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**Increased IL-4- and IL-17-producing CD8⁺ cells are related to decreased
CD39⁺CD4⁺Foxp3⁺ cells in allergic asthma**

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Keywords

allergic asthma, CD39⁺Tregs, CD8⁺IFN- γ ⁺ cells, CD8⁺IL-4⁺ cells, CD8⁺IL-17⁺ cells

Abstract

Objective: In allergic asthma, regulatory T cell (Treg) number and function are decreased.
Antigen primed CD8⁺ T cells play an indispensable role in the full development of airway

inflammation and airway hyper-responsiveness (AHR) occurring in asthma. In this study, we investigated the relationship between subpopulations of CD8⁺ T cells and CD39⁺ Tregs.

Methods: Female C57BL/6 mice were used to develop the model of allergic asthma.

Experimental mice were immunized with ovalbumin (OVA) by intra-peritoneal (i.p) injection and then challenged with OVA by intra-tracheal administration. Control mice were immunized with vehicle by i.p injection and challenged with OVA. Airway inflammation was determined by histology and AHR was measured by an invasive method. Levels of IFN- γ , IL-4 and IL-17 in bronchoalveolar lavage fluid (BALF) were determined by enzyme-linked immunosorbent assay. The frequencies of CD8⁺IFN- γ ⁺ cells (Tc1), CD8⁺IL-4⁺ cells (Tc2), CD8⁺IL-17⁺ cells (Tc17) and CD39⁺Tregs were measured by flow cytometry. The correlation between CD39⁺Tregs and Tc subsets was analyzed by Pearson's test.

Results: Experimental mice displayed phenotypes of allergic asthma, including inflammatory cell infiltration into the lungs, goblet-cell hyperplasia, increased airway resistance, and increased IL-4 and IL-17 in BALF. Compared to control mice, experimental mice displayed lower CD39⁺Tregs and Tc1 but higher Tc2 and Tc17. There was a negative correlation between CD39⁺Tregs and Tc2 or Tc17.

Conclusion: In allergic asthma, increased Tc2 and Tc17 are possibly related to insufficient CD39⁺Tregs.

Introduction

Allergic asthma is a common chronic inflammatory disease characterized by airway inflammation, reversible airway obstruction and AHR. Antigen-primed memory T cell response is crucial to the pathogenesis of airway inflammation. Although some aspects of the memory T cell response mechanism have been elucidated, attention has been focused mainly on the role of CD4⁺ T cells.

Data from a mouse model of allergen-induced asthma demonstrate that CD8⁺ T cells may also secrete Th2-type cytokines such as **interleukin** (IL)-4, IL-5, IL-9 and IL-13 and that antigen-primed CD8⁺ T cells play a central role in the full development of allergic inflammation and AHR[1]. The Tc2 subset of CD8⁺ T cells has been shown to be involved in eosinophilic airway inflammation and AHR by secreting IL-4 and IL-13 [2]. IL-9-producing CD8⁺ T cells (Tc9) strengthen Th2 cell-mediated classic airway inflammation by promoting eosinophil infiltration into airways and lungs [3]. The role of **interferon** (IFN)- γ -producing CD8⁺ T cells (Tc1) in asthma is controversial, with some studies showing that Tc1 cells attenuate eosinophilic airway inflammation[4, 5], and others suggesting that Tc1 cells induce non-eosinophilic airway inflammation [6]. IL-17-producing CD8⁺ T cells (Tc17) have been shown to be involved in airway inflammation [7]. These data suggest a potential role of CD8⁺ T cells in airway inflammation and AHR.

Regulatory T cells (Tregs), distinct from effector CD4⁺ T cells, have the capacity to maintain immune balance [8]. Naturally occurring CD4⁺CD25⁺ Tregs (nTregs) are universally acknowledged to play an essential role in preventing the development of allergic asthma by suppressing the activation and proliferation of effector CD4⁺ T cells [9]. Adaptive Tregs exert anti-inflammatory effects by generating inhibitory cytokines (IL-10, TGF- β and IL-35), as well as cytotoxic T lymphocyte antigen 4, B and T lymphocyte attenuator, and programmed cell death-1 [10]. Recent research suggests that **Adenosine Triphosphate** (ATP) hydrolysis and/or adenosine production represent another suppressive mechanism of Tregs through the co-expression of CD39 and CD73 [11, 12]. This hypothesis is supported by a report that adenosine is able to suppress allergen sensitization in early-life infections [13].

CD39, mainly expressed by immune cells and vascular endothelial cells, can hydrolyze pro-inflammatory extracellular ATP and generate the anti-inflammatory mediator adenosine in tandem with CD73 [14]. In mice CD39 is expressed on almost all Tregs. Tregs from *Cd39*-null mice are unable to fully restrain allograft rejection [12] or to prevent the development of IL-17-mediated autoimmune disease [15]. A link between increased airway bronchospasm and airway inflammation and decreased CD39 activity has been reported [16]. Our previous studies showed that Tregs from *Cd39* null mice cannot fully suppress airway inflammation or prevent CD4⁺CD25⁻ cell (responder T cell) production of cytokines such as IL-4, IL-17, and IFN- γ [17].

Together these data suggest that CD39⁺Tregs play an important role in the regulation of CD4⁺ T cell immune response associated with autoimmunity, allergy and allograft rejection.

Involvement of CD39⁺Tregs in the regulation of CD8⁺ T cells in allergic airway inflammation, however, is not firmly established. In this study, we explore the relationship between CD8⁺ T cell sub-populations and CD39⁺Tregs in a murine model of allergic airway inflammation.

Materials and Methods

Mice

Female C57BL/6 mice (Age, 6-8 weeks; **Weight**, 18-22g) were purchased from the Experimental Animal Center of Wuhan University (Wuhan, China). All mice were housed with specific pathogen-free food and water under a 12 hours light cycle. All animal experiments were approved by the Institutional Animal Ethics Committee of Wuhan University.

Animal experimental protocols

The experimental protocol for OVA-induced asthma model was performed as previously reported [17]. Briefly, experimental mice were immunized by intra-peritoneal (i.p.) injection of 20 µg OVA (Sigma-Aldrich, St Louis, USA) emulsified in 2 mg alum (Pierce, Rockford, USA) in 200 µl vehicle on days 0 and 14. Mice were then challenged by intranasal administration of 100 µg

OVA in 50 µl vehicle on days 25-27. **Control mice were immunized with vehicle by i.p injection and challenged with OVA.**

Measurement of airway hyper-responsiveness (AHR)

Twenty four hours after the final OVA challenge, AHR to methacholine (Sigma-Aldrich, St Louis, USA) was determined by an invasive method using the FinePointe RC system (Wilmington, NC). Mice were anesthetized with pentobarbital (50mg/kg) and then inserted with a tracheostomy tube, followed by mechanical ventilation. Baseline airway resistance was measured using aerosolized phosphate buffered saline (PBS). Airway resistance to increasing concentrations of aerosolized methacholine (ranging from 3.12 to 25 mg/ml) was recorded.

Bronchoalveolar lavage and lung histology

Mice were sacrificed by cervical dislocation twenty four hours after the last challenge. The lungs were lavaged with a total volume of 1.5 ml PBS containing 1 mM sodium EDTA, followed by **lung** resections, as previously described [18]. Cells in bronchoalveolar lavage fluid (BALF) were collected and were stained with Wright-Giemsa for differential cell counts (macrophages, eosinophils, neutrophils and lymphocytes).

Left lungs were fixed in 4% paraformaldehyde buffer, dehydrated, and embedded in paraffin. Lung sections were stained with hematoxylin and eosin (**H&E**) to evaluate airway inflammation and with periodic acid-Schiff (PAS) to evaluate goblet cell hyperplasia and mucus secretion.

Cell isolation and flow cytometric analysis

Twenty four hours after the final OVA challenge, lungs were removed, cut into small pieces, digested in collagenase I (1 mg/mL; Invitrogen, Carlsbad, CA) at 37 °C for 1 hour, and filtered through a 100-µm nylon net filter. Lung mononuclear cells were isolated by centrifugation at 800x g for 20 min at room temperature in a Lymphoprep gradient (density = 1.081 mg/ml; TBD, Tianjin, China), as previously described [17].

Lung mononuclear cells were plated in six-well plates at a density of 1×10^6 cells/well and cultured in RPMI-1640 medium plus 10% fetal bovine serum (FBS, Hyclone, USA). The cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (both from Sigma-Aldrich, St Louis, USA) for 2 hours and then incubated for **another** 4 hours in the presence of 3 µg/ml brefeldin A (eBioscience, St Louis, USA). **The CD3⁺CD8⁺ or CD3⁺CD4⁻ cells were gated as Tc cells in FACS, as was supported by Chen H et al in their study of Th17 and Tc17 and OVA-induced airway inflammation[7]. To identify Tc1 (CD3⁺CD4⁻IFN-γ⁺), Tc2 (CD3⁺CD4⁻IL-4⁺) and Tc17 (CD3⁺CD4⁻IL-17⁺) cells, activated lung mononuclear cells were first stained with FITC-conjugated anti-CD3 and PE-cy7-conjugated anti-CD4 antibodies. Following this surface staining for CD3 and CD4, cells were fixed and permeabilized, then divided into three aliquots. The three aliquots were stained with PE-conjugated anti-IFN-γ, anti-IL-4 and anti-IL-17 antibodies, respectively. Cells were then analyzed using a FACS Aria II flow cytometer and BD FACS Diva software. To identify CD4⁺CD39⁺Foxp3⁺ Treg**

cells, activated lung mononuclear cells were stained with PE-conjugated anti-CD39 and PE-cy7-conjugated anti-CD4 antibodies, followed by fixation, permeabilization and staining with FITC-conjugated anti-Foxp3 antibodies. Stained cells were analyzed by Beckman Coulter with Expo32 software. All antibodies were obtained from eBioscience (St Louis, USA), and all antibody incubations were done for 30 minutes in the dark at room temperature.

Detection of cytokines in BALF

Cytokine levels in BALF supernatants were measured by **enzyme-linked immunosorbent assay** (ELISA) according to the manufacturer's protocol. The lower limit of detection was 4 pg/ml for IL-4 and IL-17 and 15 pg/ml for IFN- γ (eBioscience, St Louis, USA).

Statistical analysis

Data analysis was performed with Student's *t*-test using SPSS 17.0 software. All data were represented as means + standard deviation (SD). Correlation analysis was analyzed by Pearson's test. $P < 0.05$ was regarded as statistical difference.

Results

Cardinal features of the murine model of allergic inflammation

Histological examination with H&E and PAS staining indicated typical asthma features in OVA-exposed mice (Fig. 1A-D). These mainly included inflammatory cell infiltration into the

lungs, goblet-cell hyperplasia and mucus accumulation in the lumen of bronchioles. Total cell counts in BALF were significantly higher in experimental mice than in control mice, as were absolute numbers of eosinophils, neutrophils and lymphocytes (Fig. 2A). **Levels of cytokines IL-4 and IL-17A were higher in BALF from experimental mice than from control mice (Fig. 2B).** AHR was assessed by invasive measurements of airway resistance using a Buxco RC system. In comparison to control mice, experimental mice developed higher airway resistance when stimulated with aerosolized methacholine. Significant difference was observed between the two groups at methacholine concentrations of 6.25 to 25 mg/ml (Fig. 2C).

Frequencies of Tc1, Tc2 and Tc17 in lung mononuclear cells

Similar to CD4⁺ T cells, CD8⁺ T cells also secrete cytokines such as IL-4, IFN- γ and IL-17A. Frequencies of Tc1 (CD3⁺CD4⁻IFN- γ ⁺), Tc2 (CD3⁺CD4⁻IL-4⁺) and Tc17 (CD3⁺CD4⁻IL-17⁺) cells in activated lung mononuclear cells were measured by flow cytometric analysis (Fig.3).

Representative dot plots of IFN- γ -, IL-4- and IL-17-positive CD3⁺CD4⁻ T cells from experimental and control mice are shown in Fig. 3A. The percentage of Tc2 and Tc17 in lung mononuclear cells was significantly higher in experimental mice than in control mice. However, the frequency of Tc1 in lung mononuclear cells did not differ significantly between the two groups (Fig. 3B).

Frequency of CD39⁺CD4⁺Foxp3⁺ cells in lung mononuclear cells

Tregs (CD39⁺CD4⁺Foxp3⁺ cells) play an important role in suppressing the development of allergic asthma. Our previous study demonstrated that CD39 mRNA and protein levels were reduced in allergic asthmatic mice and the inhibitory function of Tregs from *Cd39* null mice was weakened. Therefore, we investigated the frequency of Tregs in lung mononuclear cells as measured by flow cytometric analysis. A reduction of CD39⁺CD4⁺Foxp3⁺ cells was observed in experimental mice compared to controls, as shown in Fig. 4.

Correlation between CD39⁺CD4⁺Foxp3⁺ cells and CD8⁺ T cell subpopulations

To assess possible interactions between CD39⁺CD4⁺Foxp3⁺ cells and CD8⁺T cell subpopulations Tc1, Tc2 and Tc17, we analyzed the correlation between CD39⁺CD4⁺Foxp3⁺ cells and Tc1, Tc2 and Tc17 in experimental mice by Pearson's test. As shown in Fig. 5, a negative correlation was found between CD39⁺CD4⁺Foxp3⁺ cell frequency and Tc2 or Tc17 cell frequencies ($r = -0.876$ $p = 0.001$ and $r = -0.585$ $p = 0.041$, respectively), but **no significant correlation** between CD39⁺CD4⁺Foxp3⁺ cell and Tc1 cell frequencies was seen ($r = -0.106$ $p = 0.743$).

Discussion

In the present study we found that CD8⁺IL-4⁺ cells (Tc2) and CD8⁺IL-17⁺ (Tc17) cells were increased and CD4⁺CD39⁺Foxp3⁺ cells (Tregs) were decreased in a murine model of allergic airway inflammation, and that there is a negative correlation between CD39⁺Tregs and Tc2 or

Tc17 cell frequencies. These results further support the role of CD39⁺Tregs in preventing airway inflammation and AHR, perhaps through regulation of CD8⁺ and CD4⁺ T cell immune responses.

In most mouse models of allergen induced allergic asthma, experimental animals are first systemically sensitized to specific antigens and then challenged with the same allergen administered via the airways, while negative control animals are sensitized with PBS/vehicle and challenged with antigen or challenged with PBS. In this study, we chose to challenge the control mice with OVA, as was done by Idzko et al in their study of ATP and airway inflammation [19].

Antigen-primed T cell immune response is central to the pathogenesis of allergic asthma. Induced sputum from severe asthmatic patients has been shown to contain increased numbers of CD8⁺ T cells[20], and a negative correlation has been found between increased CD8⁺ T cells and lung function [21]. Once stimulated by specific antigen, CD8⁺ T cells differentiate into distinct subsets including Tc1, Tc2, Tc9 and Tc17. The role of these CD8⁺ T cell subsets in allergic asthma is not well understood. Studies using mouse models have yielded conflicting results about the role of Tc1 in allergic asthma, with one study demonstrating that Tc1 cells are pathogenic [6] and another showing that they are beneficial [22]. Thus it is possible that Tc1 cells and IFN- γ have a bidirectional role in allergic airway inflammation. Tc2 cells have been shown to aggravate allergic airway inflammation in a mouse model [23]. In the present study, we observed significantly increased frequencies of Tc2 and Tc17 in asthmatic mice, as well as increased levels of IL-4 and

IL-17 in BALF, which is consistent with the previous studies [4, 24]. However, we found no significant difference in the frequencies of Tc1 cells or in the IFN- γ levels in BALF between asthmatic and control mice, supporting the possibility of a bidirectional role for IFN- γ in allergic airway inflammation.

CD39, an ATP scavenger, is involved in the development of autoimmune diseases, infectious diseases and ischemia-reperfusion injury through its regulation of the purinergic signaling pathway [25-28]. Since Idzko et al published their findings that ATP levels are increased in BALF from asthmatic patients and from OVA-exposed mice [19] the role of CD39 in allergic asthma has been studied with interest. CD39 is required for the immunosuppressive effects of CD4⁺Foxp3⁺ regulatory cells through ATP hydrolysis and/or adenosine production. Decreased CD39 levels have been observed in lungs from asthmatic mice [29] and in the peripheral blood of asthmatic patients [30]. It has been reported that CD39 expressed by CD4⁺Foxp3⁺ regulatory cells is involved in preventing allergic airway inflammation by modifying CD4⁺ T cell response [17]. However, the immunomodulatory effect of CD39⁺CD4⁺Foxp3⁺ cells on CD8⁺ T cells in allergic airway inflammation has not been determined. We found that the frequency of local CD39⁺CD4⁺Foxp3⁺ Tregs is decreased in allergic asthma, indicating that a sufficient number of Tregs is crucial to prevent allergic airway inflammation and AHR. This, together with our finding of negative correlation between CD39⁺CD4⁺Foxp3⁺ cell frequency and Tc2 and Tc17 frequencies,

suggests that insufficient numbers and/or impaired function of CD39⁺Tregs contribute to increased effector T cells involved in the pathogenesis of allergic asthma.

This study did have some limitations, specifically in experimental method and design. The question of how CD39⁺Tregs influence CD8⁺ T cell immunity in OVA-induced allergic airway inflammation cannot be determined by the current data. The conclusions of this study could be verified and extended by using specific antigen mice such as OVA specific OT II TCR transgenic mice (OT2) to minimize the impact of other immune cells, and by having a larger sample size. In addition, the impact of CD39 expression level on regulation of CD8⁺ T cell immunity should be considered, given the fact that CD39⁺Tregs can express low, intermediate or high levels of CD39. Our study showed that CD39⁺Tregs can regulate CD8⁺ T cell immunity in a murine model of allergic airway inflammation. **However, many questions about the mechanism by which CD39⁺Tregs influence CD8⁺ T cell immunity remain to be resolved.**

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This paper received no funding from outside agencies, organizations or companies.

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Fig. 1

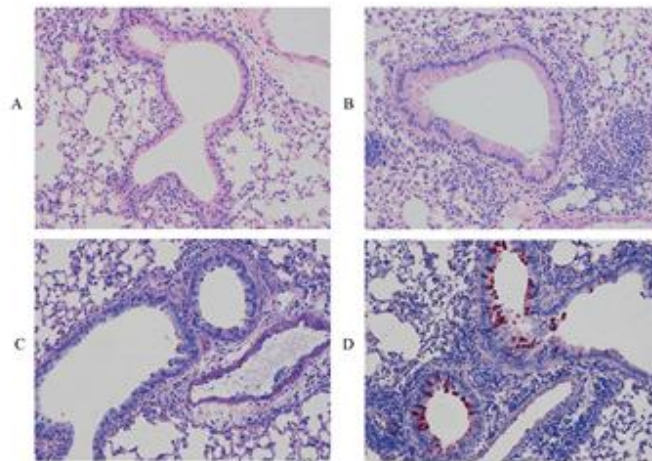


Figure 1. OVA-exposed mice show eosinophilic airway inflammation and goblet cell hyperplasia.

Representative micrographs of lung sections stained with HE (A and B) and PAS (C and D).

($\times 200$) A and C: control lung sections; B and D: experimental lung sections.

Fig. 2

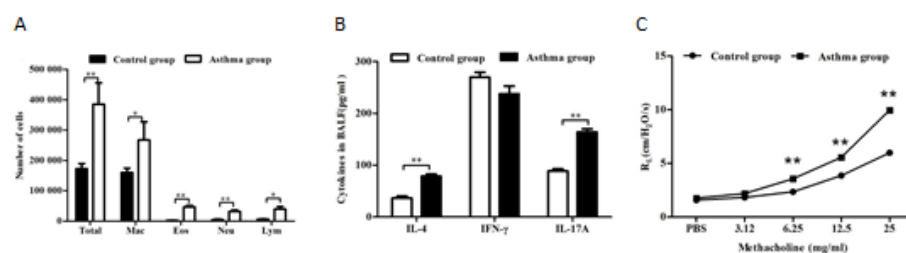


Figure 2. Inflammatory cells, cytokine levels and airway resistance are increased in OVA-exposed mice. Differential cell counts in bronchoalveolar lavage fluid (BALF). (B) Levels of cytokines in BALF. (C) Airway responsiveness to increasing concentration of methacholine. Data are expressed as means + standard deviation (SD). (n = 6-8 mice per group) * $P < 0.05$, ** $P < 0.01$

Fig. 3

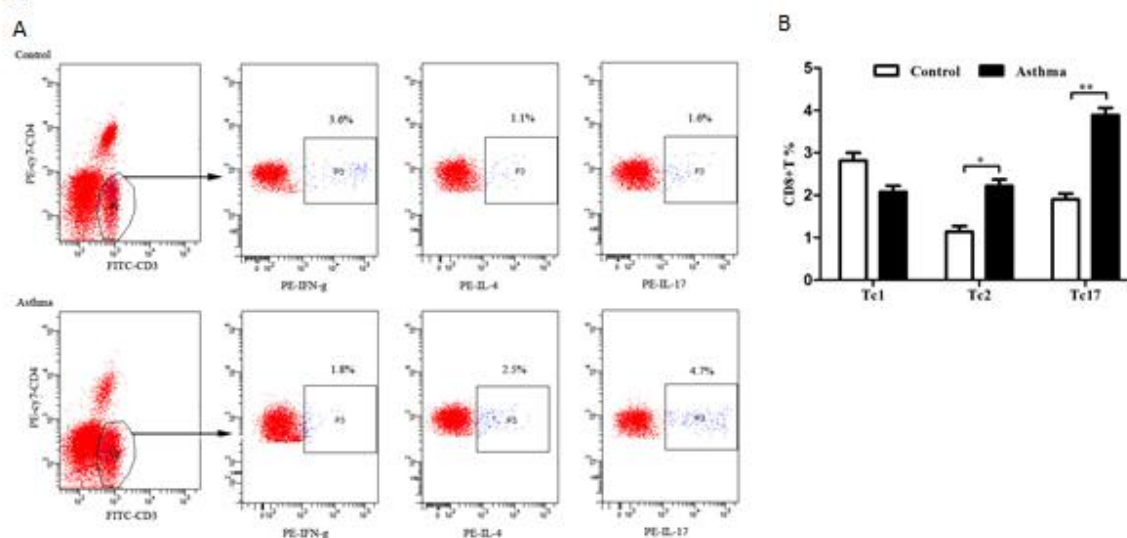


Figure 3. Frequencies of Tc1, Tc2 and Tc17 in lung mononuclear cells as measured by flow cytometric analysis. Lung mononuclear cells were stimulated with PMA and ionomycin and blocked by BFA, then pooled for flow cytometric analysis. **(A) Representative dot plots of Tc1 ($CD3^+CD4^-IFN-\gamma^+$), Tc2 ($CD3^+CD4^-IL-4^+$) and Tc17 ($CD3^+CD4^-IL-17^+$) cells from mice with allergic asthma and controls. (B) The percentages of Tc1, Tc2, and Tc17 cells as measured by flow cytometric analysis. Data are expressed as means + SD. (n = 6-8 mice per group) * $P < 0.05$, ** $P < 0.01$**

Fig. 4

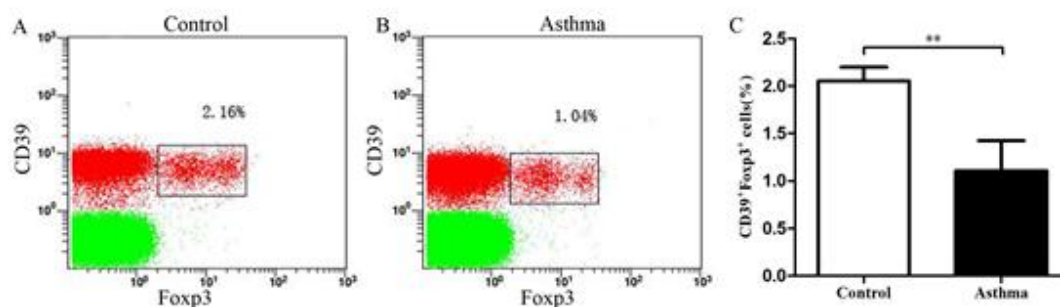


Figure 4. CD39⁺CD4⁺Foxp3⁺ cells in lung mononuclear cells. Representative dot plots of CD39⁺CD4⁺Foxp3⁺ cells from lung mononuclear cells from control mice. (B) Representative dot plots of CD39⁺CD4⁺Foxp3⁺ cells from lung mononuclear cells from experimental mice (asthma). (C) The frequency of CD39⁺CD4⁺Foxp3⁺ cells in lung mononuclear cells from experimental (asthma) and control mice. Data are expressed as means + SD. (n = 6-8 mice per group) ** $P < 0.01$

Fig. 5

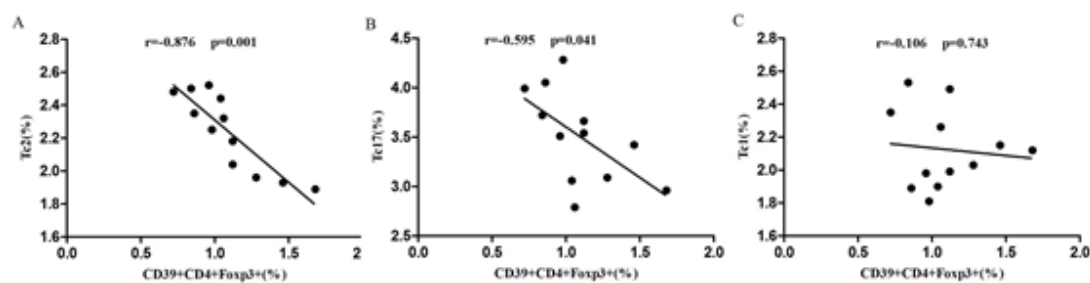


Figure 5. Correlation between frequencies of CD39⁺CD4⁺Foxp3⁺ cells and Tc cell subpopulations in experimental mice. Correlations between the frequencies of CD39⁺CD4⁺Foxp3⁺ cells and Tc2 (A), Tc17 (B) and Tc1 (C) subpopulations.