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Original Article

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

Yanchun Li[1], Fan Li[2], Xiuhua Yu[1], Li Liu[1]

[1]First Hospital of Jilin University, Changchun, China; [2]Changchun Institute of Applied Chemistry, Chinese Academy of Science, Changchun, China

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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 ($CD3^+CD4^+/CD4^-CD44^{High}CD62L^{neg}$), 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 overresponse, 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

Table 1. Antibodies for flow cytometric analysis

	FITC	PE	PE-Cy7	APC
Experimental samples	CD4	CD44	CD3e	CD62L
Isotype control	CD4	Rat IgG2bκ	CD3e	Rat IgG2bκ
Negative control	-	-	-	-

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 $CD4^{+/-}CD3^+CD44^{High}CD62L^-$ 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. Eosinophil and neutrophil counts in bronchoalveolar lavage fluid and hematoxylin and eosin staining of lung tissues were used to establish the asthmatic mouse model. Every several (2-5) days, beginning the day before the first challenge to 30 days after the last challenge, three mice in the asthma group, at each time point, were sacrificed. Spleens and lung cells were isolated for flow cytometry. At 48 hours after the last challenge, six mice in the asthma group and six mice in the normal control group were sacrificed. Their spleens and lung cells were isolated.

The current experimental protocol was approved by the Ethics Committee of Jilin University, Changchun, China.

ELISA

Serum IgE, IL-4, and IFN-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 (5×10^5 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,

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

Group	n	IL-4 (pg/mL)	IFN-γ (pg/mL)	IgE (μg/mL)
Asthma	6	47.95 ± 5.03$^{\Delta}$	2.15 ± 1.02$^{\blacktriangle}$	115.4 ± 7.09$^{\Delta}$
Control	6	13.19 ± 1.52	6.81 ± 1.94	11.29 ± 1.31

Note: Δ: 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

Group	n	Eos	Neu
Asthma	6	29.17 ± 6.18$^{\Delta}$	2 ± 0.89$^{\blacktriangle}$
Control	6	2 ± 1.41	0.33 ± 0.52

Eos: eosinophils; Neu: neutrophils; Δ: 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-Cy7-conjugated anti-mouse CD3e, PE-conjugated rat IgG2bκ, and APC-conjugated rat IgG2bκ. 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 FACSCalibur flow cytometer (BD Pharmingen). Finally, the cells were sorted for CD3 and CD4 expression, then resorted for CD44 and CD62L expression. Percentages of $CD3^+CD4^+$-$CD44^{High}CD62L^-$ TEM cells and $CD3^+CD4^-$ $CD44^{High}CD62L^-$ 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 ($CD44^{High}CD62L^-$) (**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.

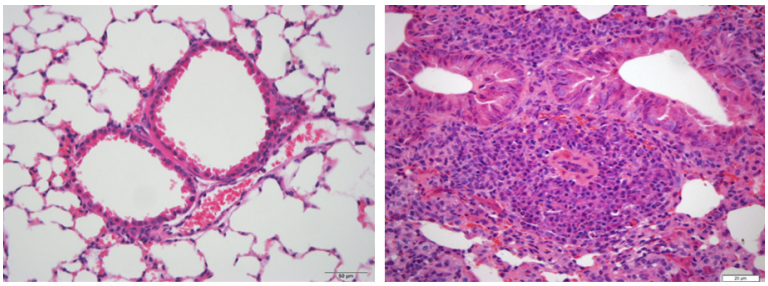

Figure 1. Hematoxylin and eosin staining of lung tissues in indicated groups (400 × magnification).

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, over time, in the 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 ($CD44^{hi}CD62L^{lo}$) and TCM cells

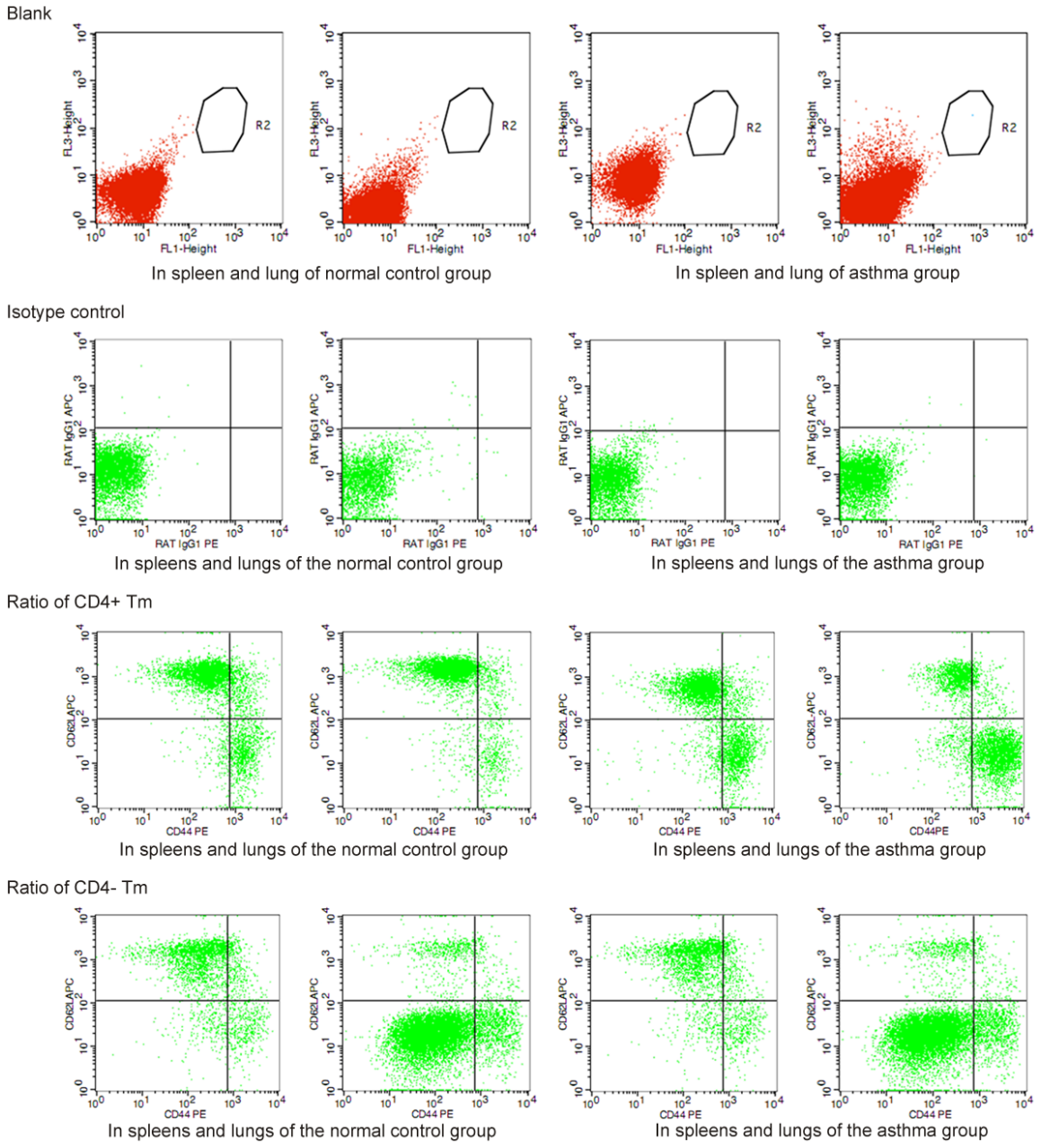

Figure 2. Identification of TEM cells by flow cytometry. Spleen and lung cells were harvested at 48 hours after the final OVA challenge (asthma group) or PBS administration (control group) and detected by flow cytometry. $CD3^{+}CD4^{+/-}CD44^{High}CD62L^{-}$ TEM cells were identified using FITC-conjugated 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.

($CD44^{hi}CD62L^{hi}$) in mice lungs [18, 19], central memory ($CD45^{RA-}CCR7^{+}$) $CD8^{+}$ T-cells, and effector memory ($CD45RA^{+/-}CCR7^{-}$) $CD8^{+}$ T-cells from human peripheral blood [20]. Therefore, in the current study, flow cytometry was applied, identifying both $CD4^{+}$ and $CD4^{-}$ TEM cell levels.

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

Table 4. Percentages of TEM cells at 48 hours after the final OVA challenge in the lungs

Group	n	Lung		*P*
		$CD4^+$ TEM cells/ $CD4^+$ T cells	$CD4^-$ TEM cells/ $CD4^-$ T cells	
Asthma	6	$55.69 \pm 2.18^{\Delta}$	61.97 ± 3.22	0.003
Control	6	$11.32 \pm 1.72^{\#}$	12.24 ± 2.18	0.434
p		< 0.0001	< 0.0001	

Note: $^{\#}P > 0.05$ compared with $CD4^-$ TEM cells/$CD4^-$ T-cells; $^{\Delta}P < 0.05$ 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

Group	n	Spleen		*P*
		$CD4^+$ TEM cells/ $CD4^+$ T cells	$CD4^-$ TEM cells/ $CD4^-$ T cells	
Asthma	6	$32.1 \pm 2.83^{\#}$	33.64 ± 3.93	0.454
Control	6	$20.48 \pm 2.38^{\#}$	18.14 ± 1.63	0.0752
p		< 0.0001	< 0.0001	

Note: $^{\#}P > 0.05$ compared with $CD4^-$ TEM cells/$CD4^-$ T-cells.

rapidly produce microbicidal lymphokines during secondary immune responses. In contrast, TCM cells proliferate to produce new effector cells. They recreate themselves during secondary immune responses [21]. The current examination of the development of TEM cells in OVA-sensitized and OVA-challenged mice demonstrated that percentages of $CD4^+$ and $CD4^-$ TEM cells were much higher in the spleens and lungs of OVA-sensitized mice, compared with those in normal control mice. These findings are consistent with the results of Mojtabavi et al. They identified allergen-specific memory Th2 cells in both the lungs and spleens in an OVA-induced asthmatic mouse model [5]. Given the important roles of antigens in the production of TEM cells, it is not surprising that mice exposed to OVA developed more TEM cells than control mice. In the control mice without OVA challenge, ratios of $CD4^+$ and $CD4^-$ TEM cells were higher in the spleens than in the lungs. In contrast, in OVA-challenged mice, the ratio was higher in the lungs than in the spleens. Current findings suggest that TEM cells accumulate in the local attacked organ rather than in the lymphatic system, even though sensitization is systematic. Circulation patterns of memory T-cells may account for these results. TEM cells can circulate between lymphoid tissues and nonlymphoid tissues during steady-state conditions. However, they are largely excluded from lymph nodes. Indeed, most TEM cells locate to nonlymphoid sites. TCM cells, in contrast, mainly circulate in lymphoid tissues (lymph nodes, spleen, and bone marrow) and the blood [22]. In addition, TEM cells detected in this study may include tissue-resident memory cells, a recently identified subset of memory cells that express low levels of CCR7 and CD62L, as with TEM cells. However, these tissue-resident memory cells have high levels of local nonlymphoid tissue-homing molecules, such as CD103 and CD69. These cells reside in the peripheral nonlymphoid tissues long after the initial immune response [23]. During the observation of quantitative TEM cells, over time, in the asthma group, it was found that $CD4^+$ TEM cells peaked at day 2 after the final OVA challenge. They were maintained at a high level until day 9 after the final OVA challenge. Moreover, levels were still slightly higher than the baseline level at day 30 after the final challenge. In a study of OVA-sensitized mice, Th2 memory cells were reported to be able to exist in the lungs for more than 400 days. This allows the mice to recover from acute diseases and recover after re-exposure to aerosolized OVA over 400 days [5]. These cells have been shown to contribute to generation, persistence, and progression of asthma [5, 24]. Present findings revealed dynamic and quantitative fluctuations of $CD4^+$ TEM cells during the generation, progression, and remission of an immune response in asthmatic mice.

In addition, $CD4^-$ 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

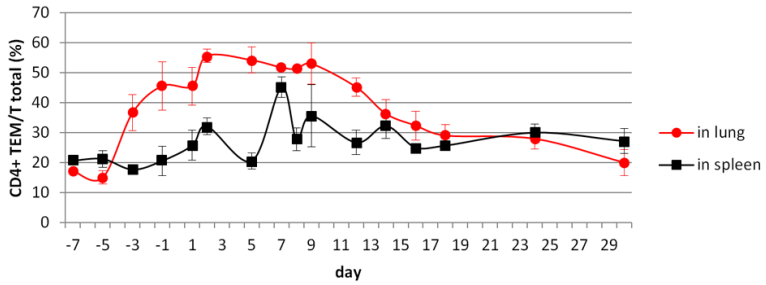

Figure 3. Alteration of percentages of $CD4^+$ TEM cells of the total $CD4^+$ T-cells during and after the OVA challenge. Spleen (red) and lung (black) cells were harvested from three mice at the indicated times, up to 30 days after the final challenge. Percentages of $CD4^+$ TEM cells ($CD3^+CD4^+CD44^{High}CD62L^-$), of the total $CD4^+$ cells, were determined using flow cytometry.

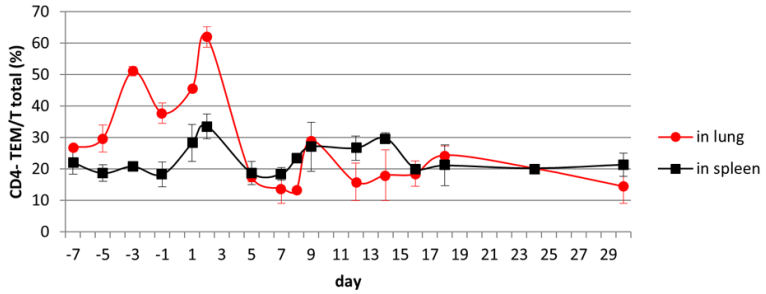

Figure 4. Alteration of percentages of $CD4^-$ TEM cells of the total $CD4^-$ T-cells during and after the OVA challenge. Spleen (red) and lung (black) cells were harvested from three mice at the indicated times, up to 30 days after the final challenge. Percentages of $CD4^-$ TEM cells ($CD3^+CD4^-CD44^{High}CD62L^-$), of the total $CD4^-$ T-cells, were determined using flow cytometry.

$CD8^+$) 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.

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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.

Address correspondence to: Li Liu, The First Hospital of Jilin University, No.71, Xinmin Street, Changchun, Jilin, China. Tel: +86-15804301885; E-mail: liuli0205@163.com

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