<td>115.4 ± 7.09*</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>13.19 ± 1.52</td>
<td>6.81 ± 1.94</td>
<td>11.29 ± 1.31</td>
</tr>
</tbody>
</table>

Note: * compared with normal control group, P < 0.0001; ▲ compared with normal control group, P < 0.001.

Table 3. Counts of eosinophil and neutrophil in BALF of each group

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Eos</th>
<th>Neu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>6</td>
<td>29.17 ± 6.18*</td>
<td>2 ± 0.89*</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>2 ± 1.41</td>
<td>0.33 ± 0.52</td>
</tr>
</tbody>
</table>

Eos: eosinophils; Neu: neutrophils; * compared with normal control group, P < 0.0001; ▲ compared with normal control group, P < 0.01.

PE-conjugated anti-mouse CD44, and APC-conjugated anti-mouse CD62L. Controls cells were labeled with FITC-conjugated anti-mouse CD4, PE-Ey7-conjugated anti-mouse CD3e, PE-conjugated rat IgG2bx, and APC-conjugated rat IgG2bx. Negative controls contained cells without antibodies. All antibodies were from BD Pharmingen (San Jose, CA, USA). Diluted BD FACS Lysing Solution (BD Pharmingen, 1 mL) was mixed with the test cells and the corresponding antibodies for 5-10 min. The cell mixtures were then centrifuged, washed with PBS, resuspended in PBS (cells from one lung were suspended in 300 µL), and sorted on a BD FACS Calibur flow cytometer (BD Pharmingen). Finally, the cells were sorted for CD3 and CD4 expression, then resorted for CD44 and CD62L expression. Percentages of CD3^+CD4^+ CD44^hi/CD62L TEM cells and CD3^+CD4^+ CD44^hi/CD62L TEM cells, of the total amount of CD3^+CD4^+ T-cells, were calculated.

Statistical analysis

Data from the asthma group and normal control group were compared using unpaired Student’s t-tests. P < 0.05 indicates statistical significance. Data are presented as mean ± standard error of the mean.

Results

Establishment of the asthmatic mouse model

The established asthmatic mouse model was verified by multiple tests. Mice in the asthma group exhibited scratching of the head and nose, as well as sneezing, on the second day after OVA inhalation. This was followed by weak shortness of breath, abdominal muscle twitching, weight loss, coat color loss, and luster deterioration. Mice in the normal group did not show any of the above symptoms. Serum levels of IgE and IL-4 in the asthma group were significantly higher than those in the normal group (P < 0.01). Serum levels of IFN-γ in the asthma group were significantly lower than those in the normal group (P < 0.01; Table 2). In addition, eosinophil and neutrophil counts in the bronchoalveolar lavage fluid in the asthma group were significantly higher than those in the normal group (P < 0.01; Table 3).

Lung tissue samples of the normal group showed that the bronchial epithelium was intact. There was no infiltration of inflammatory cells. However, samples from the asthma group presented inflammatory cell infiltration of the bronchial mucosa and submucosa on the bronchial wall and its adjacent vessels, including lymphocytes, eosinophils, and neutrophils. These were accompanied by goblet cell hyperplasia, smooth muscle thickening, lumen constriction, and adjacent small artery wall thickening and constriction (Figure 1). These alterations verified the establishment of an asthmatic mouse model.

Percentages of CD4^+ TEM cells and CD4^+ TEM cells in the OVA-challenged mice

Numbers of CD4^+ and CD4^+ T-cells (CD3^+), as well as TEM cells (CD44^hi/CD62L) (Figure 2), were evaluated by flow cytometry at 48 hours after the OVA challenge. Percentages of TEM cells are shown in Tables 4 and 5. Percentages of CD4^+ TEM cells, of the total CD4^+ T-cells (55.69 ± 2.18%), in the asthma group were significantly higher than those in the normal control group (11.32 ± 1.72%) in the lungs (P < 0.0001). The distribution of the percentages of CD4^+ TEM cells in the lungs showed a similar pattern (61.97 ± 3.22% vs. 12.24 ± 2.18%) (P < 0.0001). Present data indicates that pulmonary CD4^+ and CD4^+ TEM cells may play important roles in asthma. Notably, the percentage of CD4^+ TEM cells was obviously higher than that of CD4^+ TEM cells in the asthma group (P < 0.05). However, the number of CD4^+ TEM cells rapidly declined (Figure 4). These alterations suggest that CD4^+ TEM cells are likely involved in the onset of asthma.
In mice spleens, the percentage of CD4+ TEM cells, of the total CD4+ T cells (32.1 ± 2.83%), was significantly higher than that in the normal control group (20.48 ± 2.38%) (P < 0.0001). Similarly, the percentage of CD4+ TEM cells, of the total CD4- T cells, in the asthma group was much higher than that of the control group (33.64 ± 3.93% vs. 18.14 ± 1.63%; P < 0.0001; Table 5). Results suggest that spleen CD4+ and CD4- TEM cells are implicated in the pathogenesis of asthma. However, there were no differences between the percentages of CD4+ TEM cells and CD4- TEM cells in the asthma group in the spleens (Table 5).

Interestingly, the percentage of TEM cells in the lungs, whether they were CD4+ or CD4- TEM cells, was much lower than that in the spleens. However, after sensitization and aerosol challenge with 2% OVA by inhalation, percentages of TEM cells in the lungs, whether they were CD4+ or CD4- TEM cells, were dramatically increased. They were significantly higher than levels in the spleens (Tables 4 and 5). These findings may help explain the different roles of TEM cells in different organs, including the lungs and spleen.

**Alterations of TEM cell percentages over time in OVA-challenged mice**

Percentages of CD4+ and CD4- TEM cells in the spleen and lung cells in the asthma group were monitored on the day before the initial OVA challenge to 30 days after the final challenge. Percentages of CD4+ TEM cells, of the total CD4+ T cells, were 17.34 ± 0.22% and 20.63 ± 0.99% in the lungs and spleens, respectively, before the initial challenge. The percentage of CD4+ TEM cells in the lungs increased to 36.68 ± 6.10% on day 4 after the initial challenge. This number remained around 40-45% for several days, then elevated again after the final OVA challenge. It reached a peak (55.69 ± 2.18%) at day 2 after the final challenge, then remained over 50% for 7 days after peaking. Afterward, the ratio declined, gradually, to 20.04 ± 4.39% at day 30 after the final OVA challenge (Figure 3).

The percentage of CD4+ TEM cells in the lungs and spleens was in accord with that in the lungs. However, the overall increased value was relatively lower and happened slightly later than that in the lungs. The percentage of CD4+ TEM cells in the spleens reached a peak (45.16 ± 3.40%) at day 7 after the final OVA challenge, then decreased gradually to 25.01 ± 1.20% at day 16 after the final challenge. Levels remained at 25-30% through day 30 (Figure 3). The baseline percentage of CD4- TEM cells in the lungs and spleens was 26.99% and 22.12 ± 3.77%, respectively, before the initial challenge. The ratio in the lungs gradually elevated and reached a plateau of 51.13 ± 1.54% on day 4 after the initial challenge. Levels increased again after the final challenge, reaching a peak (61.97 ± 3.22%) on day 2 after the final challenge. Levels then decreased rapidly to less than 20% within 2 weeks after the final challenge (Figure 4). In contrast, CD4- TEM cell percentages in the spleens remained around the baseline level of approximately 20% during the OVA challenge, then increased rapidly to 33.64 ± 3.93% after the final challenge. Levels remained constant between 18-30% for 2 weeks, then returned to the baseline level by day 30 (Figure 4). The alternating pattern of CD4- TEM cells in the spleens, during and after the OVA challenge, was quite different from that in the lungs. It was also quite different from the CD4+ TEM cell alternating pattern.

**Discussion**

Flow cytometry has been widely applied for detection of cellular types, quantitatively and qualitatively, according to specific cellular markers. In the context of asthma, flow cytometry has been widely accepted for identification of TEM cells (CD44hiCD62Llo) and TCM cells...
Memory T-cells in an asthmatic mouse model

Antigens play an important role in the production of memory T-cells and activation of naive CD4+ T-cells to produce lymphokines. These are needed for proliferation and generation of lymphoblast-derived effector cells, as well as the production of memory cells [21]. TEM cells have the characteristics of effector cells. They

Figure 2. Identification of TEM cells by flow cytometry. Spleen and lung cells were harvested at 48 hours after the final OVA challenge (asthma group) or PBS administration (control group) and detected by flow cytometry. CD3^+CD4^-/CD44^hiCD62L^- TEM cells were identified using FITC-conjugated anti-mouse CD4, PE-Cy7-conjugated anti-mouse CD3e, PE-conjugated anti-mouse CD44, and APC-conjugated anti-mouse CD62L. For isotype controls, FITC-conjugated anti-mouse CD4, PE-Cy7-conjugated anti-mouse CD3e, PE-conjugated rat IgG2bκ, and APC-conjugated rat IgG2bκ were used. No antibodies were added in the control group.
Table 4. Percentages of TEM cells at 48 hours after the final OVA challenge in the lungs

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CD4⁺ TEM cells/CD4⁺ T cells</th>
<th>CD4⁻ TEM cells/CD4⁻ T cells</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>6</td>
<td>55.69 ± 2.18*</td>
<td>61.97 ± 3.22</td>
<td>0.003</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>11.32 ± 1.72*</td>
<td>12.24 ± 2.18</td>
<td>0.434</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Note: *P > 0.05 compared with CD4⁺ TEM cells/CD4⁺ T-cells;  
*P < 0.05 compared with CD4⁻ TEM cells/CD4⁻ T-cells.

Table 5. Percentages of TEM cells at 48 hours after the final OVA challenge in the spleens

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CD4⁺ TEM cells/CD4⁺ T cells</th>
<th>CD4⁻ TEM cells/CD4⁻ T cells</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>6</td>
<td>32.1 ± 2.83*</td>
<td>33.64 ± 3.93</td>
<td>0.454</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>20.48 ± 2.38*</td>
<td>18.14 ± 1.63</td>
<td>0.0752</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Note: *P > 0.05 compared with CD4⁺ TEM cells/CD4⁺ T-cells.

Memory T-cells in an asthmatic mouse model

TEM cells may participate in the pathogenesis of asthma, but with a different pattern. The percentage of CD4⁺ TEM cells reached a peak on day 2 after the final challenge. However, levels declined rapidly and completely the following two weeks. These results suggest that CD4⁺ TEM cells and CD4⁻ TEM cells may play different roles in asthma. They also suggest that pathophysiological changes in the lungs of OVA-challenged mice are more likely to be mediated by CD4⁺ TEM cells, as they remain at high levels in the lungs for a longer time than CD4⁻ TEM cells. The finding that CD8⁺ T-cells act as important helpers for CD4⁺ T-cells during asthma [25] may explain these results, to some extent. T lymphocytes include two main subsets, CD4⁺ T-cells and CD8⁺ T-cells. Thus, the majority of the CD4⁺ T-cells, in the current study, was CD8⁺ T-cells. The high number of CD4⁺ TEM (presumably

CD8+ cells observed during the onset phase of asthma in this study may act as helpers for the relatively low number of CD4+ T-cells. In later phases of the disease, as the number of CD4+ TEM cells increases, the need for helper cells would be expected to decrease, perhaps explaining the rapid decrease of CD4- cells after the onset phase of asthma.

In summary, present results suggest that both CD4+ and CD4+ TEM cells participate in the pathogenesis of asthma in a mouse model. However, their roles were shown to be different because of different dynamic alteration patterns during and after the OVA challenge. Further exploration is warranted, examining the mechanisms in which these lymphocyte subtypes affect asthma development.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

FACS, fluorescence-activated cell sorting; OVA, ovalbumin; PBS, phosphate-buffered saline; TCM cells, central memory T-cells; TEM cells, effector memory T-cells; Th, T helper; Treg cells, regulatory T-cells.

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References


