Journal of ASTHMA

http://informahealthcare.com/jas
ISSN: 0277-0903 (print), 1532-4303 (electronic)

J Asthma, 2014; 51(4): 360–365
© 2014 Informa Healthcare USA, Inc. DOI: 10.3109/02770903.2013.878954

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ENVIRONMENTAL DETERMINANTS

Infection with respiratory syncytial virus influences FasL-mediated apoptosis of pulmonary γδ T cells in a murine model of allergen sensitization

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Abstract

Background: It has been reported that adoptive transfer of γδ T cells increases the cellular infiltration, especially eosinophils, in the lungs of allergic mice, suggesting that γδ T cells may play a proinflammatory role in allergic airway inflammation. Respiratory syncytial virus (RSV) infection can decrease the number of Th2-type γδ T cells. However, the underlying mechanisms remain unknown. *Methods*: BALB/c mice were inoculated intranasally with RSV before or after sensitization to OVA. The amounts of Th1/Th2 cytokines as well as the levels of specific antibodies were determined by ELISA. The apoptotic death of pulmonary γδ T cells was analyzed by flow cytometry. *Results*: Adoptive transfer of γδ T cells increased the production of Th2 cytokines in the lungs and allergy-related antibodies in the serum, further confirming that γδ T cells act as pro-inflammatory cells or a promoter for the development of allergic asthma. RSV infection before sensitization to OVA enhanced apoptotic death of pulmonary γδ T cells. The percentage and absolute number of FasL-expressing γδ T cells in the lungs of allergic mice were elicited significantly by prior RSV infection. Blocking FasL with monoclonal antibody diminished apoptotic death of γδ T cells, suggesting that FasL is important for RSV-induced apoptosis of pulmonary γδ T cells. *Conclusions*: This work provides evidence that RSV infection suppresses the subsequent development of OVA-induced allergic responses partly by enhancing FasL-mediated apoptosis of pulmonary γδ T cells.

Keywords

Allergic responses, apoptotic death, Fas/FasL pathway, pulmonary γδ T cells, respiratory syncytial virus

History

Received 14 October 2013
Revised 18 December 2013
Accepted 21 December 2013
Published online 11 March 2014

Introduction

Allergic asthma is a Th2-associated respiratory disease characterized by the infiltration of eosinophils and the production of interleukin (IL)-4, IL-5 and IL-13 by T lymphocytes in response to certain antigens [1,2]. Although the biological role and repertoire of γδ T cells have not been defined fully, studies suggest that γδ T cells can have both proinflammatory and regulatory functions, depending on the model [3,4]. In murine model of asthma, γδ T cells are required for Th2-type eosinophilic inflammation and airway hyperresponsiveness (AHR), since a deficiency in this T cell subset results in decreased eosinophils in bronchoalveolar lavage (BAL) fluid and lung [5], but presence of this T cell subset results in enhanced allergic airway inflammation [6]. In fact, γδ T cells promote allergen-specific IgE responses and airway hyperreactivity by producing Th2 cytokines [6–8]. IL-4- and IL-5-producing γδ T cells have been found in the nasal passages of asthmatics [9]. Otherwise, in a murine model of allergic asthma, a large influx of γδ T cells can be detected in the lungs of allergic mice. Moreover, adoptive transfer of γδ T cells that obtained from OVA-sensitized and challenged mice increases the number of inflammatory cells, especially eosinophils, in the lungs of transferred mice, suggesting that under a certain condition, γδ T cells play a proinflammatory role in allergic airway inflammation [10].

Viral respiratory tract infections affect allergen-induced airway inflammation [11,12]. Respiratory syncytial virus (RSV), a member of the family *Paramyxoviridae*, genus *Pneumovirinae*, is the leading cause of lower respiratory tract disease in infants and young children [13], and has been linked to asthma and allergic sensitization. It has been reported that under a certain condition, RSV infection may play a protective role in allergen-induced airway inflammation. A diminished production of Th2-like cytokines as well as allergen-specific Th2-type antibodies has been found in the lungs of allergic mice that were infected with RSV before sensitization to an allergen [14]. γδ T cells have been associated with RSV-related diseases. Indeed, depletion of γδ T cells *in vivo* has been shown to reduce lung inflammation and disease severity in murine model that were immunized with RSV F-protein and challenged with live RSV [3].

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Interestingly, RSV infection decreases the number of pulmonary γδ T cells as well as activated γδ T cells [10], leading us to presume that RSV protects against the subsequent development of allergen-induced allergic reactions probably by enhancing apoptotic death of pulmonary γδ T cells.

Thus, in the present study, by using BALB/c mice that were inoculated intranasally with live RSV before or after sensitization to OVA, the mechanisms of reduction in pulmonary γδ T cell count are investigated. Furthermore, the role of γδ T cells for the development of OVA-induced allergic responses is further confirmed.

Methods

Mice

Pathogen-free 8-week-old female BALB/c mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Mice had fresh water and autoclaved food and were kept at 23 °C under bioclean conditions throughout all experiments. To avoid laboratory contamination, all virus- and allergen-inoculated mice were housed in negatively pressurized isolators equipped with a ventilation system through a high-efficiency particulate air filter (AH model; Nihon-Ika, Osaka, Japan). This work was approved by the Institutional Animal Care and Use Committee of China Medical University, China.

Virus

Human RSV type A2 (RSV A2) was supplied kindly by Dr. B. Wang, Center for Disease Control and Prevention, Shenyang, China. The virus was propagated in monolayer of HEp-2 cells (ATCC) grown in Eagle's minimum essential medium supplemented with 2% heat-inactivated fetal calf serum (FCS, Greiner, Frickenhausen, Germany). At the time point of the maximum cytopathic effect, cells were harvested and disrupted by sonication. The supernatant was layered on top of a sucrose gradient and centrifuged at $100\,000 \times g$ for 2 h at 10 °C. The pellet containing virus was resuspended in 10 mM phosphate-buffered saline (PBS) and stored in aliquots at −80 °C. Viral titers was expressed as a 50% tissue culture infectious dose (TCID50), calculated using the method of Reed and Muench [15].

Experimental design

Mice were sensitized by intraperitoneal injection of 20 μg of OVA (Grade V, Sigma, St. Louis, MO) emulsified in 2.25 mg aluminum hydroxide in a total volume of 100 μl on days 0 and 14, followed by aerosolized airway challenge with nebulized OVA (1% in saline) for 20 min on days 28, 29, and 30. One week before the first OVA sensitization or 1 week after the last OVA sensitization, mice, under light anesthesia (pentobarbital sodium, 0.025 mg/g body weight, intraperitoneal administration), were infected intranasally with RSV in 20 μl of suspension containing 2×10^6 TCID50 per mouse. Samples were collected 2 d after the last nebulized OVA exposure. There were four experimental groups: (1) PBS-sensitized, PBS-challenged group (expressed as saline); (2) OVA-sensitized, OVA-challenged group (expressed as OVA); (3) infection with RSV before sensitization to OVA (expressed as RSV+OVA); (4) infection with RSV after sensitization to OVA (expressed as OVA + RSV).

Preparation of single cell suspensions from lung

Mice were anesthetized and the lung was flushed in situ with 20 ml of sterile PBS via cannulation of heart to remove intravascular blood pool. Minced lung tissues were incubated at 37 °C for 60 min on a rocker with 200 mg/ml collagenase D and 40 mg/ml DNase I (Roche Molecular Biochemicals, Mannheim, Germany). Subsequently, the enzyme-digested lung tissue was passed through a stainless steel mesh. Single cell suspensions were collected by density gradient centrifugation with lymphocyte-separation solution (Antibody Institute, Gumma, Japan).

Adoptive transfer of γδ T cells

Lymphocytes were prepared from the spleens of OVA-sensitized and challenged mice at day 2 after the last challenge, and incubated with PE-labeled anti-CD3 mAb and FITC-conjugated anti-TCR γδ mAb (eBioscience, San Diego, CA) for 20 min on ice. γδ T cells were then sorted based on their expression of CD3 and TCR γδ using a MoFlo cell sorter. The ratio of γδ T/total cells was 90.5 ± 1.8% purity. Purified $CD3^+\gamma\delta^+$ T cells were resuspended to 1×10^6 cells/ml of PBS, and 2×10^4 cells/mouse in 200 μl of PBS were injected via the tail vein into the mice within 1 h before the first airway challenge.

Assay of cytokine production

Lungs were lavaged via the intratracheal tube with 1 ml PBS. The amounts of IFN-γ, IL-4 and IL-17A in the BAL fluids were assayed by using a mouse cytokine detection ELISA kit (eBioscience) in accordance with the manufacturer's instructions. The minimum detectable concentrations of IFN-γ, IL-4 and IL-17A were 15, 5 and 10 pg/ml, respectively.

Quantification of antibody level

The production of total serum IgE as well as OVA-specific serum Ig isotypes was measured by ELISA. Briefly, microplates were coated with 10 μg of OVA proteins overnight at 4 °C. Test samples were added to the well and incubated at 37 °C for 1 h. Bound antibodies were reacted with goat horseradish peroxidase-conjugated anti-mouse IgE, IgG1, or IgG2a (Sigma), respectively. After washing, the antigen–antibody complex was incubated with enzyme substrate of *O*-phenylenediamine dehydrochloride. The absorbance value at 490 nm was measured in an automated microplate reader.

Identification of $FasL^+\gamma\delta^+$ T cells in the lung

To determine the percentage and number of $FasL^+TCR\gamma\delta^+$ T cells in the lungs of allergic mice, single cell suspensions from the enzyme-digested lung tissue were collected and stained with FITC-conjugated anti-γδ TCR, APC-conjugated anti-FasL and PE-conjugated anti-CD45 Abs (eBioscience) for 20 min on ice. Then, 100 000 stained lung cells were analyzed by flow cytometry. Rat anti-mouse CD16/CD32

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monoclonal antibody was used to block non-specific Ab binding.

Apoptosis of γδ T cells

Single-cell suspensions from the lungs of mice were incubated with PE-conjugated anti-CD3 and FITC-conjugated anti-γδ TCR monoclonal antibodies (eBioscience). $CD3^+\gamma\delta^+$ T cells were isolated by MoFlo cell sorter and then stained with annexin V-FITC and propidium iodide (BD Pharmingen, SanDiego, CA), according to the manufacturer's directions. Cell debris was excluded from analysis by appropriate forward light scatter threshold setting. Cells staining with annexin V-FITC, but not propidium iodide, were regarded as early apoptotic cells. This method was used to avoid contamination of the data with debris or late apoptotic cells.

To determine whether FasL is required for the γδ T cell death, pulmonary γδ T cells were re-stimulated *in vitro* with Con A (5 μg/ml) in the presence of neutralizing FasL mAb (10 μg/ml, eBioscience) for 12 h. The percentage of apoptotic cells was determined by annexin V staining.

Statistical significance

All results shown are representative of three independent experiments. Data are presented as mean ± SD of results for each group of five mice tested. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. A p value less than 0.05 was considered significant.

Results

Adoptive transfer of γδ T cells increases the production of Th2-type cytokines in the lungs of allergic mice

It has been reported that adoptive transfer of γδ T cells augments allergen-induced airway inflammation, particularly the infiltration of eosinophilis, suggesting that γδ T cells may act as a promoter or pro-inflammatory cells in allergen-induced allergic responses [10]. To further investigate the role of γδ T cells in respiratory allergy, γδ T cells from OVA-sensitized and challenged mice were transferred into the corresponding mice and the changes in the production of Th1/Th2 cytokines were detected. As shown in Figure 1, sensitization and challenge with OVA induced a significant increase in the levels of IL-4 and IL-17 A in the lungs of allergic mice. Prior RSV infection, however, decreased the amounts of Th2 cytokines but promoted the production of Th1 cytokine. The titer of IFN-γ was enhanced from undetectable level (<15 pg/ml) in OVA mice to 49.9 ± 20.9 pg/ml in RSV+OVA mice. In contrast, RSV infection after sensitization to OVA seems to have no effect on OVA-induced cytokine production (Figure 1).

Adoptive transfer of γδ T cells increased the production of Th2 cytokines. Large amounts of IL-4 and IL-17 were detected in the lungs of transferred mice as compared with that of corresponding non-transferred mice (Figure 1A and B). However, Th1 cytokine IFN-γ, was decreased to undetectable level by adoptive transfer of the pulmonary γδ T cells (Figure 1C).

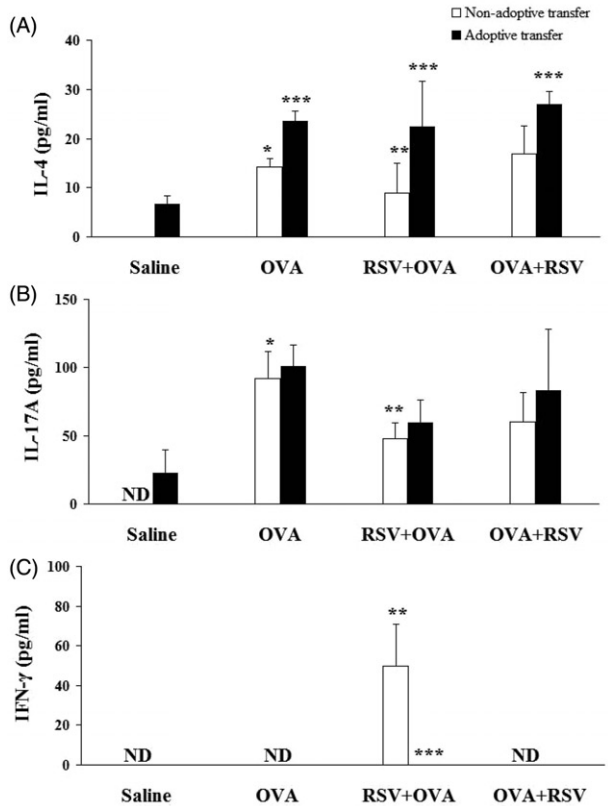

Figure 1. Cytokine production in the lungs of BALB/c mice after adoptive transfer of γδ T cells. BALF was collected 2 d after the last nebulized OVA exposure. The amounts of IL-4 (A), IL-17A (B), and IFN-γ (C) in the lungs of allergic mice were detected by ELISA kit. Data are presented as mean±SD of results for each group of five mice tested. The data shown is representative of three separate experiments. *Significant difference ($p<0.01$ by ANOVA) compared with corresponding saline group. **Significant difference ($p<0.05$ by ANOVA) compared with corresponding OVA group. ***Significant difference ($p<0.01$ by ANOVA) compared with corresponding non-transferred group, respectively. ND: Not detectable.

Adoptive transfer of γδ T cells increases the levels of Th2-type antibodies in the serum of allergic mice

As shown in Figure 2, OVA-sensitized and challenged mice exhibited Th2-dominant antibody responses, characterized by increased levels of total serum IgE, OVA-specific IgE and OVA-specific IgG1. Prior RSV infection diminished the amounts of the allergy-related antibodies (Figure 2). However, by γδ T cell transfer, these Th2-type antibodies, particularly total IgE as well as OVA-specific IgE and IgG1, were raised significantly as compared with that of corresponding non-transferred mice (Figure 2). No measurable OVA-specific IgG2a production could be detected in this study (data not shown). These results together with our previous data further confirmed that γδ T cells act as Th2-type cells and contribute to the allergy-related antibody production.

RSV infection enhances OVA-induced apoptotic death of pulmonary γδ T cells

Since prior RSV infection decreases the number of pulmonary γδ T cells as well as activated γδ T cells [10], leading us to hypothesize that RSV infection may cause activated γδ T cells

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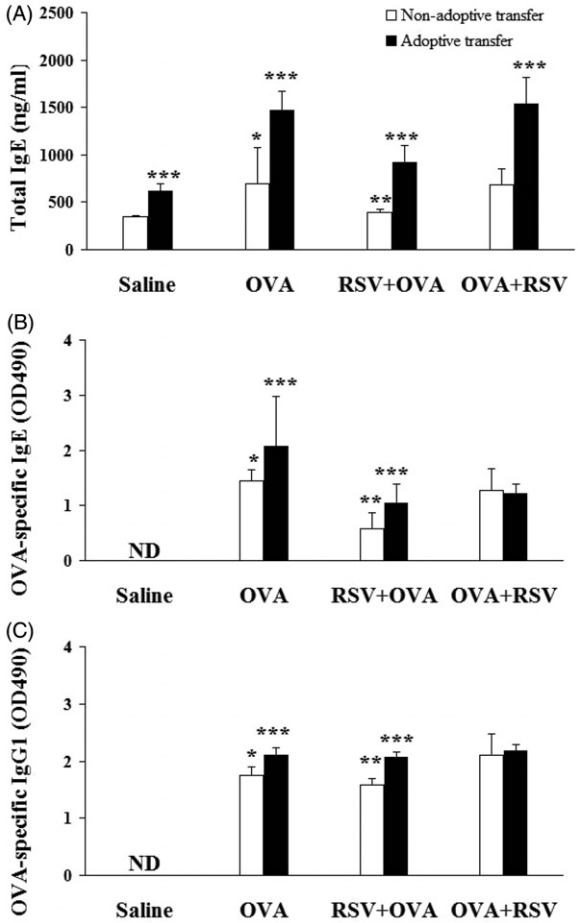

Figure 2. Production of total serum IgE and OVA-specific Ig isotypes in BALB/c mice after adoptive transfer of γδ T cells. The sera were collected 2 d after the last nebulized OVA exposure. Total IgE (A), OVA-specific IgE (B) as well as OVA-specific IgG1 (C) were detected by ELISA kit. Data are presented as mean ± SD of results for each group of five mice tested. The data shown is representative of three separate experiments. *Significant difference ($p < 0.01$ by ANOVA) compared with corresponding saline group. **Significant difference ($p < 0.01$ by ANOVA) compared with corresponding OVA group. ***Significant difference ($p < 0.01$ by ANOVA) compared with corresponding non-transferred group, respectively. ND: Not detectable.

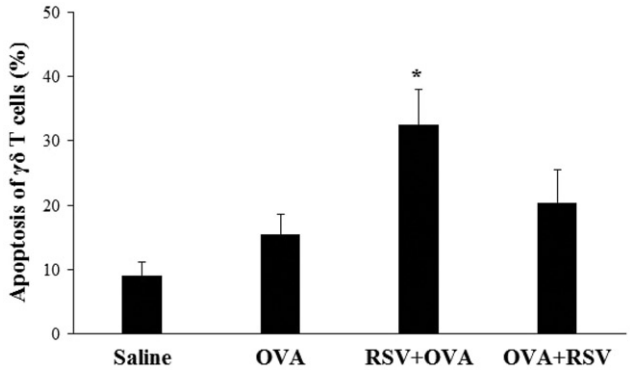

Figure 3. Apoptotic death of pulmonary γδ T cells. γδ T cells were isolated from the lungs of mice 2 d after the last nebulized OVA exposure, and restimulated *in vitro* with Con A for 24 h. The percentage of apoptotic γδ T cells was detected by annexin-V staining. Data are presented as mean ± SD of results for each group of five mice tested. The data shown is representative of three separate experiments. *Significant difference ($p < 0.01$) compared with corresponding control OVA mice.

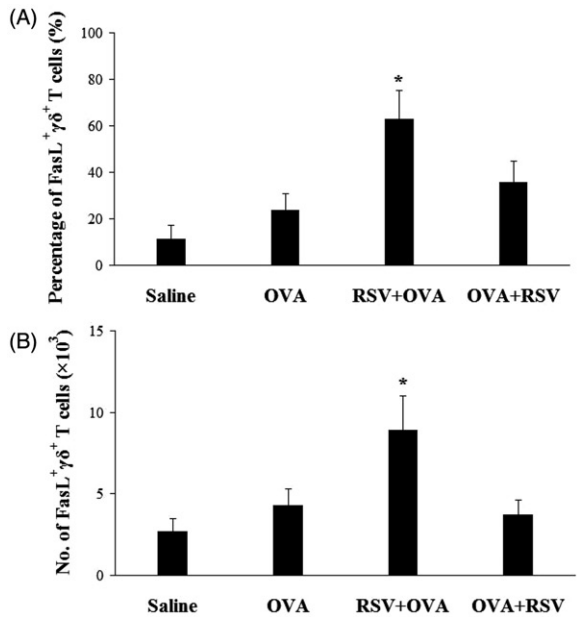

Figure 4. Prior RSV infection increases the percentage (A) and absolute number (B) of FasL-expressing γδ T cells. Lung parenchyma cells were isolated from the tested mice and innate lymphoid cells were gated from $CD45^+$ live cells, and then assessed based on the expression of TCR γδ and FasL. Data are presented as mean ± SD of results for each group of five mice tested. The data shown are representative of three separate experiments. *Significant difference ($p < 0.01$) compared with corresponding control OVA mice.

to die. To confirm this suggestion, apoptotic death of pulmonary γδ T cells was determined by flow cytometric detection of extracellular annexin V binding. As shown in Figure 3, prior RSV infection increased the percentage of apoptotic γδ T cells from 15.4 ± 3.1% in OVA mice to 32.4 ± 5.6% in RSV + OVA mice. To determine whether the apoptotic death was induced directly by RSV infection, the apoptosis of pulmonary γδ T cells isolated from the mice that were infected with RSV alone was detected. No appreciable difference in the percentage of apoptotic γδ T cells could be found between RSV mice (7.3 ± 3.5%) and control saline mice (8.9 ± 2.2%).

Apoptosis of γδ T cells depends on FasL pathway

Apoptosis can be divided into the extrinsic and intrinsic pathway [16]. The extrinsic apoptosis is mediated by the superfamily of death receptors, including Fas ligand. To provide the evidence that FasL may be involved in γδ T cells death, lung parenchyma cells were collected and the percentage and number of $FasL^+$γδ T cells were determined by flow cytometry. As shown in Figure 4, sensitization and challenge with OVA induced a infiltration of $FasL^+$γδ T cells in the lungs of allergic mice. Prior RSV infection, however, increased the infiltration. The percentage and absolute number of $FasL^+$γδ T cells were enhanced significantly in the lungs of RSV+OVA mice as compared with that of OVA mice as well as OVA+RSV mice (Figure 4). Moreover, when the pulmonary γδ T cells were re-stimulated *in vitro* with Con A in the presence of neutralizing FasL mAb, the apoptosis of γδ T cells in the lungs of RSV+OVA mice was inhibited markedly (Figure 5). These results suggest that prior RSV

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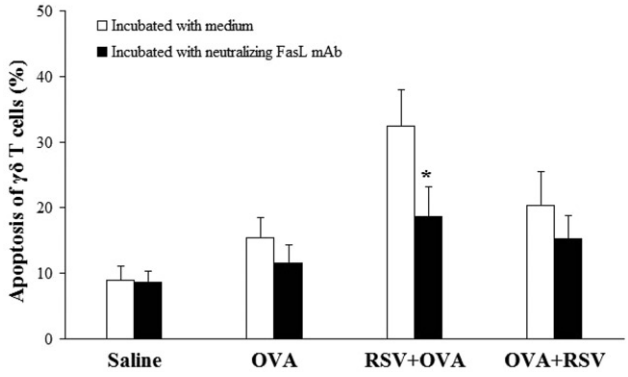

Figure 5. Prior RSV infection enhances apoptotic death of pulmonary γδ T cells via FasL-dependent pathway. Isolated γδ T cells were restimulated *in vitro* with Con A in the presence of neutralizing FasL mAb for 24 h. The apoptotic death of pulmonary γδ T cells was determined by annexin V staining. Data are presented as mean ± SD of results for each group of five mice tested. The data shown is representative of three separate experiments. *Significant difference ($p < 0.01$) compared with corresponding anti-FasL mAb-treated RSV + OVA mice.

infection augments OVA-induced apoptotic death of γδ T cells partly via FasL-dependent pathway.

Discussion

The regulatory effects of γδ T cells on allergic responses appear to be complex. Depending on the model, γδ T cells promote allergen-specific IgE responses, eosinophilic airway inflammation, and AHR [5–8]; suppress the development of specific IgE antibody and AHR [5,17]; or have no effect [18]. However, in our experimental condition, γδ T cells may act as a promoter or positive regulator in allergen-induced allergic responses, since adoptive transfer of γδ T cells from OVA-sensitized and challenged mice increases the production of Th2 cytokines as well as allergy-related Th2-type antibodies not only in allergic mice but also in saline mice (Figures 1 and 2). These findings are consistent with other reports that γδ T cells may be essential for Th2-mediated airway inflammation [19,20], based on observations of decreased inflammation, AHR and Th2 cytokine production in γδ T cell-deficient mice [6,7].

RSV infection can establish Th1 type dominant environment in adult mice [21,22], and then alters Th2 type responses subsequently induced by allergen sensitization [14]. By using BALB/c mice that were inoculated intranasally with live RSV before sensitization to OVA, it has been reported that RSV infection diminishes allergic airway inflammation by reducing the number of total pulmonary γδ T cells as well as activated γδ T cells [10]. Death receptor-mediated apoptosis is involved in the reduction of lymphocyte numbers [23,24]. Members of the TNFR family, particularly CD95, initiate activation-induced cell death (AICD) upon engagement by their specific ligands (FasL), leading to activation of a regulated cascade of caspases and ultimately to apoptosis [25,26]. It becomes clear that FasL is expressed largely on the surface of T cells to exert activation-induced cell death [27]. Guzy et al. reported that *E. coli* Nissle induces FasL expression on γδ T cells and the fact that neutralizing FasL can prevent *E. Coli* Nissle-induced γδ T cell death [28]. On the other hand, γδ T cells isolated from Mycobacteria tuberculosis-infected patients are more susceptible to AICD than normal individuals and death is mediated by Fas and FasL interaction [29]. In this study, the apoptotic death of pulmonary γδ T cells was enhanced significantly, with an increase in the number of FasL-expressing γδ T cells in the lungs of RSV-infected, OVA-sensitized mice (Figures 3 and 4), suggesting that RSV infection may affect FasL-mediated apoptotic death of pulmonary γδ T cells. Indeed, by blocking FasL with neutralizing FasL mAb, the percentage of apoptotic γδ T cells in the lungs of tested mice was diminished (Figure 5). Together, these results indicate that RSV infection may promote γδ T cells to be undergoing apoptosis via FasL-dependent pathway.

Because infection with RSV alone cannot enhance the apoptotic death of pulmonary γδ T cells, suggesting that the increased γδ T cell death in the lungs of RSV-infected, OVA-sensitized mice may not be due to virus loading. It seems likely that prior RSV infection alters the responding environment of respiratory tract and then affects allergen-induced apoptotic death of pulmonary γδ T cells. Furthermore, apoptotic cell death may remove Th1 or Th2 cells allowing Th2 or Th1 responses to become dominant [30]. Thus, in the present study, prior RSV infection enhances apoptosis of the Th2-type pulmonary γδ T cells, and then, diminishes allergic airway inflammation whish is subsequently induced by allergen sensitization.

It has been reported that two γδ T cell types are capable of either suppressing or enhancing allergen-induced airway reactions [31]. These cells express Vγ4 and Vγ1, respectively. The $V\gamma4^+$ subset can be induced to suppress AHR in allergen-sensitized and challenged mice [31–33], whereas, $V\gamma1^+$ subset can promote production of Th2 cytokine and eosinophilic infiltration [31]. Although in this article, we did not examine which subset of γδ T cells in the lungs of OVA-sensitized and challenged mice was influenced by RSV infection. However, from these results that RSV infection diminishes the number of γδ T cells and inhibits Th2 cytokine production by γδ T cells [10], together with the results of γδ T cell transfer, we suppose that under our experimental condition, Th2-type γδ T cells ($V\gamma1^+$ cell?) may be the major subset induced by OVA-sensitization and challenge. Prior RSV, however, induces apoptotic death of the Th2-type γδ T cells, and then, diminishes allergic responses in the lungs of allergic mice.

Conclusion

This work provides evidence that under a certain condition, γδ T cells play a pro-inflammatory role in allergen-induced allergic responses. RSV infection before sensitization to OVA promotes FasL-mediated apoptosis of the Th2-type γδ T cells.

Declaration of interest

This study was supported by the found from National Natural Science Foundation of China (No. 81273239/H1005). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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